**The transmembrane domains of the type III secretion system effector Tir are involved in its secretion and cellular activities.**

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Running title: The transmembrane domains of the Tir effector

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**Abstract**

Enteropathogenic Escherichia coli (EPEC) is a diarrheagenic pathogen and one of the major causes of gastrointestinal illness in developing countries. EPEC, like many other Gram-negative bacterial pathogens, possesses an essential virulence machinery called the type III secretion system (T3SS) that enables the injection of effector proteins from the bacteria into the host cytoplasm. Among these, the translocated Intimin receptor (Tir) is the first effector to be injected and its activity is essential for the formation of attaching and effacing (A/E) lesions, the hallmark of EPEC colonization. Tir belongs to a unique group of transmembrane domain (TMD)-containing secreted proteins, which have two conflicting destination indications: one for bacterial membranal integration and another for protein secretion. In this study, we examined whether Tir TMDs are involved in the protein’s ability to be secreted, translocated, and function within the host cells. For this purpose, we created Tir TMD variants with their original or alternative TMD sequence. Our results suggest that the C-terminal TMD of Tir (TMD2) is critical for Tir’s ability to escape integration into the bacterial membrane. However, the TMD sequence by itself is not sufficient and its effect is context-dependent. Moreover, we found that the N-terminal TMD of Tir (TMD1) is important for the protein post-secretion function at the host cell. Taken together, our study further supports the hypothesis that the TMD sequences of translocated proteins encode information crucial for protein secretion and their post-secretion function.

**Introduction**

Enteropathogenic *Escherichia coli* (EPEC) is a Gram-negative, facultative anaerobe, rod-shaped bacterium that infects epithelial cells in the gastrointestinal tract (GIT). EPEC is a major cause of infantile diarrhea in developing countries that has recently been re-emergenced and reported in various outbreaks in Oceania, East Asia and Northern Europe (1-3). The hallmark of EPEC colonization is the formation of distinct histopathological lesions termed “attaching and effacing” (A/E) lesions. Members of the A/E family also include the human pathogen enterohemorrhagic *E. coli* (EHEC), which causes hemorrhagic colitis and pediatric kidney failure, and the mouse pathogen *Citrobacter rodentium*, which is used in animal models (4). A/E lesions are characterized by the destruction of the intestinal microvilli, intimate bacterial attachment to the plasma membrane of enterocytes, and cytoskeletal rearrangement that result in formation of dense actin filaments beneath adherent bacteria, termed pedestals (5, 6). The development of A/E lesions occurs in three sequential stages: (i) initial bacterial adherence, (ii) cellular signal transduction, and (iii) intimate attachment (7).

To allow bacterial adherence and interference with cellular signal transduction, EPEC utilizes a specialized protein-secretion complex called the type III secretion system (T3SS). The T3SS is a syringe-shaped nanomachine that spans the bacterial membranes and extends to the host cell membrane, where it forms a pore that allows various effector proteins to be translocated across the plasma membrane and into the host cell cytoplasm. These effectors are subverting key cellular processes (such as immune response, signal transduction, vesicle transport, and cytoskeletal dynamics) in order to promote bacterial replication, survival, and transmission. In A/E pathogens, the T3SS is encoded on a 35 kbp chromosomal pathogenicity island called the locus of enterocyte effacement (LEE). The type III secretion (T3S) activity is a tightly regulated process that is divided into secretion of three substrates groups to ensure proper assembly and timely secretion. These include early (needle and inner rod proteins), intermediate (translocators), and late (effectors) substrates (4, 8).

The first effector to be translocated into the host cell is the translocated Intimin receptor (Tir). The delivery of a self-receptor to the host cell by the bacterium is considered a novel infection strategy only reported in A/E pathogens. The Tir adopts a hairpin loop topology on the plasma membrane, where the N- and C-termini are cytosolic, with two helical transmembrane domains (TMDs) traversing the host membrane (Fig. 1A). The central loop is exposed to the extracellular environment and interacts with the C-terminus of Intimin, a bacterial outer membrane adhesin presented on the EPEC membrane, with high affinity (Kd ~ 10 nM for EPEC). This interaction facilitates the tight attachment between the bacteria and the host (9). Upon Tir-Intimin interaction, Intimin dimerizes and leads to Tir clustering. This stimulates phosphorylation of the Tir at various residues at its C-terminus domain (10). While the role of serine/threonine phosphorylation on Tirs is not fully understood, it is well established that tyrosine phosphorylation of Tirs is required for rearrangement of filamentous actin into pedestal structures, leading to the formation of A/E lesions (4, 9).

Tirs belong to a unique group of secreted proteins that contains at least one TMD. Specifically in EPEC, two translocator proteins, EspB and EspD, and two effectors, Tir and EpsZ, are TMD-containing secreted proteins. These proteins have, by definition, a targeting conflict as their sequence contains signals for two incompatible pathways: On the one hand, they contain an N-terminal T3SS signal, guiding them for export through the T3SS and, on the other, they have a TMD signal that guides them to the bacterial membrane (11). This conflict requires targeting discrimination, which ensures that, post-translation, the TMD-containing secreted proteins can escape the bacterial membrane integration mechanism and remain soluble at the cytoplasm until their timely secretion. While the mechanism behind this targeting discrimination remains largely unknown, recent studies have suggested that a significant factor is the moderate hydrophobicity of the TMDs of these secreted proteins, which is sufficient for integration into eukaryotic cell membranes, yet not recognized by the bacterial signal recognition particle (SRP) (12). This was recently demonstrated by our group when the replacement of the TMDs of EPEC translocators (EspB and EspD) with a more hydrophobic sequence resulted in their mis-localization into the bacterial membrane and abolished their secretion (13).

In this study, we expanded our research to Tir as an additional TMD-containing secreted protein and examined the role of its TMDs on its secretion, translocation, and post-translocation functions. Moreover, we studied whether the context of the specific TMDs affects these properties. Our results support the previous observation that the TMD sequences of TMD-containing secreted proteins are crucial for protein secretion as well as their context within the protein sequence. Moreover, our data suggest that the TMDs affect Tir function at the host cell-membrane, post-secretion. Specifically, it appears that the C-terminal TMD of Tir (TMD2) is important for proper secretion while the N-terminal TMD (TMD1) is required for Tir’s post-secretion function.

**Materials and methods:**

**Bacterial strains -** All strains and plasmids used in this study are listed in Table S1. Wild-type EPEC O127:H6 strain E2348/69 [streptomycin-resistant] and EPEC null mutants (Δ*escN*, Δ*escV*, and Δ*tir*\*) were used to assess the T3SS and translocation activities. The strain Δ*tir*\*, a generous gift from Prof. Ilan Rosenshine (Hebrew University of Jerusalem, Israel), contains a deletion of Tir 79-873 base pairs. This modified strain has unfunctional Tir but expresses normal levels of CesT, which are critical for expression of virulence genes via inhibition of CsrA (14). *E. coli* DH10B was used for plasmid handling and *E. coli* strain BL21 (λDE3) was used for protein expression. The *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth (Sigma) supplemented with the appropriate antibiotics. Antibiotics were used at the following concentrations: streptomycin (50 μg/mL), ampicillin (100 µg/mL), and kanamycin (50 μg/mL).

**Construction of plasmids -** Cloning was performed using the Gibson assembly method (15, 16). To label Tir with V5, the pSA10 plasmid was amplified using the primer pair pSA10\_vector\_F/pSA10\_vector\_R and the Tir gene was amplified from EPEC genomic DNA and fused to a V5-tag at the C-terminus in two sequential reactions using the primer pairs Tir\_V5\_F1/Tir\_V5\_R1 and then Tir\_V5\_F1/ Tir\_V5\_R2 (Table S2). The PCR products were subjected to digestion with *Dpn*I, purified, and assembled by the Gibson assembly method.

The TMD-exchanged versions of *tir* were generated using the pTirwt-V5 (pSA10) vector as a template. To replace the TMD2 with TMD1, the TMD1 sequence was amplified using the primer pair Tir\_TMD1\_F1/Tir\_TMD1\_R1 and the 200 bp sequence downstream of TMD2 was amplified using the primer pair Tir\_TMD2\_200bp\_F/Tir\_TMD2\_200bp\_R (Table S2). Both fragments were then ligated using overlapping PCR reaction with the primer pair Tir\_TMD1\_F1/ Tir\_TMD2\_200bp\_R (Table S2). Gibson assembly was conducted by amplifying the pTirwt-V5 (pSA10) vector with the primer pair pSA10\_Tir\_TMD2\_F/ pSA10\_Tir\_TMD2\_R (Table S2), followed by *Dpn*I treatment of the reaction and subjecting the amplified vector and the TMD1 insert to ligation. The resulting construct, pTir-D1-V5 (pSA10), expressed Tir-V5 protein that contains a TMD1 sequence instead of the original TMD2 sequence.

To replace the TMD1 with TMD2, the TMD2 sequence was amplified using the primer pair Tir\_TMD2\_F1/Tir\_TMD2\_R1 and the 200 bp sequence downstream of TMD1 was amplified using the primer pair Tir\_TMD1\_200bp\_F/Tir\_TMD1\_200bp\_R (Table S2). Both fragments were then ligated using overlapping PCR reaction with the primer pair Tir\_TMD2\_F1/ Tir\_TMD1\_200bp\_R (Table S2). Gibson assembly was conducted by amplifying the pTirwt-V5 (pSA10) vector with the primer pair pSA10\_Tir\_TMD1\_F / pSA10\_Tir\_TMD1\_R (Table S2), followed by *Dpn*I treatment of the reaction and subjecting the amplified vector and the TMD2 insert to ligation. The resulting construct, pTir-D2-V5 (pSA10), expressed Tir-V5 protein that contains a TMD2 sequence instead of the original TMD1 sequence.

To generate Tir sequence where the TMDs are in a revearse order, pTir-D2-V5 (pSA10) was amplified using the primer pair pSA10\_Tir\_TMD2\_F/pSA10\_Tir\_TMD2\_R (Table S2), treated with *Dpn*I, and subjected to ligation with the previously used TMD1 insert comprised of TMD1 and the 200 bp sequence downstream of TMD2. The resulting construct, pTir-R21-V5 (pSA10), expressed Tir-V5 protein that contains TMD1 and TMD2 sequences at replaced positions.

To replace the TMD1 with the TMD of EscD, the EscD TMD sequence was amplified from EPEC genomic DNA using the primer pair EscD\_TMD\_Tir\_TMD1\_F /EscD\_TMD\_Tir\_TMD1\_R and the 200 bp sequence downstream of TMD1 was amplified using the primer pair Tir\_TMD1\_200bp\_F/Tir\_TMD1\_200bp\_R (Table S2). Both fragments were then ligated using overlapping PCR reaction with the primer pair EscD\_TMD\_Tir\_TMD1\_F/Tir\_TMD1\_200bp\_R (Table S2). Gibson assembly was conducted by amplifying the pTirwt-V5 (pSA10) vector with the primer pair pSA10\_Tir\_TMD1\_F / pSA10\_Tir\_TMD1\_R (Table S2), followed by *Dpn*I treatment of the reaction and subjecting the amplified vector and the TMD1-EscD insert to ligation. The resulting construct, pTir- EscD1-V5 (pSA10), expressed Tir-V5 protein that contains an EscD TMD sequence instead of the original TMD1 sequence.

To replace the TMD2 with TMD of EscD, the EscD TMD sequence was amplified from EPEC genomic DNA using the primer pair EscD\_TMD\_Tir\_TMD2\_F / EscD\_TMD\_Tir\_TMD2\_R and the 200 bp sequence downstream of TMD2 was amplified using the primer pair Tir\_TMD2\_200bp\_F/Tir\_TMD2\_200bp\_R (Table S2). Both fragments were then ligated using overlapping PCR reaction the primer pair EscD\_TMD\_Tir\_TMD2\_F/Tir\_TMD2\_200bp\_R (Table S2). Gibson assembly was conducted by amplifying the pTirwt-V5 (pSA10) vector with the primer pair pSA10\_Tir\_TMD2\_F/pSA10\_Tir\_TMD2\_R (Table S2), followed by *Dpn*I treatment of the reaction and subjecting the amplified vector and the TMD2-EscD insert to ligation. The resulting construct, pTir- EscD2-V5 (pSA10), expressed Tir-V5 protein that contains an EscD TMD sequence instead of the original TMD2 sequence.

To label cesT with a His-tag at its C-terminus, the *cesT* coding region was amplified from EPEC genomic DNA using the primer pair CesT\_His\_F/ CesT\_His\_R. The PCR product was then subcloned as a *Nco*I/*Xho*I fragment into *Nco*I/*Xho*I -digested, His-tagged pET28a. All constructs were verified by DNA sequencing.

***In vitro* type III secretion (T3S) assay -** T3S assays were performed as previously described (17). Briefly, EPEC strains were grown overnight in LB broth, supplemented with the appropriate antibiotics, in a shaker at 37°C. The cultures were diluted 1:40 into pre-heated Dulbecco’s modified Eagle’s medium (DMEM, Biological Industries) and grown statically for 6 h in a tissue culture incubator (with 5% CO2) to an optical density of 0.7 at 600 nm (OD600). To induce protein expression, 0.25 mM IPTG was added to bacterial cultures. The cultures were then centrifuged at 20,000 × g for 5 min to separate the bacterial pellets from the supernatants, the pellets were dissolved in SDS-PAGE sample buffer, and the supernatants were collected and filtered through a 0.22 μm filter (Millipore). The supernatants were normalized according to the bacterial OD600 and then precipitated with 10% (v/v) trichloroacetic acid (TCA) overnight at 4°C to concentrate proteins secreted into the culture medium. The samples were then centrifuged at 18,000 × g for 30 min at 4°C, the precipitates of the secreted proteins were dissolved in SDS-PAGE sample buffer, and the residual TCA was neutralized with saturated Tris. Proteins were analyzed by SDS-PAGE gels and western blotting.

**Western blot analysis -** Samples were subjected to SDS-PAGE and transferred to a nitrocellulose (pore size: 0.45 µm; Bio-Rad) membrane. The blots were blocked for 1 h with 5% (w/v) skim milk-PBST (0.1% Tween in phosphate-buffered saline), incubated with the primary antibody (diluted in 5% skim milk-PBST for 1 h at room temperature or overnight at 4°C), washed, and then incubated with the secondary antibody (diluted in 5% skim milk-PBST, for 1 h at room temperature). Chemiluminescence was detected with EZ-ECL reagents (Biological Industries). The following primary antibodies were used: mouse anti-His (Pierce), diluted 1:2,000; rabbit anti-MBP (ThermoFisher Scientific), diluted 1:1,000; mouse anti-DnaK (Abcam, Inc.), diluted 1:5,000; and mouse anti-actin (MPBio), diluted 1:10,000. Antibodies directed against T3SS components were a generous gift from Prof. B. Brett Finlay (University of British Columbia, Canada) and Prof. Rebekeh DeVinney (University of Calgary, Canada) and included mouse anti-Tir, rat anti-Intimin, and rat anti-Tir. Horseradish peroxidase-conjugated (HRP)-goat anti-mouse (Abcam Inc.), HRP-conjugated goat anti-rabbit (Abcam Inc.), and HRP-conjugated goat anti-rat (Jackson ImmunoResearch), diluted 1:10,000, were used as the secondary antibodies. Representative western blots of at least three independent experiments are presented in the Results section.

**Bacterial fractionation -** Bacterial cell fractionation was performed as previously described (18). Briefly, EPEC strains from an overnight culture were sub-cultured 1:50 in DMEM, and grown statically for 6 h at 37°C in a CO2 tissue culture incubator. To induce protein expression, 0.25 mM IPTG was added to bacterial cultures. Cells were harvested, washed in PBS, and resuspended in buffer A [50 mM Tris (pH 7.5), 20% (w/v) sucrose, 5 mM EDTA, protease inhibitor cocktail (Roche Applied Science), and lysozyme (100 µg/mL)] and incubated with rotation for 15 min at room temperature to generate spheroplasts. MgCl2 was then added to a final concentration of 20 mM and samples were centrifuged for 10 min at 8,000 × g. The supernatants containing the periplasmic fractions were collected. The pellets, which contained the cytoplasm and the membrane fractions, were resuspended in lysis buffer (20 mM Tris/HCl pH 7.5, 150 mM NaCl, 3 mM MgCl2, 1 mM CaCl2, and 2 mM β-mercaptoethanol with protease inhibitors). All subsequent steps were carried out at 4°C. RNase A and DNase I (10 µg/mL) were added and the samples were sonicated (Fisher Scientific, 3 × 15 s). Intact bacteria were removed by centrifugation (2,300 × g for 15 min) and the cleared supernatants containing cytoplasmic and membrane proteins were transferred to new tubes. To obtain the cytoplasmic fraction, the supernatants were centrifuged (in a Beckman Optima XE-90 Ultracentrifuge with an SW60 Ti rotor) for 30 min at 100,000 × g, to pellet the membranes. The supernatants, containing the cytoplasmic fraction, were collected and the pellets containing the membrane fractions were washed with lysis buffer and resuspended in 0.1 mL lysis buffer with 0.1% SDS. The protein content of all samples was determined using BCA (Cyanagen) before adding SDS-PAGE sample buffer with β-mercaptoethanol. Intimin, maltose-binding protein (MBP), and DnaK were used as markers for the membrane, periplasm and cytoplasm fractions, respectively.

**Co-elution by nickel affinity chromatography -** *E. coli* BL21, expressing His-tagged CesT or one of the various Tir-V5 variants, from an overnight culture were sub-cultured 1:50 in 50 mL LB broth, and grown shaking for 6 h, at 37°C. 0.25 mM IPTG was added to induce protein expression. Bacterial cells were collected by centrifugation (3,200 x g for 30 min) and resuspended in lysis buffer. The samples were sonicated (Fisher Scientific, 3 × 15 s) and then incubated on ice for 15 min with 0.1% NP-40 (v/v). Intact bacteria were removed by centrifugation (18,000 x g for 15 min), protease inhibitor cocktail was added to the cleared supernatants, and they were incubated rotating, at various combinations, with Ni-NTA resin at 4°C overnight. Finally, the nickel beads were collected by centrifugation (500 x g for 1 min) and washed 5 times with lysis buffer containing 10 mM imidazole. Proteins were eluted by adding sample buffer and boiling the samples for 10 min. Whole cell lysates and eluted samples were analyzed using SDS- PAGE and western blot.

**Translocation activity -** Translocation assays were performed as previously described (19). Briefly, 3 x 106 HeLa cells were infected for 3 h with EPEC strains that were grown statically overnight at a multiplicity of infection (MOI) of ~50. Infected cells were then washed in cold PBS, collected, and lysed in designated lysis buffer (PBS with: 0.5% triton X-100 (v/v), protease inhibitor and 1 mM DTT). Thereafter, samples were centrifuged at 18,000 × g for 2 min to remove non-lysed cells, and supernatants were collected, boiled with SDS-PAGE sample buffer, and subjected to western blot analysis with anti-Tir and anti-actin (loading control) antibodies. Uninfected samples and samples infected with the Δ*tir\** mutant strain were used as negative controls.

To purify the cellular membranes of infected cells, washed cells were collected by scraping the plates. They were then resuspended in lysis buffer (3 mM imidazole (pH 7.4), 250 mM sucrose, 0.5 mM EDTA and protease inhibitor) and mechanically fractioned by vigorous passage through a 25-gauge needle. The cellular lysates were centrifuged (3,000 x g for 15 min) to remove non-lysed cells and then further centrifuged (in a Beckman Optima XE-90 Ultracentrifuge with an SW60 Ti rotor at 100,000 x g for 40 min) to separate the cytoplasmic from the membrane fractions.

**Gentamicin protection assay -** HeLa cells (4.4 x 104), seeded in wells of a 96 wells plate, were infected with EPEC strains (carrying a plasmid coding for carbenicillin resistance) at a MOI of 20 for 3 h. IPTG (0.25 mM) was added 0.5 h post-inoculation to induce protein expression. The cells were then washed with PBS and incubated with fresh DMEM containing gentamicin (100 μg/mL) for 1 h. The cells were then washed and lysed with PBS containing 0.1% triton X-100 (v/v). Samples were collected and plated, at various dilutions, on LB agar plates containing carbenicillin. The plates were incubated overnight at 37°C and the numbers of colony forming units (CFUs) were counted.

**Lactate dehydrogenase (LDH) cytotoxicity assay -** HeLa cells (5 x 104 cells) were infected with the EPEC strains that were pre-induced and grown in DMEM statically for 3 h at 37°C in a CO2 tissue culture incubator with 0.25 mM IPTG. Infection was at MOI of 50 for 4 h. The cultures supernatants were then collected and subjected to CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega) to determine their LDH levels. The absorbance at 490 nm was measured and calculated as percentage of uninfected cells treated with the kit lysis buffer.

**Results**

**Replacement of Tir TMDs sequences alters protein secretion.** We have previously found that the TMDs of two TMD-containing secreted proteins are critical to promote protein secretion and prevent protein integration into the bacterial membrane (13). To examine whether Tir TMD sequences are also critical for protein secretion, we cloned Tir labeled with a V5 tag at its C-terminus (pTirwt-V5) on a plasmid and created three Tir TMDs variants with an alternative hydrophobic sequence. To minimize protein modification, we used the Tir’s original TMDs instead of unrealated TMD sequences to create: (i) Tir-D2-V5, where the central 20 residues of the N-terminal TMD (TMD1) were replaced by the 20 central residues of the C-terminal TMD (TMD2) to form a variant with double TMD2, hence called D2; (ii) Tir-D1-V5, where TMD2 was replaced by TMD1 to form a variant with double TMD1, hence called D1; and (iii) Tir-R21-V5, where Tir TMDs are in a reverse orientation, hence called R21 (Tir variants are depicted in Fig. 1A). In addition, we created two additional Tir TMD-variants, where the TMDs were replaced by the TMD sequence of a structural membrane protein called EscD, which is integrated into the bacterial membrane and is part of the T3SS complex. To that end, we generated two Tir TMD exchanged variants: Tir-EscD1-V5 (TMD1 is replaced by EscD TMD) and Tir-EscD2-V5 (TMD2 is replaced by EscD TMD), as depicted in Fig. 1A. Tirwt-V5 and the TMD-exchanged variants were transformed into EPEC Δ*tir\**, grown under T3SS-inducing conditions and examined for their ability to secrete Tir. We observed that WT EPEC secrete significant level of Tir while the Δ*escV* mutant strain, with a deletion of one of the T3SS structural genes, expressed Tir but was unable to secrete it ([Fig. 1B](https://www.tandfonline.com/doi/full/10.1080/21505594.2021.1898777#f0001)). As expected, we did not detect Tir expression in EPEC Δ*tir\** mutant. Nevertheless, transformation of this strain with pTirwt-V5 restored Tir expression and secretion (Fig. 1B). Interestingly, Δ*tir\** strains that express Tir variants with replacement of TMD1 (Tir-D2-V5 and Tir-EscD1-V5) showed similar Tir secretion to EPEC WT, while strains that expressed Tir varients with replacement of TMD2 of Tir (Tir-D1-V5 and Tir-EscD2-V5) showed reduced Tir secretion (Fig. 1B). The Δ*tir\** strain that expressed Tir-R21-V5 showed secretion phenotype like the TMD2 exchanged (Fig. 1B). Reduction of Tir secretion in these latter strains (Tir-D1-V5, Tir-R21-V5, and Tir-EscD2-V5) was accompanied by Tir enrichment at the bacterial pellets (Fig. 1B), thus suggesting that Tir secretion was altered but not its overall expression. Considering that Tir is an effector protein, it should mainly be secreted by the T3SS at the late stage, following the attachment to the host cells (4, 20). This regulation prevents premature secretion of effectors into the extracellular environment and was suggested to be sensed by a drop in calcium concentration (21). To simulate host-cell attachment, in the absence of cells, bacteria can be transferred to calcium-free DMEM medium to enhance effector secretion. Examination of Tir secretion under these conditions showed a similar secretion pattern to that in regular T3SS-inducing conditions (Fig. S1), further validating our results that alteration of Tir TMD2 reduce the ability of the protein to be secreted by the T3SS.

**The TMD sequences of Tir influence the protein sub-cellular localization**. The correlation between the reduced secretion of TMD2-exchanged Tir variants and their enhanced levels within the bacterial pellets suggested that these variants are mislocalized. To examine that, we grew the Tir variant strains under T3SS-inducing conditions. Supernatants were collected and whole cell extracts were fractionated to cytoplasmic, periplasmic, and membranal fractions. Evaluation of western blot analysis with anti-Tir antibody revealed that the TMD2-exchanged versions (Tir-D1-V5, Tir-R21-V5, and Tir-EscD2-V5) showed enriched localization to the membranal fraction compere to Tirwt-V5 and its TMD1-exchanged versions (Fig. 2A). Surprisingly, although the Tir-EscD1-V5 variant contains the only TMD sequence of a membrane protein, it did not alter dramatically the localization of Tir to the bacterial membrane. Correct bacterial fractionation was confirmed by western blots probed with anti-DnaK (a cytoplasmic marker), anti-MBP (a periplasmic marker), and anti-Intimin (a membranal marker) antibodies (Fig. 2B). Overall, the fractionation results suggest that TMD2 of Tir is more critical than TMD1 for targeting Tir for secretion and preventing it from bacterial membrane integration by the co-translational-translocation mechanism.

**Tir TMDs exchange does not affect CesT binding.** The ability of Tir to escape the co-translational pathway and membrane integration mechanism was previously suggested to be mediated through interaction with its chaperone, CesT (22). Although the minimal chaperone binding domain (CBD) of Tir was found to be located at residues 35-77, together with an additional binding region at Tir’s C-terminus (23), it was previously reported that T3SS chaperones might also interact with translocators and effectors TMDs to mask them from the SRP and prevent erroneous targeting to the bacterial membrane (12). To examine whether the replacement of Tir TMD sequences disrupted Tir-CesT interaction, we expressed WT and TMD-exchanged Tir variants in *E. coli* BL21 to prevent Tir secretion, as well as His-tagged CesT, lysed the bacteria, incubated the mixed lysates with nickel beads, and pulled down CesT-His together with its binding partners. We examined only the Tir variants that contain Tir native sequences to minimize background noise that might occur due to non-specific EscD interactions. SDS-PAGE and western blot analysis of the eluted samples using anti-His and anti-Tir antibodies indicated that all Tir variants co-eluted with CesT-His to a similar extent as WT Tir(Fig. 3). Incubation of the Tir lysates in the absence of CesT-His resulted in minor non-specific binding to the nickel beads, which was similar across all Tir variants. Taken together, these results suggested that the replacement of the TMDs did not disrupt the interaction between CesT and Tir.

**Tir TMDs affect protein translocation into host cells and plasma membrane integration.** To examine Tir variants in a system that better simulates the natural process of the Tir protein, we infected host cells with bacteria that express WT or TMD-exchanged Tir and examined their ability to properly translocate Tir into the cells. High levels of Tir were detected for EPEC WT, while no Tir was detected in uninfected cells or cells infected with the EPEC Δ*tir*\* mutant strain (Fig. 4A). Importantly, we detected similar levels of translocated Tir in cells infected with EPEC Δ*tir*\* expressing WT Tir (pTirwt-V5) as cells infected with WT EPEC (Fig. 4A), while EPEC Δ*tir*\* that express Tir-D2-V5 showed a similar translocated level to WT EPEC and EPEC Δ*tir*\* expressing Tirwt-V5, EPEC Δ*tir*\* that expressed Tir-D1-V5 and Tir-R21-V5, showed reduced level of translocated Tir in the infected cells (Fig. 4A). Actin was used as a loading control. As Tir should be presented on the host membrane to allow bacterial adhesion and pedestal formation, we further separated the infected cells to soluble and membrane fractions. In addition, since Tir phosphorylation in the membrane was suggested to be required for efficient actin polymerization, we assessed the level of Tir phosphorylation in the membrane by subjecting the membrane samples to low-acrylamide percentage gels that can separate between the unphosphorylated Tir (~75 kDa) and the phosphorylated Tir (~90 kDa). Samples of HeLa cells infected by EPEC WT showed a high level of unphosphorylated Tir and a low level of phosphorylated Tir. This phenotype was enhanced in samples infected by EPEC Δ*tir*\* expressing Tirwt-V5, probably due to Tir overexpression (Fig. 4B). Membrane samples of HeLa cells infected by EPEC Δ*tir*\* expressing Tir-D1-V5 and Tir-R21-V5 showed relatively similar overall levels of Tir as membranes of HeLa infected by EPEC Δ*tir*\* expressing Tirwt-V5, however with no apparent detection of phosphorylated Tir (Fig. 4B). The membranes of HeLa cells infected by EPEC Δ*tir*\* expressing Tir-D2-V5 showed a slightly reduced level of translocated Tir, but had a low level of phosphorylated Tir (Fig. 4B). These results suggest that the alteration of TMD sequences of Tir had a much milder effect on the translocation ability of Tir relative to the effect we observed using Tir as a model system for protein secretion.

**The second TMD sequence of Tir is involved in bacterial adhesion.** In order to examine the role of Tir TMDs in proper protein folding and function, we performed gentamicin protection assay. Although EPEC is generally considered an extracellular pathogen, it was shown to possess limited invasive abilities both *in vivo* and *in vitro* (9, 24). This bacterial invasiveness was linked to the Tir-Intimin mediated adherence, that sometime manifested in localized Tir to the intra-vacuolar bacterium (25), suggesting that EPEC invasiveness ability can indicate Tir functionality. To that end, HeLa cells were infected with the various Tir-exchanced variant strains for 3 h and then incubated for 1 h with gentamicin. The cells were then washed, lysed, and the lysates were plated on LB plates for overnight incubation. We determined the colony forming units (CFUs) and calculated them as a percentage of WT EPEC count (Fig. 5). As expected, we detected significantly lower CFUs for the ∆*escN* mutant strain, lacking the T3SS ATPase and therefore having no T3SS activity, and Δ*tir\** compared to WT EPEC. Transformation of the Δ*tir\** with pTirwt-V5 restored CFUs to WT EPEC level indicating proper functionality of Tir. The strains expressing TMD2-exchanged versions (Tir-D1-V5 and Tir-R21-V5) yielded CFUs lower or similar to WT levels, yet the Δ*tir\** expressing Tir-D2-V5 yielded significantly higher CFUs compared to WT EPEC (Fig. 5), suggesting that the Tir-D2-V5 protein induces significantly higher invasiveness activity.

**Tir TMDs are involved in the cytotoxicity induced by EPEC.** To further investigate the role of Tir TMDs in its post-secretion function, we assessed the release of lactate dehydrogenase (LDH) following the infection with bacteria that express WT or TMD-exchanged Tir variants. This method has been widely used, with a verity of cell lines, to evaluate EPEC-induced cell death, which is linked to intimate adherence and interception of apoptotic pathways (26-29). Specifically, LDH cytotoxicity assay was recently used to demonstrate the involvement of Tir’s phosphorylation and interaction with Intimin to the induction of pyroptosis via the NLRP3 inflammasome activation in THP-1 cells (30). To that end, we grew the bacteria under T3SS-inducing conditions and used them to infect HeLa cells for 4 h. The culture supernatants were then collected and the LDH levels were analyzed compered to LDH levels of lysed cells. Infection with EPEC WT showed LDH release that indicated about 60% cellular toxicity while the Δ*tir*\* mutant showed significantly less LDH release of about 30% (Fig. 6). Unexpectedly, infection with Δ*tir*\* mutant that expresses Tirwt-V5 resulted in significantly higher levels of LDH release, which reached the maximal value (Fig. 6). These results suggest over-expression of Tir enhance cellular cytotoxicity. Surprisingly, while infection with the TMD2-exchanged Tir variants (Tir-D1-V5 and Tir-R21-V5) resulted in similar or slightly lower released LDH levels as Tirwt-V5, infection with the Tir-D2-V5 variant resulted in a significant decrease of LDH release (~45%) (Fig. 6). These results suggest that the cytotoxic effect of Tir is linked to its TMDs, as either the replacement of TMD1 or the duplication of TMD2 reduced the cytotoxic response of the cells to bacterial infection.

**Discussion**

Tir is an effector protein translocated into the host via the T3SS. Post translocation, Tir is inserted into the apical plasma membrane in a yet-to-be elucidated mechanism, binds Intimin, and sequentially activates a cellular cascade resulting in the actin modulation. Tir belongs to the unique family of TMD-containing secreted proteins which manage to escape integration into the bacterial membrane and are inserted into the host plasma membrane instead. Previous research have demonstrated that the TMDs of T3SS translocators are important to the proteins’ ability to be secreted and to their post-secretion functions (13). In this study, we aimed to examine whether this observation represents a more general phenomenon common to many TMD-containing secreted proteins. Moreover, we studied whether the context of the TMDs along the protein sequence affects protein secretion and function. To that end, we chose the Tir protein, which is a secreted effector that contains two TMDs, and cloned its WT and TMD-exchanged variants. We first assessed the effects of the exchanged TMDs on Tir’s ability to be secreted throught the T3SS complex. Interestingly, we observed reduced Tir secretion of all Tir variants that contained TMD2 exchange, while replacement of TMD1 showed normal secretion (Fig. 1B). These results suggest that the second TMD of Tir is essential for the ability of Tir to be secreted. The fact that the reduced secretion of TMD2-exchanged variants was accompanied by enrichment of Tir at the bacterial pellets (Fig. 1B) suggests that the proteins’ secretion was affected by the TMD exchange, but not their expression. Since TMDs are involved in targeting proteins to the membrane and can, therefore, interfere with protein secretion, we examined the sub-cellular localization of Tir variants. We observed that the TMD2-exchange variants that have shown reduced secretion were enriched in the bacterial membrane compared to Tirwt-V5 and TMD1-exchanged variants (Fig. 2A). This result is in keeping with our previous report, whereby a replacement of the translocators TMDs with an alternative hydrophobic sequence resulted in their mislocalization into the bacterial membrane (13). Interestingly, the Tir-EscD1-V5 variant did not display enriched membrane localization, despite containing the only TMD sequence of an actual membrane protein. Taken together, these results further demonstrate that TMD2 of Tir is involved in determining Tir destination, probably by promoting protein escape from the bacterial membrane integration mechanism.

As previously mentioned, Tir and other secreted proteins interact with chaperones to prevent their premature folding, erroneous integration into the bacterial membrane, and to lead them to the injectosome for secretion. To examine whether Tir variant mislocalization results from impaired interaction with Tir’s chaperone, CesT, we examined CesT-Tir interaction using affinity chromatography. The results indicate that all Tir variants co-eluted with CesT-His to a similar level as Tirwt-V5 (Fig. 3), suggesting that the TMD replacement did not disrupt Tir-CesT binding. Nevertheless, since both Tir and CesT are known to bind the T3SS ATPase, EscN, directly and in complex (31), it is possible that, while the TMDs are not directly involved in Tir-CesT interation, they are involved in the interactions within the CesT-Tir-EscN complex or the dissociation kinetics between CesT and Tir, to allow Tir secretion.

It was previously suggested that the moderate hydrophobicity of the TMDs is a critical factor for the targeting of TMD-containing secreted proteins for secretion (12). In an attempt to explain the different phenotypes observed by the replacement of each TMD, we calculated the apparent free energy differences (ΔGapp) required for TMD insertion using Jpred (32). A negative ΔGapp value predicts its recognition as a TMD helix and its membranal integration, while a positive value does not exclude membrane integration but suggests it requires a stabilizing interaction (32). The calculated ΔGapp for Tir’s TMD1 and TMD2 were 0.938 and 2.385 respectively. While both values are positive, the ΔGapp for TMD2 is significantly higher. Since proteins with relatively low calculated ΔGapp are targeted to the bacterial membrane (for example, the calculated ΔGapp for EscD’s TMD is 0.123), the high ΔGapp of Tir TMD2 might explain the different contribution of TMD1 and TMD2 regarding the secretion phenotype of Tir. Interestingly, our results demonstrate that, even though the Tir-R21-V5 variant comprises the original WT sequence, the switched positions of the TMDs affected protein secretion. These results suggest that the location of the TMDs and not exclusively their sequence affects their contribution to protein secretion. This is further supported by the observation that. while in this study Tir with double TMD1 sequence (Tir-D1-V5) did not support full Tir secretion, replacement of EspB’s TMD with the TMD1 of Tir showed similar EspB secretion to EspB WT (13). These results suggest that the TMD sequence by itself could not stimulate protein secretion, but rather the TMD within a specific context of the targeted protein. Altogether, these results support our conclusion of the involvement of the TMD sequence of TMD-containing secreted proteins in the secretion process and suggest that the context and not only the presence of TMD *per se* within the protein sequence is also critical.

While we can use Tir to study TMD-containing secreted proteins, it is worth mentioning that Tir is an effector that is not normally secreted to the extracellular media. Using the T3SS assay, we have created a segragation between Tir’s secretion and translocation phases, which does not occur naturally. Hence, while this method allow us to assess the role of Tir TMDs in the secretion process, these results are not necessarily indicative of the TMDs’ involvement in Tir’s translocation and post-secretion functions. Therefore, to study the involvement of Tir’s TMDs on its ability to translocate into host cells, we infected HeLa cells with EPEC Δ*tir\** expressing Tirwt-V5 and TMD-exchanged variants. We observed that, while all Tir variants translocated to the host cells, the TMD2-exchanged versions (Tir-D1-V5 and Tir-R21-V5) demonstrated lower translocation levels (Fig. 4A). Membrane fractionation of the cells revealed that all Tir variants translocated into the host membrane, yet the TMD2-exchanged variants were poorly phosphorylated (Fig. 4B). A possible explanation of these results is that the TMD2-exchanged variants orient incorrectly across the membrane or interact insufficiently with Intimin to promote phosphorylation. To link these results with protein function, we performed a gentamicin protection assay that evaluated bacterial invasiveness as means to determine Tir-intimin interaction (25). We found that expression of Tirwt-V5 in EPEC Δ*tir\** restored bacterial invasiveness to EPEC WT levels, thus confirming that EPEC invasiveness is Tir-dependent (Fig. 5). Surprisingly, strains expressing TMD2-exchanged versions yielded CFUs that were not significantly different from those of WT EPEC. This, therefore, does not support our hypothesis that these Tir variants are folded incorrectly or interact with Intimin insuffiently. In addition, LDH release, which quantifies the cytotoxic effect linked to Tir phosphorylation and interaction with Intimin, showed that infection with TMD2-exchanged variants resulted in a cytotoxic effect similar to EPEC Δ*tir\** that express Tirwt-V5 (Fig. 6). This incompatablity between the Tir phosphorylation level and the functional assay suggests that either Tir phosphorylation is not critical for Tir activity or that very low phosphorylation level is sufficient for Tir function. In addition, based on our results, we can conclude that TMD2 sequence is not critical for Tir activity post-translocation.

Unexpectedly, we observed that Δ*tir\** expressing Tir-D2-V5 showed very high invasiveness while inducing significantly less LDH release compared to WT EPEC and Δ*tir\** that express Tirwt-V5 (Fig. 5 and Fig. 6). This negative correlation suggests that hyperinvasive EPEC is less virulent and, therefore, induces reduced cytotoxic effect on host cells. In addition, the results indicate that TMD1 is critical for Tir activity and therefore, replacing it with an alternative TMD sequence disrupted Tir function. Whether it was the lack of TMD1 or the addition of TMD2 that altered Tir function is yet to be determined. As TMDs were previously shown to be involved in various protein-protein interaction and membrane organization (33-35), it is possible that exchanging the sequence TMD1 by that of TMD2 sequence altered Tir interactions with other proteins or changed the membrane properties. In any event, we observed that Tir TMD1 is more critical for Tir function than TMD2.

Overall, our results support our previous finding that the TMD sequences of TMD-containing secreted proteins are different from classical TMD sequences and that this allows them to escape the bacterial membrane integration mechanism. Moreover, our results suggest that these sequences are not defined only by their hydrophobicity level but also by their adjacent protein sequence. Furthermore, our results suggest that, while Tir TMD2 is more critical for protein section, TMD1 is more critical for the activity of the protein within the host cells.

**Figure legends:**

**Figure 1: Tir TMD2 is critical for protein secretion**. **(A)** Schematic illustration of Tir folding across the host membrane and the TMDs organization in the WT and TMD-exchanged Tir variants. **(B)** Protein secretion profiles of EPEC strains grown under T3SS-inducing conditions: WT, ∆*escV*, Δ*tir*\*, and Δ*tir*\* carrying the pTirwt-V5 and Tir TMD-exchanged variants. The secreted fractions were normalized, concentrated from the supernatants of bacterial cultures, and analyzed by SDS-PAGE and western blotting with an anti-Tir antibody. Bacterial pellets were analyzed to verify Tir expression.

**Figure 2: Replacement of TMD2 leads to altered Tir localization and enhanced bacterial membrane localization.** EPEC Δ*tir*\* expressing Tirwt-V5 or TMD-exchanged Tir variants were grown in calcium-free DMEM to simulate T3SS effector secretion. Supernatants (S) were collected, filtered, and concentrated while bacterial pellets were collected, lysed, and fractioned into periplasmatic (P), cytoplasmatic (C), and membranal (M) fractions. **(A)** Western blot analysis of the samples was conducted using an anti-Tir antibody. **(B)** To confirm correct bacterial fractionation, western blots were probed with anti-MBP (periplasmatic marker), anti-DnaK (cytoplasmatic marker), and anti-intimin (membranal marker) antibodies.

**Figure 3: Tir-CesT interaction is not disrupted by the replacement of Tir’s TMDs.** *E. coli* BL21 (DE3) expressing Tirwt-V5, Tir-D1-V5, Tir-D2-V5, Tir-R21-V5 and CesT-His were grown for 6 h. The bacteria cells were collected, lysed, mixed, and incubated overnight with nickel beads. Bacterial lysates and eluted fractions were loaded on SDS-PAGE 12% gel and analyzed by western blotting with anti-His and anti-Tir antibodies.

**Figure 4: Replacement of TMD2 reduces the levels of phosphorylated Tir within the membrane fraction. (A)** HeLa cells were infected with WT, Δ*tir*\*, and EPEC Δ*tir*\* expressing Tirwt-V5, Tir-D1-V5, Tir-D2-V5, Tir-R21-V5 for 3 h or left uninfected (UI). The cells were washed and lysed using PBS with Triton X-100. Cell lysates were subjected to SDS-PAGE and western blot analysis with anti-Tir and anti-actin (control) antibodies. **(B)** The membranal fractions of HeLa cells infected with the various bacterial strains were subjected to SDS-PAGE and western blot analysis with anti-Tir and anti-actin (control) antibodies.

**Figure 5: Gentamicin protection assay:** HeLa cells were infected with EPEC WT, Δ*escN*, Δ*tir*\*, and EPEC Δ*tir\** expressing Tirwt-V5, Tir-D1-V5, Tir-D2-V5, Tir-R21-V5 for 3 h. Cells were washed and incubated in fresh DMEM with gentamicin 100 µg/mL for 1 h. Cells were then washed and lysed with triton X-100 (0.1% (v/v)). Samples were then plated in serial dilutions on LB plates with carbenicillin. The plates were incubated overnight at 37°C and bacterial colony-forming units (CFUs) were then counted. The percent of bacterial colonies relative to EPEC WT is presented. Bars represent geometric means for each strain, tested in triplicate in five independent experiments. Error bars represent standard deviation. (\*\*, p< 0.01, unpaired student’s T test. ns – not significant).

**Figure 6: Overexpression of Tir enhances cellular damage while replacement of TMD1 reduces cellular damage.** EPEC WT, Δ*tir*\*, and EPEC Δ*tir*\* expressing Tirwt-V5, Tir-D1-V5, Tir-D2-V5, Tir-R21-V5 variants were grown under T3SS-inducing conditions for 3 h before infecting HeLa cells for 4 h. The culture supernatants were then collected and analyzed for LDH release. Bars represent geometric means for each strain, tested in quintuplicate over three independent experiments. Error bars represent standard deviation (\*\* p < 0.01. unpaired student’s T test. ns – not significant).

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