The role of the membrane associated domain of the export apparatus protein, EscV (SctV), in the activity of the type III secretion system.

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Running title: SctV of the EPEC T3SS

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**Abstract**

Diarrheal diseases are still one of the major public health concerns worldwide. Many of the bacterial pathogens that cause these diseases have a specialized protein complex, called type III section system (T3SS), which delivers effector proteins directly into host cells. These effectors manipulate host-cell processes for the benefit of the infecting bacteria. The T3SS structure resembles a syringe that is anchored within the bacterial membrane and projects towards the host-cell membrane. The entry port of the T3SS substrates, called the export apparatus, is formed by five integral membrane proteins. Among the export apparatus proteins, EscV is the largest one and as it forms a nonamer, it constitutes the largest portion of the export apparatus complex. While there is considerate data on the soluble cytoplasmic domain of EscV, our knowledge on its membrane-associated section and its transmembrane-domains (TMDs) is still very limited. In this study, we found that TMD5 and TMD6 of EscV mediate strong self-oligomerization using isolated genetic reporter system. Substituting these TMDs within the full-length protein with a random hydrophobic sequence resulted in a complete loss of function of the T3SS, further suggesting that EscV TMD5 and TMD6 sequences have functional role in addition to their structural role as membrane anchors. As we observed only mild reduction in the ability of the TMD-exchanged variants to integrate into the T3SS full- or intermediate-complexes we concluded that EscV TMD5 and TMD6 are not crucial for the global assembly or stability of the T3SS complex but rather involved in promoting the proper TMD-TMD interactions within the complex and the overall TMDs’ orientation to allow channel opening for T3SS substrates.

**Introduction**

Diarrheal diseases are a major global health concern and are considered the second leading cause of death in children under the age of five. According to the World Health Organization (WHO), there are nearly 1.7 billion cases of childhood diarrheal disease per year with an estimate of 500,000 deaths. One of the main infectious agents of pediatric diarrhea is enteropathogenic *E. coli* (EPEC) (Clarke et al., 2002). This pathogen was related to a series of outbreaks of infantile diarrhea in the 1940s and 1950s (Robins-Browne, 1987). While EPEC is no longer considered to be an important cause of acute diarrhea in many countries, the occurrence of severe disease outcomes associated with its infections has recently reemerged (Croxen et al., 2013).

EPEC belongs to a family of bacteria that forms a distinctive histological lesion in the intestinal epithelium and collectively called attaching and effacing (A/E) pathogens (Goosney et al., 2000). In the A/E lesion the bacteria tightly attach to the intestinal epithelial cells of the host, causing a disruption of the brush border microvilli and promote formation of actin pedestals that elevate the pathogen above the epithelial cell. This morphology is mediated by a protein transport nanomachine termed type 3 secretion system (T3SS) (Buttner, 2012;Deng et al., 2017;Wagner et al., 2018). The T3SS delivers virulence factors directly into host cells and these manipulate host cell cytoplasm rearrangement. The injected effectors also interfere with and modify critical cellular pathway to improve bacterial survival and replication (Bhavsar et al., 2007). The core architecture of the T3SS consists of a basal body embedded within the bacterial membranes, a periplasmic inner rod, a transmembrane export apparatus, and a cytosolic platform, which includes an ATPase complex and the C-ring. In addition, a distinct hollow needle is assembled on the extracellular face of the basal body, which is linked in A/E pathogens to an extracellular long filament, and a pore complex at the host membrane to create a channel for protein secretion (Buttner, 2012).

The T3SS structural genes are encoded within the bacterial chromosome, on a large 35-kbp genomic pathogenicity island called the locus of enterocyte effacement (LEE). The LEE is organized into seven operons (LEE1 to LEE7) that encodes structural proteins, as well as regulators and several protein effectors (Elliott et al., 2000;Deng et al., 2004;Franzin and Sircili, 2015;Gaytan et al., 2016). The export apparatus, which is found at the center of the inner membrane ring and facing the cytoplasmic side, is among the most conserved substructures within the T3SS complex. This structure is essential for secretion and it acts as the entry portal for the T3SS substrates. The export apparatus is assembled from five highly conserved membrane proteins, named EscR, EscS, EscT, EscU, and EscV, which were shown to form a multimeric protein complex with a stoichiometry of 5:1:4:1:9, respectively, in the homologous T3SS of *Salmonella* Typhimurium (Kuhlen et al., 2018). The complexity of this struc­ture is illustrated by the estimation that a total of 104 transmembrane domains (TMDs) are involved in its formation (Zilkenat et al., 2016). Among the export apparatus components, EscV, which is named SctV according to the T3SS unified nomenclature (Wagner and Diepold, 2020), is the largest protein (72 kDa) and as it forms a nonamer, it constitutes the largest portion of the export apparatus complex.

EscV is divided into two large domains: a N-terminal region with 7-8 predicted TMDs and a C- terminal cytoplasmic domain (Wagner et al., 2010;Abrusci et al., 2013). The presence of a putative N-terminal cleavable signal sequence indicated that EscV is likely directed to the inner membrane through the sec pathway (Garmendia et al., 2005) and it was found that its membrane localization was independent of the T3SS (Gauthier et al., 2003). EscV and its homologs in *Salmonella* and *Shigella* (InvA and MxiA, respectively) were shown to oligomerize and form a cytoplasmic homo-nonameric ring that is located directly below the secretion pore and above the ATPase complex (Abrusci et al., 2013;Bergeron et al., 2013;Majewski et al., 2020).

EscV and its homologs in both the virulence and flagellar T3SSs have been implicated in recruitment of T3SS substrates, chaperones, and proteins from the "gate-keeper" family of proteins to the T3SS apparatus as part of the regulation process of hierarchical secretion of T3SS substrates (Diepold et al., 2012;Minamino et al., 2012;Abrusci et al., 2013;Kinoshita et al., 2013;Portaliou et al., 2017). The binding between EscV and various T3SS cargo was shown to occur via EscV's cytoplasmic C-terminus (Minamino et al., 2012;Gaytan et al., 2016;Shen and Blocker, 2016;Portaliou et al., 2017). Mutations in two amino acid residues located on the surface of MxiA, the *shigella* SctV homolog, were shown to lead to 2 to 3-fold increase of secretion of the IpaH effector compared to WT strain (Shen and Blocker, 2016).

Overall, these studies indicated that SctV family of proteins are part of the export gate complex where they form an IM pore, which is required for the assembly and proper function of the T3SS and act as a substrate selection checkpoint. Nevertheless, although the EscV is an integral membrane protein that contribute more than half of the TMDs of the export apparatus, most of the available information about this protein is related to its soluble domain. Thereupon, in this study, we investigate the role of EscV TMDs in the protein function and their involvement in the global T3SS assembly and activity.

**Results**

The soluble C-terminal region of EscV and its homologs, is well characterized (Abrusci et al., 2013;Majewski et al., 2020), yet not much is known about the N-terminal region, which is predicted to be embedded within bacterial membrane. To identify EscV TMDs, we analyzed EscV sequence in TMD prediction software (TMPred, TMHMM, and Phobius) and found seven regions with high probability to serve as TMDs; TMD1, residues 17-39, TMD2, residues 43-62, TMD3, residues 74-96, TMD4, residues 111-133, TMD5, residues 205-227, TMD6, residues 237-259, and TMD7, residues 296-329 (Figure 1A). To identify conserved motifs/residues within EscV TMDs, we performed multiple sequence alignment of EPEC EscV (B7UMA7), FlhA of *E. coli* flagella (P76298), EscV of the E. coli O157:H7(Q7DB70), YscV of the Yersinia enterocolitica (A0A2J9SJU1), MxiA of the *Shigella* T3SS (P0A1I5), and InvA of the *Salmonella* typhimurium T3SS (A0A0H3NL68) by Clustal Omega and presented them using BoxShade software (Figure S1). Among the TMDs, we found that TMD6 showed the highest sequence conservation, with 65% identity **(Figure 1B). In addition, we found that TMD5 contains** a GxxxG motif, which was previously reported to be critical for TMD-TMD interactions within the membrane (Moore et al., 2008).

**EscV TMD5 and TMD6 support TMD-TMD interactions –** As TMDs are known to be involved in protein-protein interactions, we examined the ability of isolated EscV TMDs to support self-interaction. For that purpose, we employed the ToxR assembly system (Figure 2A), which is monitor the strength of TMD-TMD interactions within the bacterial inner membrane (Langosch et al., 1996;Joce et al., 2011). We compared the oligomerization level of EscV TMDs with that of glycophorin A (GpA) TMD sequence, which contains a GxxxG motif and is used as a reference for strong homo-oligomerization (Lemmon et al., 1992;Adair and Engelman, 1994;Russ and Engelman, 2000), the N-terminal TMD of the *E. coli* aspartate receptor (Tar-1), which has moderate oligomerization (Sal-Man et al., 2004), and polyalanine (A16) sequence as a non-oligomerizing sequence (Langosch et al., 1996;Sal-Man et al., 2005). Considering that the amino acid sequence of TMD7 was significantly longer than the other TMDs, we decided to test two different forms of this TMD (named TMD7.1 and TMD7.2). The sequences of the studied TMDs are presented in Figure 2A. We observed a strong TMD self-oligomerization activity of EscV TMD5, TMD6 and TMD7.2 compared to the activities of the GpA and Tar-1 TMDs, whereas the EscV TMD1, TMD2, TMD3 TMD4, and TMD7.1 showed reduced oligomerization activities compared to GpA (Fig. 2B). As expected, the oligomerization of the A16 background control was low (Figure 2B). These findings suggested that TMD5, TMD6 and TMD7.2 of EscV might be involved in the oligomerization of the full-length protein EscV, through TMD-TMD interactions. To exclude the possibility that the high self-oligomerization activity of EscV TMD5, TMD6 and TMD7.2 resulted from higher expression level of these chimera proteins, we subjected the bacterial samples to SDS-PAGE and Western immunoblotting analysis with an anti-maltose binding protein (MBP) antibody. All samples showed comparable expression levels (Figure 2B). To verify that the ToxR-TMD-MBP chimera proteins correctly integrated into the inner membrane, we employed the maltose complementation assay. For that purpose, we used an *E. coli* strain deleted for the *malE* gene (PD28), which cannot produce endogenous MBP, and therefore cannot support bacterial growth in minimal medium with maltose as the sole carbon source (Langosch et al., 1996). Only strains that express the chimera protein ToxR-TMD-MBP and orient it across the inner membrane, with MBP facing the periplasm, will support bacterial growth. We observed that all examined strains demonstrated bacterial growth, which indicated proper membrane integration, while the negative control that does not contain a TMD (ΔTM), showed no growth, as expected (Figure 2C). Overall, these results suggest that TMD5, TMD6, and TMD 7.2 of EscV are involved in EscV self-oligomerization through TMD-TMD interactions. However, due to the high conservation of TMD6 and the GxxxG motif within TMD5, on one hand, and the unclear boundaries of TMD7, on the other hand, we decided to focus on EscV TMD5 and TMD6.

**Replacement of EscV TMDs to a non-oligomerizing sequence (7L9A) affects bacterial fitness –** To examine whether EscV TMD5 and TMD6 serves solely as a membrane anchor or have a functional role within the full-length protein, we constructed EscV mutant proteins lacking its TMD5 or TMD6 sequences. Since EscV deleted of its TMD5 or TMD6 will likely adopt an alternate protein folding compared to the native protein or have impaired localization, we constructed TMD5- and TMD6-exchanged EscV proteins, where the native core TMD5 and TMD6 sequences (16 amino acids in length) were replaced by a hydrophobic sequence. We chose a hydrophobic sequence of seven consecutive leucine residues followed by nine alanine residues (7L9A), which was previously shown to be sufficiently hydrophobic to support protein integration into the membrane yet cannot support TMD-TMD interactions (Sal-Man et al., 2005). To examine the biological effect of such replacement, we transformed the TMD5 and TMD6-exchanged EscV (EscV-TMD5ex-His and EscV-TMD6ex-His), as well as EscVwt-His, into the *escV*-null strain (Δ*escV*) to examine their ability to complement the loss of T3SS activity. However, all strains, induced by addition of IPTG to 0.25mM concentration, showed reduced growth rate upon induction of EscV overexpression (Figure 3). To determine the conditions that allow EscV expression without severe reduction of bacterial fitness, we grew WT EPEC, EPEC Δ*escV*, and EPEC Δ*escV* carrying either pEscVwt-His, pEscV-TMD5ex-His, or pEscV-TMD6ex-His in LB or in DMEM (which is used for T3SS-inducing conditions), in the presence (0.1 or 0.25 mM) or the absence of IPTG. Optical density at 600 nm was measured over time (Figure 3). We observed that expression of EscV WT and TMD-exchanged versions have fitness cost when induced with IPTG concentration higher than 0.1 mM (Figure 3). These results suggest that overexpression of EscV is toxic to bacteria and therefore negatively affects bacterial growth. Based on these results we used 0.1 mM IPTG concentration for our future experiments.

**TMD5 and TMD6 are critical for EscV activity –** To examine whether EscV TMD5 and TMD6 sequences are critical for the activity of the full-length protein, we examined whether EscV-TMD5­ex-His and EscV-TMD6ex-His can complement the T3SS activity of EPEC *escV* strain. Only functional EscV can complement the T3SS of *escV* strain, which is measured by the ability of EPEC strains to secrete three T3SS translocators (EspA, EspB, and EspD) into the culture supernatant, when grown under T3SS-inducing conditions.

First, we evaluated the ability of WT EscV to complement the T3SS activity of *escV*. We observed that expression of EscVwt-His within the Δ*escV* strain restored secretion of translocators, but also demonstrated hypersecretion of effectors (Tir and NleA) (Figure 4A). To evaluate whether this phenotype occurs due to the labeling of EscV or due to its expression from a plasmid, we examined the T3SS activity of Δ*escV* carrying plasmids with unlabeled EscV or EscV labeled with various tags and expressed from low and high copy-number plasmids. We observed that transformation of unlabeled EscVwt resulted in milder phenotype and only slight elevation in effectors' secretion was observed while expression of labeled EscV, regardless of the tag type, resulted in hypersecretion of effectors (Figure S2). Interestingly, expression of both EscV-TMD5­ex-His and EscV-TMD6ex-His failed to complement the T3SS activity of Δ*escV* strain and demonstrated secretion profile similar to that of Δ*escV* and Δ*escN* (Figure 4A). Comparable protein expression of the WT and the exchanged-versions was observed by analyzing whole-cell lysates by western blot analysis using anti-His antibody (Figure 4A).

To analyze whether the unregulated secretion of Δ*escV* complemented with pEscVwt-His affected the ability of the bacteria to infect host cells, we examined the ability of the strain to infect and translocate effectors into the HeLa cells. For this purpose, we infected HeLa cells with WT, Δ*escN*, Δ*escV*, and Δ*escV* complemented with pEscVwt-His and examined the cleavage pattern of JNK, a cellular protein that is cleaved by NleD, a translocated EPEC effector (Baruch et al., 2011). WT EPEC induced extensive degradation of JNK, as expected, relative to the uninfected sample and to the samples infected with Δ*escN* or Δ*escV* mutant strains ([Figure 4B](https://www.frontiersin.org/articles/10.3389/fmicb.2019.02551/full#F1)). EPEC Δ*escV* transformed with the plasmid encoding EscVwt-His showed a JNK degradation profile, indicating functional complementation by His-labeled EscV (Figure 4B). In addition, Δ*escV* strain transformed with EscV TMD-exchanged versions (pEscV-TMD5ex-His or pEscV-TMD6ex-His) showed no degradation of JNK, as observed for the uninfected sample (Figure 4B). Overall, our results suggest that His-labeled EscV functionally complements the T3SS activity, however, replacing the native TMD5 or TMD6 sequences of EscV to an alternative hydrophobic sequence (7L9A) impairs the function of the T3SS (Figure 4B).

**TMDs replacement does not affect EscV localization to the bacterial membrane –** To exclude the possibility that EscV-TMD5­ex-His and EscV-TMD6ex-His failed to complement the *escV* T3SS activity due to impaired subcellular localization, we grew the strains under T3SS-inducing conditions and fractionate them into periplasmic, cytoplasmic and membrane fractions. Our results showed that EscV-TMD5ex-His and EscV-TMD6ex-His localized mostly to the membrane fraction, similarly to EscVwt-His (Figure 5). Correct bacterial fractionation was confirmed by analyzing the samples with anti-MBP (periplasmic marker), anti-DnaK (cytoplasmic marker), and anti-intimin (membrane marker) antibodies. Overall, our results indicated that replacement of TMD5- and TMD6 did not disrupt EscV localization to the bacterial membrane.

**EscV TMD6 is involved in complex formation –** To investigate whether the EscV TMD-exchanged variants fail to complement the T3SS activity of the Δ*escV* null strain due to their inability to properly integrate into the T3SS complex, we prepered crude membrane samples of EPEC Δ*escV* and EPEC Δ*escV* null strain transformed with EscVwt-His, EscV-TMD5ex-His and EscV-TMD6ex-His grown under T3SS-inducing conditions. The samples were then analyzed by BN-PAGE and immunoblotting. BN-PAGE analysis revealed that EscVwt-His and EscV-TMD5ex-His preserve the ability to integrate into the T3SS complex, as they migrated primarily as a large complex (> 1Mda) at the top of the gel. However, EscV-TMD6ex-His integration into the complex appiered to be impaired (Figure 6). To verify that the modified running pattern of the EscV TMD6-exchanged version was not due to reduced protein expression, we analyzed the crude membrane extracts by SDS-PAGE and western blotting using anti-His antibody. Similar expression levels were observed for all EscV variants (Figure 6). These results, suggest that TMD5 and TMD6 are not critical for the integration of EscV into the T3SS complex, as EscV exchanged versions enabled the formation of high-molecular complexes; EscV-TMD5ex-His fully preserved the ability to integrate into the T3SS full- or intermediate-complexes, while integration of EscV-TMD6ex-His was impaired.

**A single mutation within the GxxxG motif of TMD5 abolish EPEC T3SS activity and complex formation** – To examine whether the GxxxG motif, identified within TMD5, is critical for protein activity, we mutated the glycine residues at position 213 and 217 to either alanine or leucine (G213A, G217A, G213L, and G217L). Due to expression challenges of the mutated proteins tagged with His-tag, we labeled EscV WT and single mutants with the V5 tag, that demonstrated similar secretion profile as EscVwt-His (Figure S2). The single mutants were transformed into Δ*escV* and examined for their T3SS activity. We observed that mutations G213A and G217A showed similar secretion profile as Δ*escV* strain transformed with EscVwt-V5 while the single mutation G213L completely abolish the T3SS activity (Figure 7A). *escV* strain transformed with EscV G217L mutant showed a much milder effect relative to the G213L mutation (Figure 7A). To confirm proper expression of the EscV point mutation variants, whole-cell lysates were submitted to western blot analysis using anti-His antibody. Comparable protein expression was detected for the WT and the single mutants (Figure 7A). Our results suggest that replacement of the glycine residues of the GxxxG motif found in TMD5 by a large reside (leucine) disrupts the activity of the protein while replacement by a small residue (alanine) does not.

To investigate the effect of the single mutation G213L on the assembly of the T3SS complex, we examined the effect of G213L mutation on the ability of EscV protein to properly integrate into the T3SS complex. For this purpose, we grew EPEC WT and EPEC Δ*escV* strain transformed with EscVwt-V5, EscVG213A-V5 and EscVG213L-V5 under T3SS-inducing conditions. We prepared crude membranes and analyzed them by BN-PAGE and immunobloting. BN-PAGE analysis showed that Δ*escV* mutant strain transformed with EscVwt-V5 and EscVG213A-V5 migrated mainly as a large complex at the top of the gel, while the EscVG213L-V5 integration into the complex appiered to be impaired (Figure 7B). To confirm that the altered running pattern of EscVG213L-V5 mutant form was not due to reduced protein expression, the crude membrane extracts were analyzed by SDS-PAGE and immunoblotting using anti-V5 antibody. We detected lower expression level of EscVG213L-V5 relative to EscVwt-V5 and EscVG213A-V5, but not to a level that explains the significant reduction in complex formation (Figure 7B). Overall, our results indicate that the GxxxG motif, and more specifically the glycine at position 213, are critical for the proper EscV integration into the T3SS complex.

**Discussion**

The high sequence conservation within the sequence of EscV TMD6 and the conserved GxxxG motif within TMD5 (Fig. 1B), together with the numerous studies regarding TMDs-derived oligomerization of membrane complexes (Fink et al., 2012), urged us to examine whether EscV TMDs are involved in the protein oligomerization. Results using the isolated ToxR system demonstrated that TMD5 and TMD6 exhibited strong self-oligomerization activities, with activities similar to that of the well-characterized GpA TMD sequence (Fig. 2A).

To investigate whether TMD5 and TMD6 sequences are critical for the activity of the full-length protein, we replaced each of these TMDs with an alternative hydrophobic sequence (7L9A). The plasmids encoding TMD5- or TMD6-exchanged EscV versions were transformed into Δ*escV* null strain and their T3SS activity was examined. We found that expression of either EscV-TMD5ex-His or EscV-TMD6ex-His failed to complement the T3SS activity of EPEC Δ*escV* strain while the expression of EscVwt-His restored T3SS (Fig. 4A). Infection of HeLa cells with bacterial strains that express either TMD5- or TMD6-exchanged EscV versions were non-virulent and demonstrated JNK degradation profiles comparable to uninfected cells (Fig. 4B). Since we observed that the membrane localization of EscV, both the WT and the TMD-exchanged versions, was not disrupted (Fig. 5), we concluded that EscV TMD5 and TMD6 are critical not only for proper membrane anchoring but also for T3SS activity and EPEC ability to infect host cells as they cannot be replaced by an alternative hydrophobic sequence. Based on the ToxR results, we presume that TMD5 and TMD6 are involved in protein oligomerization although we did not detect complete complex dissociation for T3SS with TMD-exchanged variants (Fig. 6). These results suggest that EscV TMD5 and TMD6 are not crucial for the global assembly or stability of the T3SS complex but rather that they are involved in promoting the proper TMD-TMD interactions within the complex and their overall orientation to allow passage of T3SS substrates.

To examine the role of the GxxxG motif, found within TMD5, on the overall activity of the T3SS, we mutated single glycine residues within the motif and replaced them with either a non-polar small amino acid (alanine) or a non-polar large amino acid (leucine). We found that the original glycine residues can be replaced by alanine residues with no effect on T3SS activity (Fig. 7A). These results concur with previous reports suggesting that the GxxxG motif is equivalent to Small-xxx-Small motif (Lock et al., 2014;Curnow et al., 2020;Wang et al., 2020). Replacement of glycine to leucine, however, abolished T3SS activity, when glycine in position 213, but not in 217, was mutated (Fig. 7A). These results suggested that the two glycine positions do not contribute equally to the activity of the protein and position 213 is more critical for EscV function within the T3SS complex. Interestingly, while we observed reduced complex formation for the G213L mutation (Fig. 7B), we did not observe similar reduction for EscV TMD5-exchanged (Fig. 6), although both contained conversion of glycine at position 213 to leucine. These results suggest that TMD-TMD packing is context-dependent and is not depended on a single residue or motif. Our results is in agreement with previous reports that demonstrated that the GxxxG motif supports TMD interactions within the context of oligo-methionine and oligo-valine sequences but not within randomized TMDs (Brosig and Langosch, 1998;Unterreitmeier et al., 2007;Langosch and Arkin, 2009).

Expression of EscVwt-His within the *escV* null strain unexpectedly resulted in hypersecretion of effectors compared to WT EPEC secretion (Fig. 4A). Interestingly, HA- and V5-tagged EscV expressed from a high copy-number plasmid (pSA10) presented similar secretion profile as well as expression of HSV-tagged EscV from a low copy-number plasmid (pACYC184, Fig. S2). A milder phenotype was observed for expression of unlabeled EscV (Fig. S2). Overall, these results suggested that overexpressing, and to a larger extent, labeling EscV at its C-terminus, regardless of the nature of the tag, interferes with substrate secretion regulation. Our results correlate well with previous studies that indicated that the EscV is involved in substrate secretion regulation through interaction with the "gate-keeper", SepL, and several T3SS chaperons (Portaliou et al., 2017;Gaytan et al., 2018). The identification that EscV interacts with SepL via its C-terminal (Portaliou et al., 2017) suggests that labeling EscV at this critical domain disrupt EscV-SepL interaction and therefore induce uncontrolled T3S. This conclusion was further supported by our inability to recapitulate EscV-SepL interaction when EscV was labeled on its C-terminal (data not shown).

Examination of the ability of EPEC Δ*escV* that expresses EscVwt-His to infect HeLa cells revealed similar infection capacity as WT EPEC strain (Fig. 4B). This result was unexpected as previous studies revealed that strains with dysregulated T3 substrate secretion (*sepL*, *sepD*, and *escP*) showed reduced infectivity and effector translocation abilities (Deng et al., 2004;Deng et al., 2015;Shaulov et al., 2017). To our knowledge, this is the first example of an EPEC strain that lack hierarchical substrate secretion regulation but show similar virulence capabilities to the WT strain. We assume that in contrast to previous strains, the amounts of secreted translocators of Δ*escV* that expresses EscVwt-His was not reduced, and therefore allowed robust infection.

In summary, in this work we have shown that TMD5 and TMD6 of EscV are critical for T3SS activity likely due to their role in TMD-TMD packing within the complex. Further investigation will be required to determine the structural organization within the bacterial inner membrane and to depict the direct interaction partners of EscV within the T3SS complex.

**Figure legends:**

Figure 1: **Prediction of TMDs of EscV**. **(A)** TMHMM software prediction analysis of the probability of each EscV amino acid to be localized with the bacterial membrane. Seven TMDs were identified (sequence is colored red). **(B)** Sequence alignment of the EscV export apparatus protein. A standard protein BLAST alignment is presented by ClustalW (Larkin et al., 2007) for EscV of EPECT3SS (B7UMA7), FlhA of *E. coli* flagella (P76298), EscV of the E. coli O157:H7(Q7DB70), YscV of the Yersinia enterocolitica (A0A2J9SJU1), MxiA of the *Shigella* T3SS (P0A1I5), and InvA of the *Salmonella* typhimurium T3SS (A0A0H3NL68). A high level of conservation was observed within TMD6 sequence and among the GxxxG motif found within TMD5.

Figure 2: **EscV TMD self-oligomerization activity**. **(A)** Schematic illustration of a ToxR assembly system. TMD-TMD interaction promotes oligomerization of the transcription activator ToxR, which then can bind (in its oligomeric form) the ctx promoter and transcribe the reporter gene, lacZ. The TMD sequences, inserted between the ToxR and the MBP are presented. **(B)** The LacZ activities of FHK12 bacterial strains expressing the ToxR-TMD-MBP chimeras of various EscV TMDs, GpA, Tar-1, and A16 TMDs. Bars represent the standard deviation of at least three independent experiments. The expression of ToxR-TMD-MBP chimera proteins containing the different TMD sequences, were analyzed on SDS-PAGE and western blotting using an anti-MBP antibody and are presented under each corresponding sample. (**C**) Growth curves of PD28 bacteria transformed with plasmids encoding ToxR-TMD-MBP chimera protein containing the GpA (\*), Tar-1 (+), A16 (△), EscV TMD1 (○), TMD2 (▲), TMD3 (-), TMD4 (♦), TMD5 (×), TMD6 (□), TMD7.1 (■), TMD7.2 (◊) or in the absence of a TMD (ΔTM, •). The bacteria were grown in a minimal medium containing maltose. All bacterial cultures showed similar growth curves, indicating proper membrane integration.

Figure 3: **Overexpression of EscV reduces bacterial fitness.** Growth curves of WT EPEC (■), Δ*escV* (□), and EPEC Δ*escV* complemented with EscVwt-His (•), EscV-TMD5ex-His (△), EscV-TMD6ex-His (▲). Strains were grown at 37°C in DMEM (left panel) and LB (right panel) media with various IPTG concentrations (0, 0.1, and 0.25 mM). Optical density at 600 nm was measured every 30 minutes and plotted over time.

Figure 4: **Replacement of EscV TMD5 and TMD6 by an alternative hydrophobic sequence abolishes T3SS activity. (A)** Protein secretion profiles of EPEC WT, Δ*escV*, Δ*escN* and EPEC Δ*escV* strain carrying the pEscVwt-His, pEscV-TMD5ex-His, or pEscV-TMD6ex-His plasmids grown under T3SS-inducing conditions with 0.1 mM IPTG. The secreted fractions were filtered and protein content was concentrated from the supernatants of bacterial cultures and analyzed by SDS-PAGE and Coomassie blue staining. The T3SS-secreted translocators and effectors EspA, EspB, EspD, NleA and Tir are marked on the right of the gel. EspC, which is not secreted via the T3SS, is also indicated. EscV expression was examined by analyzing bacterial pellets by SDS-PAGE and western blot analysis with an anti-His antibody.  Numbers on the left are molecular masses in kilodaltons. (**B**) Proteins extracted from HeLa cells infected with WT, Δ*escN*, Δ*escV*, or Δ*escV* carrying the pEscVwt-His, pEscV-TMD5ex-His or pEscS-TMD6ex-His, were subjected to western blot analysis using anti-JNK antibody and anti-actin (loading control). JNK and its degradation fragments are indicated.

Figure 5: **Replacement of EscV TMDs by an alternative hydrophobic sequence does not affect membrane localization**. EPEC Δ*escV* strain expressing EscVwt-His, EscV-TMD5ex-His and EscV-TMD6ex-His were grown under T3S-inducing conditions, were fractionated into periplasmic (P), cytoplasmic (C), and membrane (M) fractions and analyzed by western blot analysis with an anti-His antibody. Proper bacterial fractionation was confirmed by analyzing the samples by SDS-PAGE and western blotting with anti-DnaK (cytoplasmic marker), anti-MBP (periplasmic marker), and anti-intimin (membrane marker) antibodies.

Figure 6: **Association of EscV-exchanged version with the T3SS complex**. Membrane protein extracts of Δ*escV*, Δ*escV* expressing EscV-His, EscV-TMD5ex-His, and EscV-TMD6ex-His were incubated in BN sample buffer, subjected to BN-PAGE (upper panel) and SDS-PAGE (lower panel), and western blot analysis using anti-His antibodies.

Figure 7: **The glycine residue at position 213 is critical for the T3SS activity.** **(A)** Protein secretion profiles of EPEC strains grown under T3SS-inducing conditions: WT, Δ*escN*, Δ*escV*, and Δ*escV* complemented with EscVwt-V5, EscVG213A-V5, EscVG217A-V5, EscVG213L-V5, or EscVG217L-V5. The secreted fractions were concentrated from the supernatants of bacterial cultures and analyzed by SDS-PAGE and Coomassie blue staining. The expression of EscV-V5 variants were examined by analyzing the bacterial pellets by SDS-PAGE and Western blot analysis with an anti-V5 antibody. **(B)** Membrane protein extracts of WT EPEC and Δ*escV* expressing EscVwt-V5, EscVG213A-V5, or pEscVG213L-V5 were incubated in BN sample buffer and then subjected to BN-PAGE (upper panel) or SDS-PAGE (lower panel) and western blot analysis using anti-V5 antibody. Molecular masses in kilodaltons are presented on the right.

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