

# Tryptophan-mediated Dimerization of the TssL Transmembrane Anchor Is Required for Type VI Secretion System Activity

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2	Tryptophan-mediated dimerization of the TssL transmembrane
3	anchor is required for Type VI secretion system activity
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#### 1 Abstract

2

3 The Type VI secretion system (T6SS) is a multiprotein complex used by bacteria to 4 deliver effectors into target cells. The T6SS comprises a bacteriophage-like contractile 5 tail structure anchored to the cell envelope by a membrane complex constituted of the 6 TssJ outer membrane lipoprotein and the TssL and TssM inner membrane proteins. 7 TssJ establishes contact with the periplasmic domain of TssM whereas the 8 transmembrane segments of TssM and its cytoplasmic domain interact with TssL. TssL 9 protrudes in the cytoplasm but is anchored by a C-terminal transmembrane helix 10 (TMH). Here, we show that TssL TMH dimerization is required for the stability of the 11 protein and for T6SS function. Using the TOXCAT assay and point mutations of the 23 12 residues of the TssL TMH we identified Thr194 and Trp199 as necessary for TssL TMH 13 dimerization. NMR hydrogen-deuterium exchange experiments demonstrated the 14 existence of a dimer with the presence of Trp185 and Trp199 at the interface. A 15 structural model based on molecular dynamics simulations shows that TssL TMH dimer 16 formation involves  $\pi$ - $\pi$  interactions resulting from the packing of the two Trp199 rings 17 at the C-terminus and of the six aromatic rings of Tyr184, Trp185 and Trp188 at the N-18 terminus of the TMH.

#### 1 Introduction

2

3 The Type VI secretion system (T6SS) is a molecular machine widely distributed in 4 proteobacteria and responsible for the contact-dependent secretion of effector proteins into 5 target cells [1,2]. Effectors delivered by this apparatus interfere with eukaryotic host cell 6 physiology, or cause damages in bacterial cells [3-6]. The T6SS is therefore involved in 7 pathogenesis and/or bacterial competition. At the molecular level, the T6SS could be 8 represented as a nano-speargun [7-9] (Fig. 1A): an inner tube made of stacked Hcp hexameric 9 rings capped by the VgrG spike complex and wrapped by a contractile sheath is propelled into 10 the target cells during sheath contraction [10-12]. This tubular structure is assembled on a 11 sub-membrane platform - or baseplate - that comprises the TssEFGK and VgrG subunits and 12 is anchored to the cell envelope by the membrane complex [13-18].

In enteroaggregagtive Escherichia coli (EAEC), the T6SS membrane core complex is 13 14 constituted of the outer membrane-associated TssJ lipoprotein and the TssL and TssM inner 15 membrane proteins [14,19,20] (Fig. 1B and C). TssJ folds as a classical transthyretin with an 16 additional  $\alpha$ -helix and an extended loop connecting  $\beta$ -strands 1 and 2 (L1-2) [21-23]. TssM 17 comprises three transmembrane helices (TMH): TMH1 and TMH2 forms a hairpin located 18 close to the N-terminus, separated from the third helix, TMH3, by a 35-kDa cytoplasmic 19 domain [16,24] (Fig. 1C). The TssM cytoplasmic portion comprises two subdomains with 20 structural homologies to NTPases and the DPY-30 dimerization hairpin [16]. The TssM 21 cytoplasmic domain makes contact with TssL, as well as with TssG and TssK, two 22 components of the tail assembly platform [13,15,16,18]. The majority of the EAEC TssM 23 subunit (750 amino-acids) is located in the periplasm and its C-terminal extremity interacts 24 with the TssJ lipoprotein L1-2 loop [14,21]. The EAEC TssL protein is a C-tail membrane protein, anchored to the inner membrane by a C-terminal TMH that requires the YidC 25

insertase for its insertion [25,26] (Fig. 1C). The TssL cytoplasmic domain comprises two 3helix bundles [27-29] and interacts with two subunits of the baseplate, TssE and TssK [15,17].
In EAEC, this domain forms weak dimers, but the full-length protein (*i.e.*, comprising the Cterminal transmembrane anchor) is a stable dimer that resists Sodium Dodecyl Sulphate-Poly
Acrylamide Gel Electrophoresis (SDS-PAGE; [27]).

6 The TssJLM membrane complex is the first T6SS structure to be assembled. Its 7 biogenesis starts with the positioning of the TssJ lipoprotein and proceeds with the sequential 8 addition of TssM and TssL [14]. Polymerization of this hetero-trimer requires local 9 remodelling of the cell wall layer that is assured by a dedicated peptidoglycan hydrolase or a 10 housekeeping transglycosylase [30,31]. Ten copies of the hetero-trimer polymerize to form 11 the 5-fold symmetry transenvelope channel. The 12-Å negative-stain electron microscopy 12 structure of the TssJLM complex from enteroaggregative E. coli has been reported [14] (Fig. 13 1B). It comprises a large base constituted of the cytoplasmic domains and transmembrane 14 segments and a cap composed of TssJ. Base and cap are connected by pillars made of the TssM periplasmic domains [14]. The TssJLM structure has been obtained in a closed 15 16 conformation and molecular dynamics (MD) simulations have suggested that large 17 conformational modifications of the pillars cause opening of the structure for the passage of 18 the inner tube during sheath contraction [14]. To resist the strength generated during sheath 19 contraction, the TssJLM membrane complex is stably anchored to the cell wall by a peptidoglycan-binding domain either fused at the C-terminus of TssL or carried by an 20 21 accessory component [20,32].

Although the EM structure of the EAEC TssJLM complex and the X-ray structures of the soluble domains of TssL, TssM and TssJ have been reported, little is known on the transmembrane portion of the complex. It has been shown that the C-terminal anchor of TssL is required for T6SS function and for stable dimerization of TssL [27]. We therefore sought to

1 gain insights into the structure of the TssL TMH and its dimerization determinants. TOXCAT 2 analyses demonstrated that the TssL TMH is necessary and sufficient for dimerization. 3 Functional assays further showed that TMH-mediated TssL dimerization is required for TssL 4 stability and T6SS function. Finally, scanning site-directed mutagenesis coupled to TOXCAT, 5 structural Nuclear Magnetic Resonance (NMR) studies and MD simulations defined that 6 tryptophan rings engage in  $\pi$ - $\pi$  interactions to mediate TssL<sup>TM</sup> dimerization.

- 7
- 8 **Results**
- 9

#### 10 The TssL transmembrane segment is sufficient for dimerization.

Structure-function analyses of the T6SS TssL cytoplasmic domain revealed that it 11 12 weakly dimerizes [17,27]). By contrast, the full-length protein forms dimers that are stable in 13 SDS-PAGE [27]. We concluded that the C-terminal transmembrane helix strongly contributes 14 to TssL dimerization. To test this hypothesis, we used the TOXCAT assay [33], a two-hybrid 15 assay based on the reconstitution of an active ToxR dimeric regulator upon association of two 16 transmembrane helices. The dimeric ToxR regulator binds the tox promoter and activates the 17 expression of the gene encoding chloramphenicol acetyl-transferase and conferring resistance 18 to chloramphenicol [33,34]. The sequence corresponding to the transmembrane helix of TssL (TssL<sup>TM</sup>) was inserted between the ToxR' domain and the maltose-binding protein (MBP) to 19 yield the ToxR'-TssL<sup>TM</sup>-MBP fusion protein. Controls include the transmembrane helix of the 20 21 glycophorin A (GpA), considered as a model for transmembrane helix association, as well as a point mutant disrupting dimerization of GpA (GpA<sup>G83I</sup>) [33]. Cells producing ToxR'-22 TssL<sup>TM</sup>-MBP grew on minimal medium with maltose as carbon source (Fig. 2A-B), 23 24 demonstrating that the MBP localizes in the periplasm and hence that the construct is properly 25 inserted in the membrane via the TssL transmembrane helix. Chloramphenicol disk and chloramphenicol acetyl-transferase activity (Fig. 2C and 2D) assays further showed that
 TssL<sup>TM</sup> dimerizes at levels comparable to the GpA control. From these data, we conclude that
 TssL<sup>TM</sup> dimerizes by itself in the membrane.

4

### 5 Thr194 and Trp199 contribute to TssL<sup>TM</sup> dimerization.

To get further information on the driving forces that control TssL<sup>TM</sup> dimer formation, 6 7 we used the same TOXCAT approach coupled to site directed mutagenesis screening. A 8 number of motifs involved in transmembrane helix interactions have been reported, such as leucine zipper and GxxGxxxG motifs. Analyses of the TssL<sup>TM</sup> sequence reveals the presence 9 10 of a leucine zipper motif (LxxLxxxL) comprising Leu195, Leu198 and Leu202 (shown in red 11 in Fig. 3A). Leucine zippers are common protein-protein interaction motifs, and such a motif has been shown to be involved in the dimerization of the T4SS-associated VirB10 12 transmembrane segment [35]. We first engineered mutations to break the TssL<sup>TM</sup> leucine 13 14 zipper motif by substituting Leu195 and Leu198 by alanines (LALA variant). TOXCAT assays show that mutations of these two leucine residues had only a slight effect on the ability 15 of TssL<sup>TM</sup> to dimerize (Fig. 3B and 3D) and thus contradict the leucine zipper hypothesis. To 16 identify the residues of TssL<sup>TM</sup> involved in dimer formation, we performed a systematic 17 18 scanning mutagenesis, in which each individual residue has been changed to tryptophan. Tryptophan side-chains are bulky and have been previously shown to disrupt contacts 19 between tightly packed transmembrane segments [36]. Tryptophan residues of TssL<sup>TM</sup> were 20 21 substituted by alanines. TOXCAT assays demonstrated that while a number of substitutions affect the stability of the TssL<sup>TM</sup> dimer, only two mutations - Thr194Trp (T194W) and 22 Trp199Ala (W199A) — have a level of chloramphenicol acetyl transferase activity 23 comparable to the absence of TMH (Fig. 3C and 3D). 24

1 Previous data have shown that the TssL transmembrane helix stabilizes the TssL dimer. 2 which then resists SDS-PAGE analysis [27]. We hence tested whether the T194W and 3 W199A substitutions impact the stability of the TssL dimer in SDS-PAGE. As previously 4 observed, the cytoplasmic domain of TssL (TssL<sub>C</sub>) migrates as a monomer upon SDS-PAGE 5 whereas the dimer of the full-length TssL protein is detected (Fig. 4, lanes 1 and 2 6 respectively). For the T194W and W199A variants of TssL only monomers are observed indicating that the T194W and W199A mutations have a strong effect on TssL<sup>TM</sup> dimer 7 8 formation (Fig. 4, lanes 3 and 4).

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### 10 Structural analysis of the TssL<sup>TM</sup> dimer interface

To investigate the structure of the TssL<sup>TM</sup> dimer, we first conducted Nuclear Magnetic 11 Resonance (NMR) experiments using a synthetic peptide corresponding to the TssL 12 13 transmembrane segment (Met183-Ala207), stabilized by two additional lysine residues at the N- and C-termini. The TssL<sup>TM</sup> peptide was solubilized in presence of SDS because (i) the 14 TssL<sup>TM</sup> dimer has been shown to be stable in SDS-PAGE [27], (ii) in such context, SDS 15 micelles have been previously shown to mimick the membrane environment [37], and (iii) 16 17 SDS is commonly used for solution NMR analyses of transmembrane domains [38-40]. Proton assignment of the TssL<sup>TM</sup> peptide was obtained using TOCSY and NOESY data 18 19 (Table S1). On the basis of NH/NH nuclear Overhauser effect restraint measurements we concluded that the sequential NH/NH distances are in agreement with a structure of the 20 21 peptide as an extended  $\alpha$ -helix (Fig. 5A, Fig. S1) [41]. The presence of a unique set of peaks 22 in the NMR spectra suggested that the TssL peptide forms a symmetric dimer in SDS. In this 23 case, it is quite impossible to distinguish intra and intermolecular distance restraints (nOes) at 24 the interface using homonuclear NMR. To investigate the dimer interface, we used hydrogen-25 deuterium (H-D) exchange experiments by recording NOESY experiments in D<sub>2</sub>O buffer. In

1 D<sub>2</sub>O the NH groups exposed to the solvent are rapidly converted in ND groups that are not 2 observed in the proton NMR spectra. By contrast, in these conditions, the NH groups 3 protected by a hydrogen bond or by any other contact are slowly converted in ND groups and 4 are still observed in the proton NMR spectra. Fig. 5B and 5C show that the Trp185 and 5 Trp199 ring NHs are significantly more protected to the solvent exchange compared to the amide groups of the rest of the TssL<sup>TM</sup> peptide, likely because of their location at the dimer 6 7 interface. Thus, the H-D exchange experiments support the conclusion of the TOXCAT assay 8 that TssL<sup>TM</sup> dimer interface involves the Trp199 rings at the periplasmic side of the TMH and 9 in addition, provides support to the location of the Trp185 rings at the interface at the 10 cytoplasmic side of the TMH (Fig. 5D). However, rapid H-D exchange was observed for the 11 amide group of Thr194, suggesting that it is not protected and thus that Thr194 is not located at the dimer interface. However, although the NMR approach demonstrated that TssL<sup>TM</sup> has 12 13 an helical structure and is organized as a dimer in SDS with Trp185 and Trp199 at the interface, it was not possible to define the structure of TssL<sup>TM</sup> using classical approaches of 14 15 homonuclear NMR.

Toward the aim to obtain a structural model of the TssL<sup>TM</sup> dimer and to better define the 16 17 dimerization interface, we used molecular dynamics (MD) simulations. For comparison, these 18 MD simulations were conducted in conditions identical to the NMR experiments (at 300K, in 19 SDS micelle). First, a 50-ns simulation of the monomer peptide was performed in a SDS micelle containing 110 detergent molecules to generate the basic element, *i.e.*, the TssL<sup>TM</sup> 20 21 monomer. Consistent with the NMR data, the averaged structure demonstrated that the TssL<sup>TM</sup> monomer is a straight  $\alpha$ -helix in SDS environment (Fig. 5E and 6A). This structure 22 23 further shows that Trp199 and Trp185 belong to the same helix face (Fig. 5E). By contrast, 24 Thr194 and Trp199 belong to opposite faces (Fig. 5E and 6A), and hence, it is unlikely that 25 both residues participate to a homodimeric interaction without substantial structure

1 disturbance. To explore if such distortions could arise in the context of the dimeric structure, we performed MD simulations of TssL<sup>TM</sup> in the SDS micelle (*i.e.*, in conditions similar to the 2 NMR and SDS-PAGE experiments, in which the TssL<sup>TM</sup> dimer was observed). Starting from 3 4 eight different symmetric conformations (see methods), the superimposition of the eight averaged structures (Fig. 6A) shows no significant changes of the structure of TssL<sup>TM</sup>. Fig. 5 6B and 6C capture the proximity between the Thr194 or Trp199 in each dimer during the 6 7 course of the 8 simulations (numbered from 0° to 350° according to the relative orientation of 8 the helix faces). Interestingly, only the 250° simulation allows a tight proximity between the 9 Thr194 of the two monomers (Fig. 6B), whereas the 0°, 50° and 350° simulations bring the 10 Trp199 of both monomers at close proximity (Fig. 6C). These simulations also indicate that 11 the Trp199 of one monomer is never close to the Thr194 residue of the other monomer. As 12 suggested by the organization of residues on the face of the straight  $\alpha$ -helix (Fig. 5E), the positioning of Trp199 at the interface also bring aromatic residues of the TssL<sup>TM</sup> N-terminal 13 14 region (Tyr184, Trp185, Trp188) in close proximity. To further address the role of the aromatic residues at the interface, the '0' and 350° simulations were prolonged for 100 ns (Fig. 15 16 6D and 6E). The two simulations converged to a very similar structure in which eight 17 aromatic rings (Tyr184, Trp185, Trp188, Trp199 from each monomer) interact at the interface of the TMH dimer (Fig. 5F). Interestingly, Trp185 was protected in NMR H-D exchange 18 experiments and thus described as an interfacial residue. Hence, modeling of the TssL<sup>TM</sup> 19 20 dimer confirms that (i) Thr194 and Trp199 are not located on the same interface, and (ii) that Trp199 likely contributes, as well as residues Tyr184, Trp185 and Trp188, to TssL<sup>TM</sup> 21 dimerization (Fig. 5F). The implication of Trp199 to TssL<sup>TM</sup> dimerization during MD 22 simulations validates the results of the TOXCAT assay and NMR H-D exchange experiments. 23 24 By contrast to the TOXCAT assay, the NMR and MD data did not highlight a role for residue Thr194. To test whether the replacement of Thr194 by a Trp residue interfere with TssL<sup>TM</sup> 25

dimerization, we performed MD simulations using the TssL<sup>TM</sup> peptide bearing a Trp at 1 2 position 194 (Fig. S2). This substitution significantly affects the contact map calculated for the TssL<sup>TM</sup> dimer during a 50-ns simulation. Notably, the contacts that initially involved the 3 4 N-terminal aromatic cluster decrease (see for example Trp188) to the advantage of new 5 contacts that are formed on the C-terminal region of the peptide (Fig. S2A). From the initial 6 position (in red), this drift leads to a more open conformation (in blue) that moves forward the 7 two N-terminal extremities (Fig. S2B). This structural change corresponds to a huge rise in 8 the root-mean square deviation (rmsd) of monomer B relative to monomer A, that reaches a plateau at ~5-6 Å in less than 5 ns, increasing to 10 Å after 20 ns, a behavior typical of a loss 9 of structural stability (Fig. S2C). Hence, the lack of interaction between TssL<sup>TM</sup> T194W 10 variants in the TOXCAT assay might be correlated to a significant impairment of the TssL<sup>TM</sup> 11 12 dimer integrity that could arise from subtle conformational or dynamic alterations.

13

#### 14 Transmembrane-mediated TssL dimerization is required for T6SS function.

15 The T6SS assembles a tail-like structure comprising the tail tube made of Hcp hexameric 16 rings wrapped by the sheath. Contraction of the sheath is proposed to propel the Hcp tube 17 toward the target cell. The presence of the Hcp protein in the culture supernatant is therefore a 18 reporter of T6SS activity [42]. Indeed, Hcp is detected in the culture supernatant of a wild-19 type strain, whereas is absent in the culture supernatant of a  $\Delta tssL$  mutant strain (Fig. 7A). 20 The production of a plasmid-borne full-length TssL protein restores the release of Hcp. By 21 contrast, despite the observation that the W199A variant is produced at levels comparable to 22 wild-type TssL, this mutation abolishes Hcp release (Fig. 7A). The EAEC T6SS has anti-23 bacterial activity and has been shown to provide a competitive advantage against E. coli K-12 24 cells [43]. The results of the competition assays showed that cells producing the W199A TssL 25 mutant are unable to eliminate competitor E. coli K-12 cells (Fig. 7B).

Taken together, the Hcp release and anti-bacterial competition assays demonstrate that
 the TssL W199A mutation prevents proper function of the Type VI secretion apparatus in
 EAEC, suggesting that T6SS function requires dimerization of the TssL transmembrane
 anchor.

5

### 6 TssL dimerization increases TssL stability.

To gain further insights onto the role of  $TssL^{TM}$  dimerization during T6SS assembly, we tested whether disrupting  $TssL^{TM}$  dimer formation impact the stability of the full-length TssL. EAEC cells producing TssL or TssL-W199A were subjected to protein synthesis arrest using the ribosomal 30S-specific spectinomycin antibiotic, and steady-state TssL levels were monitored over time. Fig. 8 shows that TssL is a rather stable protein with a half-life of ~ 50 min. The W199A substitution causes an important decrease of TssL stability, with a measured half-time < 10 min.

14

#### 15 Ligand-induced dimerization of TssL

16 Our in vivo results, monitored in the context of the fully assembled T6SS, cannot exclude that the W199A substitution affects other functions or interactions in addition to TssL<sup>TM</sup> 17 18 dimerization. We hence engineered TssL variants in which the genetically modified FK506-19 binding protein (FKBP), F<sub>V</sub>, was fused to W199A TssL (Fig. 9A). FKBP are soluble signaling 20 proteins that dimerize in response to a chemical inducer, rapamycin [44]. Hence, the couple F<sub>V</sub> and AP20187, a non-toxic analogue of rapamycin, could be used for ligand-induced 21 22 dimerization [44,45] (Fig. 9A). F<sub>V</sub> was fused to the TssL C-terminus (Fig. 9A), which is 23 located in the periplasm and permissive to insertion, as TssL subunits fused to peptidoglycanbinding motifs are commonly found associated with T6SS [32]. TOXCAT assay showed that 24 25 the presence of AP20187 in the medium overcomes the dimerization defect caused by the TssL<sup>TM</sup> W199A mutation (Fig. 9B). Ligand-induced dimerization of the TssL W199A variant restores T6SS-mediated Hcp release (Fig. 9C) and antibacterial activity (Fig. 9D), and increases TssL W199A stability to wild-type levels (Fig. 9E-G). Taken together, these results demonstrate that Trp199 is a key residue for TssL<sup>TM</sup> dimerization and that TssL<sup>TM</sup> dimerization is essential for T6SS activity.

6

#### 7 Discussion

8 In this study, we report that the TssL transmembrane segment is a major determinant 9 of TssL dimerization. Using a combination of TOXCAT, NMR and MD assays, we show that 10 TssL<sup>TM</sup> dimer formation is mediated by  $\pi$ - $\pi$  interactions involving three aromatic residues at 11 the cytoplasmic side (Tyr184, Trp185 and Trp188) and one tryptophan at the periplasmic side 12 (Trp199). We further provide evidence for a critical role of TM-mediated TssL dimerization 13 in T6SS stability and activity.

Few data are available in the field of bacterial secretion systems regarding the role of TM segments, and more specifically how interactions between TM helices participate to the assembly of these multiprotein complexes. However, the dimerization of the *Agrobacterium tumefaciens* VirB10 protein TM helix is required for Type IV secretion [35,46].

Our results highlighted the specific and important role of TssL<sup>TM</sup> aromatic residues in 18 dimer formation. Interestingly, TssL<sup>TM</sup> dimerization is mediated by a total of eight aromatic 19 20 rings, with six of them forming a tight patch.  $\pi$ - $\pi$  packing of aromatic rings is a common 21 interaction motif in proteins [47,48], but is also a very common interface between proteins 22 and ligands [49]. The involvement of aromatic-aromatic interactions in TM helices is not well 23 documented [50]. By contrast, cation- $\pi$  interactions, such as Lys-Trp interactions, have been 24 shown to control TM interactions within the chloride intracellular channel protein 1 (CLIC1) 25 [51]. Rather, tryptophan side-chains in TMH have usually functional roles including

1 selectivity or gatekeepers such as in conjugation coupling proteins [52] and proton or 2 potassium channels [53-56], and locks for rotameric switches, such as for the ghrelin receptor 3 [57]. Interestingly, an alignment of the TMH sequences of T6SS-associated TssL homologues 4 covering the different T6SS subfamilies shows that tryptophan or aromatic residues are highly 5 conserved at the N- and C-termini of the TMHs (Fig. 10). This conservation is also shared 6 with T4bSS-associated IcmH/DotU proteins (Fig. 10), highlighting the importance of 7 aromatic rings at these positions. It is noteworthy that, by contrast to the EAEC TssL TMH, 8 only one aromatic residue is usually conserved at the N-terminus. This observation suggests 9 that the packing between two aromatic rings might be sufficient to stabilize TssL TMH 10 formation at the N-terminus, and thus may explain why single substitution of the Y184, W185 11 or W188 residue does not significantly impact dimer formation in the TOXCAT assay.

12 It is worthy to note that NMR H-D experiments did not fully copy the results of the 13 TOXCAT assay. However, the MD simulations provided molecular explanations for all the discrepancies. First, no role of residue Thr194 in TssL<sup>TM</sup> dimerization could be inferred by H-14 15 D experiments, whereas the T194W substitution strongly interferes with dimerization using TOXCAT. However, MD simulations using a peptide corresponding to the T194W variant 16 showed that this substitution induces a destabilization of the TssL<sup>TM</sup> dimer packing and a 17 18 significant motion of one monomer relative to the other. Interestingly, the threonine side-19 chain tends to bend  $\alpha$ -helices by the formation of an additional hydrogen bond between its side-chain yO atom and the i-3 or i-4 peptide carboxyl oxygen [58]. This additional hydrogen 20 bond also alters the dynamic of the helix bending [59] in a way that, in the TssL<sup>TM</sup> peptide, 21 22 opposes to the formation of a coiled-coil on the dimerization face. Hence, incorporating a 23 small disturbance in bending flexibility at one side of the TMH results in a significant 24 displacement of the residues located at the other side [58], such as we observed in the case of the TssL<sup>TM</sup> dimer. In conclusion, although Thr194 does not participate directly to the TssL<sup>TM</sup> 25

1 interface, the additional hydrogen bond contributed by the side-chain is important to maintain the TssL<sup>TM</sup> in a conformation competent for dimerization. The quantitative TOX-CAT assays 2 also indicated that several substitutions along the helix affect TssL<sup>TM</sup> dimer formation, albeit 3 to a lower extent compared to T194W. While we did not conduct MD simulations for each 4 variant, it is likely that these substitutions slightly impact TssL<sup>TM</sup> stability and/or 5 6 conformation, and thus indirectly affect its dimerization. Second, the packing of the six 7 aromatic rings (Tyr184, Trp185 and Trp188) at the N-terminal portion of the TssL  $\alpha$ -helix is an important determinant of TssL<sup>TM</sup> dimerization, but none of the Y184W, W185A and 8 9 W188A substitutions affects dimerization by TOXCAT. As noted above, the lack of effect of these single substitutions on TOXCAT can be explained by the redundancy of the  $\pi$ - $\pi$ 10 11 interactions between these aromatic rings, an hypothesis in agreement with the observation that only one aromatic residue is usually conserved at the TssL<sup>TM</sup> N-termini. 12

13 In between the N-terminal Tyr184, Trp185 and Trp188 and the C-terminal Trp199 residues of the dimer interface are located the three leucine residues ascertained as a potential 14 leucine zipper. However, the TOXCAT assay showed that mutation of this potential leucine 15 zipper did not impact TssL<sup>TM</sup> dimerization. Interestingly, contrarily to the prototypical motif 16 found in the CGN4 leucine zipper [60], the structure of the TssL<sup>TM</sup> peptide revealed that it 17 18 does not present a coiled-coil structure. As a consequence, the leucine side-chains point 19 toward different directions and hence there is room to accommodate Trp substitutions owing to Trp flexibility due to the free rotation along the C $\alpha$ -C $\beta$  and C $\beta$ -C $\gamma$  bonds. 20

The development of  $TssL-F_V$  fusions to control TssL dimer levels and hence T6SS assembly is a remarkable tool. Because the assembly of the TssJLM membrane complex represents an early stage of T6SS biogenesis [13,14,61], the ligand-inducible TssLdimerization could be used to synchronize T6SS assembly and activities.

1 Finally, a better understanding of the interactions between the TssL TMH provides the 2 basis for the development of synthetic peptides that would inhibit TssL dimerization and thus 3 T6SS assembly. Hydrophobic peptides interfering with transmembrane domains have been 4 used to block the action of neuropilin-1, an actor of the human Hedgehog signalling pathway that is involved in development disorders and cancers [62,63]. TssL<sup>TM</sup>-targeting peptides that 5 6 mimick the dimerization interface might be of specific interest to diminish the virulence or the 7 anti-bacterial activity of important bacterial pathogens that use the T6SS to colonize the host, 8 such as Vibrio cholerae, Pseudomonas aeruginosa, or Salmonella enterica [64-68].

9

#### 10 Material and Methods

11

#### 12 Bacterial strains, media, growth conditions and chemicals

13 The bacterial strains used in this study are listed in Table S2. Escherichia coli K-12 DH5a, W3110 14 and NT326 [69] were used for cloning procedures, anti-bacterial competition and TOXCAT assays, 15 respectively. The enteroaggregative E. coli strain 17-2 and its  $\Delta tssL$  (previously described as  $\Delta sciP$ ; 16 [20]) derivative were used for this study. Strains were routinely grown in LB at 37°C with aeration. 17 The TOXCAT assay was performed in M9 minimal medium with glucose or maltose as carbon source. 18 Expression of the T6SS gene cluster was induced in Sci1-inducing medium (SIM; M9 minimal medium supplemented with LB 10%, glycerol 0.4%, casaminoacids 40 µg.mL<sup>-1</sup>, MgCl<sub>2</sub> 2 mM, CaCl<sub>2</sub> 19 0.1 mM, Vitamin B1 200 µg.mL<sup>-1</sup> [70]). Plasmids and mutations were maintained by the addition of 20 ampicillin (100 µg.mL<sup>-1</sup> for *E. coli* K-12, 200 µg.mL<sup>-1</sup> for EAEC) or kanamycin (50 µg.mL<sup>-1</sup> for *E.* 21 22 *coli* K-12). Protein synthesis was arrested by addition of spectinomycin (200 µg.mL<sup>-1</sup>). Induction of 23 genes cloned into pIBA vectors was obtained with anhydrotetracyclin (AHT - used at 0.02 µg.mL<sup>-1</sup> 24 throughout the study; IBA Technologies). The AP20187 dimerization inducer was purchased from 25 Clinisciences and used at 20 mM. The anti-TolB polyclonal antibodies are from the laboratory 26 collection while the anti-FLAG (clone M2, Sigma Aldrich) and anti-HA (clone HA-7, Sigma-Aldrich)

monoclonal antibodies and the goat secondary anti-mouse antibodies coupled to alkaline phosphatase (Jackson Immuno) or AlexaFluor<sup>®</sup> 680 (Molecular Probes) are commercially available.

3

2

#### 4 Plasmid construction

5 The plasmids used in this study are listed in Table S2. Polymerase Chain Reactions (PCR) were 6 performed with a Biometra thermocycler, using the Pfu Turbo DNA polymerase (Stratagene). Custom 7 oligonucleotides were synthesized by Sigma-Aldrich and are listed in Table S2. Plasmids pIBA-TssL 8 and pIBA-TssL<sub>C</sub> producing the full-length and the cytoplasmic domain (residues 1-183) of TssL 9 protein fused to a N-terminal FLAG sequence have been previously described [25]. The sequence 10 encoding the TssL transmembrane helix has been inserted into the pcckan vector [69] after PCR-11 amplification from the EAEC 17-2 chromosome DNA using primers 5-TssLTM-NheI and 3-TssLTM-12 BamHI. The NheI-BamHI fragment was then inserted into pcckan digested by the same restriction 13 enzymes to yield pcc-TssL-TM. FK506-binding protein (FKBP) fusion chimeras were engineered by a 14 double PCR technique, allowing amplification of the FKBP(F36V) sequence (from plasmid 15 pPSV35CV-ppkA-Fv; [45]) flanked by extensions annealing to the target vector [20,71]. The product 16 of the first PCR has then been used as oligonucleotides for a second PCR using the pIBA-TssL or pcc-17 TssL-TM as template. Site-directed mutagenesis was performed using complementary 18 oligonucleotides bearing the desired substitutions. All constructs have been verified by colony PCR, 19 restriction analyses and DNA sequencing (Genome Express).

20

#### 21 Antibacterial competition assay

The antibacterial growth competition assay was performed as previously described [43]. Briefly, the wild-type *E. coli* strain W3110 bearing the pUA66-*rrnB* plasmid (Kan<sup>R</sup> [72]) was used as prey in the competition assay. The pUA66-*rrnB* plasmid provides a strong constitutive green fluorescent (GFP<sup>+</sup>) phenotype. Attacker and prey cells were grown for 16 h in SIM medium, and then diluted in SIM to allow maximal expression of the *sci1* gene cluster [70]. Once the culture reached an absorbance at  $\lambda$ =600 nm ( $A_{600}$ ) of 0.8, cells were harvested and normalized to  $A_{600}$ =10 in SIM. Attacker and prey cells were mixed to a 4:1 ratio and 20-µL drops of the mixture were spotted in triplicate onto a pre-

warmed dry SIM agar plate supplemented with 0.02 µg.mL<sup>-1</sup> of AHT. After 4-hour incubation at 37°C, 1 2 fluorescent images were recorded with a LI-COR Odyssey imager. The bacterial spots were scratched 3 off, and cells were resuspended in LB medium and normalized to an  $A_{600}$ =0.5. Triplicates of 200 µL were transferred into wells of a black 96-well plate (Greiner) and the  $A_{600}$  and fluorescence (excitation, 4 5 485 nm; emission, 530 nm) were measured with a Tecan Infinite M200 microplate reader. The relative 6 fluorescence was expressed as the intensity of fluorescence divided by the  $A_{600}$ , after subtracting the 7 values of a blank sample. For estimating bacterial prey survival, colony-forming units (cfu) were 8 enumerated after plating serial dilutions on LB plates supplemented with kanamycin. The experiments 9 were done in triplicate, with identical results, and we report here the results of a representative 10 experiment.

11

#### 12 Hcp release assay

13 Supernatant and cell fractions have been separated as previously described [20] except that cells were 14 grown in SIM medium. When necessary, AHT was added 30 minutes before harvesting the cells. Briefly, 2×10<sup>9</sup> cells producing HA epitope-tagged Hcp (from plasmid pOK-Hcp<sub>HA</sub>; [20]) were 15 16 harvested and collected by centrifugation at 2,000  $\times$  g for 5 min. The supernatant fraction was then 17 subjected to a second low-speed centrifugation, a high-speed centrifugation (16,000  $\times$  g for 15 min) 18 and filtered on sterile polyester membranes with a pore size of 0.2 µm (membrex 25 PET, 19 membraPure GmbH) before precipitation with trichloroacetic acid (TCA) 15%. Cells and precipitated 20 supernatant were resuspended in loading buffer and analysed by SDS-PAGE and immunoblotting with 21 the anti-HA antibody. As control for cell lysis, Western blots were probed with antibodies raised 22 against the periplasmic TolB protein.

23

#### 24 TOXCAT assay

The TOXCAT assay was performed as described [33,34,69]. *MalE complementation assay*: NT326 cells producing the TOXCAT fusion were streaked onto M9 minimal medium plates supplemented with 0.4% glucose or with 0.4% maltose as the sole carbon source. Plates were incubated for 2 days at 37°C. *Disk diffusion assay*: 20 µL of chloramphenicol (90 mg.mL<sup>-1</sup>) was added on a nitrocellulose 10-

1 mm filter disk (Bio-Rad, 0.2 µm pore size) disposed onto a sterile empty petri dish. After drying, the 2 filter was deposited at the center of a dried LB plate supplemented with ampicillin. Plates were 3 incubated for 6 hours at 37°C before removing the filter disk. 2 mL of a culture of NT326 cells 4 producing the TOXCAT fusion were used to make a lawn and plates were incubated ON at 37°C. The 5 experiments have been performed in triplicate. TOXCAT quantitative assay: NT326 cells producing 6 the TOXCAT fusion were inoculated into 10 mL of LB supplemented with ampicillin and grown to an  $A_{600}=1$  at 37°C with vigorous shaking. 10<sup>10</sup> cells (~ 6  $A_{600}$  units) were harvested by centrifugation and 7 8 washed with 1 mL of sonication buffer (25 mM Tris-HCl, 2 mM EDTA, pH8.0). Cells were then 9 resuspended in 1 mL of sonication buffer and lysed by sonication. CAT activity measurements: 12 µL of lysate were mixed with 300 µL of reaction buffer (0.1 mM acetyl-CoA, 0.4 mg.mL<sup>-1</sup> 5,5'-dithiobis-10 11 (2-nitrobenzoic acid), 0.1 M Tris-HCl, pH7.8), and the absorbance was acquired at  $\lambda$ =412 nm at 10-12 sec intervals for a minimum of 4 min to measure the background rate of acetyl-CoA hydrolysis. Then, 13 12 µL of 2.5 mM chloramphenicol was added, and the absorbance was measured every 10 sec. for 1 14 min. Each lysate was assayed in triplicate, and the slopes of the recorded graphs were converted to 15 enzyme activity units ( $\epsilon$ = 13,600 at  $\lambda$ =412 nm).

16

#### 17 AP20187-mediated FK506-binding protein dimerization

18 Dimerization of FKBP(F36V) was induced by the addition of 20 mM AP20187 (Clinisciences,
19 resuspended in dimethylsulfoxide) in the culture medium.

20

#### 21 **Protein stability assay**

Cultures were grown at 37°C to an  $A_{600} = 0.8$ , and 200 µg.mL<sup>-1</sup> of spectinomycin was added to stop protein synthesis [73]. A sample was harvested at time zero and equivalent samples were harvested 5, 10, 15, 30, 60, 120, 240 and 480 min. after spectinomycin addition. The bacterial density was measured at different time points to verify that no growth occurred. Cell pellets were resuspended in Laemmli loading buffer, heated for 15 min at 96°C, and subjected to 12.5% SDS-PAGE. Westernblotting was performed with goat secondary anti-mouse antibodies coupled to AlexaFluor<sup>®</sup> 680 and

1 images were recorded at  $\lambda$ =700 nm using an LI-COR Odyssey imager. Image analyses were 2 performed with the ImageJ processing program using the Fiji interface [74]. The image was first 3 converted to grayscale in .jpg format. The rectangle tool of ImageJ was used to select a rectangular 4 area of the size corresponding to the lane width, in order to cover the minimal area to contain the 5 whole of the largest band. The same frame was used to select each TssL band of the nitrocellulose 6 membrane. For each selection, the number of pixels was calculated. A control region with no band 7 was also selected to subtract the background. The number of pixels of each band, relative to time 0 8 was then plotted against time. The degradation rate constant (k) was calculated as the slope of the 9 linear curve obtained by plotting the Napierian logarithm of each intensity value against time and 10 fitting to a first-order decay function. Half-life values ( $\tau_{k}$ ) were obtained using the equation  $\tau_{k}$ = 11  $\ln(2)/k$ .

12

#### 13 TssL<sup>TM</sup> peptide synthesis and structural analysis by NMR

14 A synthetic peptide, corresponding to the TssL transmembrane segment (residues Met183-Ala207) 15 with two additional lysine residues at the N- and C-terminus of the peptide (sequence: 16 KKMYWLSWGAGIVTLAGLWCVLSSVLAKK) was synthesized by Schafer-N (Danemark). Two 17 samples were prepared, one in H<sub>2</sub>O and one in D<sub>2</sub>O for proton/deuterium exchange experiments. For 18 each sample 2 mg of peptide were solubilized in 500 µL of D25-Sodium Dodecyl Sulfate 1%, in 10 19 mM KH<sub>2</sub>PO4, 1 mM D10-Dithiothreitol, pH 4.5. TOCSY and NOESY experiments were recorded at 20 300 K using a Bruker Avance III 600 spectrometer equipped with a TCI cryoprobe. Mixing time for 21 the NOESY experiments was 300 ms. The NMR spectra were processed with Topsin software (Bruker) 22 and proton assignment was obtained using the CARA software [75].

23

#### 24 Molecular dynamics simulations

On the basis of NMR data, the sequence corresponding to the synthetic TssL peptide was modelled as
a canonical α-helix using the Deepview software [76]. The structure was duplicated using VMD and a
Tcl script was used to build 8 symmetric dimer conformations by translating and rotating monomers.

1 The resulting structures were in close contact and in parallel arrangements. A peptide-detergent 2 complex was built using the Charmm-gui server [77]. The resulting micelles contained 112 SDS 3 molecules and 17,600 water molecules. The system was neutralized using 112 Na<sup>+</sup> and 8 Cl<sup>-</sup> ions. All 4 the simulations were performed using Gromacs 5.1 with the Charmm 36 forcefield. For the 5 simulations, the default protocol and MD parameters generated by Charmm-gui were conserved. 6 Long range electrostatic forces were computed above 1.2 nm, and a switching function was used for 7 short-range electrostatics forces between 1 and 1.2 nm. The system was minimized using the steepest 8 descent algorithm until the maximum force converges under 1000 kJ/mol/nm. The micelle and the 9 water environment around the peptide were relaxed using several rounds of simulations starting from 10 constrained peptide, then relaxing forces on side-chains and finally on the main chain. The system was 11 thermalized and equilibrated at 300 K using several rounds of simulation over 375 ps. A timestep of 1 12 fs, with the LINCS algorithm applied to constraint bonds that involves H atoms. The temperature was 13 controlled using a multi-coupling scheme separating the peptide and the micelle, with an isotropic 14 Berendsen thermostat and a coupling time constant of 5 ps and 1 ps for heating and equilibration 15 respectively. For the production, a coupling scheme with 10-ns trajectories was produced for the 16 various systems. The simulations were filtered on the basis of MD analysis, TOXCAT and NMR data 17 that resulted on the selection of two simulations that were extended to 100 ns. Finally, simulations of 18 the T194W variant were started from an equilibrated conformation of the wild-type peptide. MD 19 simulations of Thr194 variants were performed after substitution of Thr194 by Trp on the TssL<sup>TM</sup> 20 structure using the Depview software for 20 ns respectively.

21

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#### 14 Legend to Figures

Figure 1. Schematic representation of the T6SS membrane complex and TssJLM proteins. (A) Representation of the T6SS highlighting the cytoplasmic tail structure anchored to the cell envelope by the membrane complex. (B) Negative-stain electron microscopy structural model of the TssJLM membrane complex [14]. (C) Localisation, topology and structure of the TssJ outer membrane lipoprotein, and of the TssL and TssM inner membrane proteins. IM, inner membrane; OM, outer membrane.

Figure 2. TssL<sup>TM</sup> dimerizes. TOXCAT assay. NT316 reporter cells producing the indicated
ToxR-X-MBP fusions (empty, no TMH sequence cloned; GpA<sup>TM</sup>, Glycophorin A TMH;
GpA<sup>TM(G83I)</sup>, GpA<sup>TM</sup> with G83I substitution; TssL<sup>TM</sup>, TssL TMH) were streaked on M9
minimal medium supplemented with glucose (A) or maltose (B) as sole carbon source.

Resistance to chloramphenicol (cam) conferred by the TM-mediated dimerization of the ToxR transcriptional regulator was measured by the disk assay (C, halo diameter mean ± standard deviation [SD] in mm) and quantitative chloramphenicol acetyltransferase (Cat) assay (D). The mean (value relative to the value obtained for the "empty" vector) is indicated on top on each bar.

Figure 3. Thr194 and Trp199 contribute to TssL<sup>TM</sup> dimerization. (A) Sequence of the 6 TssL<sup>TM</sup> peptide. The numbering is relative to the full-length TssL protein. The transmembrane 7 segment is indicated by the green frame and the leucine residues participating to a putative 8 9 leucine zipper are coloured red. The two leucine residues substituted in the LALA mutant ware underlined. (B) TOXCAT disk assay of the TssL<sup>TM</sup> fragment and its LALA derivative. 10 11 The diameter of the halo is indicated on bottom (mean  $\pm$  SD in mm). The quantitative Cat 12 assay measures are reported in panel D. TOXCAT disk (C) and quantitative Cat (D) assays of the TssL<sup>TM</sup> fragments bearing the indicated substitutions. The level of activity corresponding 13 14 to the empty plasmid is indicated by the red dotted line.

Figure 4. TM-mediated contacts stabilize TssL dimer on SDS-PAGE.  $2 \times 10^8 \Delta tssL$  cells producing FLAG-tagged full-length TssL or the indicated TssL variants (TssL<sub>C</sub>, cytoplasmic domain of TssL; T194W and W199A, substitution variants of the TM Thr194 and Trp199 residues respectively) were subjected to 12.5% SDS-PAGE and immunoblotting with the monoclonal anti-FLAG antibody. The position of TssL, TssL<sub>C</sub> and TssL dimers (\*) are indicated on right. Molecular weight markers are indicated on left.

Figure 5. The Trp185 and Trp199 rings are protected in NMR Hydrogen-Deuterium exchange experiments. (A) Zoom-in of the H<sub>N</sub>/H<sub>N</sub> region of the NOESY spectrum of the TssL<sup>TM</sup> peptide (2 mM) at 300K. The NMR sample was in phosphate buffer pH4.5 and SDS

1% (H<sub>2</sub>O conditions). Cross peaks (nuclear Overhauser effects) corresponding to coupling 1 2 between H<sub>N</sub> of residues i and i+1 are observed. The assignment is shown in Table S1 and Fig. S1. (B and C) Overlay of the NOESY spectra of the TssL<sup>TM</sup> peptide in H<sub>2</sub>O (blue) and D<sub>2</sub>O 3 (red). H<sub>N</sub> chemical shift assignment was indicated by residues sequence numbering. In D<sub>2</sub>O, 4 5 the exposed protons are exchanged with Deuterium and thus disappeared; however the 6 protected protons are not exchanged and thus are still observable. In (B) the regions of H<sub>N</sub> (x 7 axis)/ H<sub>CA</sub> (y axis) correlations is shown, and in (C) H<sub>N</sub> of rings of tryptophan residues are shown. (D) Sequence of the TssL<sup>TM</sup> peptide. The numbering is relative to the full-length 8 9 TssL protein. The transmembrane segment is indicated by the green frame, and the tryptophan 10 residues protected in H-D exchange experiments (Trp185 and Trp199) are coloured red. (E) Structural model of the TssL<sup>TM</sup> monomer in SDS micelles. The orientation of the 11 transmembrane segment relative to the membrane is shown. Residues located at the TssL<sup>TM</sup> 12 13 interface (indicated on right), as well as residue Thr194 are represented by spheres 14 (tryptophan residues in grey, leucine zipper residues in pink). (F) Representation of the structural model of TssL<sup>TM</sup> dimer in SDS micelles obtained by MD simulations of the TssL<sup>TM</sup> 15 16 monomer. The orientation of the two transmembrane segments relative to the membrane is 17 shown. The Trp185, Trp188 and Trp199 rings are represented by spheres and pointed with 18 arrowheads, highlighting the packing of the two Trp199 rings at the periplasmic side and of 19 the four rings from Trp185 and Trp188 at the cytoplasmic side.

Figure 6. The Trp185 and Trp199 rings control tight packing of the TssL<sup>TM</sup> dimer. (A) Superimposition of the averaged structures of TssL<sup>TM</sup> monomerA obtained from the molecular dynamic simulations of 8 different TssL<sup>TM</sup> dimers. The Thr194 and Trp199 residues are shown in spheres. (B and C) For the eight simulations of the dimer, (labeled from 0° to 350°), the superimposed structure of monomer A (ribbon) is represented at the center. The Thr194 and Trp199 side chains corresponding to the reference simulation (0°) are shown in spheres. The trajectory traces of the Thr194 (B) and Trp199 (C) side chains of monomer B
are shown at the periphery of the central helix. (D and E) Top panels: distances (in nm)
between the two residues Y184 (black), Trp185 (red), Trp188 (green), Thr194 (blue), or
Trp199 (yellow) in the dimer during 100-ns MD simulations starting from the 0° (D) and 350°
(E) conformations. Bottom panels: final structures obtained after the 100-ns MD simulations
starting from the 0° (D) and 350° (E) conformations. Residues are shown in spheres with the
same color code than top panels.

8 Figure 7. Trp199 is required for T6SS function. (A) Hcp release assay. The cell pellet (C) and supernatant (S) fractions from  $5 \times 10^8$  cells of the wild-type 17-2 or  $\Delta tssL$  derivative, or 9 10  $\Delta tssL$  producing FLAG-tagged full-length TssL (tssL+) or the indicated TssL variants, and 11 HA-tagged Hcp, were subjected to 12.5% SDS-PAGE and immunoblotted with anti-TolB 12 (periplasmic leakage control, upper panel), anti-HA (to detect Hcp, middle panel) and anti-13 FLAG (to detect TssL, lower panel) antibodies. Molecular weigh markers are indicated on the 14 left. (B) Anti-bacterial activity. The indicated attacker strains were mixed with kanamycin resistant GFP<sup>+</sup> prey cells and spotted on SIM medium. After incubation, the fluorescent image 15 16 of the spot was recorded (middle panel) and level of surviving prev cells was estimated by measuring the fluorescence (upper graph) and by counting the number of colonies on 17 18 kanamycin plates (lower graph).

**Figure 8. Trp199 stabilizes TssL.** (A)  $5 \times 10^8$ -cell samples of the  $\Delta tssL$  strain producing FLAG-tagged full-length TssL or the indicated TssL variants were harvested at the indicated time (in min) after protein synthesis arrest and were subjected to 12.5% SDS-PAGE and immunoblotting with anti-FLAG antibody, and secondary antibody coupled to AlexaFluor 680. Molecular weigh markers are indicated on the left. The relative intensity of each band 1 was plotted against time (B) and half-times of TssL and TssL variants (in min) were
2 calculated from the slope of the first-order decay function (C).

Figure 9. Trp199-mediated TssL<sup>TM</sup> dimerization is required for TssL stability and T6SS 3 4 function. (A) Schematic representation of the TssL-W199A-F<sub>V</sub> chimera protein. The TssL 5 full-length protein carrying the W199A substitution (schematized with the red star) was fused 6 to the FKBP F36V variant domain (F<sub>V</sub>, green). F<sub>V</sub> dimerization could be induced by the 7 rapamycin homologue AP20187 (AP, pink diamond). P, periplasm; C, cytoplasm. (B) TOXCAT disk (upper panel) and quantitative Cat (lower panel) assays of W199A TssL<sup>TM</sup> 8 9 fused to  $F_V$  (W199A- $F_V$ ) in absence (-) or presence (+) of the FKBP ligand inducer AP20187 (AP). (C) Hcp release assay. The cell pellet (C) and supernatant (S) fractions from  $5 \times 10^8$ 10  $\Delta tssL$  cells producing FLAG-tagged W199A-F<sub>V</sub> and HA-tagged Hcp, grown in absence (-) or 11 12 presence (+) of AP were subjected to 12.5% SDS-PAGE and immunoblotted with anti-TolB 13 (periplasmic leakage control, upper panel), anti-HA (to detect Hcp, middle panel) and anti-FLAG (to detect TssL-F<sub>V</sub>, lower panel) antibodies. Molecular weigh markers are indicated on 14 15 the left. (D) Anti-bacterial activity. The  $\Delta tssL$  attacker strain producing W199A-F<sub>V</sub> was mixed with kanamycin resistant GFP<sup>+</sup> prey cells and spotted on SIM medium supplemented 16 17 (+) or not (-) with AP. After incubation, the fluorescent image of the spot was recorded 18 (middle panel) and level of surviving prey cells was estimated by measuring the fluorescence 19 (upper graph) and by counting the number of colonies on kanamycin plates (lower graph). (E)  $5 \times 10^8$ -cell samples of the  $\Delta tssL$  strain producing TssL-W199A-F<sub>V</sub>, grown in absence (-) or 20 presence (+) of AP were harvested at the indicated time (in min) after protein synthesis arrest 21 22 and were subjected to 12.5% SDS-PAGE and immunoblotting with anti-FLAG antibody, and secondary antibody coupled to AlexaFluor<sup>®</sup> 680. Molecular weigh markers are indicated on 23 24 the left. The relative intensity of each TssL-F<sub>V</sub> band was plotted against time (F) and halftimes of W199A- $F_V$  were calculated from the slope of the first-order decay function (G). 25

1 Figure 10. Sequence alignment of the TMHs from selected TssL homologues. The 2 sequences of the TMHs of the T6SS-associated TssL proteins and T4bSS-associated 3 IcmH/DotU proteins from strains listed on left (phylogeny realised based on the full-length 4 TssL/IcmH sequences using the server at www.phylogeny.fr) were aligned using Clustal W 5 (right panel). The position of the TMH (from experimental data for EAEC [25] or identified 6 using the TMHMM and TMPred TMH prediction servers) is underlined in green. Tryptophan 7 and other aromatic residues located at the N- and C-termini of the TMH sequences are 8 indicated in red and blue respectively. The residues participating in  $\pi$ - $\pi$  packing for EAEC 9 TssL TMH dimerization (Y184, W185, W188 and W199; this study) are underlined.

### 1 Legend to Supplementary Figures

Supplementary Figure S1. Assignment of the TssL<sup>TM</sup> NOESY spectrum. The H<sub>N</sub>/H<sub>N</sub>
region of the NOESY spectrum of the TssL<sup>TM</sup> peptide in H<sub>2</sub>O is shown (same as Fig. 5A).
The cross peak correlations are indicated by sequential numbering of the TssL<sup>TM</sup> residues.

Supplementary Figure S2. Instability in the TssL<sup>TM</sup> T194W mutant. (A) Contact map for
the TssL<sup>TM</sup> dimer (left) and the T194W mutant dimer (right). (B and C) Lateral (B, left panel)
and top (B, right panel) views and root mean square deviation (RMSD, in Å) (C) of the
orientation of monomer B relative to the fixed monomer A during the course of the 20-ns
molecular dynamics simulation of the T194W mutant.























Figure 8











Residue	$\mathbf{H}_{\mathbf{N}}$	Ha	Hb	Others
Lys-2	7.62	3.70		Hg 1.03; 0.87; He 2.13
Lys-1	8.44	3.85		Hd 1.62; Hg 1.44;0.85
Met183	8.30	3.57	3.38; 1.60	
Tyr184	7.36	4.50	3.96; 4.18	Hd 7.49; 7.00 He 7.33; 6.90
Trp185	8.60	4.48	3.34; 3.3	Hd 7.14 He 9.84; 7.81 Hh 6.98 Hz 7.37; 6.80
Leu186	8.11	4.04	1.77; 1.67	Hg 1.52 Hd 0.90; 0.80
Ser187	7.14	4.10	3.85	
Trp188	7.78	4.37	3.12; 3.29	Hd 7.18 He 9.87; 7.48 Hh 6.99 Hz 7.37; 6.80
Gly189	8.32	3.64		
Ala190	7.88	4.13	1.33	
Gly191	8.11	3.85		
Ile192	7.71	3.99	1.75	Hg 1.59; 1.34; Hd 0.64; 0.79
Val193	7.97	4.23	2.2	Hg 1.76
Thr194	7.78	4.16		Hg 1.42
Leu195	7.50	4.09	2.04	Hd 0.89; Hg 1.35
Ala196	8.33	4.10	1.40	
Gly197	8.17	3.75	3.62	
Leu198	7.70	4.13		Hd 0.98;0.82; Hg 2.13
Trp199	7.45	4.18	4.02	Hd 7.16; He 9.79;7.46; Hh 6.97; Hz 7.31;6.63
Cys200	7.99	3.98	2.18; 1.73	
Val201	8.52	4.1	1.64	Hg 1.31
Leu202	7.98	4.15	1.81; 1.60	Hg 1.31
Ser203	7.70	4.13	3.70	
Ser204	8.10	3.85	3.08; 3.00	
Val205	8.21	3.75		Hg 1.6
Leu206	8.29	3.86	1.85; 1.61	Hg 1.38
Ala207	7.69	4.13	0.98	
Lys+1	7.67	4.26	1.69	Hd 0.98; He 2.12
Lys+2	7.16	4.67	1.60	He 2.89

Table S1. Sequential <sup>1</sup>H assignment of the TssL<sup>TM</sup> peptide (residues 183-207) at 300K and pH4.5

Supplementary Table S2. Strains, plasmids and oligonucleotides used in this study.

### Strains

Strains	Description and genotype	Source
<u>E. coli K-12</u>		
DH5a	F-, $\Delta(argF-lac)$ U169, phoA, supE44, $\Delta(lacZ)$ M15, relA, endA, thi, hsdR	New England Biolabs
W3110	F-, lambda- IN( <i>rrnD-rrnE</i> )1 <i>rph-</i> 1	Laboratory collection
NT326	F-, Δ(argF-lac)U169, rpsL, relA, rbsR, flbB, ptsF,thi, deoC, ΔmalE444, recA	[68]
Enteroaggregative E. coli		
17-2	WT enteroaggregative Escherichia coli	Arlette Darfeuille-Michaud
$17-2\Delta tssL$	17-2 deleted of the <i>tssL</i> gene of the <i>sci1</i> T6SS gene cluster	[20]
	-	

# Plasmids

Vectors	Description	Source
Expression vectors		
pASK-IBA37	cloning vector, Ptet, Amp <sup>R</sup>	IBA Technology
pASK-IBA37-TssL <sub>FL</sub>	scil tssL cloned into pASK-IBA37, C-terminal FLAG epitope	[25]
pASK-IBA37-TssL <sub>C</sub>	Cytoplasmic domain (residues 1-183) of scil tssL cloned into pASK-IBA37,	
	N-terminal FLAG epitope	[25]

pASK-IBA37-TssL <sub>FL</sub> -T194W	Thr194 to Trp into pASK-IBA37-TssL <sub>FL</sub>	This study
pASK-IBA37-TssL <sub>FL</sub> -W199A	Trp199 to Ala into pASK-IBA37-TssL <sub>FL</sub>	This study
pASK-IBA37-TssL <sub>FL</sub> -W199A-FK	FK506 binding protein fused to TssL into pASK-IBA37-TssL <sub>FL</sub>	This study
pOK-HcpHA	scil hcp cloned into pOK12, C-terminal HA epitope	[20]
pUA66-rrnb	<i>PrrnB</i> ::gfpmut2 transcriptional fusion in pUA66, Kan <sup>R</sup>	[71]

### Vectors for TOXCAT assay

pcckan	ToxR-dependent expression of <i>cat</i>	[33]
pccGpA	pcckan plasmid with glycophorin A TMH inserted between ToxR and MBP	[33]
pccGpG83I	Gly83 to Ile into pccGpA	[33]
pcc-TssL-TM	pcckan plasmid with TssL TMH (residues 174-217) inserted between ToxR and MBP	This study
pcc-TssL-LALA	Leu-195 and Leu-198 to Ala into pcc-TssL-TM	This study
pcc-TssL-M183W	Met-183 to Trp into pcc-TssL-TM	This study
pcc-TssL-Y184W	Tyr-183 to Trp into pcc-TssL-TM	This study
pcc-TssL-W185A	Trp-185 to Ala into pcc-TssL-TM	This study
pcc-TssL-L186W	Leu-186 to Trp into pcc-TssL-TM	This study
pcc-TssL-S187W	Ser-187 to Trp into pcc-TssL-TM	This study
pcc-TssL-W188A	Trp-188 to Ala into pcc-TssL-TM	This study
pcc-TssL-G189W	Gly-189 to Trp into pcc-TssL-TM	This study
pcc-TssL-A190W	Ala-190 to Trp into pcc-TssL-TM	This study
pcc-TssL-G191W	Gly-191 to Trp into pcc-TssL-TM	This study
pcc-TssL-I192W	Ile-192 to Trp into pcc-TssL-TM	This study
pcc-TssL-V193W	Val-193 to Trp into pcc-TssL-TM	This study
pcc-TssL-T194W	Thr-194 to Trp into pcc-TssL-TM	This study
pcc-TssL-L195W	Leu-195 to Trp into pcc-TssL-TM	This study
pcc-TssL-A196W	Ala-196 to Trp into pcc-TssL-TM	This study
pcc-TssL-G197W	Gly-197 to Trp into pcc-TssL-TM	This study
pcc-TssL-L198W	Leu-198 to Trp into pcc-TssL-TM	This study
pcc-TssL-W199A	Trp-199 to Ala into pcc-TssL-TM	This study
pcc-TssL-C200W	Cys-200 to Trp into pcc-TssL-TM	This study

pcc-TssL-V201W	Val-201 to Trp into pcc-TssL-TM	This study
pcc-TssL-L202W	Leu-202 to Trp into pcc-TssL-TM	This study
pcc-TssL-S203W	Ser-203 to Trp into pcc-TssL-TM	This study
pcc-TssL-S204W	Ser-204 to Trp into pcc-TssL-TM	This study
pcc-TssL-V205W	Val-205 to Trp into pcc-TssL-TM	This study
pcc-TssL-L206W	Leu-206 to Trp into pcc-TssL-TM	This study
pcc-TssL-W199A-FK	FK506 binding protein fused to TssL-TM into pcc-TssL-W199	This study

# Oligonucleotides

Name	Destination	Sequence (5'	· → 3')
For plasmid construction			
5-TssLTM-NheI 3-TssLTM-BamHI	insertion of T insertion of T	ssL-TM into pcckan ssL-TM into pcckan	GGTGGTGGTGCGTGCTAGCGCACCGGGTTACCGTG CGTACGTGCATGGTTAGGATCCCTCCCTGCCCGGTA AGCC
5-pcckn-L-TM-FKBP	FK506 bindir	g protein fused to TssL-TM	CAGGTGGCACGGCTTACCGGGCAGGGAGGGCTC GAGATGGCTTCTAGAGGAGTGCAGGTG
3-pcckn-L-TM-FKBP	FK506 bindir	g protein fused to TssL-TM	GTTTAAAGCTGGATTGGCTTGGGTTGATCAGGA TTCATTCCAGTTTTAGAACCTTCACATCG
5-IBA-TssL-LFKBP	FK506 bindir	g protein fused to TssL	CAGGTGGCACGGCTTACCGGGGCAGGGACTCGAGATGG CTTCTAGAGGAGTGCAGG
3-IBA-TssL-FKBP	FK506 bindir	g protein fused to TssL	GATGGTGATGCGATCCTCTGCTAGCTCATTCCAGTTTT AGAACCTTCACATCGAAG
For site directed mutagenesis			
5-TssL-L195A-L198A	Leu195 to Ala	a and Leu198 to Ala	GGGGCGGGTATTGTGACG <u>GCG</u> GCTGGT <u>GCC</u> TGGTGTG TGCTTTCTTC

3-TssL-L195A-L198A	Leu195 to Ala and Leu198 to Ala	GAAGAAAGCACACACCAG <u>GCA</u> CCAGCC <u>GCC</u> GTCACAA TACCCGCCCC
5-A-M183W	Met183 to Trp	GTTACCGTGCTGGCAGAACG <u>TGG</u> TACTGGTTGTCATGG GGG
5-A-Y184W	Tyr183 to Trp	GTTACCGTGCTGGCAGAACGATG <u>TGG</u> TGGTTGTCAT GGGGGGCG
5-A-W185A	Trp185 to Ala	GTTACCGTGCTGGCAGAACGATGTAC <u>GCG</u> TTGTCATG GGGGGCGGG
5-A-L186W	Leu186 to Trp	GTTACCGTGCTGGCAGAACGATGTACTGG <u>TGG</u> TCAT GGGGGGCGGGTAT
5-A-S187W	Ser187 to Trp	GTTACCGTGCTGGCAGAACGATGTACTGGTTG <u>TGG</u> TG GGGGGCGGGTATTGTG
3-A-M183-S187		TCTGCCAGCACGGTAACCCGGTGCACGCAC
5-B-W188A	Trp188 to Ala	GAACGATGTACTGGTTGTCA <u>GCG</u> GGGGGGGGGGTATTGT GAC
5-B-G189W	Gly189 to Trp	GAACGATGTACTGGTTGTCATGG <u>TGG</u> GCGGGTATTGTG ACG
5-B-A190W	Ala190 to Trp	GAACGATGTACTGGTTGTCATGGGGGG <u>TGG</u> GGTATTGT GACGCT
5-B-G191W	Gly191 to Trp	GAACGATGTACTGGTTGTCATGGGGGGGGGCG <u>TGG</u> ATTGT GACGCTGGC
5-B-I192W	Ile192 to Trp	GAACGATGTACTGGTTGTCATGGGGGGGGGGGGT <u>TGG</u> GT GACGCTGGCTGG
3-B-W188-I192		CAACCAGTACATCGTTCTGCCAGCACGGTA
5-C-V193W	Val193 to Trp	TGTCATGGGGGGGGGGGGGTATT <u>TGG</u> ACGCTGGCTGGTCTC TGG
5-C-T194W	Thr194 to Trp	TGTCATGGGGGGGGGGGGGTATTGTG <u>TGG</u> CTGGCTGGTCTC TGGTG
5-C-L195W	Leu195 to Trp	TGTCATGGGGGGGGGGGGGTATTGTGACG <u>TGG</u> GCTGGTCTC TGGTGTG
5-C-A196W	Ala196 to Trp	TGTCATGGGGGGGGGGGGGTATTGTGACGCTG <u>TGG</u> GGTCTC TGGTGTGTG

5-C-G197W	Gly197 to Trp	TGTCATGGGGGGGGGGGGTATTGTGACGCTGGCT <u>TGG</u> CTC TGGTGTGTGCTT
3-C-V193-G197		ACCCGCCCCCATGACAACCAGTACATCGT
5-D-L198W	Leu198 to Trp	GTATTGTGACGCTGGCTGGT <u>TGG</u> TGGTGTGTGCTTTCT TCT
5-D-W199A	Trp199 to Ala	GTATTGTGACGCTGGCTGGTCTC <u>GCG</u> TGTGTGCTTTCT TCTGTG
5-D-C200W	Cys200 to Trp	GTATTGTGACGCTGGCTGGTCTCTGG <u>TGG</u> GTGCTTTCT TCTGTGCT
5-D-V201W	Val201 to Trp	GTATTGTGACGCTGGCTGGTCTCTGGTGT <u>TGG</u> CTTTCTT CTGTGCTTGC
5-D-L202W	Leu202 to Trp	GTATTGTGACGCTGGCTGGTCTCTGGTGTGTG <u>TGG</u> TCT TCTGTGCTTGCAG
3-D-L198-L202		AGCCAGCGTCACAATACCCGCCCCCATGA
5-E-S203W	Ser203 to Trp	CTGGTCTCTGGTGTGTGTGCTT <u>TGG</u> TCTGTGCTTGCAGAC CAG
5-E-S204W	Ser204 to Trp	CTGGTCTCTGGTGTGTGTGCTTTCT <u>TGG</u> GTGCTTGCAGAC CAGGTG
5-E-V205W	Val205 to Trp	CTGGTCTCTGGTGTGTGTGCTTTCTTCT <u>TGG</u> CTTGCAGACC AGGTGGCA
5-E-L206W	Leu206 to Trp	CTGGTCTCTGGTGTGTGTGCTTTCTTCTGTG <u>TGG</u> GCAGAC CAGGTGGCACG
3-E-S203-L206		CACACCAGAGAGACCAGCCAGCGTCACAAT