

Coevolution-Guided Mapping of the Type VI Secretion Membrane Complex-Baseplate Interface

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Abstract

The type VI secretion system (T6SS) is a multiprotein weapon evolved by Gram-negative bacteria to deliver effectors into eukaryotic cells or bacterial rivals. The T6SS uses a contractile mechanism to propel an effector-loaded needle into its target. The contractile tail is built on an assembly platform, the baseplate, which is anchored to a membrane complex. Baseplate-membrane complex interactions are mainly mediated by contacts between the C-terminal domain of the TssK baseplate component and the cytoplasmic domain of the TssL inner membrane protein. Currently, the structural details of this interaction are unknown due to the marginal stability of the TssK-TssL complex. Here we conducted a mutagenesis study based on putative TssK-TssL contact pairs identified by co-evolution analyses. We then evaluated the impact of these mutations on T6SS activity, TssK-TssL interaction and sheath assembly and dynamics in enteroaggregative *Escherichia coli*. Finally, we probed the TssK-TssL interface by disulfide cross-linking, allowing to propose a model for the baseplate-membrane complex interface.

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Introduction

The type VI secretion system (T6SS) is a molecular nanoweapon that uses a contractile mechanism to propel an effector-loaded needle into bacterial and/or eukaryotic target cells.^{1–4} The T6SS is comprised of two complexes.^{2,4–6} The tail structure is constituted of the baseplate (BP) complex on which the tube-sheath complex is polymerized.^{7–9} The BP complex is made of six TssEFGK wedges surrounding the VgrG spike.^{7–11} The tail tube-sheath complex is composed of the inner tube, made of stacked Hcp hexamers, wrapped by the TssBC sheath.^{12–14} The base of the VgrG spike serves as initiator for the polymerization of the inner

tube, while the wedge complexes prime sheath polymerization.^{15–16} The sheath polymerizes under an extended conformation, and contracts upon contact with the target cells or in response to other signals, propelling the tube-spike needle, loaded with effectors, into the target.^{6,13,17–23} The passage of the tube-spike needle across the cell envelope of the attacker cell necessitates the membrane complex (MC), which also serves as docking station for the baseplate.^{8,24} The membrane complex is composed of the TssL and TssM inner membrane proteins, and of the TssJ outer membrane lipoprotein.^{24–29} Interestingly, the tail and membrane complexes present different evolutionarily histories. The TssL and TssM subunit of the MC share

homologies with components of the Type IVb secretion system.^{30–32} By contrast, with the exception of the TssK subunit, the tail proteins are conserved within the large family of contractile injection systems, which also include members of the Myoviridae bacteriophages and R-type pyocins.^{9,13,16,33–}

³⁵ The trimeric TssK wedge subunit is functionally and structurally related to receptor-binding proteins (RBP) of short non-contractile phages.³⁶ ⁷ RBPs are surface proteins that recognize cell surface receptors allowing specific phage binding to target cells.^{38–39} RBPs are comprised of an N-terminal shoulder domain that is anchored into the baseplate, and a C-terminal head domain that binds specifically to receptors.³⁹ The T6SS TssK proteins has a similar organization, with an N-terminal domain anchored to the baseplate, and a Cterminal domain that binds to the MC.7-8,36-37,40 This interaction allows the BP to be recruited to the MC docking station but also properly positions the tail structure in order to propel the tube-spike complex across the MC channel.8,36-37 While recent studies have provided important information on MC and BP composition and structures, molecular details on how these two T6SS complexes are connected remains incomplete. Boyer et al. noted that the tssK and tssL genes co-occur in most T6SS gene clusters,³² suggesting that they have coevolved to properly adapt the baseplate onto the membrane complex. Indeed, protein-protein interaction studies have shown that TssK interacts with the TssL cytoplasmic domain.^{8,36} Further works demonstrated that TssK-TssL complex formation involve the TssK C-terminal head domain and the TssL L2-L3 loop.^{37,40} Here, to gain further information on MC-BP contacts, we conducted a rationally-guided mutagenesis based on coevolution prediction of TssK/TssL residue pairs. The coevolution strategy proposed four sites of contacts between the two proteins that were tested by phenotypic and protein-protein interaction assays, allowing to propose a model of the MC-BP interface.

Material and methods

Bacterial strains, media, chemicals and growth conditions. Strains used in this study are listed in Table S1. E. coli K-12 strain DH5a was used for all cloning procedures, BTH101 for bacterial two-hybrid assays, and W3110 for coimmunoprecipitation experiments. The enteroaggregative E. coli (EAEC) strains used in this study are all derivatives of the wild-type strain 17-2. The 17-2 tssB-sfGFP, ΔtssK, ΔtssK tssBsfGFP, $\Delta tssL$, and $\Delta tssL$ tssB-sfGFP have been previously described.^{8,24,26,36} E. coli cells were routinely grown in Lysogeny Broth (LB) supplemented with antibiotics when necessary (kanamycin 50 µg. mL⁻¹, chloramphenicol 40 μ g.mL⁻¹, ampicillin 100 μ g.mL⁻¹. For T6SS expression, EAEC cells were grown in Sci1-inducing medium (SIM; M9 minimal medium supplemented with glycerol 0.25%, vitamin B1 200 μ g.mL⁻¹, casaminoacids 40 μ g.mL⁻¹, MgCl₂ 2 mM, CaCl₂ 0.1 mM, and LB (10% v/ v)).⁴¹ Expression from pBAD and pTrc99A vectors was induced with L-arabinose (0.05%) and Isopropyl β -D-1-thiogalactopyranoside (IPTG, 200 μ M), respectively. Dichloro (1,10-phenanthroline) copper(II) (Cu-oP) and *N*-ethylmaleimide (NEM) were purchased from Sigma-Aldrich.

Plasmid construction. Plasmids used in this study are listed in Table S1. Polymerase Chain Reactions (PCR) were performed using a Biometra thermocycler using Q5 DNA polymerase (New England Biolabs). Custom oligonucleotides, listed in Table S1, were synthesized by Sigma Aldrich. Bacterial two-hybrid vectors producing TssK or TssL fused to the Bordetella adenviate cyclase T18 or T25 domains, and plasmid pBAD-TssK^V, producing TssK fused to a C-terminal VSV-G, have been previously published.³⁶ Plasmid pTrc-TssLST, producing TssL fused to a C-terminal Streptag II, was constructed by restriction free (RF) cloning.⁴² Briefly, the DNA fragment corresponding to the *tssL* gene was amplified using primers that introduced extensions annealing to the target vector. The double-stranded product of the first PCR has then been used as primer for a second PCR using the target vector as template. PCR products were then treated with DpnI to eliminate template plasmids and transformed into DH5α-competent cells. The pKO3-tssL vector was engineered by restriction/ligation cloning of a BamHI-Sall DNA fragment encompassing the tssL gene into pKO3.43 Substitutions were introduced by site-directed mutagenesis using complementary oligonucleotides bearing the desired mutation. All plasmids have been verified by DNA sequencing (Eurofins).

Strain engineering. The $\Delta tssKL$ strain was constructed bv the one-step inactivation procedure⁴⁴ using λ -red recombination carried by vector pKOBEG,⁴⁵ and a PCR product corresponding to the pKD4 kanamycin cassette⁴⁴ flanked by 50-bp extensions annealing upstream and downstream the region to be deleted. Recombinant strains were selected on LB plates supplemented with kanamycin, and the kanamycin cassette was excised mutations by the Flp FRT recombinase carried by vector pCP20.44 Strains 17-2 bearing point mutations at the chromosomal tssL locus have been engineered using the suicide vector pKO3.43 The first and second recombination events were selected on LB plates supplemented with chloramphenicol at 42 °C, and supplemented with sucrose (5%), respectively, as previously described.⁴³ All strains were verified by colony-PCR using various oligonucleotide pair combinations. Strains with chromosomal point mutations were further verified by PCR amplification of the region of interest and DNA sequencing.

Protein-protein interaction assays. Bacterial two-hybrid assays were conducted as previously

described.³⁶ Pull-down experiments were performed as previously described⁴⁰ with modifications: protein samples were subjected to pull-down on StrepTactin sepharose (Cytiva) and CellLytic B buffer (diluted 1/5, Sigma-Aldrich) was added in the washing buffer.

Disulfide bond formation assay. Disulfide bond formation assays were performed as previously published.^{14–15} Briefly, cells producing the *tssK* and *tssL* cysteine derivatives were grown to an absorbance at $\lambda = 600$ nm (A_{600}) of ~0.6 and then incubated with 0.3 mM of Cu-oP for 20 min without agitation. About 3×10^{10} cells were then harvested by centrifugation and resuspended in 0.5 mL of 10 mM HEPES (pH7.4), 30% sucrose, 1 mM EDTA and 2.5 mM NEM for 30 min on ice to block free thiol groups. Cells were pelleted by centrifugation and mixed with loading buffer prior to analysis by SDS-PAGE and immunoblotting.

Anti-bacterial assays. The antibacterial growth competition assay was performed as described⁴⁶ with modifications. The WT E. coli strain W3110 bearing the pUA66-rrnB plasmid 47 was used as recipient in the competition assay. The kanamycin-resistant pUA66-rrnB plasmid provides a strong constitutive green fluorescent (GFP⁺) phenotype. Attacker and recipient cells were grown in SIM medium to an A_{600} of 0.6–0.8, harvested and normalized to a A_{600nm} of 0.6 in SIM. Attacker and recipient cells were mixed to a 4:1 ratio and 10-µL drops of the mixture were spotted in triplicate onto a prewarmed dry SIM agar plate. After 4-hour incubation at 37 °C, fluorescent images were recorded with a LI-COR Odyssey imager. The bacterial spots were scratched off, cells were resuspended in LB medium supplemented with kanamycin, normalized to an A₆₀₀ of 0.5 and serially diluted. One hundred μL of serial dilutions were then spread in triplicate on LB plates supplemented with kanamycin to select surviving recipients. Colony forming units were numbered after overnight incubation at 37 ° C. The experiment was done in triplicate.

Live fluorescence microscopy. Cells were grown in SIM medium at 37 °C to an A₆₀₀ of 0.6-0.8, resuspended in fresh SIM to an $A_{600 \text{ nm}}$ of 10, and 5 µL were spotted onto a thin pad of SIM supplemented with 2% agarose covered with a glass coverslip. Phase contrast and fluorescence were recorded on a Nikon Eclipse Ti2 microscope equipped with a 100 \times NA 1.45 Ph3 objective, an Orca-Fusion digital camera (Hamamatsu) and a perfect focus system. All fluorescence images were acquired with a minimal exposure time to minimize bleaching and phototoxicity effects. Exposure times were typically 30 ms for phase for contrast. 100 ms TssB-sfGFP. The experiments were performed at least in triplicate and representative results are shown. Images were analyzed using ImageJ (https://imagej. nih.gov/ij/) and the MicrobeJ v5.11y plugin (https:// www.microbej.com/). Statistical dataset analyses were performed with several representative fields from at least three independent biological replicates, using Excel and the R software environment. The number of measured cells or events (n) is indicated on each figure.

Bioinformatics analyses. To predict residue pairs in contact between two protein domains, we combined the RaptorX complex contact prediction web server⁴⁸ and the GREMLIN monomer contact prediction web server.⁴⁹ We queried the sequences of the two protein domains in RaptorX, obtaining a multiple sequence alignment (MSA) of the two concatenated sequences. The MSA is used as input in GREMLIN, which returns a list of coevolving residue pairs ranked by their contact probability.

To obtain accurate interactions, we determined a contact probability threshold using the EAEC T6SS wedge complex structure¹⁰ as a benchmark in a binary classification study. First, we obtained pairs of coevolving residues between TssK and TssG and between TssG and TssF. The known structure of the wedge complex (PDB: 6N38) was used to calculate the distance between the residues of each pair (minimum distance between all heavy atoms). The pairs of residues were classified into close pairs, when the corresponding distance was below 8 Å, and distant pairs, when the distance was above 8 Å. Additionally, by defining an arbitrary contact probability threshold, the pairs were classified as predicted interacting pairs, when the corresponding contact probability was above the threshold, and predicted non-interacting pairs, contact probability below the threshold. Hence, given a contact probability threshold, close pairs predicted as interacting were considered as true positives (TP), and close pairs predicted as non-interacting are false negative (FN). Similarly, false positives (FP) and true negatives (TN) correspond to distant pairs predicted as interacting or non-interacting, respectively. Finally, we computed the precision (TP/(TP + FP)) of the binary classification for a given contact probability threshold. To determine the high-confidence threshold, we maximized TPs while minimizing the FPs. Setting a probability threshold of 0.9 and selecting all pairs with a probability above this threshold allowed us to select no FP's (Supplementary Figure 1(a)) and a maximal precision (Supplementary Figure 1(b)). Hence, we used the high-confidence threshold to filter the interactions predicted for TssK-Ct, and TssL_C. All the data and scripts used for this methods section were deposited in Git repository (https://github.com/ishon92/ CoevolutionTssKL.git).

Information-driven docking simulations were performed with the standard parameters on the HADDOCK 2.4 web server,⁵⁰ implemented by imposing ambiguous distance constraints based on the results of disulfide cross-linking data (active residues) using GenTBL.⁵¹ AlphaFold2 structural models were generated using the Colabfold server using standard parameters.^{52–54} **Miscellaneous.** Standard methods were used for sodium dodecyl-sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) and protein transfer on nitrocellulose membranes. Membranes were probed with Strep-Tag Classic (clone Strep-tag II, Bio-Rad), and anti-VSV-G (clone P5D4, Sigma-Aldrich) monoclonal antibodies, and goat antimouse secondary antibodies conjugated to Alkaline Phosphatase (Beckman Coulter). Alkaline phosphatase signal was developed in alkaline buffer (pH 9) in the presence of 10 mM MgCl₂, 0.15 mg/mL of 5-bromo-4-chloro-3-indolylpho sphate and 0.3 mg/mL of nitroblue tetrazolium.

Results and discussion

Co-evolution analyses propose a potential interface between the TssK C-terminal domain and the TssL cytoplasmic domain

To better understand how the T6SS baseplate is connected to the membrane complex (Figure 1(a)), we first conducted bioinformatic analyses. Previous analyses have shown that

the *tssK* gene co-occurs with the *tssL* gene,³² and further protein-protein interaction studies demonstrated that the C-terminal domain of TssK (TssK-Ct) engages interaction with the cytoplasmic domain of TssL, TssL_C.36-37,40 We thus predicted the residue-residue interactions between TssK-Ct and TssL_C by combining coevolution analysis with binary classification. First, the predicted contacts were ranked based on the probability scores obtained from web servers dedicated to estimating residue-residue covariation. Second, enforcing a binary classification on the known T6SS baseplate wedge complex structure,¹⁰ four high-confidence interacting pairs were selected between TssK-Ct and TssL_c: TssK-Ala381/TssL-TssK-Ala381/TssL-Gln94. TssK-Val390/ L76, TssL-Ara141. and TssK-Pro429/TssL-Pro92 (Table 1) (Figure 1(b) and (c)). Interestingly, TssK residues in the predicted pairs are located into loops that protrude on the top of the T6SS wedge structure,¹⁰ while TssL residues in these pairs are located in close proximity to a region of the TssL cytoplasmic domain previously shown to be required for TssK-L interaction.



Figure 1. Schematic representation of the type VI secretion system and positions of the co-evolved residues in the TssL and TssK structures. (a) Schematic organization of the type VI secretion system representing the different subcomplexes (pink/purple, membrane complex; blue, baseplate; green, tail tube/sheath complex). IM, inner membrane; OM, outer membrane. (b) Structures of the enteroaggregative *E. coli* TssL cytoplasmic domain (orange, PDB: 3U66⁵⁶) and TssK trimer (grey/blue, adapted from PDB: 5M30³⁷). The C-terminal domain of one monomer of TssK is indicated (TssK-Ct). (c) Magnification of the TssL cytoplasmic (orange) and TssK C-terminal (blue) domains highlighting the positions of the residues identified by co-evolution analyses and targeted in this study (TssL: residues L76, P92, Q94 and R141, red spheres; TssK: residues A381, V390 and P429, orange spheres).

Rank	TssK-CTD residue	TssL residue	Contact Probability
1/515	A381	Q94	0.983
2/515	V390	R141	0.965
3/515	A381	L76	0.943
4/515	P429	P92	0.904
5/515	L399	S142	0.892
6/515	S351	W188	0.883

Table 1 **Coevolving residue pairs between TssK-CTD and TssL.** High-confidence contacts (green) have a contact probability above 0.90 and low-confidence contacts (red) have a probability below 0.90.

Impact of TssK and TssL mutations on T6SS antibacterial activity

We first tested the importance of the TssK and TssL potential interfacial residues for T6SS activity. The EAEC Sci1 T6SS was previously shown to confer a competitive advantage against a K-12 laboratory strain of E. coli, through the secretion of the Tle1 phospholipase.⁴⁶ A *E. coli* K-12 strain carrying a vector that confers constitutive GFP fluorescence and kanamycin resistance was used as recipient. The EAEC T6SS Sci1 activity was estimated by the fluorescence of the attacker/ recipient mixture after 4 hours of co-incubation (Figure 2, lower panels), and quantified by counting the number of surviving recipients on selective kanamycin plates (Figure 2, upper panels). The tssK and tssL mutations were introduced on the chromosome, at the native locus. Figure 2 shows that TssK substitution A381W, and TssL substitutions L76W. Q94W and P92A abolish T6SS activity, whereas TssK substitutions V390W and P429A and TssL substitution R141D significantly decrease T6SS activity without abolishing it.

Impact of mutations on T6SS sheath assembly, dynamics and stability

To gain further insights onto the impact of these mutation on T6SS assembly and function, we conducted fluorescence microscopy analyses, using a fusion of the superfolder-GFP (sfGFP) to TssB, one of the T6SS sheath subunits. As previously described, about ~60% of wild-type EAEC TssB-sfGFP cells produce T6SS sheath structures (Figure 3(a) and (b)).⁵⁵ While the majority of these cells produce a single T6SS structure, up to 4 structures can be observed per cell (Figure 3(b)). The *tssK* and *tssL* mutants presented different behaviors. While almost no sheath structure was observed in TssK-A381W and TssL-L76A, TssL-P92A and TssL-Q94W mutant cells, about of 20%

of TssK-V390W cells assembled sheath structures. and TssK-P429A cells behave similarly to the wildtype (Figure 3(a) and (b)). With the exception of TssK-P429A, the impact of these mutations on T6SS sheath assembly is in agreement with the T6SS-dependent antibacterial activities of the corresponding mutant cells (Figure 2). The overall antibacterial activity of TssK-P429A mutant cells was significantly decreased (Figure 2) although they presented a wild-type phenotype in terms of number and distribution of T6SS sheath structures (Figure 3(b)). This difference prompted us to further study the impact of this mutation on T6SS dynamics. Time-lapse recordings showed that, contrarily to wild-type cells, TssK-P429A mutant cells presented aberrant contraction events (Figure 3(c)). Although no significant difference was observed in the residence time, *i.e.*, the time for which sheaths remain in the extended conformation prior to contraction (Figure 3(d)), a significant proportion of TssK-P429A sheath structures contracts in the opposite direction (i.e., contraction towards the distal extremity) or in both direction (bidirectional contraction) (Figure 3(c) and (e)). These observations suggest that the sheath structures break in the middle or at the basal extremity, reflecting defects in membrane complex/baseplate stability.

Impact of TssK and TssL mutations on TssK-TssL complex formation

To provide information on the role of the coevolved residue pairs on MC-BP complex formation, the TssK-TssL_C interaction was assayed by bacterial two-hybrid (BACTH, Figure 4 (a) and (b)) and pull-down on StrepTactin sepharose (Figure 4(c)). BACTH analyses showed all the TssK mutants are still capable to form oligomers with wild-type TssK (Figure 4(a)). Similarly, all the mutations introduced in the cytoplasmic domain of TssL did not impact TssL_C



Figure 2. Impact of co-evolved residue substitutions on T6SS antibacterial activity. *E. coli* K-12 competitor cells (W3110 gfp^+ , kan^R) were mixed with the indicated attacker cells, spotted onto SIM agar plates and incubated for 4 h at 37 °C. The fluorescence of the bacterial spots are shown and the numbers of surviving *E. coli* competitor cells (counted on selective kanamycin medium) are indicated in the upper graph (c.f.u., colony-forming unit) as boxplots/ scatterplots. The dots indicate the twelve values from three independent assays. The bold horizontal bar indicates the median value, the lower and upper boundaries of the internal box plot indicate the 25th and 75th percentiles, respectively, and the whiskers indicate the 10th and 90th percentiles. Statistical significance relative to the WT strain is indicated above the plots (***, p < 0.001; one-tailed Wilcoxon's *t*-test).

oligomer formation (Figure 4(b)). These results suggest that the TssK-T25 and TssL_C-T18 fusion variants are produced and properly folded. BACTH and pull-down analyses revealed that TssK mutation A381W and TssL mutations L76W

and Q94W abolish interactions with TssL_C and TssK, respectively, while TssK substitutions V390W and P429A, and TssL substitutions P92A and R141D impact the TssK-TssL_C interaction without abolishing it (Figure 4(a)–(c)).

Figure 3. Impact of co-evolved residue substitutions on T6SS sheath assembly and dynamics. (a) Representative fields of fluorescence microscopy recording of the indicated wild-type (WT) and mutant EAEC cells producing TssB-sfGFP. Scale bar (lower right panel), 2 µm. (b) Number of TssB-sfGFP sheaths per cell. The percentage of cells with 0, 1, 2 or >2 sheaths per cell (from dark to light blue) is shown as well as standard deviations from three biological replicates. The number of analyzed cells (n, total number of analyzed cells from three biological replicates) is indicated on top of each bar. (c) Violin plot representation of the sheath residence time in WT (red) and TssK P429A mutant (green) cells (bold vertical bar, median values; closed red circles, mean; lower and upper boundaries of the internal box plot, 25th and 75th percentiles, respectively; whiskers, 10th and 90th percentiles; outliers, black circles). The number of analyzed sheath assembly/contraction events (n) is indicated on the right. The mean and s.d. are indicated on top of each violin plot. Statistical significance relative between WT and TssK-P429A cells is indicated on the right (*, p < 0.05; two-tailed Wilcoxon's t-test; p-value: 0.029). (d) Fluorescence microscopy time-lapse recordings of EAEC TssK P429A cells producing TssB-sfGFP (the time from the initial image is indicated on each panel) highlighting opposite contraction (contraction towards the distal extremity of the sheath reflecting break at the basal extremity, yellow arrows) and bidirectional contraction (contraction towards both extremities reflecting break in the center of the sheath, blue arrows). The quantitative analyses (canonical, opposite and bidirectional contractions in blue, dark green and light green, respectively) are shown in panel (e), as well as standard deviations from three biological replicates. The number of sheath events analyzed for each strain (n, total number of analyzed cells from three biological replicates) is indicated on top of each bar.

To gain detailed insights onto the TssK–TssL interface, we substituted the coevolved pair residues by cysteines. Thiol groups of cysteine side-chains form disulfide bonds if located at appropriate distances (C α -C α distance <7 Å) leading to covalent complex formation that can be observed by non-reducing SDS-PAGE analyses.

TssK and TssL possess five native cysteine residues each, with two cysteines in the TssK C-terminal domain, and four on the TssL cytoplasmic domain. All these cysteine residues are buried into the structure and not accessible. In agreement with this observation, pilot experiments showed that no covalent TssK-TssL





Figure 4. Impact of co-evolved residue substitutions on TssK-TssL interaction. (a and b) Bacterial two-hybrid analysis. BTH101 reporter cells producing the indicated domains/proteins and variants (a, TssK variants; b, TssL variants) fused to the T18 and T25 domain of the *Bordetella* adenylate cyclase were spotted on X-Gal-IPTG reporter LB agar plates. The blue color of the colony reports interaction between the two partners. Controls include T18 and T25 fusions to Pal and TolB, two proteins that interact but unrelated to the T6SS. (c) Pull-down assay. Soluble lysates of *E. coli* cells producing the wild-type (WT) or variant VSV-G-tagged TssK (TssK^V) and Strep-tagged TssL (TssLST) proteins were subjected to pull-down on StrepTactin beads. The total lysates (T) and pulled-down material (P) were separated by 12.5%-acrylamide SDS-PAGE and immunodetected with anti-VSV-G (upper panel) and anti-StrepTag II (lower panel) monoclonal antibodies. The positions of TssK^V and TssLST are indicated on the right. Note that TssK migrates as a doublet, as previously observed.^{36,40} Molecular weight markers (in kDa) are indicated on the left.

complex can be visualized upon addition of the oxidative agent copper(II) orthophenanthroline, suggesting that none of the native cysteines engages in disulfide bond formation (Supplementary Figure S2(a)). Figure 5(a) shows that higher-molecular-weight species (denoted with *) are observable for three of the four combinations: TssK-A381C/TssL-L76C, TssK-A381C/TssL-Q94C and TssK-P429C/TssL-P92C. The apparent molecular weight of these species $(\sim 68 \text{ kDa})$ is compatible with a complex between TssK (theoretical weight: 49 kDa) and TssL (theoretical weight: 23 kDa). These complexes are likely to be covalently bound by disulfide bridges as they dissociated upon the addition of a reducing agent (Figure 5(a)) and likely comprise both TssK and TssL as they are recognized by both the anti-VSV-G and -StrepTag antibodies (Figure 5(b)). As control experiments for specificity, we also tested all combinations corresponding to non-coevolved pairs. In these conditions, no TssKL complex was observed (Supplementary Figure S2(b)), demonstrating the specificity of disulfide bridge formation between the TssK-A381C/TssL-L76C, TssK-A381C/TssL-Q94C and TssK-P429C/TssL-P92C coevolved pairs, but also that none of the native cysteine residues of TssK and TssL engage interaction with the substituted cysteines. A summary of the contacts is shown in Figure 5(c).



Figure 5. Disulfide cross-linking identifies TssK-TssL contacts. (a and b) Cells producing the indicated VSV-Gtagged TssK (TssK^V) and Strep-tagged TssL (TssLST) cysteine variants were subjected to *in vivo* oxidative treatment with copper-orthophenanthroline. After quenching with *N*-ethyl maleimide, total cell extracts were subjected to 11%acrylamide SDS-PAGE and immunodetected with the anti-VSV-G (upper panel in panel a; "V" panels in panel b) and anti-StrepTagII (lower panel in panel a; "ST" panels in panel b) monoclonal antibodies. In panel a, samples were treated (+) or not (-) with β -mercaptoethanol. In panel b, each lane was cut vertically in two halves after protein transfer on nitrocellulose membrane (white lines) and each half was immuno-detected with the anti-VSV-G (V) or anti-StrepTagII (ST) antibody. The positions of TssK^V, TssLST and of the TssK^V-TssLST complex (*) are indicated on the right. Molecular weight markers (in kDa) are indicated on the left. (c) Summary of the contacts (bold lines) identified by disulfide cross-linking between the TssK (blue) and TssL (orange) cysteine variants. The co-evolved residue pairs are indicated by the colored shades (TssK-P429/TssL-P92, green; TssK-A381/TssL-L76 and TssL-A381/TssL-Q94, cyan; TssK-V390/TssL-R141, pink).

Concluding remarks

Although the biogenesis and mechanism of action of the T6SS are well described, details are yet missing regarding the interaction between subcomplexes, and notably subcomplexes with different evolutionarily histories. The phage related TssEFGK baseplate complex is recruited to the T4bSS-like TssJLM membrane complex via interactions between the C-terminal domain of TssK and the cytoplasmic domain of TssL. In this study, we used coevolution programs to identify potential contact pairs between the TssK and TssL proteins, suggesting a model for the interface of the heterodimer. The residues involved in the contacts were targeted by mutagenesis and the impact of the corresponding assayed mutations by interbacterial was competition, fluorescence microscopy and interaction assays. These protein-protein analyses revealed that residues TssK A381 and TssL L76 and Q94 participate to TssK-L complex formation and are hence required for efficient assembly of the T6SS. The TssK V390 and P429 and TssL P92 and R141 mutations caused a reduced number or aberrant behavior of T6SS sheaths, which could be caused by the instability of the MC-BP complex. The results of the disulfide cross-linking assays showed that TssK residue A381 and TssL residues L76 and Q94, and TssK residue P429 and TssL residue P92 are at close proximity and can engage in disulfide bond formation when substituted by cysteines.

Comparison of the protein–protein interaction and phenotypic analyses suggest that substituting TssK-A381, TssL-L76 and TssL-Q94 by bulky side-chain residues has a strong impact on complex formation, as well as on T6SS function, suggesting that this interface is critical for a functional BP-MC interaction. By contrast, substituting TssL residue P92 did not have a significant impact while substituting the coevolved partner TssK residue P429 decreased T6SS function. Fluorescence microscopy recordings showed that sheaths from TssK-P429A cells are



Figure 6. Model of the EAEC TssK–TssL_c complex. (a) Disulfide cross-linking constrained model of the TssK-Ct (blue) / TssL_c (orange) complex. The coevolved residue pairs engaged in disulfide cross-linking are shown in green and cyan (TssK-P429/TssL-P92, green; TssK-A381/TssL-L76 and TssL-A381/TssL-Q94, cyan) while the pair that does not form disulfide bond (TssK-V390/TssL-R141) is shown in pink. (b) Overlayed comparison of the experimental-based constrained model (same color as in panel a) with the model generated by the AlphaFold2 prediction program (grey). less stable and can break at the base (opposite contraction) or in the middle (bidirectional contraction). We suggest that the P429A mutation locally affects the TssK-Ct structure, leading to the destabilization of the MC-BP interface which cannot resist the strength engendered by sheath polymerization.

By imposing the restraints derived from the disulfide cross-linking data for the TssK-A381/ TssL-L76, TssK-A381/TssL-Q94, and TssK-P429/ TssL-P92 residue pairs, a model of the TssK-Ct-TssL_C complex can be proposed (Figure 6(a)). Interestingly, with a root-mean-square deviation (r. m.s.d) of 0.91 Å, this model is very close to the one proposed for the TssK-Ct/TssL_C complex by released AlphaFold2 structure the recently prediction program⁵²⁻⁵⁴ (Figure 6(b)). To completely understand the interaction between the BP and MC, some unresolved issue remains to be addressed, notably how 36 TssK C-terminal domains are connected to 10 TssL cytoplasmic domains. Nevertheless, this refined model of the TssK-TssL interface provides detailed information on BP-MC contacts, and helps to better understand how the TssK RBP-like protein has evolved to adapt to the TssL receptor in order to properly dock the baseplate onto the MC.

Data availability

All data are shown in the manuscript or in the supplemental material.

CRediT authorship contribution statement

Etienne Vanlioğlu: Investigation, Writing review & editing. Yoann G. Santin: Investigation, Writing - review & editing. Isaac Filella-Merce: Investigation, Formal analysis, Writing – review & editing. Riccardo Pellarin: Conceptualization, Supervision, Investigation, Software, Writing review editing. Cascales: & Eric Conceptualization, Investigation, Project administration, Supervision, Funding acquisition, Writing – original draft.

Acknowledgements

We thank members of the Cascales laboratory and Yassine Cherrak for discussions, and Moly Ba, Isabelle Bringer, and Annick Brun for technical assistance. This work was supported by the CNRS, the Aix-Marseille Université and by grants from the Agence Nationale de la Recherche (ANR-20-CE11-0017), the Fondation pour la Recherche Médicale (DEQ20180339165) and the Fondation Bettencourt-Schueller to EC. EV was supported by the Fondation pour la Recherche Médicale.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary Data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmb.2022. 167918.

> Received 25 October 2022; Accepted 6 December 2022; Available online 9 December 2022

Keywords:

protein transport; bacterial competition; protein-protein interaction; coevolution; contact sites

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Supplementary Information

Coevolution-guided mapping of the Type VI secretion membrane complex-baseplate interface

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Figure S1. Coevolution analysis contact and precision plots

Figure S2. Disulfide cross-linking assay.

Table S1. Strains, plasmids and oligonucleotides used in this study.



Figure S1. Coevolution analysis contact and precision plots. (a) Contact plot. Blue dots represent pairs of residues between TssG and TssF or between TssK and TssG, classified as close or distant using a distance threshold of 8 Å. These dots are plotted along the x-axis using their contact probability. The orange line displays the isotonic regression obtained by assigning a 1 to the close pairs and a 0 to the distant pairs in the y-axis. The black dotted line indicates the location of the selected high-confident probability threshold (0.9), which divides the plot between predicted non-interacting pairs (dots with a probability below the threshold) and predicted interacting pairs (dots with a probability above the threshold). (b) Precision plot. Blue dots represent values of precision for multiple contact probability thresholds. The orange line depicts the isotonic regression obtained with the values of precision for multiple probability thresholds. The black dotted line displays the selected high-confident threshold.



Figure S2. Disulfide cross-linking assays. (a and b) Cells producing the indicated VSV-G-tagged TssK (TssK^V) and Strep-tagged TssL (TssLST) cysteine variants were subjected to *in vivo* oxidative treatment with copper-orthophenanthroline. After quenching with *N*-ethyl maleimide, total cell extracts were subjected to 11%- (upper panels) or 13.5% (bottom panels) -acrylamide SDS-PAGE and immunodetected with the anti-VSV-G (upper panels) and anti-StrepTagII (lower panels) monoclonal antibodies. The positions of TssK^V, TssLST and of the TssK^V-TssLST complex (*) are indicated on the right. TssK^V degradation products are indicated with black arrows in the top panels. A non-specific band immunodetected with the anti-StrepTag antibody is indicated with a closed circle in the bottom panels. Molecular weight markers (in kDa) are indicated on the left.

Supplemental Table S1. Strains, Plasmids and Oligonucleotides used in this study.

Strains	Description and genotype	Source/References
Escherichia coli K12		
DH5a	F-, $\Delta(argF-lac)U169$ phoA supE44 $\Delta(lacZ)M15$ recA relA endA thi hsdR gyr	Laboratory Collection
W3110	F-, lambda-IN(<i>rrnD-rrnE</i>)1 <i>rph-</i> 1	Laboratory Collection
BTH101	F-, cya99, araD139, galE15, gakK16, rpsL, hsdR, mcrAB	Karimova et al., 1998
Enteroaggregative Escherichic	a coli	
17-2	Enteroaggregative Escherichia coli, wild-type	Arlette Darfeuille-Michaud
$\Delta tssK$	Deletion of the <i>tssK</i> gene (ECO42 4526) in 17-2	Zoued et al., 2013
$\Delta tssL$	Deletion of the tssL gene (ECO42 4527) in 17-2	Aschtgen et al., 2010
tssK-A381W	Chromosomal <i>tssK</i> point mutation (codon at position 381, Ala-to-Trp substitution) in 17-2	This study
tssK-V390W	Chromosomal tssK point mutation (codon at position 390, Val-to-Trp substitution) in 17-2	This study
tssK-P429A	Chromosomal tssK point mutation (codon at position 429, Pro-to-Ala substitution) in 17-2	This study
tssL-L76W	Chromosomal tssL point mutation (codon at position 76, Leu-to-Trp substitution) in 17-2	This study
tssL-P92A	Chromosomal <i>tssL</i> point mutation (codon at position 92, Pro-to-Ala substitution) in 17-2	This study
tssL-Q94W	Chromosomal tssL point mutation (codon at position 94, Gln-to-Trp substitution) in 17-2	This study
tssL-R141D	Chromosomal <i>tssL</i> point mutation (codon at position 141, Arg-to-Asp substitution) in 17-2	This study
tssB-sfGFP	Chromosomal insertion of <i>sfGFP</i> sequence in frame with <i>tssB</i> in 17-2	Zoued <i>et al.</i> , 2013
$\Delta tssK tssB$ -sfGFP	Chromosomal insertion of sfGFP sequence in frame with tssB in 17-2 $\Delta tssK$	Zoued <i>et al.</i> , 2013
$\Delta tssL tssB$ -sfGFP	Chromosomal insertion of sfGFP sequence in frame with tssB in 17-2 Δ tssL	Durand <i>et al.</i> , 2015
tssB-sfGFP tssK-A381W	Chromosomal tssK point mutation (codon at position 381, Ala-to-Trp substitution) in tssB-sfGFP	This study
tssB-sfGFP tssK-V390W	Chromosomal tssK point mutation (codon at position 390, Val-to-Trp substitution) in tssB-sfGFP	This study
tssB-sfGFP tssK-P429A	Chromosomal <i>tssK</i> point mutation (codon at position 429, Pro-to-Ala substitution) in <i>tssB-sfGFP</i>	This study
tssB-sfGFP tssL-L76W	Chromosomal tssL point mutation (codon at position 76, Leu-to-Trp substitution) in tssB-sfGFP	This study
tssB-sfGFP tssL-P92A	Chromosomal tssL point mutation (codon at position 92, Pro-to-Ala substitution) in tssB-sfGFP	This study
tssB-sfGFP tssL-Q94W	Chromosomal tssL point mutation (codon at position 94, Gln-to-Trp substitution) in tssB-sfGFP	This study
tssB-sfGFP tssL-R141D	Chromosomal tssL point mutation (codon at position 141, Arg-to-Asp substitution) in tssB-sfGFP	This study

Plasmid	Description and main characteristics	Source
pKO3	Thermosensitive suicide vector for chromosomal gene replacement, ts pSC101 ori, sacB, Cm ^K	Link <i>et al.</i> , 1997
pKO3-TssK	EAEC DNA fragment encompassing <i>tssK</i> , cloned into pKO3	This study
pKO3-TssK-A381W	<i>tssK</i> point mutation (codon at position 381, Ala-to-Trp substitution) into pKO3-TssK	This study
pKO3-TssK-V390W	tssK point mutation (codon at position 390, Val-to-Trp substitution) into pKO3-TssK	This study
pKO3-TssK-P429A	tssK point mutation (codon at position 429, Pro-to-Ala substitution) into pKO3-TssK	This study
pKO3-TssL	EAEC DNA fragment encompassing <i>tssL</i> , cloned into pKO3	This study
pKO3-TssL-L76W	tssL point mutation (codon at position 76, Leu-to-Trp substitution) into pKO3-TssL	This study
pKO3-TssL-P92A	tssL point mutation (codon at position 92, Pro-to-Ala substitution) into pKO3-TssL	This study
pKO3-TssL-Q94W	tssL point mutation (codon at position 94, Gln-to-Trp substitution) into pKO3-TssL	This study
pKO3-TssL-R141D	tssL point mutation (codon at position 141, Arg-to-Asp substitution) into pKO3-TssL	This study
pUT18	BACTH vector, ColE1 origin, Plac, T18 domain of <i>Bordetella</i> adenviate cyclase, Amp ^R .	Karimova <i>et al.</i> , 1998
pT18-Pal	<i>E coli pal</i> without signal sequence cloned downstream T18 into pUT18C	Battesti and Bouveret 2008
nTssK-T18	EAEC tssK cloned upstream T18 into nUT18C	Zoued <i>et al</i> 2013
pTssLc-T18	EAEC tssL cytoplasmic domain (amino-acids 1-184) cloned upstream T18 into pUT18C	Zoued <i>et al.</i> , 2013
pTssLc-T18-L76W	<i>tssL</i> point mutation (codon at position 76. Leu-to-Trp substitution) into pTssL _c -T18	This study
nTssLc-T18-P92A	tssL point mutation (codon at position 92) Pro-to-Ala substitution) into pTssLc-T18	This study
pTssLc-T18-O94W	<i>tssL</i> point mutation (codon at position 94, Gln-to-Trn substitution) into pTssL _c -T18	This study
pTssL _C -T18-R141D	<i>tssL</i> point mutation (codon at position 94), our to Trp substitution) into pTssLc T18	This study
VT25	DACTH i DI TOSI i CD i i i i i i	
pK125	BACTH vector, PISA origin, Plac, 125 domain of <i>Bordetella</i> adenylate cyclase, Kan ⁴	Karimova <i>et al.</i> , 1998
p125-10lB	<i>E. coli tolB</i> without signal sequence cloned downstream 125 into pK125	Battesti and Bouveret, 2008
pTssK-T25	EAEC tssK cloned upstream 125 into pK 125	Zoued <i>et al.</i> , 2013
pTssK-125-A381W	<i>tssK</i> point mutation (codon at position 381, Ala-to-Trp substitution) into pTssK-125	This study
pTssK-125-V390W	<i>tssK</i> point mutation (codon at position 390, Val-to-Trp substitution) into pTssK-125	This study
pTssK-T25-P429A	<i>tssK</i> point mutation (codon at position 429, Pro-to-Ala substitution) into pTssK-T25	This study
pTssL _C -T25	EAEC <i>tssL</i> cytoplasmic domain (amino-acids 1-184) cloned upstream T25 into pKT25	Zoued <i>et al.</i> , 2013
pBAD33	Expression vector, AraC, ParaBAC, arabinose-inducible, Cm ^R	Guzman et al., 1995
pBAD-TssK-VSVG	EAEC tssK gene (EC042 4526) cloned into pBAD33, rbs, C-terminal VSV-G tag	Zoued <i>et al.</i> , 2013
pBAD-TssK-A381C	tssK point mutation (codon at position 381, Ala-to-Cys substitution) into pBAD-TssK-VSVG	This study
pBAD-TssK-V390C	tssK point mutation (codon at position 390, Val-to-Cys substitution) into pBAD-TssK-VSVG	This study
pBAD-TssK-P429C	tssK point mutation (codon at position 429, Pro-to-Cys substitution) into pBAD-TssK-VSVG	This study

pTrc99A	Expression vector, Ptrp'- 'lacUVS, IPTG-inducible, Amp ^R	Pharmacia
pTrc-TssL-ST	EAEC tssL gene (EC042_4527) cloned into pTrc99A, C-terminal StrepTag	This study
pTrc-TssL-L76C	tssL point mutation (codon at position 76, Leu-to-Cys substitution) into pTrc-TssL-ST	This study
pTrc-TssL-P92C	tssL point mutation (codon at position 92, Pro-to-Cys substitution) into pTrc-TssL-ST	This study
pTrc-TssL-Q94C	tssL point mutation (codon at position 94, Gln-to-Cys substitution) into pTrc-TssL-ST	This study
pTrc-TssL-R141C	tssL point mutation (codon at position 141, Arg-to-Cys substitution) into pTrc-TssL-ST	This study

Oligonucleotide Sequence (5' to 3')

Plasmid construction a,b,c

5-Trc-TssL	<u>GGATAACAATTTCACACAGGAAACAGACC</u> ATGAATAAACCTGTTATCTCCCGGGCTG
3-Trc-TssL-ST	<u>CCCGGGTACCGAGCTCGAATTC</u> TTA <i>TTTTCAAACTGCGGATGGCTCCAAGCGCT</i> TCCCTGCCCGGTAAGCCGTGCCACC
5-BAD-TssK	CTCCATACCCGTTTTTTTGGGCTAGCAGGAGGTATTACACCATGAAGATTTATCGCCCATTATGGGAAGACG
3-BAD-TssK-VSVG	<u>GACTCTAGAGGATCCCCGGGTACC</u> TTA <i>TTTTCCTAATCTATTCATTTCAATATCTGTATA</i> TGTCCGCAGCACCGCAAAAAG
5-KO3-TssK-Gibson	GGATCGCGGCCGGGACCGGATCCATCCGCTGCGTGACGGAAAAGTGGTGG
3-KO3-TssK-Gibson	CGTCCCATTCGCCACCGGTCCGCGCTGTCAGTGCGCG
5-KO3-TssK-Gibson-v	<u>CGCGCACTGACAGCGCG</u> GACCGGTGGCGAATGGGACG
3-KO3-TssK-Gibson-v	CACCCTTTTGTTTCAGTTCAATGGATCCGGGTCCGCGGGCCGCGATCC
5-KO3-TssL-Gibson	GGATCGCGGCCGGGACCGGATCCATTGAACTGAAACAAAAGGGTG
3-KO3-TssL-Gibson	CGTCCCATTCGCCACCGGTCGACGGCTGTACGACAACCGTTCTGCC
5-KO3-TssL-Gibson-v	<u>GGCAGAACGGTTGTCGTACAGCCGTC</u> GACCGGTGGCGAATGGGACG
3-KO3-TssL-Gibson-v	CACCCTTTTGTTTCAGTTCAATGGATCCGGGTCCGCGGGCCGCGATCC

Site-directed mutagenesis a,b,c

A-TssK-A381W	GTGTCTGAGGTGGTGAATGTGtggCTGAGCGGGGTCATTATCCG
B-TssK-A381W	CGGATAATGACCCCGCTCAGccaCACATTCACCACCTCAGACAC
A-TssK-V390W	GCGGGGTCATTATCCGTCCGtggACGCATGTACCGGCGGCCATTC
B-TssK-V390W	GAATGGCCGGCGGTACATGCGTccaCGGACGGATAATGACCCCGC
A-TssK-P429A	GGCGTTGTACTTTCTACACCgcgGCATCGCTGGGAGATGTGAAAC
B-TssK-P429A	GTTTCACATCTCCCAGCGATGCcgcGGTGTAGAAAGTACAACGCC
A-TssL-L76W	CCCTGCTGGACGAGAGTGTAtggAACCGCGAAAAAACAGACGATG
B-TssL-L76W	ATCGTCTGTTTTTTCGCGGTTccaTACACTCTCGTCCAGCAGGG

GGCGCACCTGGCAGCAGGACgcgCTGCAGGCTCATTTTTTGG
CCAAAAAATGAGCCTGCAGcgcGTCCTGCTGCCAGGTGCGCC
CCTGGCAGCAGGACCCGCTGtggGCTCATTTTTTGGTACGCTC
GAGCGTACCAAAAAATGAGCccaCAGCGGGTCCTGCTGCCAGG
CTCGGTTTTACCGGTCAGTACgatTCGCAGGATGATGAGCGTCG
CGACGCTCATCATCCTGCGAatcGTACTGACCGGTAAAAACCGAG
GTGTCTGAGGTGGTGAATGTGtgcCTGAGCGGGGTCATTATCCG
CGGATAATGACCCCGCTCAGgcaCACATTCACCACCTCAGACAC
GCGGGGTCATTATCCGTCCGtgcACGCATGTACCGGCGGCCATTC
GAATGGCCGCCGGTACATGCGTgcaCGGACGGATAATGACCCCGC
GGCGTTGTACTTTCTACACCtgcGCATCGCTGGGAGATGTGAAAC
GTTTCACATCTCCCAGCGATGCgcaGGTGTAGAAAGTACAACGCC
CCCTGCTGGACGAGAGTGTAtgcAACCGCGAAAAAACAGACGATG
CATCGTCTGTTTTTTCGCGGTTgcaTACACTCTCGTCCAGCAGGG
GGCGCACCTGGCAGGACtgcCTGCAGGCTCATTTTTTGG
CCAAAAAATGAGCCTGCAGgcaGTCCTGCTGCCAGGTGCGCC
CCTGGCAGCAGGACCCGCTGtgcGCTCATTTTTTGGTACGCTC
GAGCGTACCAAAAAATGAGCgcaCAGCGGGTCCTGCTGCCAGG
CTCGGTTTTACCGGTCAGTACtgcTCGCAGGATGATGAGCGTCG
CGACGCTCATCCTGCGAgcaGTACTGACCGGTAAAACCGAG

^a sequence annealing on target vector underlined.

^b FLAG- or VSV-G-coding sequence italicized

[°] Shine-Dalgarno in bold.

^d Mutagenized codon in lower case.

References for Supplementary Table S1

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