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3 **Coevolution-guided mapping of the Type VI secretion membrane complex-**
4 **baseplate interface.**

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12 Running head: T6SS baseplate-membrane complex interaction.
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40 **ABSTRACT**

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

54 INTRODUCTION

55 The type VI secretion system (T6SS) is a molecular nanoweapon that uses a contractile
56 mechanism to propel an effector-loaded needle into bacterial and/or eukaryotic target cells¹⁻⁴.
57 The T6SS is comprised of two complexes^{2, 4-6}. The tail structure is constituted of the baseplate
58 (BP) complex on which the tube-sheath complex is polymerized⁷⁻⁹. The BP complex is made
59 of six TssEFGK wedges surrounding the VgrG spike⁷⁻¹¹. The tail tube-sheath complex is
60 composed of the inner tube, made of stacked Hcp hexamers, wrapped by the TssBC sheath¹²⁻
61 ¹⁴. The base of the VgrG spike serves as initiator for the polymerization of the inner tube, while
62 the wedge complexes prime sheath polymerization^{15,16}. The sheath polymerizes under an
63 extended conformation, and contracts upon contact with the target cells or in response to other
64 signals, propelling the tube-spike needle, loaded with effectors, into the target^{6,13,17-23}. The
65 passage of the tube-spike needle across the cell envelope of the attacker cell necessitates the
66 membrane complex (MC), which also serves as docking station for the baseplate^{8,24}. The
67 membrane complex is composed of the TssL and TssM inner membrane proteins, and of the
68 TssJ outer membrane lipoprotein²⁴⁻²⁹. Interestingly, the tail and membrane complexes present
69 different evolutionary histories. The TssL and TssM subunit of the MC share homologies with
70 components of the Type IVb secretion system³⁰⁻³². By contrast, with the exception of the TssK
71 subunit, the tail proteins are conserved within the large family of contractile injection systems,
72 which also include members of the Myoviridae bacteriophages and R-type pyocins^{9,13,16,33-35}.
73 The trimeric TssK wedge subunit is functionally and structurally related to receptor-binding
74 proteins (RBP) of short non-contractile phages^{36,37}. RBPs are surface proteins that recognize
75 cell surface receptors allowing specific phage binding to target cells^{38,39}. RBPs are comprised
76 of an N-terminal shoulder domain that is anchored into the baseplate, and a C-terminal head
77 domain that binds to specific receptors³⁹. The T6SS TssK proteins has a similar organization,
78 with an N-terminal domain anchored to the baseplate, and a C-terminal domain that binds to

79 the MC^{7,8,36,37,40}. This interaction allows the BP to be recruited to the MC docking station but
80 also properly positions the tail structure in order to propel the tube-spike complex across the
81 MC channel^{8,36,37}. While recent studies have provided important information on MC and BP
82 composition and structures, molecular details on how these two T6SS complexes are connected
83 remains incomplete. Boyer *et al.* noted that the *tssK* and *tssL* genes co-occur in most T6SS gene
84 clusters³², suggesting that they have coevolved to properly adapt the baseplate onto the
85 membrane complex. Indeed, protein-protein interaction studies have shown that TssK interacts
86 with the TssL cytoplasmic domain^{8,36}. Further works demonstrated that TssK-TssL complex
87 formation involve the TssK C-terminal head domain and the TssL L2-L3 loop^{37,40}. Here, to gain
88 further information on MC-BP contacts, we conducted a rationally-guided mutagenesis based
89 on coevolution prediction of TssK/TssL residue pairs. The coevolution strategy proposed four
90 sites of contacts between the two proteins that were tested by phenotypic and protein-protein
91 interaction assays, allowing to propose a model of the MC-BP interface.

92

93 MATERIAL AND METHODS

94 **Bacterial strains, media, chemicals and growth conditions.** Strains used in this study are
95 listed in Table S1. *E. coli* K-12 strain DH5 α was used for all cloning procedures, BTH101 for
96 bacterial two-hybrid assays, and W3110 for co-immunoprecipitation experiments. The
97 enteroaggregative *E. coli* (EAEC) strains used in this study are all derivatives of the wild-type
98 strain 17-2. The 17-2 *tssB-sfGFP*, $\Delta tssK$, $\Delta tssK tssB-sfGFP$, $\Delta tssL$, and $\Delta tssL tssB-sfGFP$ have
99 been previously described^{8,24,26,36}. *E. coli* cells were routinely grown in Lysogeny Broth (LB)
100 supplemented with antibiotics when necessary (kanamycin 50 $\mu\text{g.mL}^{-1}$, chloramphenicol 40
101 $\mu\text{g.mL}^{-1}$, ampicillin 100 $\mu\text{g.mL}^{-1}$). For T6SS expression, EAEC cells were grown in Sci1-
102 inducing medium (SIM; M9 minimal medium supplemented with glycerol 0.25 %, vitamin B1

103 200 $\mu\text{g}\cdot\text{mL}^{-1}$, casaminoacids 40 $\mu\text{g}\cdot\text{mL}^{-1}$, MgCl_2 2 mM, CaCl_2 0.1 mM, and LB (10% v/v))⁴¹.
104 Expression from pBAD and pTrc99A vectors was induced with L-arabinose (0.05%) and
105 Isopropyl β -D-1-thiogalactopyranoside (IPTG, 200 μM), respectively. Dichloro (1,10-
106 phenanthroline) copper(II) (Cu-oP) and *N*-ethylmaleimide (NEM) were purchased from Sigma-
107 Aldrich.

108 **Plasmid construction.** Plasmids used in this study are listed in Table S1. Polymerase Chain
109 Reactions (PCR) were performed using a Biometra thermocycler using Q5 DNA polymerase
110 (New England Biolabs). Custom oligonucleotides, listed in Table S1, were synthesized by
111 Sigma Aldrich. Bacterial two-hybrid vectors producing TssK or TssL fused to the *Bordetella*
112 adenylate cyclase T18 or T25 domains, and plasmid pBAD-TssK^V, producing TssK fused to a
113 C-terminal VSV-G, have been previously published³⁶. Plasmid pTrc-TssLST, producing TssL
114 fused to a C-terminal Streptag II, was constructed by restriction free (RF) cloning⁴². Briefly,
115 the DNA fragment corresponding to the *tssL* gene was amplified using primers that introduced
116 extensions annealing to the target vector. The double-stranded product of the first PCR has then
117 been used as primer for a second PCR using the target vector as template. PCR products were
118 then treated with DpnI to eliminate template plasmids and transformed into DH5 α -competent
119 cells. The pKO3-*tssL* vector was engineered by restriction/ligation cloning of a *Bam*HI-*Sal*I
120 DNA fragment encompassing the *tssL* gene into pKO3⁴³. Substitutions were introduced by site-
121 directed mutagenesis using complementary oligonucleotides bearing the desired mutation. All
122 plasmids have been verified by DNA sequencing (Eurofins).

123 **Strain engineering.** The $\Delta tssKL$ strain was constructed by the one-step inactivation
124 procedure⁴⁴ using λ -red recombination carried by vector pKOBEG⁴⁵, and a PCR product
125 corresponding to the pKD4 kanamycin cassette⁴⁴ flanked by 50-bp extensions annealing
126 upstream and downstream the region to be deleted. Recombinant strains were selected on LB

127 plates supplemented with kanamycin, and the kanamycin cassette was excised mutations by the
128 Flp FRT recombinase carried by vector pCP20⁴⁴. Strains 17-2 bearing point mutations at the
129 chromosomal *tssL* locus have been engineered using the suicide vector pKO3⁴³. The first and
130 second recombination events were selected on LB plates supplemented with chloramphenicol
131 at 42°C, and supplemented with sucrose (5%), respectively, as previously described⁴³. All
132 strains were verified by colony-PCR using various oligonucleotide pair combinations. Strains
133 with chromosomal point mutations were further verified by PCR amplification of the region of
134 interest and DNA sequencing.

135 **Protein-protein interaction assays.** Bacterial two-hybrid assays were conducted as previously
136 described³⁶. Pull-down experiments were performed as previously described⁴⁰ with
137 modifications: protein samples were subjected to pull-down on StrepTactin sepharose (Cytiva)
138 and CellLytic B buffer (diluted 1/5, Sigma-Aldrich) was added in the washing buffer.

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

146 **Anti-bacterial assays.** The antibacterial growth competition assay was performed as
147 described⁴⁶ with modifications. The WT *E. coli* strain W3110 bearing the pUA66-*rrnB* plasmid
148 (Kan^R,⁴⁷) was used as recipient in the competition assay. The pUA66-*rrnB* plasmid provides a
149 strong constitutive green fluorescent (GFP⁺) phenotype. Attacker and recipient cells were
150 grown in SIM medium to an A_{600} of 0.6-0.8, harvested and normalized to a $A_{600\text{nm}}$ of 0.6 in SIM.

151 Attacker and recipient cells were mixed to a 4:1 ratio and 10- μ L drops of the mixture were
152 spotted in triplicate onto a prewarmed dry SIM agar plate. After 4-hour incubation at 37°C,
153 fluorescent images were recorded with a LI-COR Odyssey imager. The bacterial spots were
154 scratched off, cells were resuspended in LB medium supplemented with kanamycin,
155 normalized to an A_{600} of 0.5 and serially diluted. One hundred μ L of serial dilutions were then
156 spread in triplicate on LB plates supplemented with kanamycin to select surviving recipients.
157 Colony forming units were numbered after overnight incubation at 37°C. The experiment was
158 done in triplicate.

159 **Live fluorescence microscopy.** Cells were grown in SIM medium at 37°C to an A_{600} of 0.6-
160 0.8, resuspended in fresh SIM to an $A_{600\text{ nm}}$ of 10, and 5 μ L were spotted onto a thin pad of SIM
161 supplemented with 2% agarose covered with a glass coverslip. Phase contrast and fluorescence
162 were recorded on a Nikon Eclipse Ti2 microscope equipped with a 100 \times NA 1.45 Ph3 objective,
163 an Orca-Fusion digital camera (Hamamatsu) and a perfect focus system. All fluorescence
164 images were acquired with a minimal exposure time to minimize bleaching and phototoxicity
165 effects. Exposure times were typically 30 ms for phase contrast, 100 ms for TssB-sfGFP. The
166 experiments were performed at least in triplicate and representative results are shown. Images
167 were analyzed using ImageJ (<http://imagej.nih.gov/ij/>) and the MicrobeJ v5.11y plugin
168 (<http://www.microbej.com/>). Statistical dataset analyses were performed with several
169 representative fields from at least three independent biological replicates, using Excel and the
170 R software environment. The number of measured cells or events (n) is indicated on each figure.

171 **Bioinformatics analyses.** To predict residue pairs in contact between two protein domains, we
172 combined the RaptorX complex contact prediction web server⁴⁸ and the GREMLIN monomer
173 contact prediction web server⁴⁹. We queried the sequences of the two protein domains in
174 RaptorX, obtaining a multiple sequence alignment (MSA) of the two concatenated sequences.

175 The MSA is used as input in GREMLIN, which returns a list of coevolving residue pairs ranked
176 by their contact probability.

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

198 Information-driven docking simulations were performed with the standard parameters on the
199 HADDOCK 2.4 web server⁵⁰, implemented by imposing ambiguous distance constraints based
200 on the results of disulfide cross-linking data (active residues) using GenTBL⁵¹. AlphaFold2
201 structural models were generated using the Colabfold server using standard parameters⁵²⁻⁵⁴.

202 **Miscellaneous.** Standard methods were used for sodium dodecyl-sulfate poly-acrylamide gel
203 electrophoresis (SDS-PAGE) and protein transfer on nitrocellulose membranes. Membranes
204 were probed with Strep-Tag Classic (clone Strep-tag II, Bio-Rad), and anti-VSV-G (clone
205 P5D4, Sigma-Aldrich) monoclonal antibodies, and goat anti-mouse secondary antibodies
206 conjugated to Alkaline Phosphatase (Beckman Coulter). Alkaline phosphatase signal was
207 developed in alkaline buffer (pH 9) in the presence of 10 mM MgCl₂, 0.15 mg/mL of 5-bromo-
208 4-chloro-3-indolylphosphate and 0.3 mg/mL of nitroblue tetrazolium.

209

210 **RESULTS AND DISCUSSION**

211 **Co-evolution analyses propose a potential interface between the TssK C-terminal domain** 212 **and the TssL cytoplasmic domain.**

213 To better understand how the T6SS baseplate is connected to the membrane complex (Figure
214 1a), we first conducted bioinformatic analyses. Previous co-occurrence analyses have shown
215 that the *tssK* gene co-occurs with the *tssL* gene³², and further protein-protein interaction studies
216 demonstrated that the C-terminal domain of TssK (TssK-Ct) engages interaction with the
217 cytoplasmic domain of TssL, TssL_C^{36,37,40}. We thus predicted the residue-residue interactions
218 between TssK-Ct and TssL_C by combining coevolution analysis with binary classification. First,
219 the predicted contacts were ranked based on the probability scores obtained from web servers
220 dedicated to estimating residue-residue covariation. Second, enforcing a binary classification

221 on the known T6SS baseplate wedge complex structure¹⁰, four high-confidence interacting
222 pairs were selected between TssK-Ct and TssL_C: TssK-Ala381/TssL-L76, TssK-Ala381/TssL-
223 Gln94, TssK-Val390/TssL-Arg141, and TssK-Pro429/TssL-Pro92 (Table 1) (Figure 1b and 1c).
224 Interestingly, TssK residues in the predicted pairs are located into loops that protrude on the
225 top of the T6SS wedge structure¹⁰, while TssL residues in these pairs are located in close
226 proximity to a region of the TssL cytoplasmic domain previously shown to be required for
227 TssK-L interaction⁴⁰.

228 **Impact of TssK and TssL mutations on T6SS antibacterial activity.**

229 We first tested the importance of the TssK and TssL potential interfacial residues for T6SS
230 activity. The EAEC Sci1 T6SS was previously shown to confer a competitive advantage against
231 a K-12 laboratory strain of *E. coli*, through the secretion of the Tle1 phospholipase⁴⁶. A *E. coli*
232 K-12 strain carrying a vector that confers constitutive GFP fluorescence and kanamycin
233 resistance was used as recipient. The EAEC T6SS Sci1 activity was estimated by the
234 fluorescence of the attacker/recipient mixture after 4 hours of co-incubation (Figure 2, lower
235 panels), and quantified by counting the number of surviving recipients on selective kanamycin
236 plates (Figure 2, upper panels). The *tssK* and *tssL* mutations were introduced on the
237 chromosome, at the native locus. Figure 2 shows that TssK substitution A381W, and TssL
238 substitutions L76W, Q94W and P92A abolish T6SS activity, whereas TssK substitutions
239 V390W and P429A and TssL substitution R141D significantly decrease T6SS activity without
240 abolishing it.

241 **Impact of mutations on T6SS sheath assembly, dynamics and stability.**

242 To gain further insights onto the impact of these mutation on T6SS assembly and function, we
243 conducted fluorescence microscopy analyses, using a fusion of the superfolder-GFP (sfGFP) to

244 TssB, one of the T6SS sheath subunits. As previously described, about ~ 60% of wild-type
245 EAEC TssB-sfGFP cells produce T6SS sheath structures (Figure 3a and 3b)⁵⁵. While the
246 majority of these cells produce a single T6SS structure, up to 4 structures can be observed per
247 cell (Figure 3b). The *tssK* and *tssL* mutants presented different behaviors. While almost no
248 sheath structure was observed in TssK-A381W and TssL-L76A, TssL-P92A and TssL-Q94W
249 mutant cells, about of 20% of TssK-V390W cells assembled sheath structures, and TssK-
250 P429A cells behave similarly to the wild-type (Figure 3a and 3b). With the exception of TssK-
251 P429A, the impact of these mutations on T6SS sheath assembly is in agreement with the T6SS-
252 dependent antibacterial activities of the corresponding mutant cells (Figure 2). The overall
253 antibacterial activity of TssK-P429A mutant cells was significantly decreased (Figure 2)
254 although they presented a wild-type phenotype in terms of number and distribution of T6SS
255 sheath structures (Figure 3b). This difference prompted us to further study the impact of this
256 mutation on T6SS dynamics. Time-lapse recordings showed that, contrarily to wild-type cells,
257 TssK-P429A mutant cells presented aberrant contraction events (Figure 3c). Although no
258 significant difference was observed in the residence time, *i.e.*, the time for which sheaths remain
259 in the extended conformation prior to contraction (Figure 3d), a significant proportion of TssK-
260 P429A sheath structures contracts in the opposite direction (*i.e.*, contraction towards the distal
261 extremity) or in both direction (bidirectional contraction) (Figure 3c and 3e). These
262 observations suggest that the sheath structures break in the middle or at the basal extremity,
263 reflecting defects in membrane complex/baseplate stability.

264

265 **Impact of TssK and TssL mutations on TssK-TssL complex formation.**

266 To provide information on the role of the coevolved residue pairs on MC-BP complex
267 formation, the TssK-TssL_C interaction was assayed by bacterial two-hybrid (BACTH, Figure

268 4a and 4b) and pull-down on StrepTactin sepharose (Figure 4c). BACTH analyses showed all
269 the TssK mutants are still capable to form oligomers with wild-type TssK (Figure 4a). Similarly,
270 all the mutations introduced in the cytoplasmic domain of TssL did not impact TssL_C oligomer
271 formation (Figure 4b). These results suggest that the TssK-T25 and TssL_C-T18 fusion variants
272 are produced and properly folded. BACTH and pull-down analyses revealed that TssK mutation
273 A381W and TssL mutations L76W and Q94W abolish interactions with TssL_C and TssK,
274 respectively, while TssK substitutions V390W and P429A, and TssL substitutions P92A and
275 R141D impact the TssK-TssL_C interaction without abolishing it (Figure 4a-c).

276 To gain detailed insights onto the TssK–TssL interface, we substituted the coevolved pair
277 residues by cysteines. Thiol groups of cysteine side-chains form disulfide bonds if located at
278 appropriate distances ($C\alpha$ - $C\alpha$ distance $<7 \text{ \AA}$) leading to covalent complex formation that can
279 be observed by non-reducing SDS-PAGE analyses. TssK and TssL possess five native cysteine
280 residues each, with two cysteines in the TssK C-terminal domain, and four on the TssL
281 cytoplasmic domain. All these cysteine residues are buried into the structure and not accessible.
282 In agreement with this observation, pilot experiments showed that no covalent TssK-TssL
283 complex can be visualized upon addition of the oxidative agent copper(II) orthophenanthroline,
284 suggesting that none of the native cysteines engages in disulfide bond formation
285 (Supplementary Figure S2a). Figure 5a shows that higher-molecular-weight species (denoted
286 with *) are observable for three of the four combinations: TssK-A381C/TssL-L76C, TssK-
287 A381C/TssL-Q94C and TssK-P429C/TssL-P92C. The apparent molecular weight of these
288 species (~ 68 kDa) is compatible with a complex between TssK (theoretical weight: 49 kDa)
289 and TssL (theoretical weight: 23 kDa). These complexes are likely to be covalently bound by
290 disulfide bridges as they dissociated upon the addition of a reducing agent (Figure 5a) and likely
291 comprise both TssK and TssL as they are recognized by both the anti-VSV-G and -StrepTag
292 antibodies (Figure 5b). As control experiments for specificity, we also tested all combinations

293 corresponding to non-coevolved pairs. In these conditions, no TssKL complex was observed
294 (Supplementary Figure S2b), demonstrating the specificity of disulfide bridge formation
295 between the TssK-A381C/TssL-L76C, TssK-A381C/TssL-Q94C and TssK-P429C/TssL-P92C
296 coevolved pairs, but also that none of the native cysteine residues of TssK and TssL engage
297 interaction with the substituted cysteines. A summary of the contacts is shown in Figure 5c.

298

299 **CONCLUDING REMARKS**

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

316 Comparison of the protein-protein interaction and phenotypic analyses suggest that substituting
317 TssK-A381, TssL-L76 and TssL-Q94 by bulky side-chain residues has a strong impact on
318 complex formation, as well as on T6SS function, suggesting that this interface is critical for a
319 functional BP-MC interaction. By contrast, substituting TssL residue P92 did not have a
320 significant impact while substituting the coevolved partner TssK residue P429 decreased T6SS
321 function. Fluorescence microscopy recordings showed that sheaths from TssK-P429A cells are
322 less stable and can break at the base (opposite contraction) or in the middle (bidirectional
323 contraction). We suggest that the P429A mutation locally affects the TssK-Ct structure, leading
324 to the destabilization of the MC-BP interface which cannot resist the strength engendered by
325 sheath polymerization.

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

337

338 **CRedit authorship contribution statement**

339 Etienne Vanlioglu: Investigation, Methodology; Writing – review & editing. Yoann G. Santin:
340 Investigation, Methodology, Writing – review & editing. Isaac Merce-Filella: Investigation,
341 Methodology, Writing – review & editing. Riccardo Pellarin: Conceptualization, Supervision,
342 Investigation, Methodology, Writing – review & editing. Eric Cascales: Conceptualization, Supervision,
343 Methodology, Funding acquisition, Writing – original draft, Writing – review & editing.

344

345 **Data availability**

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

347

348 **Declaration of competing interest**

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

351

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358

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532

533 LEGEND TO FIGURES

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

544 **Figure 2. Impact of co-evolved residue substitutions on T6SS antibacterial activity.** *E. coli*
545 K-12 competitor cells (W3110 *gfp*⁺, kan^R) were mixed with the indicated attacker cells, spotted
546 onto SIM agar plates and incubated for 4 h at 37 °C. The fluorescence of the bacterial spots are
547 shown and the numbers of surviving *E. coli* competitor cells (counted on selective kanamycin
548 medium) are indicated in the upper graph (c.f.u., colony-forming unit) as boxplots/scatterplots.
549 The dots indicate the twelve values from three independent assays. The bold horizontal bar
550 indicates the median value, the lower and upper boundaries of the internal box plot indicate the
551 25th and 75th percentiles, respectively, and the whiskers indicate the 10th and 90th percentiles.
552 Statistical significance relative to the WT strain is indicated above the plots (***, $p < 0.001$;
553 one-tailed Wilcoxon's *t*-test).

554 **Figure 3. Impact of co-evolved residue substitutions on T6SS sheath assembly and**
555 **dynamics.** (a) Representative fields of fluorescence microscopy recording of the indicated

556 wild-type (WT) and mutant EAEC cells producing TssB-sfGFP. Scale bar (lower right panel),
557 2 μm . (b) Number of TssB-sfGFP sheaths per cell. The percentage of cells with 0, 1, 2 or >2
558 sheaths per cell (from dark to light blue) is shown as well as standard deviations from three
559 biological replicates. The number of analyzed cells (n , total number of analyzed cells from three
560 biological replicates) is indicated on top of each bar. (c) Violin plot representation of the sheath
561 residence time in WT (red) and TssK P429A mutant (green) cells (bold vertical bar, median
562 values; closed red circles, mean; lower and upper boundaries of the internal box plot, 25th and
563 75th percentiles, respectively; whiskers, 10th and 90th percentiles; outliers, black circles). The
564 number of analyzed sheath assembly/contraction events (n) is indicated on the right. The mean
565 and s.d. are indicated on top of each violin plot. Statistical significance relative between
566 WT and TssK-P429A cells is indicated on the right (*, $p < 0.05$; two-tailed Wilcoxon's t -test;
567 p -value: 0.029). (d) Fluorescence microscopy time-lapse recordings of EAEC TssK P429A
568 cells producing TssB-sfGFP (the time from the initial image is indicated on each panel)
569 highlighting opposite contraction (contraction towards the distal extremity of the sheath
570 reflecting break at the basal extremity, yellow arrows) and bidirectional contraction
571 (contraction towards both extremities reflecting break in the center of the sheath, blue arrows).
572 The quantitative analyses (canonical, opposite and bidirectional contractions in blue, dark green
573 and light green, respectively) are shown in panel (e), as well as standard deviations from three
574 biological replicates. The number of sheath events analyzed for each strain (n , total number of
575 analyzed cells from three biological replicates) is indicated on top of each bar.

576 **Figure 4. Impact of co-evolved residue substitutions on TssK-TssL interaction.** (a and b)
577 Bacterial two-hybrid analysis. BTH101 reporter cells producing the indicated domains/proteins
578 and variants (a, TssK variants; b, TssL variants) fused to the T18 and T25 domain of the
579 *Bordetella* adenylate cyclase were spotted on X-Gal-IPTG reporter LB agar plates. The blue
580 color of the colony reports interaction between the two partners. Controls include T18 and T25

581 fusions to Pal and TolB, two proteins that interact but unrelated to the T6SS. (c) Pull-down
582 assay. Soluble lysates of *E. coli* cells producing the wild-type (WT) or variant VSV-G-tagged
583 TssK (TssK^V) and Strep-tagged TssL (TssLST) proteins were subjected to pull-down on
584 StrepTactin beads. The total lysates (T) and pulled-down material (P) were separated by 12.5%-
585 acrylamide SDS-PAGE and immunodetected with anti-VSV-G (upper panel) and anti-StrepTag
586 II (lower panel) monoclonal antibodies. The positions of TssK^V and TssLST are indicated on the
587 right. Note that TssK migrates as a doublet, as previously observed^{36,40}. Molecular weight
588 markers (in kDa) are indicated on the left.

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

604 **Figure 6. Model of the EAEC TssK–TssL_C complex.** (a) Disulfide cross-linking constrained
605 model of the TssK-Ct (blue) / TssL_C (orange) complex. The co-evolved residue pairs engaged
606 in disulfide cross-linking are shown in green and cyan (TssK-P429/TssL-P92, green; TssK-
607 A381/TssL-L76 and TssL-A381/TssL-Q94, cyan) while the pair that does not form disulfide
608 bond (TssK-V390/TssL-R141) is shown in pink. (b) Overlaid comparison of the
609 experimental-based constrained model (same color as in panel a) with the model generated by
610 the AlphaFold2 prediction program (grey).

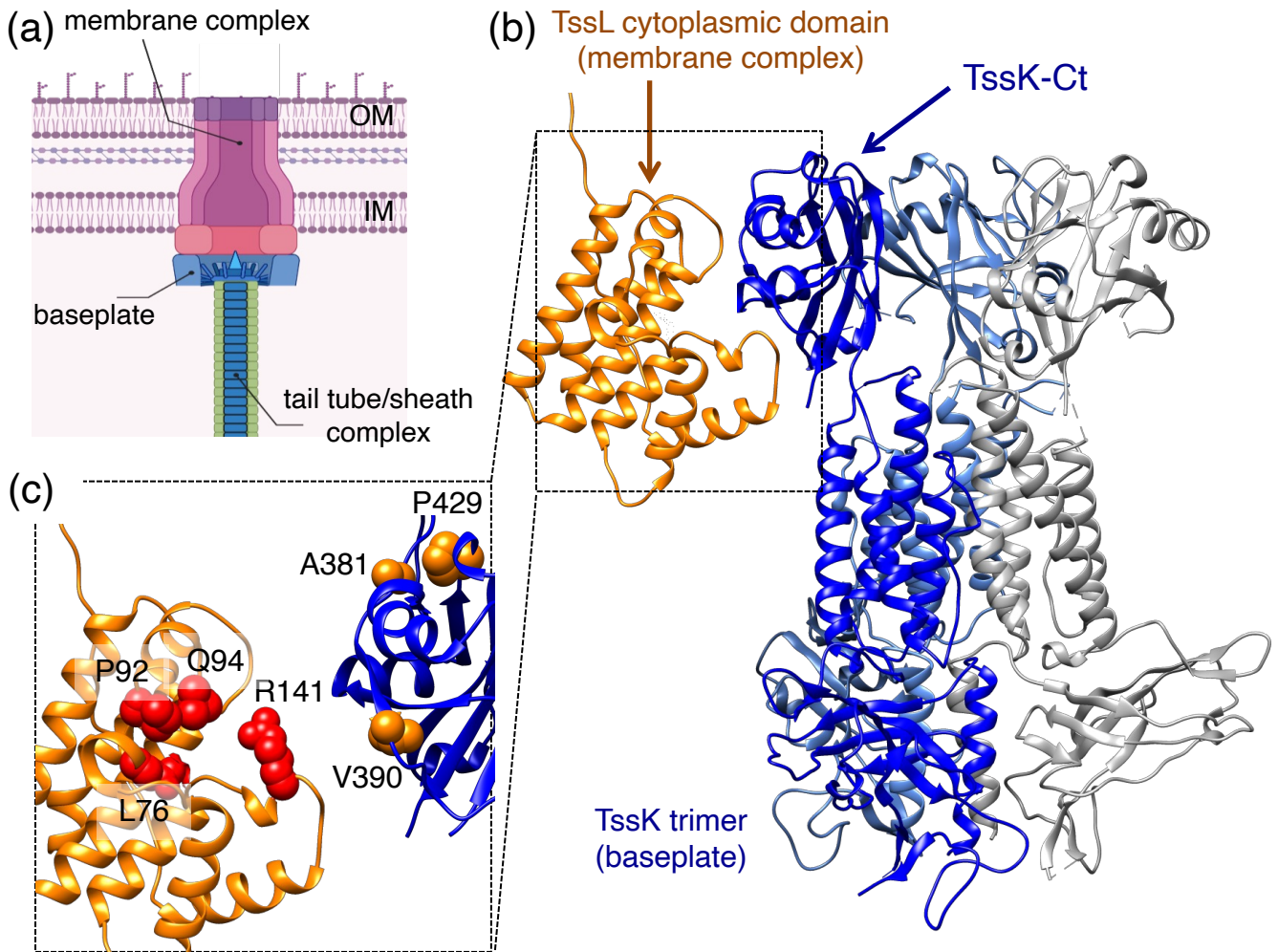


Figure 1

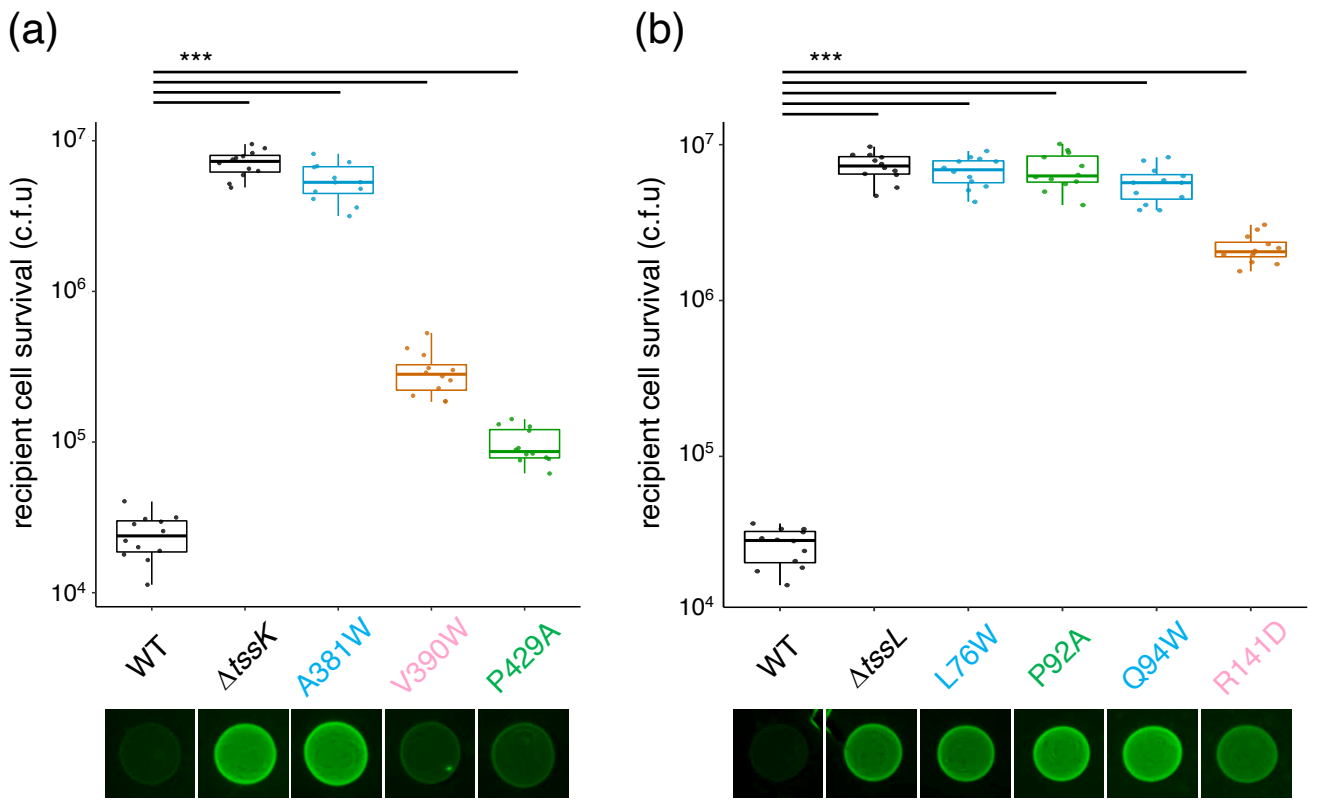


Figure 2

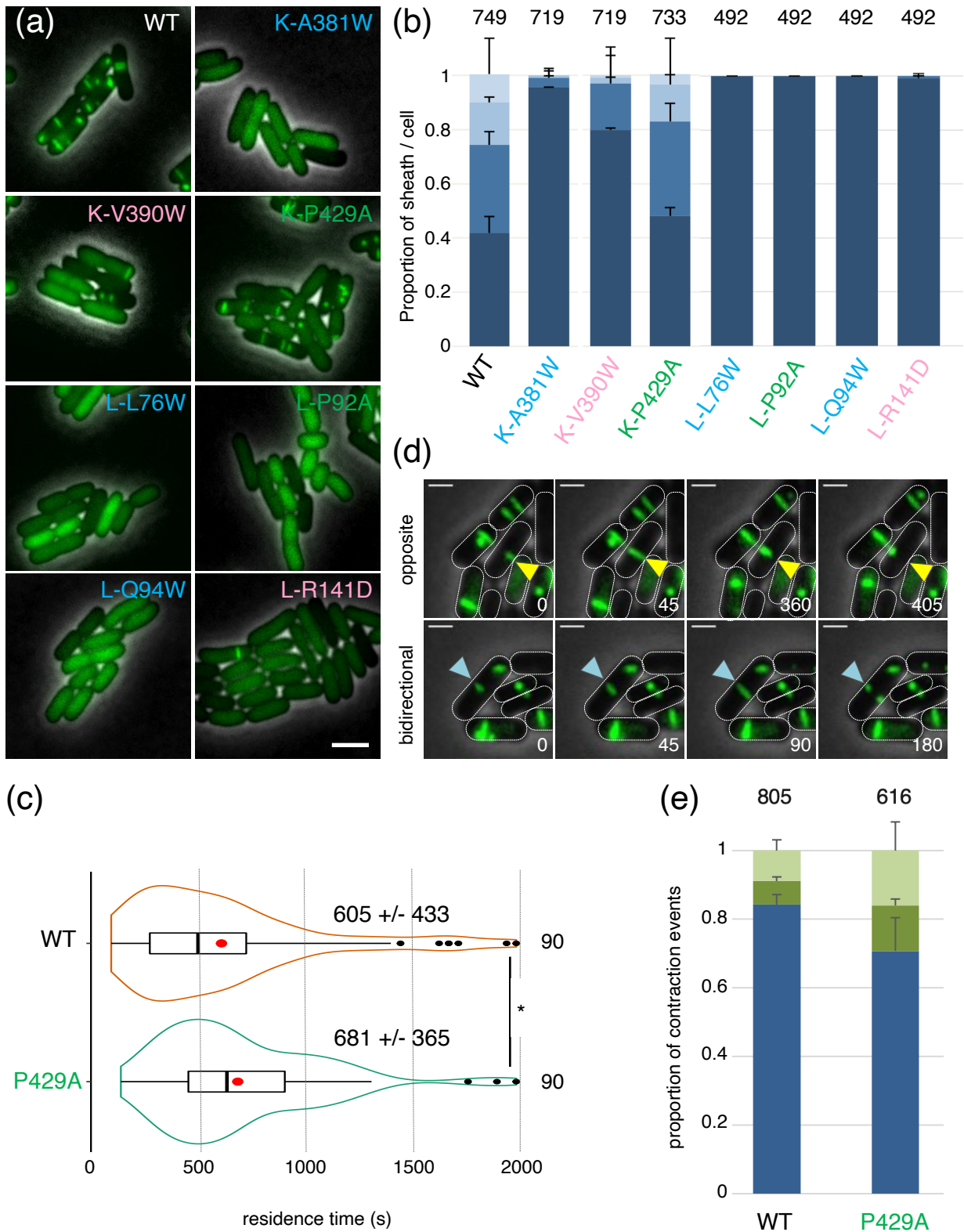


Figure 3

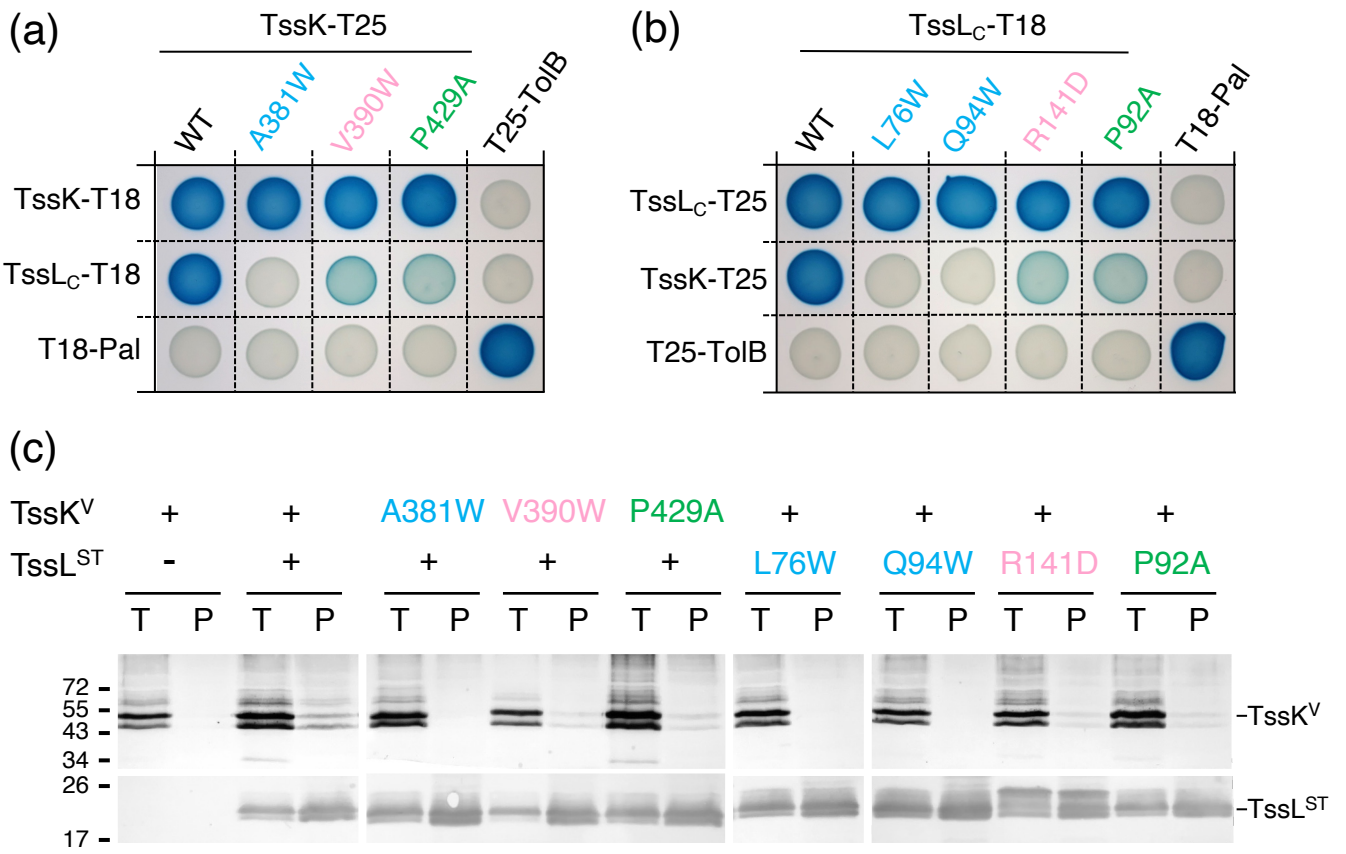


Figure 4

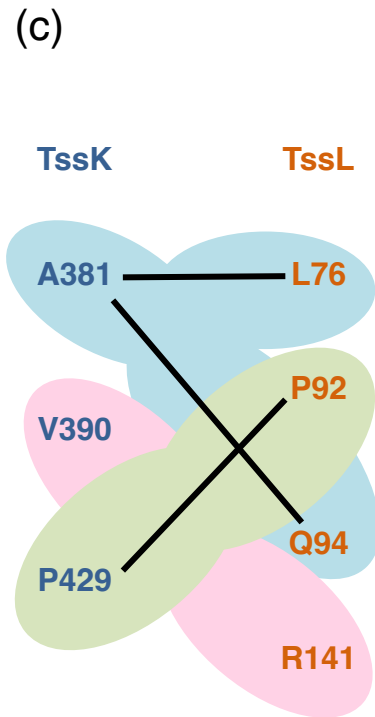
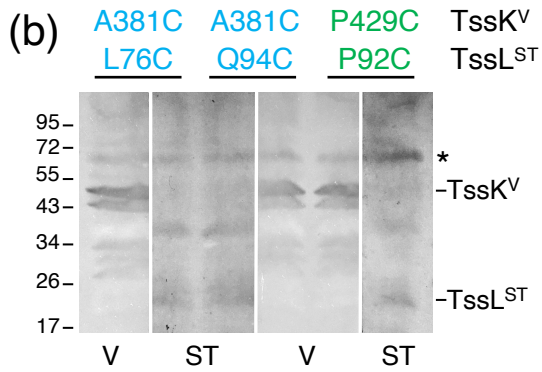
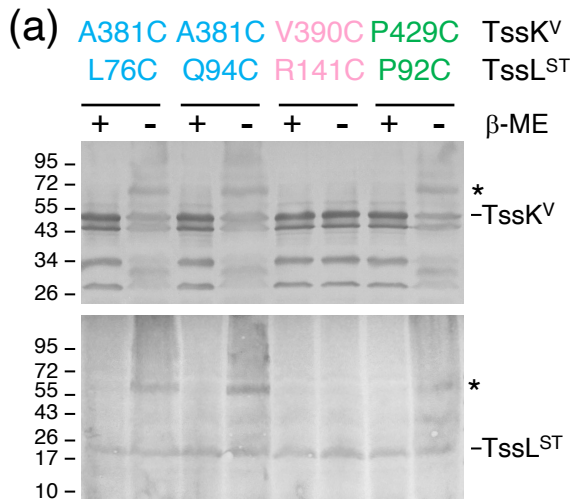
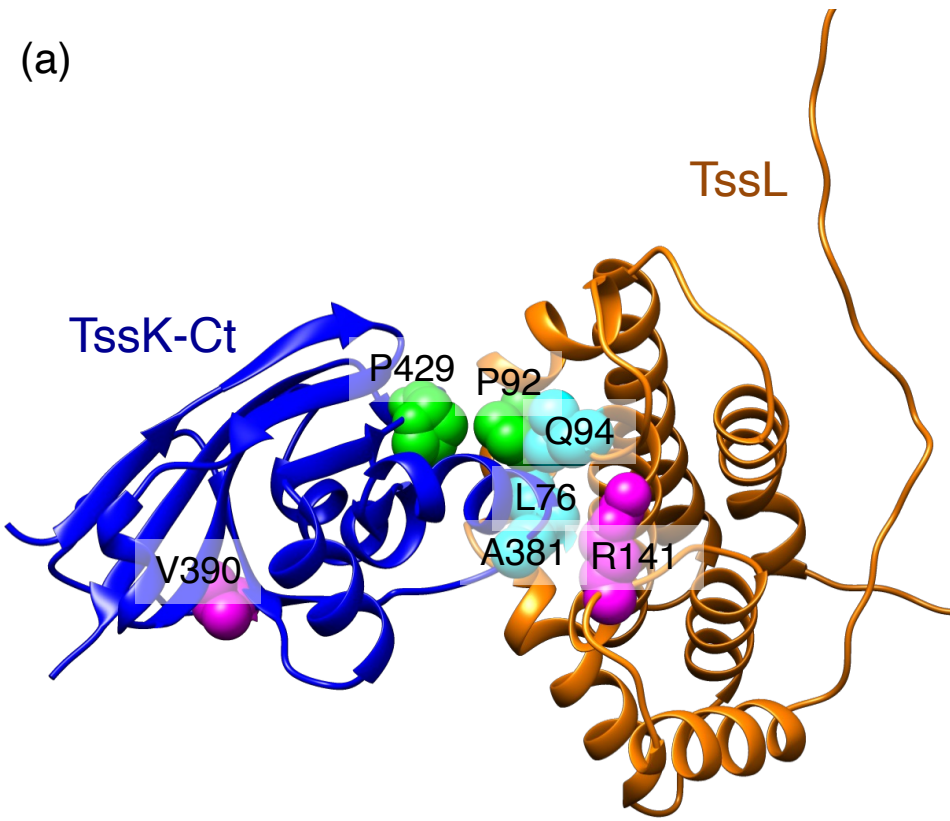


Figure 5

(a)



(b)

Co-evolution/cross-linking driven model
AlphaFold2 model

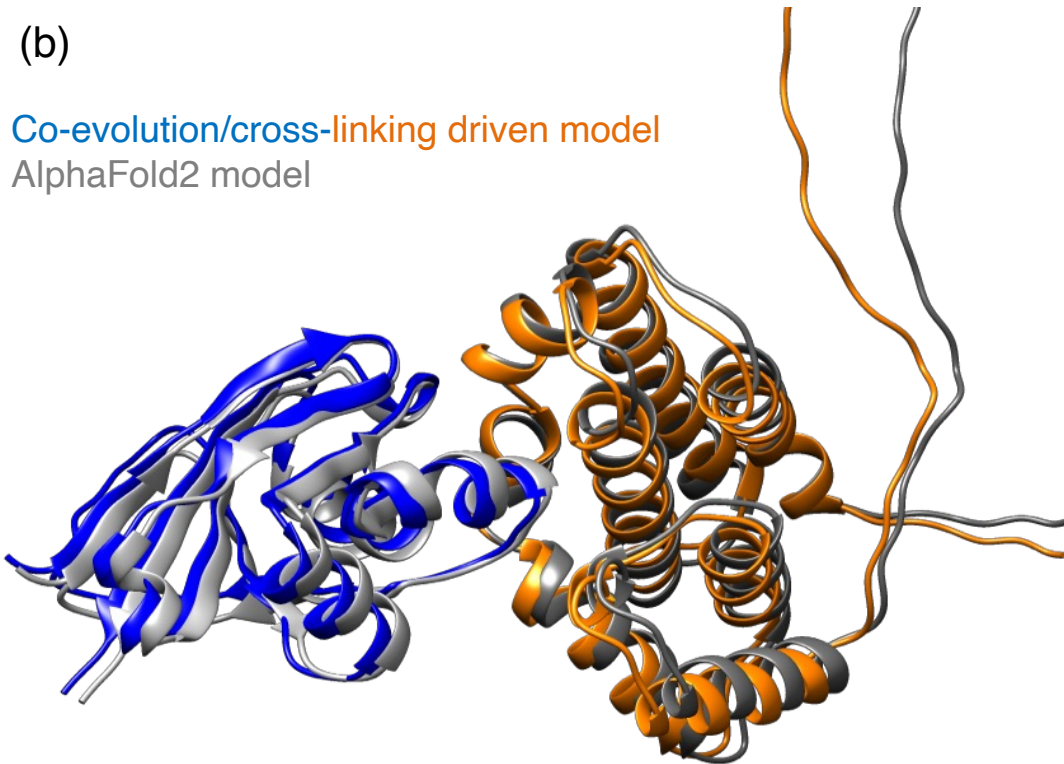


Figure 6