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1 ***In vivo* TssA proximity labeling reveals temporal interactions during Type VI secretion**  
2 **biogenesis and TagA, a protein that stops and holds the sheath.**

3 Yoann G. Santin<sup>1</sup>, Thierry Doan<sup>1</sup>, Régine Lebrun<sup>2</sup>, Leon Espinosa<sup>3</sup>, Laure Journet<sup>1</sup>  
4 & Eric Cascales<sup>1,\*</sup>

5  
6 <sup>1</sup> Laboratoire d'Ingénierie des Systèmes Macromoléculaires, Institut de Microbiologie de la  
7 Méditerranée, CNRS UMR7255, Aix-Marseille Université, 31 Chemin Joseph Aiguier, 13402  
8 Marseille Cedex 20, France

9 <sup>2</sup> Plateforme Protéomique, Marseille Protéomique (MaP) IBiSA labelled, Institut de Microbiologie de  
10 la Méditerranée, CNRS, Aix-Marseille Université, 31 Chemin Joseph Aiguier, 13402 Marseille Cedex  
11 20, France.

12 <sup>3</sup> Plateforme de biophotonique appliquée à la microbiologie, Laboratoire de Chimie Bactérienne,  
13 Institut de Microbiologie de la Méditerranée, CNRS UMR7283, Aix-Marseille Université, 31 Chemin  
14 Joseph Aiguier, 13402 Marseille Cedex 20, France

15 \* Contact information: cascales@imm.cnrs.fr  
16

17 **The Type VI secretion system (T6SS) is a multiprotein weapon used by bacteria to**  
18 **destroy competitor cells. The T6SS contractile sheath wraps an effector-loaded syringe**  
19 **that is injected into the target cell. This tail structure assembles onto the baseplate that**  
20 **is docked to the membrane complex. In entero-aggregative *Escherichia coli* TssA plays a**  
21 **central role at each stage of the T6SS assembly pathway by stabilizing the baseplate and**  
22 **coordinating the polymerization of the tail. Here we adapted an assay based on APEX2-**  
23 **dependent biotinylation to identify the proximity partners of TssA *in vivo*. By using**  
24 **stage-blocking mutations, we define the temporal contacts of TssA during T6SS**  
25 **biogenesis. This proteomic mapping approach also revealed an additional partner of**  
26 **TssA, TagA. We show that TagA is a cytosolic protein tightly associated with the**  
27 **membrane. Analyses of sheath dynamics further demonstrate that TagA captures the**  
28 **distal end of the sheath to stop its polymerization and to maintain it under the extended**  
29 **conformation.**

30 The bacterial Type VI secretion system (T6SS) is a tail structure that uses a contractile  
31 mechanism to inject a molecular syringe loaded with effectors into target cells<sup>1-8</sup>. This  
32 sophisticated apparatus is widespread in Gram-negative bacteria, and could be deployed to  
33 deliver effectors to the milieu, or into eukaryotic host cells or competitor bacterial cells. The  
34 T6SS helps to establish symbiosis, to collect metals or to disable or kill target cells<sup>5,9-13</sup>. At  
35 the molecular level, the T6SS requires a minimum set of 12 proteins that are indispensable for  
36 its assembly and function whereas additional, accessory proteins such as peptidoglycan-

37 binding proteins, peptidoglycan remodelling enzymes, disassembly ATPases and spike  
38 sharpeners might be necessary to improve its efficiency<sup>4,6,15-17</sup>.

39 The assembly of the T6SS is a coordinated process in which each subunit is recruited  
40 in a definite order. The TssA protein is required at each stage of T6SS biogenesis and  
41 mediates specific contacts with each of the T6SS sub-complexes<sup>18</sup>. In entero-aggregative *E.*  
42 *coli* (EAEC), the outer membrane lipoprotein TssJ positions first and recruits the inner  
43 membrane proteins TssM and TssL<sup>19-21</sup>. Polymerization of the TssJLM heterotrimer into a  
44 1.7-MDa trans-envelope channel, named membrane complex, comprising 10 copies of each  
45 protein, occurs after local remodelling of the cell wall which is assured by a transglycosylase  
46 that is recruited to and activated by TssM<sup>21,22</sup>. Binding of the TssA protein onto the  
47 membrane complex recruits and stabilizes a second multi-protein complex, the baseplate<sup>18</sup>.  
48 The baseplate comprises five different proteins: TssE, -F, -G, -K and VgrG<sup>23-26</sup>. The  
49 baseplate constitutes the assembly platform for the tail and shares structural and functional  
50 homologies with the baseplates of other contractile structures such as bacteriophages<sup>25,26</sup>. The  
51 T6SS baseplate is a mosaic structure that groups subunits evolutionarily related to the  
52 minimal myophage baseplate (TssE, -F and -G are homologues of the Mu phage Mup46,  
53 Mup47 and Mup48 proteins respectively, whereas VgrG is a fusion protein between  
54 homologues of Mup44 and Mup45), and TssK, a protein related to siphophage receptor-  
55 binding proteins that has evolved a C-terminal domain that anchors the baseplate to the  
56 membrane complex<sup>25-30</sup>. Once the baseplate is docked to the membrane complex<sup>23,25,31,32</sup>, the  
57 tail is built by the processive addition of inner tube and contractile sheath building blocks<sup>3,33-  
58 35</sup>. Tail assembly is a coordinated process, in which tube and sheath polymerizations are  
59 interdependent but it has been proposed that the assembly of one tube hexamer immediately  
60 precedes the polymerization of one sheath row<sup>18,33</sup>. The coordinated assembly of the sheath  
61 with that of the tube is dictated by the TssA subunit<sup>18,36</sup>. During the assembly process, TssA  
62 remains at the distal end of the tail, at which new subunits are incorporated<sup>18,37</sup>. Fluorescence  
63 microscopy recordings revealed that the length of the tail is not controlled but rather that its  
64 polymerization stops once the distal end is at proximity of the membrane<sup>1,2,18,25</sup>. Once the  
65 biogenesis of the tail is completed, TssA remains associated to the distal end, and it has been  
66 proposed that TssA remains until the last row of sheath contracts<sup>18,36</sup>. However, the molecular  
67 mechanism that controls the arrest of sheath polymerization and how the sheath is maintained  
68 in the extended conformation for long periods of time are not known.

69

70 **APEX2-dependent labeling of TssA proximity partners.**

71 Because TssA is located at the distal end of the sheath, we reasoned that definition of  
72 the TssA interactome may provide insights onto the late stages of T6SS assembly. To identify  
73 TssA partners in living cells, we developed a spatially resolved proteomic-mapping assay  
74 based on proximity-dependent biotinylation. APEX2-dependent biotinylation is a recent  
75 technology used in eukaryotic cells to define protein sub-cellular localization, membrane  
76 protein topology or protein partners<sup>38,39</sup>. APEX2 is an engineered variant of the monomeric  
77 ascorbate peroxidase that oxidizes phenol derivatives to phenoxy radicals in presence of  
78 hydrogen peroxyde (H<sub>2</sub>O<sub>2</sub>)<sup>38</sup>. Hence APEX2 converts biotin-phenol to short-lived, small-  
79 distance diffusive biotin-phenoxy radicals that covalently react with electron-rich amino-acid  
80 side chains. Based on biotin-phenoxy radicals half-life and diffusion rates, it is proposed that  
81 APEX2 biotinylates macromolecules located within 20 nm radius<sup>40</sup>. Once fused to a bait  
82 protein, APEX2 labels proteins present within the diffusion radius, and hence proteins in  
83 contact with the bait and proteins that share the same space. These proximity-biotinylated  
84 proteins can be then enriched on streptavidin and identified by mass spectrometry. In the  
85 recent years, this approach has been successfully employed to define the mitochondrial matrix  
86 proteome, to identify partners of G-protein coupled receptors and to map the *Chlamydia*  
87 *trachomatis* inclusion membrane<sup>39-46</sup>. However, studies have been restricted to eukaryotic  
88 cells and no example of APEX2-dependent labeling is yet available inside bacterial cells. We  
89 engineered a pKD4 vector derivative, pKD4-APEX2 (Supplementary Fig. 1a), allowing  
90 insertion of the *apex2* sequence at the locus of interest on the bacterial genome using the one-  
91 step procedure<sup>47</sup>. The *apex2*-coding sequence was genetically fused to *tssA* (gene accession  
92 number GI:284924261), at the native chromosomal locus in the EAEC genome to generate a  
93 APEX2-TssA fusion protein (Supplementary Fig. 1b). APEX2 was fused at the N-terminus of  
94 TssA, as we previously showed that the presence of GFP at this position did not prevent T6SS  
95 activity<sup>18</sup>. Indeed, anti-bacterial competition assay showed that cells producing APEX2-TssA  
96 eliminate competitor *E. coli* K-12 cells at a level comparable to the wild-type strain,  
97 demonstrating that fusion of APEX2 at the N-terminus of TssA does not impact T6SS activity  
98 (Supplementary Fig. 1c). Western-blot analyses of lysates of wild-type cells producing  
99 APEX2-TssA using a streptavidin-coupled antibody showed that several proteins are  
100 specifically biotinylated in presence of biotin-phenol and H<sub>2</sub>O<sub>2</sub>, demonstrating that APEX2 is  
101 functional in the bacterial cytoplasm (Supplementary Fig. 1d).

102 Wild-type cells producing the chromosomal APEX2-TssA fusion were mixed with *E.*  
103 *coli* K-12 competitor cells and incubated on plates containing 10 mM biotin-phenol. This  
104 concentration of biotin-phenol does not impact bacterial growth (Supplementary Fig. 2a) nor  
105 T6SS activity (Supplementary Fig. 2b). Live cells were then treated for 1 min with H<sub>2</sub>O<sub>2</sub> and  
106 biotinylated TssA proximity partners were enriched on streptavidin beads after quenching and  
107 cell lysis. The total eluate was subjected to mass spectrometry for protein identification (Table  
108 1, first column). The highest hits correspond to T6SS subunits, including the TssM and TssL  
109 membrane proteins, the TssK, VgrG, TssF and TssG baseplate components, the Hcp and TssC  
110 tail proteins, as well as a protein of unknown function, encoded by the *EC042\_4550* gene  
111 (accession number GI: 284924271; hereafter named TagA). Interestingly, TssK, VgrG, Hcp  
112 and TssC are known interacting partners of TssA<sup>18</sup> whereas TssF and TssG form a stable  
113 complex with TssK<sup>24,25</sup> and are likely at close proximity to the TssK-bound TssA. Finally,  
114 while no direct contacts have been identified between TssA and TssM and TssL, the TssA  
115 protein was previously shown to co-purify with the TssJLM complex<sup>18</sup>. The *EC042\_4550*  
116 gene, located at close proximity to the T6SS core component genes (Supplementary Fig. 1b),  
117 encodes a protein of the TssA family, which shares the N-terminal ImpA domain but has a  
118 different C-terminal extension and was thus named TagA (Type VI accessory gene with  
119 ImpA domain)<sup>36</sup>. The two T6SS integral inner membrane proteins, TssM and TssL, were not  
120 retrieved when detergent was omitted during preparation of the lysate sample prior to  
121 streptavidin enrichment (Table 1, second column). No significant hits were recovered from  
122 mass spectrometry analysis of streptavidin-enriched samples from wild-type cells that do not  
123 produce APEX2-TssA (Table 1, third column). In addition, no T6SS proteins were  
124 biotinylated when APEX2 was produced from the *tssA* locus but not fused to TssA (Table 1,  
125 fourth column). Finally, we generated a EAEC strain producing a fusion between APEX2 and  
126 GspE, a cytosolic ATPase that transiently associates with the membrane-anchored Type II  
127 secretion system (T2SS)<sup>48</sup>. Although two known T2SS partners of GspE, GspF and GspL<sup>49</sup>,  
128 were labeled, none of the T6SS subunit was recovered in the enriched fraction (Table 1, fifth  
129 column). All these controls experiments demonstrate the specificity of APEX2 labeling when  
130 fused to TssA.

131

132 **Stage-specific blocking mutations define the temporal TssA contacts during T6SS**  
133 **biogenesis.**

134 Previous data have suggested that TssA mediates sequential contacts with the  
135 membrane complex, the baseplate and the tail, during T6SS biogenesis<sup>18,36</sup>. We sought to test  
136 this model by arresting T6SS assembly at each stage, using non-polar mutants we previously  
137 generated. In absence of TssL, the membrane complex does not assemble and T6SS assembly  
138 is blocked at an early stage<sup>21</sup>. Indeed, no significant hit was recovered in a *tssL* strain (Table  
139 2; first column). The observation that both TssM and TssL are biotinylated in *tssK* and *vgrG*  
140 cells (Table 2, second and third columns) is in agreement with the fact that the membrane  
141 complex is assembled in absence of baseplate components<sup>25</sup> and with the proposal that TssA  
142 is bound at the cytosolic face of the membrane complex<sup>18,36</sup>. Finally, the biotinylation of  
143 membrane complex and baseplate components in *hcp* cells (Table 2, fourth column) correlates  
144 with the observation that the absence of Hcp prevents polymerization of the tail but does not  
145 impact membrane complex and baseplate assembly<sup>25</sup>. A schematic summary of proteins  
146 biotinylated by APEX2-TssA during T6SS biogenesis is shown in Figure 1.

147

#### 148 **TagA associates at the distal end of the sheath.**

149 The identification of TagA, a protein of unknown function, as a proximity partner of  
150 TssA and the location of the *tagA* gene at close proximity to T6SS core component genes led  
151 us to further characterize this protein. Interestingly, TagA was not biotinylated in *tssL*, *tssK*,  
152 *vgrG* or *hcp* cells (Table 2), suggesting that it contacts TssA in the late stage (*i.e.*, tail  
153 polymerization) of T6SS biogenesis. Bacterial two-hybrid and co-immunoprecipitation assays  
154 demonstrated that TagA directly interacts with TssA (Fig. 2a and 2b). Competition assays  
155 further showed that the *tagA* mutant strain retains  $12 \pm 4$  % of activity (Fig. 2c). Anti-bacterial  
156 activity was restored to wild-type levels upon *cis*-complementation with wild-type TagA or  
157 VSV-G-tagged TagA ( $\nu$ TagA; Fig. 2c). Hence, TagA is not an essential protein for T6SS  
158 action but rather increases the efficiency of the Type VI apparatus. To gain further insights  
159 into TagA localization, we performed fractionation and fluorescence microscopy experiments.  
160 In EAEC wild-type and  $\Delta$ *scil* (*i.e.*, a strain deleted of the T6SS gene cluster, from *tssB* to  
161 *tssJ*) cells, as well as in *E. coli* K-12 (devoid of T6SS gene cluster) cells,  $\nu$ TagA co-  
162 fractionates with the EF-Tu cytoplasmic elongation factor and with membrane-associated  
163 proteins such as OmpA, TolA and TolB (Fig. 2d). However, by contrast to OmpA and TolA,  
164  $\nu$ TagA behaves similarly to the peripherally-associated membrane protein TolB:  $\nu$ TagA is  
165 partly released from the membrane upon urea treatment (Fig. 2d), suggesting that TagA is a  
166 cytosolic protein that associates with the membrane independently of T6SS subunits. In

167 agreement with this conclusion, fluorescence microscopy imaging of a functional TagA  
168 fusion to superfolder GFP (sfGFP-TagA; Supplementary Fig. 3) revealed that TagA  
169 assembles sub-membrane punctate foci (Fig. 3a and 3b). The association of these punctate  
170 foci with the membrane was confirmed by deconvolution analyses (Supplementary Fig. 4a).  
171 Interestingly, these foci are not randomly distributed but rather cluster at the  $\frac{1}{4}$  of the cell (Fig.  
172 3c). Time-lapse recordings in wild-type showed that these foci dynamically associate - or  
173 appear close to the  $\frac{1}{4}$  and  $\frac{3}{4}$  of the cell (Fig. 3d). Interestingly, the formation of static or  
174 dynamic sfGFP-TagA foci is dependent upon the T6SS subunits, as sfGFP-TagA localizes at  
175 diffuse membrane patches in  $\Delta sciI$  cells (Supplementary Fig. 4b).

176 To better define the localization of TagA, we performed co-localization studies with a  
177 mCherry-tagged TssB sheath subunit. Fig. 3e shows that TagA localizes at one extremity of  
178 the sheath. Because the sheath extends from the baseplate to the opposite side of the cell, we  
179 next asked whether TagA partitions with the baseplate or locates at the distal end of the sheath.  
180 Co-localization experiments with the TssK baseplate subunit fused to the mCherry defined  
181 that sfGFP-TagA and TssK-mCherry do not co-localize, demonstrating that TagA is not a  
182 component of the baseplate (Fig. 3f and Supplementary Fig. 5). From these results, we  
183 conclude that TagA associates with the distal end of the sheath. This conclusion is in  
184 agreement with the APEX2-TssA-mediated biotinylation of TagA only in cells authorizing  
185 sheath polymerization. To gain further insights into TagA dynamics, we imaged sheath  
186 assembly in cells producing sfGFP-TagA. Examination of full cycles of sheath  
187 extension/contraction (see blue arrow in Fig. 3g) established that TagA is captured by the  
188 distal end of the sheath once it approaches the membrane (Fig. 3g, Supplementary Fig. 5,  
189 Supplementary Video 1).

190

### 191 **TagA stops sheath polymerization and stabilizes the extended sheath conformation.**

192 To determine the role of TagA in sheath dynamics, we imaged wild-type, *tagA* and  
193 complemented *tagA* cells producing TssB-sfGFP. Time-lapse fluorescence recordings  
194 revealed some remarkable sheath dynamics in *tagA* cells: about fifty percent of the sheaths  
195 extend toward the opposite side of the membrane but do not stop, and hence undergo a  
196 distortion event allowing their extension parallel to the membrane (Fig. 4a-b; Supplementary  
197 Video 2), ultimately leading to sheaths break or detachment from the membrane  
198 complex/baseplate (see red arrowheads in Fig. 4a). This observation suggests that in absence

199 of TagA, the polymerization of some of the sheaths does not stop at the opposite membrane  
200 (*i.e.*, the location of TagA) and thus that TagA may constitute the stopper – or part of the  
201 stopper – for T6SS sheath extension. These sheaths are likely to be non-productive,  
202 explaining the decreased T6SS activity in *tagA* cells. The remaining sheaths undergo usual  
203 extension/contraction cycles. However, we noticed that most of these sheaths contract rapidly  
204 after completion of their extension. Comparison of the residence time of extended sheaths (*i.e.*,  
205 duration of the extended sheath conformation prior to contraction) in wild-type, *tagA* and  
206 complemented *tagA* cells revealed that sheaths contract significantly more rapidly after tail  
207 competition in absence of TagA (Fig. 4c, Supplementary Fig. 6). Interestingly, the distribution  
208 of the residence time differs considerably between wild-type and *tagA* cells: the number of  
209 sheaths that contract immediately after extension is significantly increased in *tagA* cells (>  
210 60% of the events) compared to wild-type or complemented *tagA* cells (~ 5% of the events)  
211 (Fig. 4c, Supplementary Fig. 6). These results provide support to an additional role of TagA  
212 as a clamp to stabilize the extended sheath and/or to maintain the sheath under the extended  
213 conformation.

214

## 215 **Concluding remarks**

216 In this study we have adapted the APEX2-dependent biotin ligation technology  
217 initially developed in eukaryotic cells to study bacterial complexes. We report the  
218 identification of the *in vivo* proximity partners of the T6SS-associated TssA protein and use  
219 this approach in stage-specific blocking mutant cells to temporally resolve the TssA contacts  
220 during T6SS biogenesis. We show that TssA successively engages with different complexes:  
221 it interacts first with the inner membrane proteins of the membrane complex (TssL, TssM),  
222 and then the baseplate (TssF, TssG, TssK and VgrG,) and the tail (Hcp, TssC). This powerful  
223 approach recapitulates the known interacting partners of TssA previously identified by  
224 bacterial two-hybrid, co-precipitation and surface-plasmon resonance assays<sup>18</sup>. This  
225 technology also detected two known partners of the T2SS-associated GspE ATPase, GspF  
226 and GspL<sup>49-52</sup>. To our knowledge, this is the first report of the use of APEX2-dependent  
227 proximity biotinylation inside living bacterial cells. This approach is specifically powerful to  
228 dissect contacts of a dynamic protein that engages in different complexes during assembly of  
229 a multiprotein system or that is involved in different processes during the cell cycle.  
230 Interestingly, APEX2 fusion to TssA revealed an additional player in Type VI secretion in  
231 EAEC, TagA. TagA could not be considered as a T6SS core component as the *tagA* gene is



232 found associated with a limited number of T6SS gene clusters, including that of *Xenorhabdus*  
233 and *Photorhabdus* species as well as *Aeromonas hydrophila* and *Vibrio cholerae*<sup>36</sup>. In  
234 agreement with the phenotypes associated with the deletion of the *tagA* gene in *V. cholerae*,  
235 VCA0121<sup>53</sup>, competition experiments provided evidence that TagA is not essential for T6SS  
236 activity in EAEC. However, the presence of TagA optimizes T6SS efficiency as a *tagA*  
237 mutant retains  $12 \pm 4$  % of anti-bacterial activity compared to the wild-type strain. Domain  
238 analyses of TagA showed that it shares the N-terminal ImpA domain found in proteins of the  
239 TssA family, including EAEC TssA and *P. aeruginosa* TssA1<sup>18,36,54</sup>, and a C-terminal  
240 extension of unknown function. These two domains are separated by a long linker comprising  
241 a stretch of hydrophobic residues predicted to be arranged as an amphipatic  $\alpha$ -helix<sup>36</sup>. Indeed,  
242 TagA is a cytosolic protein that peripherally but tightly associates with the membrane.  
243 Interestingly, TagA localizes at specific positions in the cell, halfway between midcell and the  
244 cell poles. Although we do not know the cellular determinants that control this specific  
245 positioning, TagA is captured by the distal end of the sheath, likely by directly interacting  
246 with the TssA cap protein. TagA-TssA contacts might be mediated by their N-terminal ImpA  
247 domains, which have been previously shown to dimerize<sup>18</sup>. It is noteworthy that the *tagA* gene  
248 is usually present within T6SS gene clusters encoding TssA, but not TssA1. This observation,  
249 the interaction between the TssA and TagA, and the presence of TagA at proximity of TssA  
250 when sheath extension is completed as revealed by the *in vivo* APEX2 assay, support a  
251 functional relationship between TssA (and the distal extremity of the sheath) and TagA.  
252 Indeed, sfGFP-TagA was shown to present a diffuse membrane pattern in absence of T6SS  
253 subunits and to assemble foci at the site of contact of the distal end of the sheath with the  
254 opposite membrane. The observation that TagA is captured by the distal end of the sheath  
255 suggests a role of this accessory protein during the late stages of sheath assembly. In  
256 agreement with this suggestion, fluorescence microscopy recordings of the T6SS sheath  
257 showed two different consequences of the absence of TagA. First, aberrant sheath  
258 polymerization was observed, in which sheath polymerization does not stop at the membrane,  
259 leading to sheath distortion, bending and, ultimately, breaking. These sheaths do not contract  
260 and hence are unfruitful to destroy rival bacteria, likely explaining the decreased T6SS  
261 activity in *tagA* cells. The second consequence of the absence of TagA is the rapid contraction  
262 after sheath extension. One explanation to these two distinct events might be how the sheath  
263 hits the membrane. It has been proposed that incorporation of new Hcp hexamers in the  
264 growing tail occurs by opening the central hexaflexagon structure of TssA<sup>18,36</sup>. Therefore, one  
265 may hypothesize that if the sheath arrives perpendicular to the membrane, there is no room to

266 allow incorporation of new Hcp hexamers, hence arresting sheath polymerization. In absence  
267 of TagA, these sheaths will contract rapidly. By contrast, if the sheath does not arrive  
268 perpendicular, new Hcp hexamers can enter the TssA central lumen, allowing the sheath  
269 polymerization to proceed and causing sheath distortion and bending. One alternative  
270 hypothesis is that the absence of TagA is partly compensated by an unknown factor for  
271 preventing uncontrolled sheath extension. Using the APEX2 technology to identify proteins at  
272 proximity of TagA may provide further insights into T6SS tail completion.

273         Based on our results, we propose that TagA associates with the cytoplasmic side of the  
274 inner membrane. Once the distal end of the sheath approaches the opposite membrane, TagA  
275 is captured, stops sheath polymerization and clamps the extended sheath to the membrane. By  
276 linking both the sheath distal extremity and the membrane, TagA prevents the immediate  
277 contraction of the sheath and maintains the tensile forces required for efficient killing. TagA  
278 is thus employed by TssA<sup>+</sup> T6SS as a sheath stopper and clamp.

279

## 280 **METHODS**

### 281 **Bacterial strains, media and chemicals**

282 Enteroaggregative *E. coli* was used as model micro-organism. *Escherichia coli* K-12 strains DH5 $\alpha$ , W3110 and  
283 BTH101 were used for cloning procedures and fractionation, and bacterial two-hybrid assay, respectively.  
284 Enteroaggregative *E. coli* (EAEC) strains used in this work are isogenic derivatives of the wild-type O3:H2 17-2  
285 strain. *E. coli* K-12 and EAEC cells were routinely grown in LB broth at 37°C, with aeration. For induction of  
286 the *scil* T6SS gene cluster, cells were grown in *SciI*-inducing medium [SIM: M9 minimal medium  
287 supplemented with glycerol (0.2%), vitamin B1 (1  $\mu\text{g}\cdot\text{mL}^{-1}$ ), casaminoacids (40  $\mu\text{g}\cdot\text{mL}^{-1}$ ), LB (10% v/v)]<sup>55</sup>.  
288 Plasmids and chromosomal deletions and insertions were maintained by the addition of ampicillin (100  $\mu\text{g}\cdot\text{mL}^{-1}$   
289 for K-12), kanamycin (50  $\mu\text{g}\cdot\text{mL}^{-1}$  for K-12, 50  $\mu\text{g}\cdot\text{mL}^{-1}$  for chromosomal insertion on EAEC, 100  $\mu\text{g}\cdot\text{mL}^{-1}$  for  
290 plasmid bearing EAEC), or chloramphenicol (40  $\mu\text{g}\cdot\text{mL}^{-1}$ ). Expression of genes from pBAD or pASK-IBA  
291 vectors was induced at  $A_{600\text{ nm}} \approx 0.6$  with 0.02% of L-arabinose (Sigma-Aldrich) for 1 h or with 0.1  $\mu\text{g}\cdot\text{mL}^{-1}$  of  
292 anhydrotetracyclin (IBA Technologies) for 45 min, respectively. For BACTH experiments, gene expression was  
293 induced by the addition of iso-propyl- $\beta$ -D-thio-galactopyranoside (IPTG, Sigma-Aldrich, 0.5 mM) and plates  
294 were supplemented with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal, Eurobio, 40  $\mu\text{g}\cdot\text{mL}^{-1}$ ).

### 295 **Strain construction**

296 The *tagA* gene (*EC042\_4550*) was deleted in the enteroaggregative *E. coli* 17-2 strain using  $\lambda$ -red  
297 recombination<sup>47</sup> as previously described<sup>19</sup> using plasmid pKOBEG<sup>56</sup>. In brief, a kanamycin cassette was  
298 amplified from plasmid pKD4 using oligonucleotides carrying 50-nucleotide extensions homologous to regions  
299 adjacent to *tagA*. After electroporation of 600 ng of column-purified PCR product, kanamycin-resistant clones  
300 were selected and verified by colony-PCR. The kanamycin cassette was then excised using plasmid pCP20<sup>47</sup> and  
301 confirmed by colony-PCR. The same procedure was used to introduce APEX2 on the chromosome. The APEX2-  
302 coding sequence was amplified using the pKD4-Nter-APEX2 vector and inserted downstream of the start codon  
303 of the *tssA* (APEX2-TssA) or *gspE* (APEX2-GspE) genes or upstream the start codon of *tssA* (APEX2 in the  
304 T6SS gene cluster). Fluorescent reporter genes were amplified from the pKD4-Nter-sfGFP (N-terminal sfGFP  
305 fusions) or pKD4-Cter-mCherry (C-terminal mCherry fusions) vectors<sup>18,25</sup> and inserted on the chromosome  
306 downstream of the start codon of the *tagA* gene (sfGFP-TagA fusion) or upstream of the stop codon of the *tssK*  
307 gene (TssK-mCh fusion). For *cis*-complementation, a pKD4 derivative vector was engineered in which the *tagA*  
308 gene (or the *tagA* gene encoding an N-terminally VSV-G-tagged version of *tagA*) was placed under the control

309 of the *scil* T6SS promoter. The cassette was then introduced on the chromosome of the  $\Delta tagA$  and  $\Delta tagA$   
310 *tssB<sub>sfGFP</sub>* strains, at the *lacZ* locus, by  $\lambda$ -red recombination using pKOBEG.

### 311 **Plasmid construction**

312 PCR was performed using a Biometra thermocycler using the Q5 DNA polymerase (New England Biolabs).  
313 Enteroaggregative *E. coli* 17-2 chromosomal DNA or the pcDNA3-APEX2-NES plasmid (Addgene #49386)<sup>38</sup>  
314 were used as template for PCR amplification. All the plasmids have been constructed by restriction-free  
315 cloning<sup>57</sup>. Primers used in this study are listed in Supplementary Table 1. Briefly, the gene of interest was  
316 amplified using oligonucleotides introducing extensions annealing to the target vector. The double-stranded  
317 product of the first PCR has then been used as oligonucleotides for a second PCR using the target vector as  
318 template. PCR products were then treated with DpnI to eliminate template plasmids. All constructs have been  
319 verified by PCR and DNA sequencing (Eurofins Genomics). Plasmid pKD4-Nter-APEX2 was deposited in the  
320 Addgene plasmid repository under accession number 112868.

### 321 **In vivo APEX2-dependent biotin labeling and identification of biotinylated proteins by mass spectrometry**

322 *Biotin proximity labeling*. Experiments were initially conducted to optimize the conditions of biotin proximity  
323 labeling, by varying the concentration of biotin phenol, the concentration of H<sub>2</sub>O<sub>2</sub>, and the duration of the H<sub>2</sub>O<sub>2</sub>  
324 pulse. EAEC wild-type and APEX2 derivative cells were mixed with *E. coli* K-12 competitor cells and spotted  
325 on SIM plates supplemented with 10 mM biotin phenol (Biotine Tyramide; BP, Iris Biotech). After 4 h at 37°C,  
326 cells were washed with 1 mL of SIM medium to eliminate residual BP, and treated with 1 mM hydrogen  
327 peroxide (H<sub>2</sub>O<sub>2</sub>; Sigma Aldrich) for 1 min before quenching by washing with TSEN buffer (Tris-HCl 20 mM pH  
328 8, sucrose 30%, EDTA 1 mM, NaCl 100 mM) supplemented with egg-white lysozyme 10  $\mu\text{g}\cdot\text{mL}^{-1}$ , 10 mM  
329 sodium ascorbate and 10 mM sodium azide. Cells were either analyzed by SDS-PAGE and Streptavidin western-  
330 blotting or lysed by resuspension in CellLytic<sup>TM</sup> B (Sigma-Aldrich) supplemented or not with 0.2% Igepal<sup>®</sup> CA-  
331 630 (Sigma-Aldrich). After 1 hour on a wheel, lysates were clarified by centrifugation at 20,000 $\times g$  for 10 min.

332 *Streptavidin pull-down of biotinylated proteins*. The clarified cell lysate was incubated for 30 min at room  
333 temperature with 2 mg of Streptavidin-coated magnetic beads, equilibrated in CellLytic<sup>TM</sup> B buffer  
334 supplemented with Igepal CA-630. After three washes with CellLytic<sup>TM</sup> B supplemented with Igepal CA-630,  
335 the beads were resuspended in Laemmli loading dye and loaded on a SDS-PAGE gel. The migration was  
336 stopped when the sample reached the interface between concentrating and separating gels, and the band  
337 containing the total biotinylated proteins was cut out.

338 *Mass spectrometry analyses*. The protein-containing SDS-PAGE gel bands were washed with 100 mM  
339 acetonitrile/ammonium bicarbonate pH 7.5, reduced by 10 mM dithiothreitol in 100 mM ammonium bicarbonate  
340 pH 7.5, alkylated by 55 mM iodoacetamide in 100 mM ammonium bicarbonate pH 7.5, and overnight digested  
341 at 37 °C by Trypsin/Lys-C Mix from *Pseudomonas aeruginosa* (Promega) at 10  $\text{ng}\cdot\mu\text{L}^{-1}$  in 25 mM ammonium  
342 bicarbonate pH 7.5/proteaseMAX<sup>TM</sup> surfactant 0.025% (v/v). Tryptic peptides were extracted from gels by 0.1%  
343 (vol/vol) trifluoroacetic acid (TFA)/0.01% (vol/vol) proteaseMAX<sup>TM</sup>/50% (vol/vol) acetonitrile, and dried by  
344 speed vacuum. Solubilized samples in 0.05% (vol/vol) TFA /2 % (vol/vol) acetonitrile were analyzed on a ESI-  
345 Q-Exactive Plus (ThermoFisher) mass spectrometer coupled to a nanoliquid chromatography (Ultimate 3000,  
346 Dionex). Peptides were eluted from a C18 column (Acclaim PepMap RSLC, 75  $\mu\text{m} \times 150 \text{ mm}$ , 2  $\mu\text{m}$ , 100  $\text{\AA}$ ,  
347 Dionex) by a 6-40% linear gradient of mobile phase B (0.1% (vol/vol) formic acid (FA)/80% (vol/vol)  
348 acetonitrile) in mobile phase A (0.1% (vol/vol) FA) for 52 min. The peptides were detected in the mass  
349 spectrometer in a positive ion mode using a Top 10 Data Dependent workflow. One scan event full MS in the  
350 Orbitrap at 70,000, in a 350-1900  $m/z$  range was followed by a fragmentation MS/MS step, at 17,500, of the 10  
351 top ions, in the Higher Energy Collisional Dissociation cell set at 27. The spectra were processed by Proteome  
352 Discoverer software (ThermoFisher, version: 2.1.0.81) using the Sequest HT algorithm with the search following  
353 parameters: enteroaggregative *E. coli* 042 database (Taxonomy ID 216592 downloaded from NCBI by Protein  
354 Center, 4921 entries); trypsin enzyme (maximum 2 missed cleavages); fixed modification: carbamidomethyl  
355 (Cys); variable modification: oxidation (Met); mass values specific for monoisotopic; precursor mass tolerance:  
356  $\pm 10$  ppm; fragment mass tolerance:  $\pm 0.02$  Da. Peptide validation was based on the best Peptide Spectrum  
357 Match (PSM) defined at a 0.05 maximum Delta Cn and a 0.01 Strict Target False Discovery Rate. Proteins were  
358 identified if minimum 2 unique peptide sequences more than 6 amino-acids passed the high confidence filter.  
359 Human keratin being considered as a common contaminant in protein identification by mass spectrometry, only  
360 hits with a number of PSM higher than keratin were considered significant. Mass spectrometry datasheets are  
361 available in Supplementary Datasheets 1-9. The experiments were done in triplicate and a representative result is  
362 shown.

### 363 **Cell fractionation**

364 Cell fractionation assay was performed as previously described<sup>15,23,25</sup>. Briefly,  $5 \times 10^{10}$  exponentially growing  
365 cells were resuspended in 750  $\mu\text{L}$  of TSEN buffer and incubated for 10 min on ice. After addition of 100  $\mu\text{g}\cdot\text{mL}^{-1}$

366 of lysozyme and further incubation for 20 min on ice, 750  $\mu$ L of TN buffer (Tris-HCl 10 mM, pH 8.0, NaCl 100  
367 mM) was added, and cells were lysed by three cycles of freezing and thawing and four cycles of sonication.  
368 Unbroken cells were removed by centrifugation, and soluble and membrane fractions were separated by  
369 ultracentrifugation for 45 min at 45,000 $\times$ g. Membranes were washed once with TE buffer and resuspended in 1  
370 mL of TN buffer supplemented with 8 M urea, incubated on a wheel for 1 h at 25°C, and then centrifuged for 45  
371 min at 45,000 $\times$ g to separate integral membrane and peripherally membrane associated proteins. Soluble and  
372 membrane-associated fractions were resuspended in loading buffer and subjected to SDS-PAGE and  
373 immunoblotting. Anti-EF-Tu (HyCult Biotech, clone mAb900), anti-VSV-G (Sigma-Aldrich, clone P5D4), anti-  
374 OmpA, anti-TolA and anti-TolB (laboratory collection) antibodies were used to identify the cytoplasmic Tu  
375 elongation factor, the VSV-G-tagged TagA protein, the outer membrane OmpA protein, the inner membrane  
376 TolA protein, and the outer membrane-peripherally-associated periplasmic TolB protein, respectively. The  
377 experiments were done in triplicate and a representative result is shown.

#### 378 **Bacterial two-hybrid assay (BACTH)**

379 The adenylate cyclase-based bacterial two-hybrid technique<sup>58</sup> was used as previously published<sup>59</sup>. Briefly, the  
380 proteins to be tested were fused to the isolated T18 and T25 catalytic domains of the *Bordetella* adenylate  
381 cyclase. After introduction of the two plasmids producing the fusion proteins into the BTH101 reporter strain,  
382 plates were incubated at 30°C for 24 h. Three independent colonies for each transformation were inoculated into  
383 600  $\mu$ L of LB medium supplemented with ampicillin, kanamycin, and IPTG (0.5 mM). After overnight growth at  
384 30°C, 10  $\mu$ L of each culture was spotted onto LB plates supplemented with ampicillin, kanamycin, IPTG, and X-  
385 gal and incubated at 30°C. Controls include interaction assays with TolB and Pal, two protein partners unrelated  
386 to the T6SS. The experiments were done in triplicate and a representative result is shown.

#### 387 **Co-immuno-precipitation**

388 Soluble lysates from 2 $\times$ 10<sup>10</sup> cells producing VSV-G-tagged TagA or VSV-G-tagged TagA and FLAG-tagged  
389 TssA were obtained using the cell fractionation procedure, and subjected to immuno-precipitation on anti-FLAG  
390 M2 affinity gel (Sigma-Aldrich) for 1 h at 20°C. Beads were washed three times with TN buffer, resuspended in  
391 non-reducing Laemmli loading dye, and subjected to SDS-PAGE and immuno-blot analyses using monoclonal  
392 anti-VSV-G (Sigma-Aldrich, clone P5D4) and anti-FLAG (Sigma-Aldrich, clone M2) antibodies. The  
393 experiments were done in triplicate and a representative result is shown.

#### 394 **Inter-bacterial competition**

395 The anti-bacterial growth competition assay was performed as described<sup>60</sup>. The WT *E. coli* strain W3110 bearing  
396 the kanamycin resistant pUA66-*rrmB* plasmid<sup>61</sup> was used as prey in the competition assay. The pUA66-*rrmB*  
397 plasmid provides a strong constitutive green fluorescent (GFP<sup>+</sup>) phenotype. Attacker and prey cells were grown  
398 in SIM medium to a  $A_{600nm} \approx 0.6-0.8$ , harvested and normalized to a  $A_{600nm}$  of 10 in SIM. Attacker and prey cells  
399 were mixed to a 4:1 ratio and 15- $\mu$ L drops of the mixture were spotted in triplicate onto a prewarmed dry SIM  
400 agar plate. After 4-hour incubation at 37°C, fluorescent images were recorded with a LI-COR Odyssey imager.  
401 The bacterial spots were scratched off, and cells were resuspended in LB medium and normalized to a  $A_{600nm}$  of  
402 0.5. For fluorescence measurements, triplicates of 200  $\mu$ L were transferred into wells of a black 96-well plate  
403 (Greiner) and the  $A_{600nm}$  and fluorescence (excitation: 485 nm; emission: 530 nm) were measured with a TECAN  
404 infinite M200 microplate reader (9 measures per mixture per experiment). The relative fluorescence was  
405 expressed as the intensity of fluorescence divided by the  $A_{600nm}$ , after subtracting the values of a blank sample.  
406 For enumeration of viable *E. coli* K-12 cells, serial dilutions were plated on kanamycin plates and the number of  
407 colonies were counted after 16-h incubation at 37°C. The experiments were done in triplicate and a  
408 representative result is shown. Statistical analyses of inter-bacterial competition assays were performed by  
409 Student's *t*-test. Significant differences were defined as  $p < 0.05$  (\*),  $p < 0.01$  (\*\*), and  $p < 0.001$  (\*\*\*)

#### 410 **Fluorescence microscopy.**

411 Cells were grown in SIM to a  $A_{600nm} \approx 0.4-0.6$ , harvested and resuspended in fresh SIM to a  $A_{600nm} \approx 10$ . Cell  
412 mixtures were spotted on a thin pad of SIM supplemented with 2% agarose, covered with a cover slip and  
413 incubated for 20-30 min at room temperature before microscopy acquisition. Fluorescence microscopy was  
414 performed with a Nikon Eclipse Ti microscope equipped with an Orcaflash 4.0 LT digital camera (Hamamatsu)  
415 and a perfect focus system (PFS) to automatically maintain focus so that the point of interest within a specimen  
416 is always kept in sharp focus at all times despite mechanical or thermal perturbations. All fluorescence images  
417 were acquired with a minimal exposure time to minimize bleaching and phototoxicity effects. Exposure times  
418 were typically 20 ms for phase contrast, 100 ms for TssB-GFP, 2000 ms for sfGFP-TagA and 800 ms for TssB-  
419 mCherry and TssK-mCherry. The experiments were done in triplicate and a representative result is shown. For  
420 fluorescence microscopy, statistical analyses were performed using several representative fields from three  
421 independent biological replicates. Images were analyzed using ImageJ (<http://imagej.nih.gov/ij/>) and the

422 MicrobeJ plugin (<http://www.microbej.com/>)<sup>62</sup> or Zen (Carl Zeiss). Statistical dataset analysis was performed  
423 using Excel and the R software environment (<https://www.r-project.org/>). Contractile sheath residence time was  
424 determined as the number of frames during which the sheath remained fully extended. Only sheaths that  
425 performed visible extension and contraction within the movie period were considered.

#### 426 **Data availability.**

427 Excel sheets with the raw mass-spectrometry data are provided as Supplementary Datasheets 1-9. Plasmid  
428 pKD4-Nter-APEX2 has been deposited in the Addgene plasmid repository under accession number 112868. All  
429 data that support the findings of this study are available from the corresponding author upon request.

430

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575

576 **Correspondence and requests for materials** should be addressed to E.C. (cascales@imm.cnrs.fr).

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## 587 **Authors Contributions**

588 Y.G.S. & E.C. designed and conceived the experiments. Y.G.S., T.D., R.L., L.E. performed the  
589 experiments. Y.G.S. performed all the experiments, with the help of T.D. and L.E. for fluorescence



590 microscopy. R.L. performed the mass spectrometry analyses. E.C supervised the execution of the  
591 experiments. L.J. & E.C. provided tools. E.C. wrote the paper with contributions of Y.G.S, T.D., R.L.  
592 and L.J.

593 **Additional Information**

594 **Supplementary Information** is available for this manuscript. It includes one Supplementary Table,  
595 seven Supplementary Figures, two Supplementary Videos, and nine Supplementary Datasheets.

596

597 **Competing Interests**

598 The authors declare no competing financial interests.

599

600 **Legend to Figures**

601 **Figure 1 | Summary of TssA proximity partners.** The different stages of T6SS assembly  
602 are depicted (1, assembly of the membrane complex; 2, baseplate recruitment and docking; 3,  
603 polymerization of the tail), as well as the stage-blocking mutations used in this study (deletion  
604 of the *tssK* or *vgrG* gene stops T6SS assembly at stage 1 whereas deletion of the *hcp* gene  
605 stops T6SS assembly at stage 2). The TssA protein is shown in red and the other T6SS  
606 subunits are indicated (J corresponds to TssJ). The blue color highlights the subunits that are  
607 biotinylated by the functional APEX2-TssA fusion protein.

608 **Figure 2 | TagA is an accessory T6SS cytosolic component that associates with the**  
609 **membrane. a and b, TagA interacts directly with TssA. a, BACTH assay.** BTH101 reporter  
610 cells producing the indicated proteins fused to the T18 and T25 domain of the *Bordetella*  
611 adenylate cyclase were spotted on X-Gal-IPTG reporter LB agar plates. The BACTH  
612 experiment was performed in triplicate with identical results. **b, Co-immunoprecipitation**  
613 **assay.** Soluble lysates of *E. coli* cells producing VSV-G-tagged TagA ( $\sqrt{\text{TagA}}$ ) alone or  
614  $\sqrt{\text{TagA}}$  with FLAG-tagged TssA (TssA<sub>F</sub>) were subjected to immunoprecipitation with anti-  
615 FLAG-coupled beads. The total lysates (T) and immunoprecipitated (IP) material were  
616 separated by 12.5% acrylamide SDS-PAGE and immunodetected with anti-FLAG (upper  
617 panel) and anti-VSV-G (lower panel) monoclonal antibodies. Molecular weight markers (in  
618 kDa) are indicated on left. The co-immunoprecipitation experiment was performed in  
619 triplicate with identical results. **c, TagA optimizes T6SS activity.** Antibacterial competition  
620 assay. *E. coli* K-12 competitor cells (W3110 *gfp*<sup>+</sup>, kan<sup>R</sup>) were mixed with the indicated  
621 attacker cells, spotted onto SIM agar plates, and incubated for 4 h at 37 °C. The fluorescence  
622 of the bacterial spot (in arbitrary units, bars represent the average, standard deviation are  
623 indicated, dot plots (grey circles) are overlaid) is shown on top. The number of surviving *E.*  
624 *coli* competitor cells (counted on selective kanamycin medium) is indicated in the lower  
625 graph (in log<sub>10</sub> of colony forming units). The circles indicate the values from three  
626 independent assays, and the average is indicated by the bar. Statistical significance relative to  
627 the wild-type strain is indicated (*p*-values; NS, non significant; \*, *p* < 0.05; \*\*, *p* < 0.01, two-  
628 sided Student's *t*-test; *p*-values for fluorescence measurements (upper graph):  $\Delta tssA$ ,  
629 0.000196;  $\Delta tagA$ , 0.0086;  $\sqrt{tagA}^+$ , 0.119;  $tagA^+$ , 0.746; *p*-values for *E. coli* K-12 survival  
630 (lower graph):  $\Delta tssA$ , 0.00219;  $\Delta tagA$ , 0.0194;  $\sqrt{tagA}^+$ , 0.531;  $tagA^+$ , 0.831). **d, TagA**  
631 **fractionates with soluble and peripherally-associated membrane proteins.** Fractionation assay.

632 Total extracts (T) of WT or  $\Delta sciI$  EAEC, or *E. coli* K-12 cells producing  $\nu$ TagA were  
633 subjected to fractionation to separate the soluble (S) and membrane (M) fractions.  
634 Peripherally-associated (pM) and integral (iM) membrane proteins were separated by  
635 treatment with 8 M urea. Control markers include the integral outer membrane OmpA protein,  
636 the integral inner membrane TolA protein, the peripherally-associated membrane TolB  
637 protein, and the EF-Tu cytoplasmic elongation factor. Molecular weight markers (in kDa) are  
638 indicated on left. Fractionation experiments were performed in triplicate with identical results.  
639 Uncropped blots are shown in Supplementary Fig. 7.

640 **Figure 3 | TagA localizes at the cell quarters and binds the distal end of the sheath. a,**  
641 Fluorescence microscopy recording of wild-type EAEC cells producing sfGFP-TagA (upper  
642 panel, phase channel; lower panel, GFP channel). Localization of TagA clusters are indicated  
643 by white arrowheads. Scale bar, 1  $\mu$ m. Fluorescence microscopy recordings have been  
644 performed thirty times with identical results. A deconvolution analysis of WT EAEC cells  
645 producing sfGFP-TagA is shown in Supplementary Fig. 4a. **b,** Number of sfGFP-TagA foci  
646 per cell. The percentage of cells with 0, 1, 2 or >2 foci is indicated ( $n= 1171$  cells from three  
647 biological replicates, bars represent the average, standard deviation are indicated, dot plots  
648 (grey circles) are overlaid). The mean number of foci per cell is  $0.74 \pm 0.24$ . **c,** Spatial  
649 repartition of sfGFP-TagA foci. Shown is a projection of the foci from  $n = 316$  cells on a  
650 single cell (from blue (low abundance) to yellow (high abundance)). **d,** Fluorescence  
651 microscopy time-lapse recording of EAEC cells producing sfGFP-TagA. Individual images  
652 were taken every 40 s. The localization of TagA is indicated by the white arrowhead. A  
653 schematic diagram representating the dynamics of TagA is shown below. Scale bar, 1  $\mu$ m.  
654 Time-lapse recordings have been performed thirty times with identical results. A statistical  
655 analyses of the distribution of sfGFP-TagA dynamics in wild-type and  $\Delta sciI$  cells is shown in  
656 Supplementary Fig. 4b. **e and f,** Co-localization of sfGFP-TagA with TssB-mCherry (**e**) or  
657 TssK-mCherry (**f**). From top to bottom are shown the GFP, and mCherry channels, an overlay  
658 of the GFP and mCherry channels and a schematic representation. Scale bar, 1  $\mu$ m. Statistical  
659 analyses of TagA co-localization with TssK and TssB are shown in Supplementary Figure 5.  
660 Co-localization recordings have been performed three and five times for TssK-mCherry and  
661 TssB-mCherry, respectively, with identical results. **g,** Fluorescence microscopy time-lapse  
662 recording of EAEC cells producing sfGFP-TagA and TssB-mCherry. Individual images were  
663 taken every 30 s. White arrowheads indicate assembly or contraction events whereas the blue  
664 arrowheads point at a complete assembly-contraction cycle. A schematic diagram

665 representing a complete cycle is shown below. Scale bar, 1  $\mu\text{m}$ . See also Supplementary  
666 Video 1. Statistical analyses of co-localization of sfGFP-TagA with TssK-mCherry or with  
667 the distal end of the sheath are shown in Supplementary Fig. 5. Time-lapse co-localization  
668 recordings have been performed five times with identical results.

669 **Figure 4 | TagA stops sheath elongation and maintains the sheath under the extended**  
670 **conformation. a**, Sheath dynamics. Fluorescence microscopy time-lapse recording of EAEC  
671 wild-type (WT),  $\Delta\text{tagA}$  cells or  $\Delta\text{tagA}$  cells expressing *tagA* ( $\Delta\text{tagA tagA}^+$ ), producing TssB-  
672 sfGFP. Individual images were taken every 40 s. White arrowheads indicate complete  
673 assembly-contraction cycles whereas blue arrowheads indicate sheaths that do not stop at the  
674 opposite membrane and bend. Red arrowheads point sheath detachment from the membrane  
675 complex/baseplate. Scale bar, 1  $\mu\text{m}$ . See also Supplementary Video 2. Time-lapse recordings  
676 have been performed in triplicate with identical results. **b**, Quantification of the different  
677 sheath assembly events (blue, sheaths that stop at the opposite membrane; green, sheaths that  
678 do not stop at the opposite membrane, bend and break). The number of sheath events  
679 analyzed for each strain (*n*) is indicated on top, standard deviation are indicated by the vertical  
680 bar, dot plots (grey circles) are overlaid. **c**, Violin plot representation of the sheath residence  
681 time. The distribution of the sheath residence time in wild-type (WT, blue),  $\Delta\text{tagA}$  (red) cells  
682 and  $\Delta\text{tagA}$  expressing *tagA* ( $\Delta\text{tagA tagA}^+$ , green) is represented as a violin plot. The bold  
683 horizontal bar represents the median values; the closed circle represents the mean; the lower  
684 and upper boundaries of the internal box plot correspond to the 25% and 75% percentiles  
685 respectively; the whiskers represent the 10% and 90% percentiles. Outliers are shown as black  
686 stars. The distribution is indicated by the outer shape. The number of sheath  
687 assembly/contraction events (*n*) is indicated on right, as well as the mean and the standard  
688 deviation, and the statistical significance relative to WT (NS, non significant; \*\*\*\*,  $p <$   
689  $0.0001$ ; two-sided Wilcoxon's *t*-test; *p*-values:  $\Delta\text{tagA}$ ,  $2.2 \times 10^{-16}$ ;  $\text{tagA}^+$ , 0.62). The  
690 distribution is shown as a graph in Supplementary Fig. 6.

**Table 1. Mass spectrometry identification of proteins after APEX2-dependent proximity biotinylation.**

The highest ranked proteins are indicated, as well as the number of Peptide Spectral Matches (PSM), *i.e.*, the number of validated peptides for the corresponding protein, indicating its relative abundance. The mass spectrometry datasheets are available in supplemental data files.

APEX-TssA <sup>a</sup>		APEX-TssA <sup>a,b</sup>		none <sup>c</sup>	APEX <sup>d</sup>	APEX-GspE <sup>e</sup>		
TssM	760	VgrG	405		AceF	120	GspL	377
TssK	311	TssK	365				GspF	323
VgrG	306	Hcp	349					
Hcp	258	TagA	234					
TssF	211	TssF	222					
TagA	193	TssC	87					
TssL	163	TssG	89					
TssG	64*							
TssC	54*							

<sup>a</sup> *apex2* fused at the 5' of *tssA*.

<sup>b</sup> no detergent used in the preparation of cell lysate.

<sup>c</sup> no *apex2* inserted on the chromosome.

<sup>d</sup> *apex2* inserted in the T6SS gene cluster.

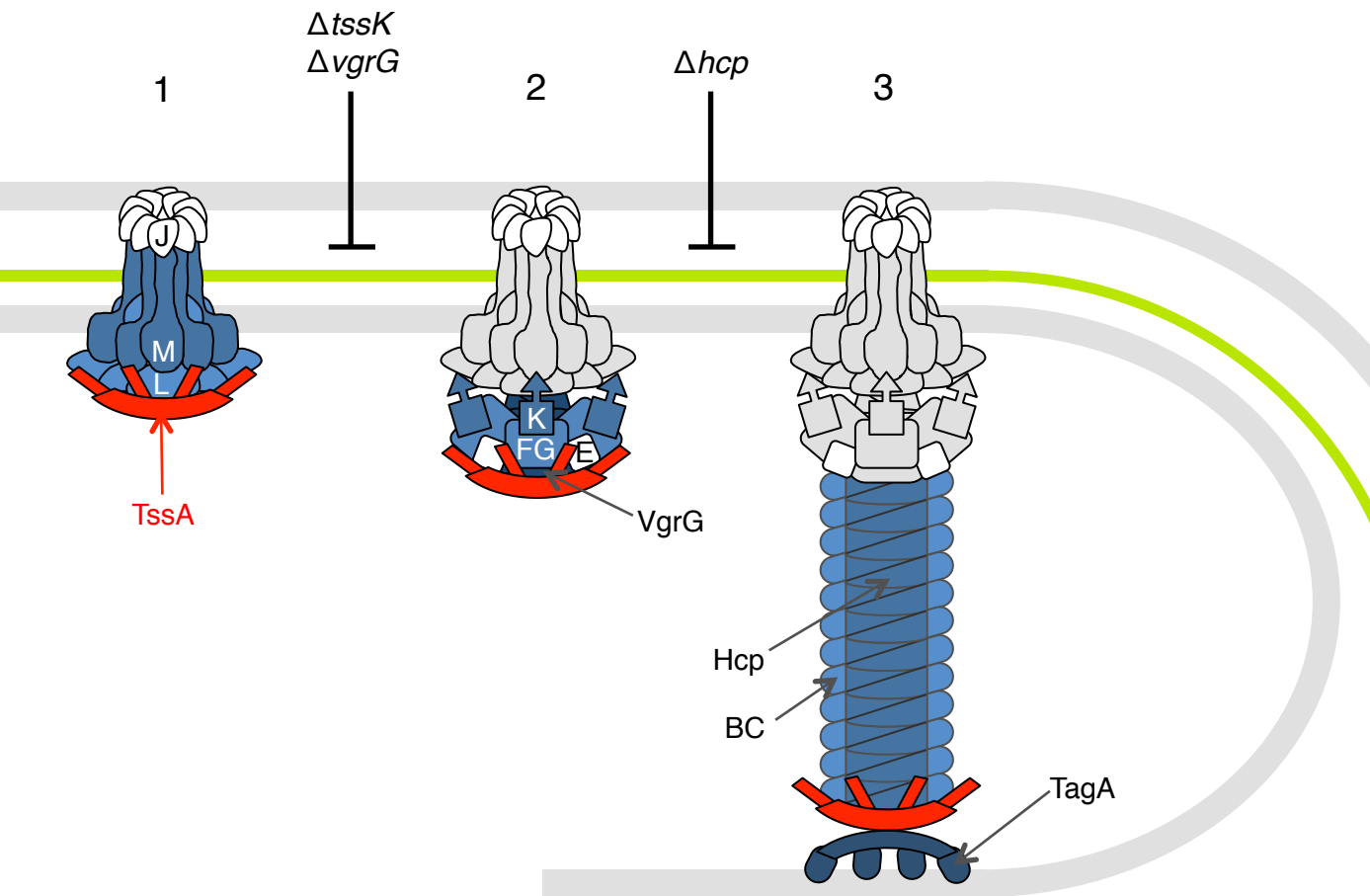
<sup>e</sup> *apex2* fused at the 5' of *gspE*.

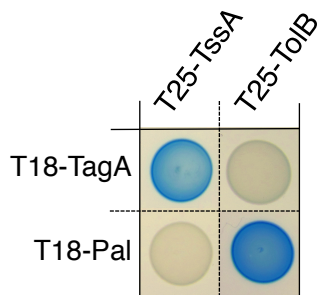
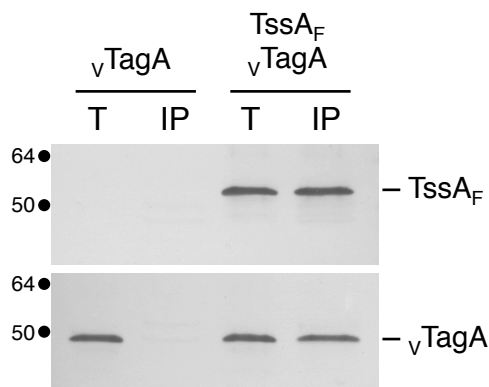
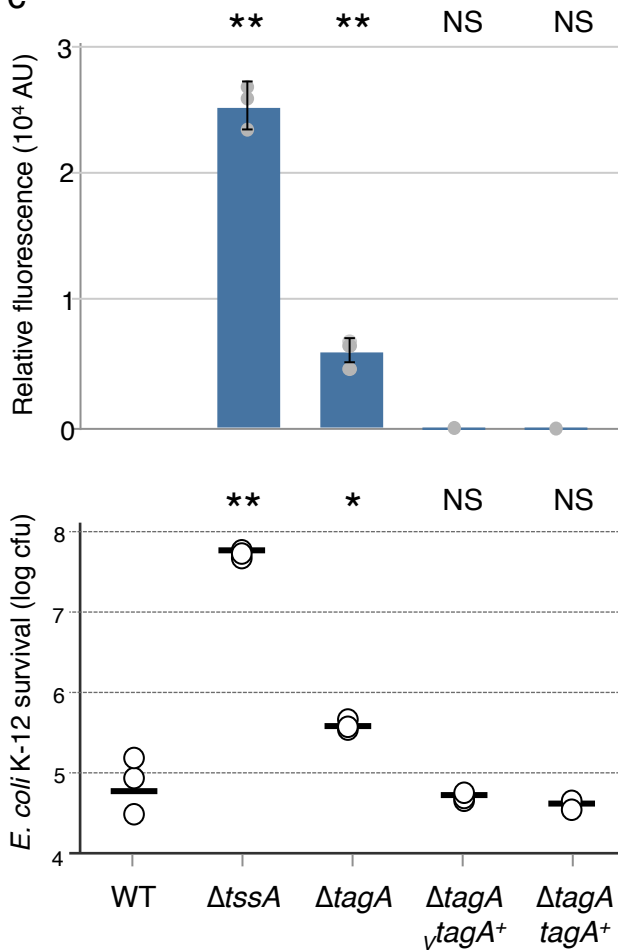
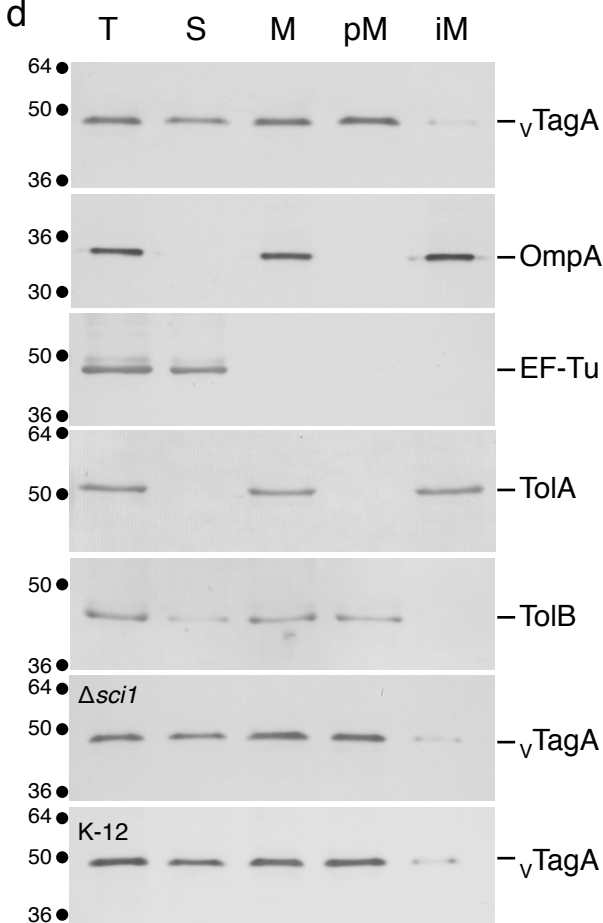
\* T6SS proteins with lower abundance than human keratin

**Table 2. Mass spectrometry identification of proteins after APEX2-dependent proximity biotinylation in stage-blocking mutant cells.**

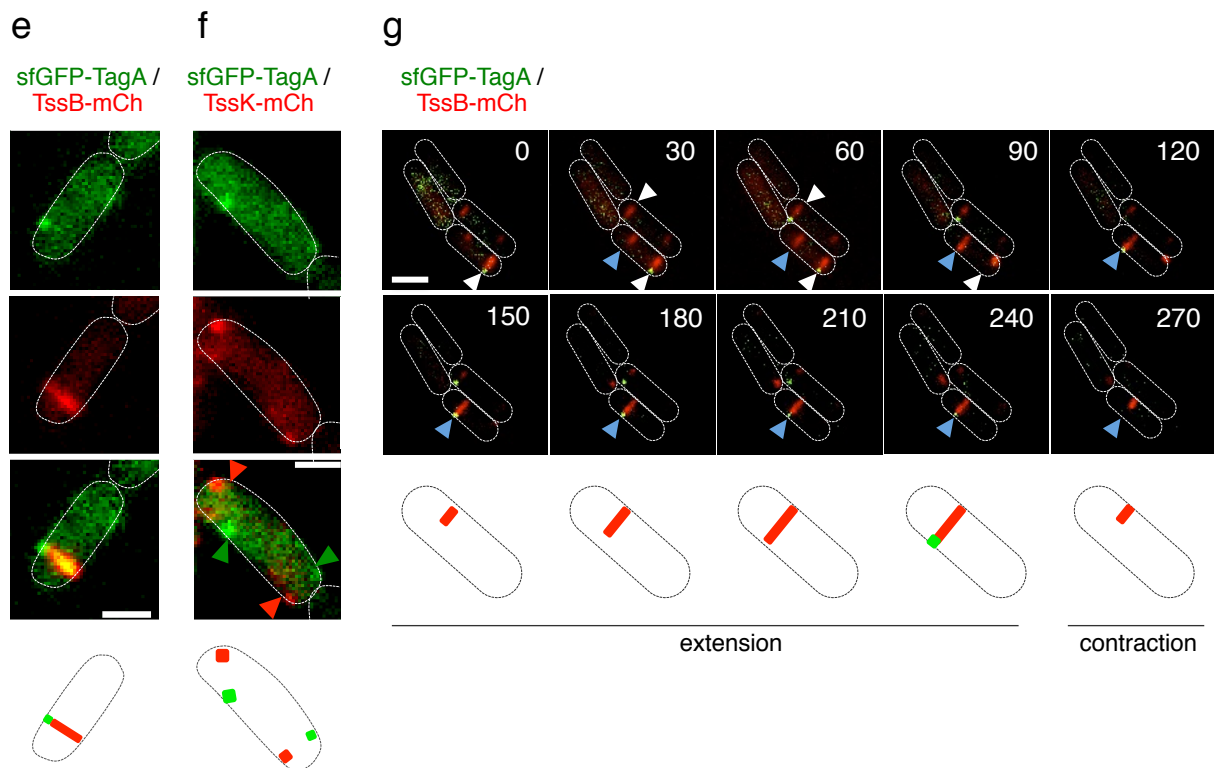
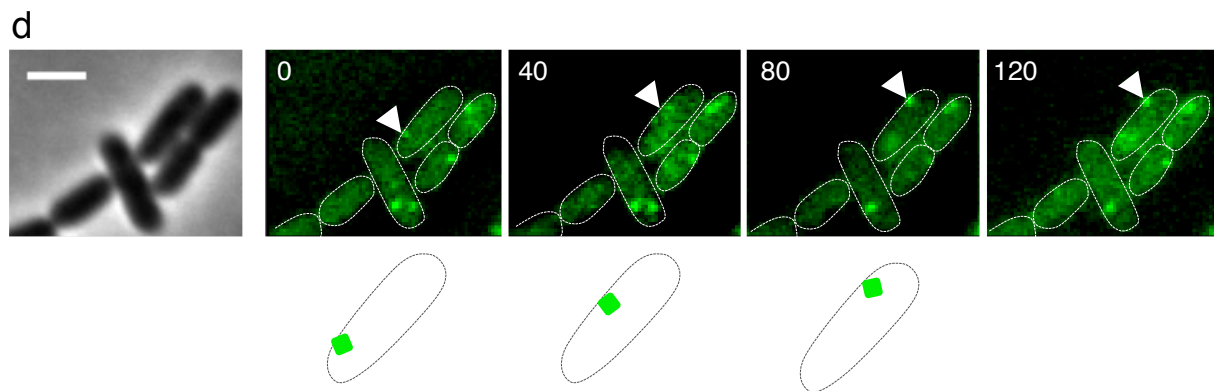
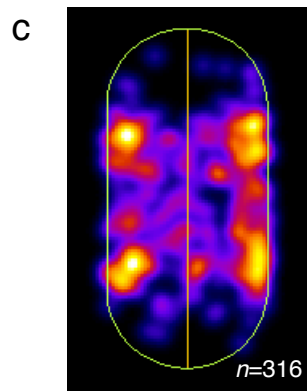
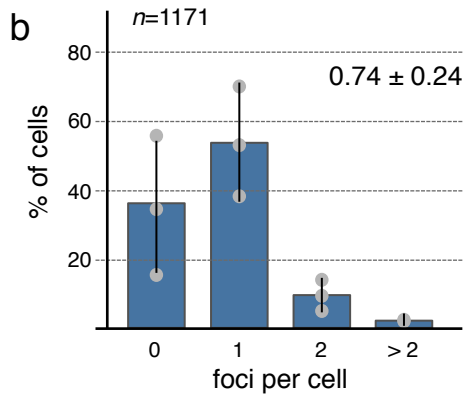
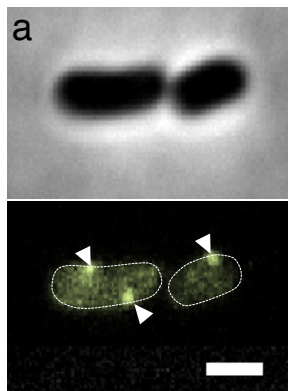
The highest ranked proteins are indicated, as well as the number of Peptide Spectral Matches (PSM), *i.e.*, the number of validated peptides for the corresponding protein, indicating its relative abundance. The mass spectrometry datasheets are available in supplemental data files.

$\Delta tssL$	$\Delta tssK$	$\Delta vgrG$	$\Delta hcp$
AceF 39	TssM 893	TssM 1343	TssM 1173
	TssL 222	TssL 296	TssK 460
	AceF 76	AceF 67	VgrG 448
			TssF 241
			TssL 235
			TssG 102



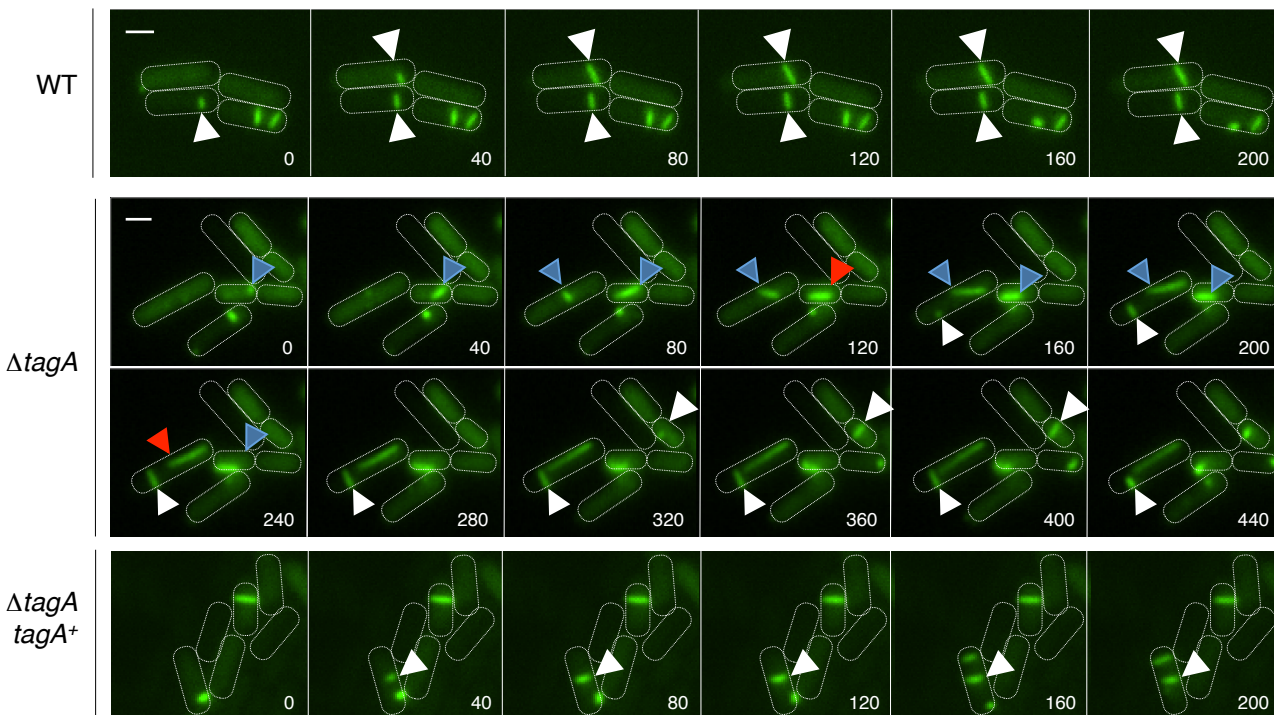
**a****b****c****d**



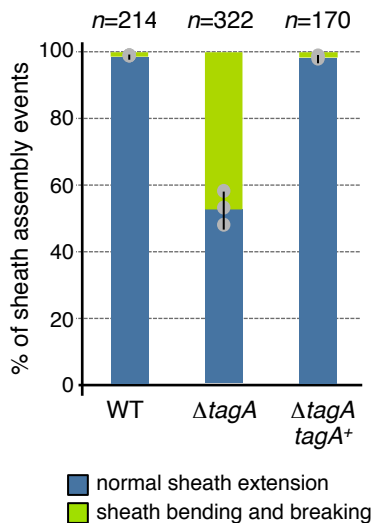


a

TssB-sfGFP



b



c

