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

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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.

The bacterial Type VI secretion system (T6SS) is a tail structure that uses a contractile mechanism to inject a molecular syringe loaded with effectors into target cells¹⁻⁸. This sophisticated apparatus is widespread in Gram-negative bacteria, and could be deployed to deliver effectors to the milieu, or into eukaryotic host cells or competitor bacterial cells. The T6SS helps to establish symbiosis, to collect metals or to disable or kill target cells^{5,9-13}. At the molecular level, the T6SS requires a minimum set of 12 proteins that are indispensable for its assembly and function whereas additional, accessory proteins such as peptidoglycanbinding proteins, peptidoglycan remodelling enzymes, disassembly ATPases and spike
 sharpeners might be necessary to improve its efficiency^{4,6,15-17}.

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 mediates specific contacts with each of the T6SS sub-complexes¹⁸. In entero-aggregative E. 41 42 coli (EAEC), the outer membrane lipoprotein TssJ positions first and recruits the inner membrane proteins TssM and TssL¹⁹⁻²¹. Polymerization of the TssJLM heterotrimer into a 43 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 that is recruited to and activated by TssM^{21,22}. Binding of the TssA protein onto the 46 membrane complex recruits and stabilizes a second multi-protein complex, the baseplate¹⁸. 47 The baseplate comprises five different proteins: TssE, -F, -G, -K and VgrG²³⁻²⁶. The 48 baseplate constitutes the assembly platform for the tail and shares structural and functional 49 homologies with the baseplates of other contractile structures such as bacteriophages^{25,26}. The 50 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 membrane complex²⁵⁻³⁰. Once the baseplate is docked to the membrane complex^{23,25,31,32}, the 56 tail is built by the processive addition of inner tube and contractile sheath building blocks^{3,33-} 57 ³⁵. Tail assembly is a coordinated process, in which tube and sheath polymerizations are 58 59 interdependent but it has been proposed that the assembly of one tube hexamer immediatly precedes the polymerization of one sheath $row^{18,33}$. The coordinated assembly of the sheath 60 with that of the tube is dictated by the TssA subunit^{18,36}. During the assembly process, TssA 61 remains at the distal end of the tail, at which new subunits are incorporated^{18,37}. Fluorescence 62 microscopy recordings revealed that the length of the tail is not controlled but rather that its 63 64 polymerization stops once the distal end is at proximity of the membrane^{1,2,18,25}. Once the biogenesis of the tail is completed, TssA remains associated to the distal end, and it has been 65 proposed that TssA remains until the last row of sheath contracts^{18,36}. However, the molecular 66 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.

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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 protein topology or protein partners^{38,39}. APEX2 is an engineered variant of the monomeric 76 77 ascorbate peroxidase that oxidizes phenol derivatives to phenoxyl radicals in presence of hydrogen peroxyde (H₂O₂)³⁸. Hence APEX2 converts biotin-phenol to short-lived, small-78 79 distance diffusive biotin-phenoxyl radicals that covalently react with electron-rich amino-acid 80 side chains. Based on biotin-phenoxyl radicals half-life and diffusion rates, it is proposed that APEX2 biotinvlates macromolecules located within 20 nm radius⁴⁰. Once fused to a bait 81 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 trachomatis inclusion membrane³⁹⁻⁴⁶. However, studies have been restricted to eukaryotic 87 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 onestep procedure⁴⁷. The *apex2*-coding sequence was genetically fused to *tssA* (gene accession 91 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 activity¹⁸. Indeed, anti-bacterial competition assay showed that cells producing APEX2-TssA 95 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 H₂O₂, 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_2O_2 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 and TssC are known interacting partners of TssA¹⁸ whereas TssF and TssG form a stable 112 complex with TssK^{24,25} and are likely at close proximity to the TssK-bound TssA. Finally, 113 114 while no direct contacts have been identified between TssA and TssM and TssL, the TssA protein was previously shown to co-purify with the TssJLM complex¹⁸. The EC042 4550 115 116 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 ImpA domain)³⁶. The two T6SS integral inner membrane proteins, TssM and TssL, were not 119 retrieved when detergent was omitted during preparation of the lysate sample prior to 120 121 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 GspE, a cytosolic ATPase that transiently associates with the membrane-anchored Type II 126 secretion system (T2SS)⁴⁸. Although two known T2SS partners of GspE, GspF and GspL⁴⁹, 127 128 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 T6SS133 biogenesis.

134 Previous data have suggested that TssA mediates sequential contacts with the membrane complex, the baseplate and the tail, during T6SS biogenesis^{18,36}. We sought to test 135 136 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 is blocked at an early stage²¹. Indeed, no significant hit was recovered in a *tssL* strain (Table 138 139 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 complex is assembled in absence of baseplate components²⁵ and with the proposal that TssA 141 is bound at the cytosolic face of the membrane complex 18,36 . Finally, the biotinvlation of 142 143 membrane complex and baseplate components in hcp cells (Table 2, fourth column) correlates 144 with the obervation that the absence of Hcp prevents polymerization of the tail but does not impact membrane complex and baseplate assembly²⁵. A schematic summary of proteins 145 146 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 ± 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 (vTagA; 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 $\Delta sci1$ (*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, vTagA co-162 fractionnates 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 vTagA behaves similarly to the peripherally-associated membrane protein TolB: vTagA 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 agreeement 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 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 $\Delta sci1$ 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.

To determine the role of TagA in sheath dynamics, we imaged wild-type, *tagA* and complemented *tagA* cells producing TssB-sfGFP. Time-lapse fluorescence recordings revealed some remarkable sheath dynamics in *tagA* cells: about fifty percent of the sheaths extend toward the opposite side of the membrane but do not stop, and hence undergo a distortion event allowing their extension parallel to the membrane (Fig. 4a-b; Supplementary Video 2), ultimately leading to sheaths break or detachment from the membrane 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 competion 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 immediatly after extension is significantly increased in tagA cells (> 210 60% of the events) compared to wild-type of 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: it interacts first with the inner membrane proteins of the membrane complex (TssL, TssM), 221 222 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 bacterial two-hybrid, co-precipitation and surface-plasmon resonance assays¹⁸. This 224 225 technology also detected two known partners of the T2SS-associated GspE ATPase, GspF and GspL⁴⁹⁻⁵². To our knowledge, this is the first report of the use of APEX2-dependent 226 227 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* and Photorhabdus species as well as Aeromonas hydrophila and Vibrio cholerae³⁶. In 233 agreement with the phenotypes associated with the deletion of the *tagA* gene in *V. cholerae*, 234 235 VCA0121⁵³, competition experiments provided evidence that TagA is not essential for T6SS activity in EAEC. However, the presence of TagA optimizes T6SS efficiency as a tagA 236 237 mutant retains 12 ± 4 % of anti-bacterial activity compared to the wild-type strain. Domain analyses of TagA showed that it shares the N-terminal ImpA domain found in proteins of the 238 TssA family, including EAEC TssA and P. aeruginosa TssA1^{18,36,54}, and a C-terminal 239 extension of unknown function. These two domains are separated by a long linker comprising 240 a stretch of hydrophobic residues predicted to be arranged as an amphipatic α -helix³⁶. Indeed, 241 TagA is a cytosolic protein that peripherally but tightly associates with the membrane. 242 243 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 domains, which have been previously shown to dimerize¹⁸. It is noteworthy that the *tagA* gene 247 248 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 growing tail occurs by opening the central hexaflexagon structure of TssA^{18,36}. Therefore, one 264 265 may hypothesize that if the sheath arrives perpendicular to the membrane, there is no room to

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

Based on our results, we propose that TagA associates with the cytoplasmic side of the inner membrane. Once the distal end of the sheath approaches the opposite membrane, TagA is captured, stops sheath polymerization and clamps the extended sheath to the membrane. By linking both the sheath distal extremity and the membrane, TagA prevents the immediate contraction of the sheath and maintains the tensile forces required for efficient killing. TagA is thus employed by TssA⁺ 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 α . 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 strain. E. coli K-12 and EAEC cells were routinely grown in LB broth at 37°C, with aeration. For induction of 285 286 the scil T6SS gene cluster, cells were grown in Scil-inducing medium [SIM: M9 minimal medium 287 supplemented with glycerol (0.2%), vitamin B1 (1 µg.mL⁻¹), casaminoacids (40 µg.mL⁻¹), LB (10% v/v)]⁵⁵. Plasmids and chromosomal deletions and insertions were maintained by the addition of ampicillin (100 μ g.mL⁻¹ 288 for K-12), kanamycin (50 μ g.mL⁻¹ for K-12, 50 μ g.mL⁻¹ for chromosomal insertion on EAEC, 100 μ g.mL⁻¹ for 289 plasmid bearing EAEC), or chloramphenicol (40 µg.mL⁻¹). Expression of genes from pBAD or pASK-IBA 290 291 vectors was induced at $A_{600 \text{ pm}} \approx 0.6$ with 0.02% of L-arabinose (Sigma-Aldrich) for 1 h or with 0.1 µg.mL⁻¹ of 292 anhydrotetracyclin (IBA Technologies) for 45 min, respectively. For BACTH experiments, gene expression was 293 294 induced by the addition of iso-propyl-β-D-thio-galactopyranoside (IPTG, Sigma-Aldrich, 0.5 mM) and plates were supplemented with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal, Eurobio, 40 µg.mL⁻¹).

295 Strain construction

296 The tagA gene (EC042 4550) was deleted in the enteroaggregative E. coli 17-2 strain using λ -red 297 recombination⁴⁷ as previously described¹⁹ using plasmid pKOBEG⁵⁶. 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 $pCP20^{47}$ 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 fusions) or pKD4-Cter-mCherry (C-terminal mCherry fusions) vectors^{18,25} and inserted on the chromosome 305 306 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 *sci1* T6SS promoter. The cassette was then introduced on the chromosome of the $\Delta tagA$ and $\Delta tagA$
- 310 $tssB_{sfGFP}$ strains, at the *lacZ* locus, by λ -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)³⁸ 314 were used as template for PCR amplification. All the plasmids have been constructed by restriction-free 315 cloning⁵⁷. 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_2O_2 , and the duration of the H_2O_2 324 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 (H₂O₂; 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 µg.mL⁻¹, 10 mM 329 sodium ascorbate and 10 mM sodium azide. Cells were either analyzed by SDS-PAGE and Streptavidin westernblotting or lysed by resuspension in CellLytic[™] B (Sigma-Aldrich) supplemented or not with 0.2% Igepal[®] CA-330 630 (Sigma-Aldrich). After 1 hour on a wheel, lysates were clarified by centrifugation at $20,000 \times g$ for 10 min. 331

Streptavidin pull-down of biotinylated proteins. The clarified cell lysate was incubated for 30 min at room temperature with 2 mg of Streptavidin-coated magnetic beads, equilibrated in CellLyticTM B buffer supplemented with Igepal CA-630. After three washes with CellLyticTM B supplemented with Igepal CA-630, the beads were resuspended in Laemmli loading dye and loaded on a SDS-PAGE gel. The migration was stopped when the sample reached the interface between concentrating and separating gels, and the band 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 ng.µL⁻¹ in 25 mM ammonium bicarbonate pH 7.5/proteaseMAXTM surfactant 0.025% (v/v). Tryptic peptides were extracted from gels by 0.1% 342 343 (vol/vol) trifluoroacetic acid (TFA)/0.01% (vol/vol) proteaseMAXTM/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 µm × 150 mm, 2 µm, 100 Å, 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

Cell fractionation assay was performed as previously described^{15,23,25}. Briefly, 5×10^{10} exponentially growing

cells were resuspended in 750 μl of TSEN buffer and incubated for 10 min on ice. After addition of 100 μg.mL⁻

366 of lysozyme and further incubation for 20 min on ice, 750 µL 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)

The adenylate cyclase-based bacterial two-hybrid technique⁵⁸ was used as previously published⁵⁹. Briefly, the 379 380 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 µL of LB medium supplemented with ampicillin, kanamycin, and IPTG (0.5 mM). After overnight growth at 384 30°C, 10 µL 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

Soluble lysates from 2×10¹⁰ cells producing VSV-G-tagged TagA or VSV-G-tagged TagA and FLAG-tagged TssA were obtained using the cell fractionation procedure, and subjected to immuno-precipitation on anti-FLAG M2 affinity gel (Sigma-Aldrich) for 1 h at 20°C. Beads were washed three times with TN buffer, resuspended in non-reducing Laemmli loading dye, and subjected to SDS-PAGE and immuno-blot analyses using monoclonal anti-VSV-G (Sigma-Aldrich, clone P5D4) and anti-FLAG (Sigma-Aldrich, clone M2) antibodies. The 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 described⁶⁰. The WT E. coli strain W3110 bearing 396 the kanamycin resistant pUA66-rrnB plasmid⁶¹ was used as prey in the competition assay. The pUA66-rrnB 397 plasmid provides a strong constitutive green fluorescent (GFP⁺) 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-µL 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 µl 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 $A_{600 \text{ nm}} \approx 0.4$ -0.6, harvested and resuspended in fresh SIM to a $A_{600 \text{ nm}} \approx 10$. 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/)⁶² 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
- 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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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

Figure 1 | Summary of TssA proximity partners. The different stages of T6SS assembly are depicted (1, assembly of the membrane complex; 2, baseplate recruitment and docking; 3, polymerization of the tail), as well as the stage-blocking mutations used in this study (deletion of the *tssK* or *vgrG* gene stops T6SS assembly at stage 1 whereas deletion of the *hcp* gene stops T6SS assembly at stage 2). The TssA protein is shown in red and the other T6SS subunits are indicated (J corresponds to TssJ). The blue color highlights the subunits that are 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 (vTagA) alone or 614 $_{\rm V}$ TagA with FLAG-tagged TssA (TssA_F) 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^+ , kan^R) were mixed with the indicated attacker cells, spotted onto SIM agar plates, and incubated for 4 h at 37 °C. The fluorescence 621 622 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_{10} 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 the wild-type strain is indicated (*p*-values; NS, non significant; *, p < 0.05; **, p < 0.01, two-627 sided Student's *t*-test; *p*-values for fluorescence measurements (upper graph): $\Delta tssA$, 628 0.000196; $\Delta tagA$, 0.0086; $v_t tagA^+$, 0.119; $tagA^+$, 0.746; p-values for E. coli K-12 survival 629 (lower graph): $\Delta tssA$, 0.00219; $\Delta tagA$, 0.0194; $vtagA^+$, 0.531; $tagA^+$, 0.831). **d**, TagA 630 631 fractionates with soluble and peripherally-associated membrane proteins. Fractionation assay.

632 Total extracts (T) of WT or Ascil EAEC, or E. coli K-12 cells producing VTagA 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 µm. 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 ± 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 µm. 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 $\Delta sci1$ 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 µm. 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. \mathbf{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 representating a complete cycle is shown below. Scale bar, 1 μm. See also Supplementary
Video 1. Statistical analyses of co-localization of sfGFP-TagA with TssK-mCherry or with
the distal end of the sheath are shown in Supplementary Fig. 5. Time-lipse co-localization
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), $\Delta tagA$ cells or $\Delta tagA$ cells expressing tagA ($\Delta tagA$ 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 complex/baseplate. Scale bar, 1 µm. See also Supplementary Video 2. Time-lapse recordings 675 676 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), $\Delta tagA$ (red) cells 682 and $\Delta tagA$ expressing tagA ($\Delta tagA$ 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 deviation, and the statistical significance relative to WT (NS, non significative; ****, p < p688 0.0001; two-sided Wilcoxon's *t*-test; *p*-values: $\Delta tagA$, 2.2×10^{-16} ; $tagA^+$, 0.62). The 689 690 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 ^a	APEX-TssA ^{a,b}	none ^c	$APEX^{d}$	APEX-GspE ^e
TssM 760	VgrG 405		AceF 120	GspL 377
TssK 311	TssK 365			GspF 323
VgrG 306	Нср 349			
Hcp 258	TagA 234			
TssF 211	TssF 222			
TagA 193	TssC 87			
TssL 163	TssG 89			
TssG 64^*				
TssC 54^*				

^a *apex2* fused at the 5' of *tssA*. ^b no detergent used in the preparation of cell lysate.

^c no *apex2* inserted on the chromosome. ^d *apex2* inserted in the T6SS gene cluster.

^e *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.

$\Delta tssL$	$\Delta tssK$	$\Delta v g r G$	Δhcp
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





b











d









С











e f sfGFP-TagA / sfG TssB-mCh Ts

sfGFP-TagA / TssK-mCh

sfGFP-TagA / TssB-mCh





а

TssB-sfGFP





sheath bending and breaking

