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1 **The tip protein PAAR is required for the function of the type VI secretion system.**

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3 Solène G. Beauvois^a, Nicolas Flaugnatti^{a,c}, Marianne Ilbert^b, Marie Boyer^a, Esther Gavello-

4 Fernandez^{c,d}, Rémi Fronzes^{c,d}, Dukas Jurénas^{a,f} and Laure Journet^{a#}

5

6 ^aLaboratoire d'Ingénierie des Systèmes Macromoléculaires, Institut de Microbiologie,

7 Bioénergies et Biotechnologie, Institut de Microbiologie de la Méditerranée, Aix-Marseille

8 Université - CNRS UMR7255, Marseille, France

9 ^bLaboratoire de Bioénergétique et Ingénierie des Protéines, Institut de Microbiologie,

10 Bioénergies et Biotechnologie, Institut de Microbiologie de la Méditerranée, Aix-Marseille

11 Université—CNRS UMR7281, Marseille, France.

12 ^cInstitut Européen de Chimie et Biologie, University of Bordeaux, Pessac, France /

13 ^dCNRS UMR 5234 Microbiologie Fondamentale et Pathogénicité, Bordeaux, France

14

15 [#]Address correspondence to Laure Journet, ljournet@imm.cnrs.fr

16 ^ePresent address: Nicolas Flaugnatti, Laboratory of Molecular Microbiology, School of Life

17 Sciences, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland.

18 ^fPresent address: Dukas Jurénas, Laboratoire de Génétique et Physiologie Bactérienne,

19 Département de Biologie Moléculaire Université Libre de Bruxelles, Gosselies, Belgium.

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21 **Running title:** The type VI secretion system PAAR tip protein

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26 **ABSTRACT**

27 Bacteria are constantly competing to colonize crowded ecological niches, such as the human
28 gut. The Type VI secretion system (T6SS) is a critical bacterial weapon in this warfare. It
29 resembles a crossbow with a poisoned arrow allowing bacteria to inject toxic effectors
30 directly into target cells. This machinery is formed by an envelope-spanning complex which
31 recruits the baseplate, an assembly platform allowing the polymerization of a contractile
32 structure. The tail consists of a tube surrounded by a sheath and topped by the needle complex
33 composed of the VgrG and PAAR proteins. In the enteric pathogen enteroaggregative
34 *Escherichia coli* (EAEC), the Tle1 phospholipase toxin ensures the antibacterial activity of
35 the T6SS-1. For its transport, Tle1 interacts directly with the trimeric spike protein VgrG.
36 However, the importance and the function of the tip protein PAAR in the T6SS remains
37 unclear. Here we characterized the PAAR protein of EAEC using biochemical, fluorescence
38 microscopy and antibacterial competition approaches. Using pull-down assays and cryo-EM
39 analysis of the (VgrG)₃-(Tle1)₃-PAAR complex we show that PAAR tops and closes the β-
40 prism structure of the VgrG spike. The PAAR protein structure is further tightened by the
41 zinc atom coordinated via conserved residues essential for its function. We provide evidence
42 that PAAR is necessary for T6SS-1 mediated killing due to its requirement for proper T6SS
43 baseplate assembly and further sheath polymerization. Our results suggest that the PAAR
44 protein is an essential component of T6SS.

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52 **IMPORTANCE**

53 The Type VI secretion system (T6SS) is a bacterial contractile injection system involved in
54 bacterial competition by the delivery of antibacterial toxins. The T6SS consists of an
55 envelope-spanning complex that recruits the baseplate, allowing the polymerization of a
56 contractile tail structure. The tail is a tube wrapped by a sheath and topped by the tip of the
57 system, the VgrG spike/PAAR complex. Effectors loaded onto the puncturing tip or into the
58 tube are propelled in the target cells upon sheath contraction. The PAAR protein tips and
59 sharpens the VgrG spike. However, the importance and the function of this protein remain
60 unclear. Here we provide evidence for association of PAAR at the tip of the VgrG spike. We
61 also found that the PAAR protein is a T6SS critical component required for baseplate and
62 sheath assembly.

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64 **Keywords:** Type VI secretion system; cryo-electron microscopy; bacterial competition;
65 protein secretion; VgrG; bacterial toxin; zinc

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78 **INTRODUCTION**

79 Bacteria constantly compete for colonization of the ecological niches granting the access to
80 nutrient sources. The best armed bacteria will have greater chances to win this warfare and
81 plenty of weapons are deployed to succeed in this competition. The type VI secretion system
82 is one of the crucial weapons allowing bacteria to inject toxic effectors directly into the target
83 cells. There are two categories of T6SS toxins. T6SS periplasmic-acting toxins can degrade
84 peptidoglycan, membrane phospholipids or form pores in the inner membrane. T6SS
85 cytoplasmic effectors target DNA, deplete energy resources, inhibit cell division or inhibit
86 translation (1-4). T6SS can also target eukaryotic cells, employing effectors mainly targeting
87 the eukaryotic cytoskeleton and membranes (1, 2, 5).

88 The T6SS machinery is composed of 13-14 core components arranged in gene clusters (6, 7).
89 The architecture can be subdivided into three subcomplexes: the membrane complex, the
90 baseplate and the tail tube/sheath complex (TTC). The membrane complex is a 1.7 MDa
91 complex that anchors the system to the bacterial envelope (8, 9). It is composed of outer
92 membrane lipoprotein TssJ and inner membrane proteins TssM and TssL (in a 15:10:10
93 stoichiometry). The membrane complex recruits the baseplate which serves as an assembly
94 platform required for the polymerization of the contractile phage tail-like tubular structure
95 (10-15). The baseplate is assembled from six wedges composed of Tss-K, -E, -F, -G proteins
96 that arrange around a central hub, formed by the trimeric spike protein VgrG (14,15). With
97 the help of TssA protein, the baseplate directs the polymerization of the contractile TTC
98 structure (11,16). The TTC is composed of an internal tube made of Hcp protein hexamers,
99 surrounded by a contractile sheath, made of TssB and TssC subunits. This cytoplasmic tail is
100 about 1 μ m long and assembles within roughly 40 seconds through the TssA protein-

101 coordinated polymerization of Hcp and TssB/C components (10, 16, 17). Upon contact with
102 prey, the sheath contracts and expels the tube surmounted by the spike, together with the
103 effectors into the target cell. Indeed, the effectors are fused or interact with these structural
104 components (1, 3, 4, 18, 19). The enteroaggregative *Escherichia coli* T6SS cluster 1 (EAEC
105 T6SS-1) delivers the phospholipase toxin Tle1 (20). Three Tle1 cargo effectors are loaded on
106 the sides of the VgrG trimer via direct protein-protein interactions (20, 21). The N-terminal β -
107 strand of Tle1 interacts with the C-terminal transthyretin (TTR) extension of VgrG through
108 fold complementation. VgrG-Tle1 interaction is further stabilized by additional zones of
109 contact on the sides and at the base of the gp5-like domain of VgrG. The Tle1 phospholipase
110 A1 (PLA1) activity is inhibited by the interaction with the VgrG protein (21). Almost entire
111 T6SS, except the membrane complex, structurally resembles contractile bacteriophages, such
112 as T4 (22-25). The T6SS spike protein VgrG is structurally homologous to the T4 puncturing
113 device composed of gp27 and gp5 protein trimers (22, 23). Precisely, the triangular base of
114 VgrG also known as a hub domain is homologous to gp27. It is followed by an OB-fold
115 domain which is homologous to gp5*- domain, and extended by a β -helical gp5C-like
116 domain (21-23, 26, 27). The blunt end of the VgrG β -helical prism is covered by a single
117 PAAR protein that knots the trimeric structure into a uniform sharp tip (28). PAAR, named
118 after proline-alanine-alanine-arginine repeats, is structurally homologous to the gp5.4 protein
119 of bacteriophage T4, that similarly caps the central spike complex formed by gp5C (12, 28,
120 29).

121 PAAR was shown to be either important or absolutely required for T6SS secretion or killing
122 activity in different systems (28, 30-32). Its role remains to be determined, although some
123 hypotheses proposed that it stabilizes the trimeric VgrG complex or sharpens the T6SS for
124 target cell puncturing.

125 Here we report structural and functional analysis of EC042_4537 gene of the EAEC T6SS-1
126 that is predicted to code for a PAAR protein. Single particle cryo-EM analysis of the VgrG-
127 Tle1-PAAR complex from EAEC revealed that PAAR caps the blunt end of the VgrG needle.
128 ICP-OES analysis of purified PAAR protein shows that a zinc atom is coordinated by
129 conserved histidines and cysteines (H14, H46, C41, C74). Bacterial competition assays
130 indicate that PAAR protein and its metal coordinating residues H46, C41 and C74 are
131 essential for T6SS-1 antibacterial activity. Finally, using fluorescence microscopy, we further
132 demonstrate that PAAR is required for correct baseplate localization and thus for sheath
133 assembly. Taken together, our results confirm that the PAAR protein is an essential core
134 component of the T6SS.

135

136 **RESULTS**

137 **PAAR caps the C-terminal needle domain of VgrG spike complex.**

138 T6SS-1 encodes a unique predicted PAAR protein, downstream the *tliI* gene coding for
139 immunity protein against the Tle1 toxin (20). Our previous cryo-EM structure of the EAEC
140 (VgrG)₃-(Tle1)₃ complex revealed that the toxins are loaded on the sides of the β -prism of the
141 VgrG spike, leaving the top of the β -prism free to accommodate the PAAR protein (21). To
142 test this hypothesis, we aimed to determine where precisely does the PAAR localize on this
143 structure. We heterologously co-produced PAAR^{FLAG} (PAAR^{FL}) and ^{Strep}VgrG (^SVgrG), as
144 well as its truncated versions deleted for the TTR domain (^SVgrG1-778 called ^SVgrG Δ TTR)
145 or the whole β -prism (needle) part (^SVgrG1-490 called ^SVgrG Δ Needle) and tested direct
146 interactions by streptactin pull-down assays (Fig. 1AB). As shown in Fig. 1B, PAAR^{FLAG} co-
147 precipitated with ^SVgrG and ^SVgrG Δ TTR but not with VgrG deleted for the whole β -prism
148 (^SVgrG Δ Needle). We conclude that PAAR directly interacts with the C-terminal domain of
149 VgrG without implication of the TTR domain. A complete spike complex loaded with toxin

150 PAAR^{FL}-^SVgrG-Tle1^H was purified using a double-affinity and size exclusion
151 chromatography (Fig. 1C) and analyzed using single particle cryo-EM (Fig. 1DE, Fig. S1A).
152 For comparison we have repeated the analysis of a previously reported ^SVgrG-Tle1^H complex
153 without PAAR purified and analyzed in the same conditions. As previously reported, the
154 (VgrG)₃-(Tle1)₃ complexes dimerized through interactions at the top of the VgrG needle to
155 produce violin body-shaped 2D classes ((21), Fig. 1D, Fig. S1B). Such dimerization was
156 completely absent in the VgrG-Tle1-PAAR complex, suggesting that PAAR prevents the two
157 blunt ends of VgrG β-prisms from sticking together (Fig. 1D, Fig. S1A). A single conical
158 density on top of the trimeric VgrG needle prism was readily recognizable and likely
159 corresponds to the PAAR protein (Fig. 1D). Surprisingly, the 2D classification revealed
160 heterogeneity in particles yielding classes where 3, 2, 1 or none of the Tle1 toxins were
161 visible judging from the side and the top views (Fig. S1A). This is in contrast with the violin-
162 body-shaped 2D classes of the (VgrG)₃-(Tle1)₃ complex (without PAAR) where sharp
163 densities for all the toxins were typically observed (Fig. S1B) (21). This suggests that the
164 toxin positions are locked by the unnatural dimerization of the (VgrG)₃-(Tle1)₃ complexes
165 that occurs due to the absence of the PAAR protein. This dimerization seems to lock the TTR
166 regions in fixed position, that are extended from the VgrG structure and hang on the flexible
167 linkers not visible in the structure (21). It is therefore likely that the toxins loaded on the
168 VgrG are indeed quite flexible, which explains the partial absence of the Tle1 densities in
169 VgrG-Tle1-PAAR complex where TTRs are not fixed.

170 Due to very small size and lack of symmetry the resolution was not sufficient to build an
171 experimental atomic model of the PAAR protein in the obtained cryo-EM density maps. We
172 have therefore produced an AlphaFold2 co-folded model of the needle domain of VgrG
173 together with PAAR and aligned it to the experimental VgrG-Tle1 model (PDB:6SJL). The
174 resulting complete model could be readily docked into best resolved cryo-EM 3D volumes

175 representing (VgrG)₃-(Tle1)₂-PAAR (4.2 Å) or (VgrG)₃-(Tle1)₁-PAAR (at 3,7 Å) structures
176 (Fig. 1E; Fig S1C). The triangular shape of PAAR model perfectly fits into the extra conical
177 density that we assigned to PAAR (Fig. 1E).

178

179 **PAAR binds a zinc atom via conserved cysteine and histidine residues.**

180 The 88 amino-acid length protein PAAR (EC042_4537) from EAEC T6SS-1 belongs to class
181 1 PAAR family (28) carrying a PAAR_CT_2 domain (cd14744 of NCBI-CDD). The
182 PAAR^{EAEC} amino acid sequence was aligned with the sequences of structurally characterized
183 PAAR proteins from *Vibrio cholerae* and *E. coli* CFT073 (Fig. 2A) (28). These two proteins
184 were shown to bind a zinc atom that is coordinated by three histidines (His) and one cysteine
185 (Cys) localized at the extremity of the tip (28). Three motifs with hydrophobic residues that
186 could functionally resemble PAAR-motifs (Pro-Ala-Ala-Arg) could be identified using
187 sequence alignment (Fig. 2A). In the PAAR^{EAEC} protein, two His (His14 and His46) and two
188 Cys (Cys41 and Cys74) residues are conserved and localize at the sharp end of the
189 AlphaFold2 structural model of PAAR (Fig. 2B). We therefore hypothesized that these
190 residues could be involved in metal binding (Fig. 2AB) as previously reported for *V. cholerae*
191 and *E. coli* PAAR proteins (28). PAAR^{EAEC} protein was fused to a SUMO protein to increase
192 its solubility and a 6×histidine tag for purification with affinity chromatography (Fig. 2C).
193 ICP-OES analysis of the purified protein showed that 74.2 % (+/- 13.7 SD) of PAAR^{EAEC}
194 contains zinc. (Fig. 2D). Only small amounts (< 0.75% of total protein) of iron, copper or
195 nickel were found in the samples (Fig. S2). PAAR^{EAEC} proteins with single and double
196 alanine substitutions in conserved cysteines C41 and C74 exhibited a significant decrease in
197 zinc content compared to the wild type (Fig. 2D). Only 34.7% (+/- 10,9 SD) of PAAR^{C41A},
198 20.1% (+/- 12. 7 SD) of PAAR^{C74A} and 25.1 % (+/- 7.0) of PAAR^{C41AC74A} were found to
199 coordinate a zinc atom (Fig. 2D). To rule out the possibility that the overall fold of the small

200 proteins was impaired, we have assayed the interaction of the mutants with VgrG. The
201 PAAR^{EAEC} proteins with single C41A and C74A, and to lesser extent double C41A/C74A,
202 substitutions retained their capacity to interact with VgrG suggesting that these mutations
203 mainly affect metal coordination (Fig. S3). Overall, these results indicate that PAAR^{EAEC}
204 protein binds a zinc atom via at least the two conserved cysteines C41 and C74.

205

206 **PAAR is required for the antibacterial activity of T6SS.**

207 In the systems with multiple T6SS tip complexes at least one PAAR protein was shown to be
208 required for functionality of the T6SS but some reports suggested that it is not always
209 necessary (28, 30-33). Our model provides unambiguous case to study the role of PAAR,
210 since only one tip complex exists in EAEC T6SS-1 and the loading of toxins on the VgrG
211 spike does not require PAAR (Fig. 1D). To determine the importance of PAAR protein in the
212 function of T6SS of EAEC we have tested the capacity of a *PAAR* deletion mutant (Δ PAAR
213 Ω) to kill *E. coli* W3110 prey, using two different methods (34) (Fig. 3). First, the predator
214 strains and the prey cells were grown separately in T6SS-1 inducing medium (SIM) before
215 being mixed at a 1:4 ratio (for colorimetric method) or 4:1 ratio (for emergence time method)
216 and spotted on SIM-agar plates. In the case of the colorimetric method, after two hours of
217 contact, yellow CPRG substrate was deposited on the mixed-cell spots. CPRG substrate was
218 degraded into purple CPR product by β -galactosidase released from the prey cells upon cell
219 lysis induced by the secreted phospholipase toxin Tle1 by the wild type (WT) strain. On the
220 contrary, competition with deletion strain of the whole T6SS-1 cluster (Δ T6SS) used for
221 control retains yellow color of the mixed spot reporting the absence of cell lysis. Interestingly,
222 the Δ PAAR Ω mutant was not able to kill W3110 strain. However, its antibacterial activity
223 could be restored by complementing the expression of PAAR protein from a plasmid
224 (PAAR⁺) (Fig. 3A). To perform quantitative analysis, we have further pursued the emergence

225 time method (34). After two hours of contact each cell spot was scraped from agar plates and
226 diluted into a liquid medium selective for prey cells. The re-growth of prey cells was
227 evaluated by measuring A_{600} for 15 hours using a TECAN. The emergence time, being the
228 time for the cultures to reach an A_{600} equal to 0.4, was plotted for each condition (Fig. 3B).
229 After being exposed to an EAEC WT strain or a complemented Δ PAAR Ω mutant strain, prey
230 cells take seven hours to reach an A_{600} of 0.4 indicating very little prey cells have survived to
231 restore the growth in culture. On the contrary, prey cells exposed to Δ T6SS-1 or Δ PAAR Ω
232 mutants took less than five hours to reach the same A_{600} , indicating that much more prey cells
233 remained viable in the mixed-cell spot. Both methods confirm that the PAAR protein is
234 required for the EAEC T6SS-1 antibacterial activity. We have also tested whether expression
235 of PAAR with the single amino acid substitutions in conserved metal-coordination residues
236 could complement lack of wild-type PAAR protein. Interestingly, except for histidine H14,
237 substitutions in all the other residues predicted to be involved in zinc binding, i.e., C41, C74
238 and H46 could not complement PAAR deletion. This further suggests the importance of zinc
239 binding at the tip of the PAAR for its function (Fig. 3). The functionality of H14 mutant may
240 suggest that it is not involved in zinc binding. However, it is not unusual that the deletion of a
241 single metal coordinating residue is not enough to affect metal binding. The Cys76 is well
242 located in the predicted structure to compensate the absence of His14 for metal binding (Fig
243 S4A). We thus constructed and analyzed C76A and His14AC76A mutants. Both H14A and
244 C76A single mutants, but not the double mutant H14AC76A, could complement PAAR
245 deletion in the killing assay (Fig S4B). Accordingly, the zinc content of the double mutant
246 H14AC76A, but not that of C76A or H14A, seems to be impaired compared to the wild type
247 (Fig S4C). All together, these data suggest that C41, C74, H46 and likely H14 (or C76) are
248 important for zinc binding and function of PAAR^{EAEC}. As a control, we have assayed alanine
249 substitutions of other cysteine residues of PAAR^{EAEC} protein, i.e., C70 and C72, that are

250 positioned elsewhere in the structure and are thus not likely to contribute to metal binding
251 (Fig. S4AB). As expected, no effect on bacterial killing was observed using those mutants.
252 The production in $\Delta PAAR\Omega$ of the different versions of PAAR protein from pBAD plasmids
253 was confirmed by Western Blot (Fig. S4F).

254

255 **PAAR is required for the polymerization of T6SS sheath.**

256 We wanted to further understand whether the killing defect in the absence of PAAR is due to
257 its requirement for T6SS assembly or in the later stages such as target cell penetration. We
258 have therefore used fluorescence microscopy to follow the dynamics of TssB sheath protein
259 fused to the fluorescent protein sfGFP, that allows observation of sheath elongation and
260 contraction events. Time lapse fluorescence microscopy revealed that as compared to wild-
261 type strain that could assemble T6SS sheaths, the PAAR mutant ($\Delta PAAR\Omega$) displayed a
262 diffuse fluorescence indicating that TssB-sfGFP proteins are produced but T6SS sheaths are
263 not assembled (Fig. 4A). However, the complementation of PAAR protein expression from a
264 plasmid ($\Delta PAAR\Omega + pBAD-PAAR$) could restore the wild-type phenotype (Fig. 4A) which
265 confirms that PAAR protein is necessary for the assembly of T6SS sheaths in EAEC. In
266 addition, PAAR function is not affected by the presence of a C-terminal VSVG tag (Fig 3 and
267 Fig 4A).

268 To assess the role of conserved cysteines and histidines of PAAR^{EAEC}, that we have shown to
269 be involved in the binding of a zinc atom (C41/C74/H46) and required for T6SS mediated
270 killing (C41/C74/H46), we tried to complement the *tssB-sfGFP* $\Delta PAAR\Omega$ by plasmid
271 expression of PAAR^{H14A}, PAAR^{C41A}, PAAR^{H46A} or PAAR^{C74A}, as well as PAAR^{C76A} or
272 PAAR^{H14AC76A}. Strains producing PAAR_{C41A}, PAAR_{H46A} or PAAR_{C74A} showed diffuse
273 fluorescence confirming that these three residues are crucial for PAAR function in the T6SS
274 sheath assembly (Fig. 4BC). In contrast, wild type-like sheath assembly was observed by

275 complementing with PAAR^{H14A}, PAAR^{C70A}, PAAR^{C72A}, or PAAR^{C76A} which is consistent
276 with antibacterial competition assays indicating that these residues do not play an important
277 role in the function of PAAR protein (Fig. 4BC, Fig. S4E). Again, expression of the
278 PAAR^{H14AC76A} double mutant could not restore sheath assembly, suggesting that the presence
279 of at least H14 or C76 residue in PAAR is necessary for T6SS function.

280

281 **PAAR is necessary for the correct localization of the T6SS baseplate, but not of the**
282 **membrane complex.**

283 The membrane complex recruits the baseplate complex that is required for the coordinated
284 assembly of the tube and sheath components (8, 11, 13, 16). To determine at which step of the
285 T6SS biogenesis PAAR protein is required, we followed the localization of the membrane
286 complex component TssM and the baseplate component TssK by fluorescence microscopy.
287 We observed the wild type and *PAAR* mutant (Δ PAAR Ω Kan) cells producing sfGFP-TssM or
288 TssK-sfGFP (Fig. 5). As described previously, in T6SS-active strains, the sfGFP-TssM and
289 TssK-sfGFP present foci at the membrane ((8, 11), Fig. 5). We noted that in the absence of
290 PAAR, TssM was correctly localized at the membrane, suggesting that PAAR is not required
291 for proper localization of sfGFP-TssM and thus for membrane complex formation (Fig. 5AB).
292 However, the localization of TssK-sfGFP became diffuse in the absence of PAAR, suggesting
293 that PAAR is required for proper formation of the baseplate and its recruitment to the
294 membrane complex (Fig. 5CD). Consequently, PAAR was also required for downstream
295 events – the TssB/C sheath assembly (Fig. 4A) and the T6SS-1 mediated killing (Fig. 3).

296

297 **DISCUSSION**

298 There is a long-standing debate about the necessity of the PAAR protein for the T6SS-
299 mediated secretion and killing. Reports using different bacteria as T6SS models yielded

300 contradictory results (28, 30-33). Most recently, it was concluded that PAAR is only
301 necessary when transporting toxic effectors (32). In this work we have employed a model
302 system that can tackle this question since (i) under well described conditions (ii) it has only
303 one T6SS firing complex (iii) which has a single PAAR protein that is (iv) uncoupled from
304 toxin loading. Using the well-studied EAEC model we have demonstrated that PAAR^{EAEC}
305 protein is required for antibacterial activity mediated by the T6SS-1. Our results indicate that
306 PAAR^{EAEC} is indispensable for T6SS-mediated killing by participating in the upstream event
307 – the recruitment of the baseplate that allows polymerization of contractile sheath of T6SS
308 (Fig. 5).

309 Ten years ago, Shneider and colleagues have first reported structures of a small protein PAAR
310 that sharpens the T6SS spike (28). It was then proposed to stabilize the baseplate hub protein
311 VgrG, by nucleating the folding of its trimers, or to regulate the incorporation of the VgrG
312 into the T6SS machinery (28). However, we have found that the steady-state levels of
313 VgrG^{EAEC} protein are not significantly different without the PAAR^{EAEC}, and that the
314 overproduction of VgrG^{EAEC} in the absence of PAAR^{EAEC} does not restore the T6SS-1
315 assembly and activity (Fig. S5, Fig. S6). Similarly, the intracellular level of VgrG2 is not
316 impaired in the absence of PAAR proteins in *Serratia marcescens* (30). Nevertheless, we
317 have previously observed that purified VgrG^{EAEC} without PAAR^{EAEC} tends to precipitate in
318 some buffers (21) and therefore we cannot disregard the idea that PAAR could facilitate the
319 folding of the VgrG trimer or its incorporation into the baseplate (28). It is important to
320 mention that previous PAAR structures were obtained in chimeric complexes that were
321 prepared using a soluble fragment of T4 gp5 β -helix and grafting the terminal β -helices of
322 various VgrG spikes. Our attempts to crystallize PAAR from EAEC, alone or in complex
323 with the gp5C part of VgrG were unsuccessful. Nevertheless, our protein interaction studies
324 supported by cryo-EM density maps docked with AlphaFold2 model presented in this study

325 provide ultimate proof of the localization of PAAR protein at the top of the β -prism of the
326 gp5-domain of VgrG (Fig. 1). The spike complex of the bacteriophage T4 adopts highly
327 similar structure where a small conical gp5.4 protein sits on top of the β -prim of the gp5C
328 (12). Strikingly, similar structures found in other phages such as P2 or Phi92 sharpen the apex
329 of their needle by tying the ends of the gp5 β -helix into the knot that overall resembles the
330 conical PAAR protein (Fig. 6) (35). Moreover, all these needle end structures are supported
331 by the coordinated metal at the far end of the sharp structures (Fig. 6). We unambiguously
332 showed that the purified PAAR^{EAEC} protein contains zinc. At least two conserved cysteines
333 and one histidine, and likely a second histidine are involved in the binding of this zinc atom.
334 Their essentiality *in vivo* (C41, C74 and H46, Fig. 3, Fig. 4) supports the crucial role of metal
335 coordinated tip structure in the T6SS function. While the AlphaFold2 model of PAAR shows
336 histidine residues oriented slightly outward, it is likely that they constitute the coordination
337 center for the zinc atom and are hence pulled inwards to yield compact and sharp tip (Fig. 2).
338 Similarly, 3 histidine and 1 cysteine residues were shown to be involved in zinc binding in the
339 homologous PAAR proteins from *Vibrio* or UPEC and in the gp5.4 protein of phage T4 (12,
340 28). In the case of *Francisella* PAAR-like T6SS tip protein IglG, 4 cysteines were shown to
341 be important for binding of iron or zinc (31). Interestingly, the apex domains of gp138 of
342 Phi92 phage, gpV of P2 phage, and gpV of the R2 pyocin all contain iron, hexacoordinated by
343 6 histidines symmetrically provided by 3 monomers (Fig. 6, PDB:4s3; (35, 36)). In the light
344 of these observations, our results further support the role of metal coordinating tip structures
345 in sharpening spikes of membrane attacking contractile systems.

346 While the sharp tip might ensure the efficient piercing of the membrane it does not readily
347 explain the requirement of the PAAR for the assembly of T6SS^{EAEC}. PAAR could be required
348 for the recruitment of the spike to the membrane complex. In this case, the sharp tip
349 maintained by the zinc atom may be important for this function. Interestingly, expression of

350 the VgrG^{EAEC}-Tle1^{EAEC} complex yielded virtually all spike complexes stuck head-to-head
351 through the hydrophobic blunt end of the VgrG β -prisms as observed by cryo-EM (Fig. 1D,
352 Fig. S1B). These interactions involve the hydrophobic patch otherwise covered by PAAR
353 ((21), Fig. 1, Fig. S1B). Indeed, the presence of PAAR^{EAEC} seems to prevent this aberrant
354 behavior of VgrG^{EAEC} (Fig. 1DE, Fig. S1A). Strikingly, the same head-to-head interaction
355 occurs in the case of T4 phage gp5 needle when produced alone. Without the PAAR homolog
356 gp5.4, the β -prism of gp5 dimerizes through its C-terminal hydrophobic tips and these
357 dimers of trimers can form in two different orientations (37). We could also observe different
358 2D classes of twin VgrG^{EAEC}-Tle1^{EAEC} complexes likely representing continuous or 60°
359 rotated interactions of β -prisms (Fig. S1B). These observations further suggest that the
360 amyloid-like structure of gp5 domain must be concluded to achieve a closed sharp end.
361 Indeed, formation of a baseplate and its attachment to the membrane complex could be
362 corrupted in the case of self-dimerization of VgrG^{EAEC} trimers. Moreover, this proposed role
363 of PAAR protein in T6SS assembly could explain previous results showing that gp27 and
364 gp5-OB domain of VgrG are sufficient for T6SS assembly and firing (20, 38). Indeed,
365 without its β -prism, the needle-less trimeric VgrG may not dimerize this way and thus PAAR
366 may not be required for T6SS assembly in this case.

367 Of note, despite the addition of PAAR protein, no interpretable density was again observed
368 for the base of VgrG corresponding to the gp27-gp5 OB fold -like domains (21). Only one
369 cryo-EM 2D class of particles displayed a distinct triangular shape structure at the base of the
370 needle, suggesting that the flexibility of this domain is not stabilized by the presence of
371 PAAR (Fig. S1AB). On the contrary, in the presence of PAAR most of the cryo-EM 2D
372 classes were missing densities for one, two or three Tle1 toxins. Closer observations revealed
373 that the EM densities around the middle of the gp5 structure were frequently visible and
374 overall more intense. The missing toxin densities are therefore likely a result of the flexibility.

375 It is possible that the head-to-head dimer of VgrG-Tle1 fixes the Tle1 toxins in a more rigid
376 conformation than the VgrG-Tle1-PAAR complex. In particular, the TTR domain of VgrG
377 that is hanging on a flexible linker might be less constrained in the presence of PAAR than in
378 the head-to-head VgrG-Tle1 dimer. Gel filtrations consistently show uniform peak for VgrG-
379 Tle1-PAAR complex and Tle1 seems to be in a stoichiometric equilibrium with VgrG based
380 on band intensities in SDS-PAGE gels (Fig S7). This suggests that Tle1 toxins are efficiently
381 loaded on the spike but are likely more flexible than previously observed. This flexibility
382 might play an important role when encountering the target and liberating the toxin from spike.

383 To conclude, we suggest that while the overall large structure of the T6SS firing tube
384 is built on the hexameric symmetry, it has to be resolved in order to be sealed and sharpened.
385 First symmetry transition is enabled by the VgrG-trimer that starts with pseudo-hexameric
386 symmetry at its base and wraps it up to a trimeric β -prism. Further, PAAR protein initiates by
387 a pseudo-trimeric symmetry that connects to the trimeric β -prism. Towards the extremity
388 PAAR is sealed into a sharp end with the help of a metal coordinated by conserved cysteines
389 and histidines. The importance of such architecture is supported by the fact that the closure of
390 the puncturing tip with the help of metal coordinating center is structurally conserved across
391 contractile injection systems that carry gp5-like β -prism domains (12, 28, 31, 35, 36). In
392 some, but not all cases, the final conical tip is encoded as a separate protein. One has to take
393 into account, that in the case of T6SS, such separation provides an additional anchoring point
394 for toxins. Hence, the separate ORF coding for the tip of puncturing device might have been
395 evolutionary favored. Nevertheless, as demonstrated in this work, even without carrying toxic
396 domains, sharpening the T6SS tip by PAAR protein is essential for the assembly and activity
397 of the T6SS machinery in EAEC.

398

399 **MATERIALS AND METHODS**

400 **Bacterial strains, growth conditions and chemicals.**

401 Strains used in this study are listed in Table S1. *Escherichia coli* DH5 α , BL21(DE3) and
402 W3110 were used for cloning procedures, protein production and as a prey for antibacterial
403 competition assays, respectively. EAEC strain 17-2 and its isogenic derivatives were used for
404 functional studies. Cells were grown at 37°C with aeration in LB with antibiotics when
405 required (ampicillin (100 μ g/ml), kanamycin (50 μ g/ml) or chloramphenicol (30 μ g/ml)) or in
406 T6SS-1-inducing medium (SIM; M9 minimal medium, 0.2% glycerol, 1 μ g/ml vitamin B1,
407 100 μ g/ml casamino acids, 10% LB, supplemented or not with 1.5% Bacto agar) (39). Gene
408 expression was induced by the addition of 0.02–0.2% of L-arabinose (Sigma-Aldrich) for
409 pBAD (40) and by 0.5 or 1 mM of isopropyl-thio-b-D-galactopyranoside (IPTG; Eurobio) for
410 pETDuet-1, pRSFDuet-1 and pACYCDuet-1 (Novagen) derivative vectors.

411

412 **Cloning procedures, plasmid and strain constructions.**

413 The plasmids and primers (obtained from Merck or IDT) used in this study are listed in Table
414 S1. Cloning was performed by standard restriction-ligation procedures – DNA fragments
415 coding for PAAR and VgrG were amplified from EAEC 17-2 chromosomal DNA using Q5
416 high-fidelity DNA polymerase (New England Biolabs), PCR fragments were purified on
417 NucleoSpin Gel and PCR Clean-up columns (Macherey-Nagel), digested as recommended by
418 the manufacturer (NEB), and purified before ligation. Recombinant plasmids were
419 transformed into *E. coli* DH5 α cells and constructions were verified by colony-PCR and by
420 DNA sequencing (Eurofins) after extraction of the plasmids using Wizard Plus SV Minipreps
421 kit (Promega).

422 For pACYC-PAAR^{FL} construct, the sequence encoding PAAR was amplified by PCR using
423 5-NdeI-PAAR and 3-XhoI-PAAR^{FL} primers introducing NdeI and XhoI sites, respectively,
424 and cloned into the pACYCDuet-1 (Novagen) multiple cloning site 2. The 3- XhoI -PAAR^{FL}

425 primer introduces a sequence encoding a FLAG-tag to allow in-frame fusion of PAAR with a
426 C-terminal FLAG extension. For pET-^HSUMO-PAAR construct, the sequence encoding
427 PAAR was amplified by PCR using primers 5-BmtI-PAAR and 3-HindIII-PAAR introducing
428 BmtI and HindIII sites, respectively, and cloned into the pETDuet-HIS-SUMO multiple
429 cloning site.

430 The pBAD-PAAR^{VSVG} was constructed by megaprimering. The 3pBAD-4537-VSVG primer
431 introduces a sequence encoding a VSVG-tag to allow in-frame fusion of PAAR with a C-
432 terminal VSVG extension. Site directed mutagenesis of *PAAR* was performed on pACYC-
433 PAAR^{FLAG}, pETDuet^{His-SUMO}-PAAR or pBAD33-PAAR^{VSVG} by QuickChange PCR- based
434 targeted mutagenesis using complementary pairs of oligonucleotides and the PfuTurbo high-
435 fidelity polymerase (Agilent Technologies).

436 For strain construction, the *PAAR* gene (EC042_4537) was deleted into the EAEC 17-2 wild-
437 type strain, the *tssB-sfGFP*, the *tssK-sfGFP* and the *sfGFP-tssM* using a modified one-step
438 inactivation procedure (41) as previously described (42) using pKD4 plasmid and
439 oligonucleotide pairs DEL-4537-5-DW/DEL-4537-3-DW. Kanamycin resistant clones were
440 selected and verified by colony-PCR and sequencing of the region (Eurofins genomics).

441

442 **SDS PAGE and Western blot analyzes.**

443 Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis (SDS-PAGE) was performed
444 using standard protocols. Proteins were stained using InstantBlueTM (Sigma-Aldrich) or
445 transferred onto 0.2 m nitrocellulose membrane (Amersham Protran). Immunoblots were
446 probed with anti-Strep-Tag Classic (clone Strep-tag II, Bio-Rad catalogue #MCA2489), anti-
447 FLAG (clone M2, Sigma-Aldrich catalogue #F3165), anti-His-Tag (clone 1B7G5, Proteintech
448 catalogue #66005-1-Ig), anti-VSVG (clone P5D4; Sigma-Aldrich) or anti-RecA (clone M2,
449 Sigma-Aldrich catalogue #F3165) primary monoclonal antibodies, and alkaline phosphatase-

450 conjugated goat anti-mouse secondary antibodies, and revealed in alkaline buffer (pH 9) using
451 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) in the presence of
452 10 mM MgCl₂.

453

454 **Protein production and pull-down assays.**

455 An overnight culture of *E. coli* BL21(DE3) co-transformed with pACYC-PAAR^{FL} (or
456 pACYC-PAAR^{FL} derivatives) and pET-^SVgrG (or pET-^SVgrG derivatives) was diluted 1/100
457 into 50 ml of LB supplemented with the required antibiotics, grown to $A_{600}=0.8$ and induced
458 with 1 mM IPTG for 18 h at 16°C. Cells were pelleted by centrifugation at $5000\times g$,
459 resuspended in buffer A (50 mM Tris-HCl pH8.5, 150 mM NaCl, 1 mM TCEP, cOmplete
460 EDTA-free protease inhibitor cocktail (Sigma)). After cell lysis by sonication, the cell extract
461 was cleared by centrifugation for 45 min at $15\ 000\times g$ and incubated for 1h at 4°C with
462 gentle mixing with 100 µl of Strep-Tactin Superflow resin (IBA Technology) previously
463 equilibrated in buffer A. The resin was washed 5 times with 300 µl of buffer A and the
464 ^SVgrG-PAAR^{FL} were eluted with 100 µl of buffer B (50 mM Tris-HCl pH8.5, 150 mM NaCl,
465 1 mM TCEP, 2.5 mM desthiobiotin). Ten microliters of the elution fractions were separated
466 by 12.5%-acrylamide SDS-PAGE, stained using InstantBlue™ (Sigma-Aldrich) or analyzed
467 by Western blot with Strep-Tag Classic or anti-FLAG antibodies.

468 For the production of Tle1-VgrG-PAAR complex, BL21(DE3) was co-transformed with
469 pRSF-Tle1^H, pET-^SVgrG and pACYC-PAAR^{FL} plasmids. ^SVgrG-Tle1^H-PAAR^{FL} complex
470 was then produced and purified using the same double step affinity procedure as for the
471 purification of the ^SVgrG-Tle1^H complex described in (21).

472

473 **Cryo-EM sample preparation, data collection and modelling.**

474 Quantifoil Cu300 mesh R2/2 grids were glow discharged at 0.2 mbar vacuum, 2mA current
475 for 45 seconds (in an ELMO glow discharge system (Cordouan Technologies). Four μ l of
476 protein samples at concentration of 0.5 ml/ml were blotted and plunge-frozen in liquid ethane
477 using Vitrobot instrument (FEI) at 4°C and 100% humidity, using blot force 0 and blotting
478 time 3s. Grids were transferred to liquid nitrogen for storage. Observation and data were
479 collected with Talos Arctica (FEI) 200 kV microscope equipped with K2 camera (Gatan).
480 Data were collected at 45k magnification, pixel size of 0.93 Å, with 4.4 s exposition and total
481 dose of 50 e-/Å with the help of SerialEM version 3.8 software. 2180 movies were collected
482 for VgrG-Tle1 complex (without PAAR) dataset. 3237 movies were collected for VgrG-Tle1-
483 PAAR dataset. Data was processed with CryoSPARC v4.0.0. Particle picking was assisted by
484 4 iterations of Topaz (43) training for 20 epochs, each time resubmitting best quality particles
485 for new iteration of training. For VgrG-Tle1 (without PAAR) dataset: 256,139 particles were
486 extracted with 550-pixels box size. For VgrG-Tle1-PAAR dataset: 436,572 particles were
487 extracted with 400-pixels box size. Best defined 2D classes were used for generation 4 ab-
488 initio models and best models were refined using heterologous refinement. For high
489 resolution VgrG-Tle1-PAAR map, particles used for best class refinement were again used to
490 generate 4 ab initio models and best model was then refined using best defined 2D classes.
491 Final step of non-uniform refinement with 31,647 particles was used to gain resolution in best
492 regions.

493 VgrG-Tle1-PAAR model was generated as follows: gp5 domain of VgrG (3 copies) and
494 PAAR were co-folded using AlphaFold2 (44, 45). Using UCSF ChimeraX (46), the best
495 ranked AlphaFold2 model was aligned to VgrG-Tle1 model (PDB:6S JL, (21)) via the gp5
496 domains. This model was then fitted into cryo-EM density maps (46).

497

498 **His-SUMO-PAAR purification and samples preparation for ICP-OES.**

499 Overnight cultures of *E. coli* BL21(DE3) transformed with pETDuet-H³⁵SUMO-PAAR or its
500 mutated derivatives were diluted 1/100 into 2L of LB supplemented with the required
501 antibiotics. Cells were grown to an $A_{600} = 0.8$ and induced with 1 mM IPTG for 18 h at
502 16°C. Cells were pelleted by centrifugation at $5000 \times g$, resuspended in buffer C (20 mM
503 HEPES pH8, 250 mM NaCl, 1 mM TCEP, cOmplete EDTA-free protease inhibitor cocktail
504 (Sigma) 100 µg/ml DNase I, 100 µg/ml lysozyme, and 10 mM MgCl₂). After cell lysis using
505 an Emulsiflex-C5 (Avestin), the cell extract was cleared by centrifugation for 40 min at 40
506 $000 \times g$ and incubated for 1h at 4°C with gentle mixing with 1 ml of Strep-Tactin Superflow
507 resin (IBA Technology) previously equilibrated in buffer C. The resin was washed 5 times
508 with 8 ml of buffer C and the H³⁵SUMO-PAAR was eluted with 100 µl of buffer D (20 mM
509 HEPES pH8, 250 mM NaCl, 1 mM TCEP, 500 mM imidazole. Ten microliters of the elution
510 fractions were separated by 15%-acrylamide SDS-PAGE, stained using InstantBlue™ or
511 transferred onto nitrocellulose membrane and immunodetected with anti- His-Tag antibodies.
512

513 **Metal quantification by Inductively Coupled Plasma analysis - Optical Emission**
514 **Spectrometry (ICP-OES).**

515 50 to 300 µM of proteins in 500 µl of PBS were diluted in 500 µl of 69% nitric acid. Samples
516 were boiled for 40 minutes and 4 ml of 3% nitric acid was added to each sample. These
517 samples were analyzed for copper, nickel, zinc, and iron content with an ICAP™ 6000 series
518 optical emission spectrometer (Thermo Scientific). Serial dilutions of pure copper, nickel,
519 iron, and zinc standard solutions were used for calibration. Results are expressed in
520 percentage of total proteins in samples, each value representing the mean of three technical
521 replicates from different fractions of two to three different purification preparations. As a
522 control, 500µl of purification buffer was submitted to the same metal content analysis. For
523 statistical analysis, one-way ANOVA followed by Dunnett's multiple comparisons test was

524 performed using GraphPad Prism version 9.3.1 for macOS (GraphPad Software, San Diego,
525 California USA).

526

527 **Fluorescence Microscopy.**

528 Overnight cultures grown in LB medium with the appropriate antibiotics were diluted 1/100
529 into SIM to an $A_{600} = 0.8$, then 500 μ l of the culture was centrifuged and resuspended in fresh
530 SIM to a $A_{600} = 10$. Cells were spotted on a thin 2% agarose pad, prepared with SIM medium
531 in Frame-Seal™ slide chambers (Bio-Rad), and covered with a cover slip. After 20 min at
532 28°C, Hilo fluorescence microscopy was performed with a Nikon Eclipse Ti2 microscope
533 equipped with a 100x NA 1.45 Ph3 objective, an Orca-Fusion digital camera (Hamamatsu), a
534 perfect focus system, and an Ilas2 TIRF/FRAP module (Gataca Systems). Fluorescence
535 images were acquired with an exposure time of 100 ms for phase contrast, sfGFP-TssM,
536 TssK-sfGFP and 50 ms for TssB- sfGFP. The experiments were conducted in triplicate and a
537 representative result is shown. The microscopy images were analyzed using ImageJ
538 (<http://imagej.nih.gov/ij/>).

539

540 **Antibacterial competition assay by the colorimetric method.**

541 Overnight cultures grown in LB medium with the appropriate antibiotics were diluted 1/100
542 into SIM medium to an $A_{600} = 0.4$. Then, 50 μ M of IPTG was added in the culture of W3110
543 bacterial prey cells to induce the production of β -galactosidase. WT or mutated *PAAR* genes
544 expression was induced from pBAD33-*PAAR*^{VSVG} using 0,2% arabinose. At $A_{600} = 0.8$, the
545 predator and the prey cultures were mixed at 1:4 ratio and spotted on SIM-agar plates
546 supplemented with 50 μ M of IPTG and 0,2 % arabinose. After 2 hours of incubation at 37 °C,
547 drops of 2 mM CPRG solution were deposited on the cell spots. Pictures were taken after the
548 emergence of the coloration. Experiment was performed at least three times and a

549 representative image is presented. For quantification of CPR, slices of agar-containing spots
550 were vortexed in 500 μ L PBS and further centrifuged at 8,000 \times g for 1 min to pellet cells and
551 agar. 100 μ l of the supernatant were dropped into a 96-well plate. After addition of 10 μ l of 2
552 mM CPRG into the supernatant, absorbance at $\lambda = 572$ was measured every 30 seconds for 1
553 hour with a TECAN.

554

555 **Antibacterial competition assay by the emergence time method.**

556 Overnight cultures grown in LB medium with the appropriate antibiotics were diluted 1/100
557 into SIM medium to an $A_{600} = 0.4$. Arabinose 0,2% was added into predator strains cultures to
558 induce *PAAR* gene expression from pBAD33-*PAAR*^{VSVG} (or pBAD33-*PAAR*^{VSVG} mutants). At
559 $A_{600} = 0.8$, the predator strain and the prey were mixed at 4:1 ratio and spotted on SIM-agar
560 plates supplemented 0,2% arabinose. After 4h of incubation at 37 °C, each spot was
561 resuspended in 1 ml of selective medium for prey and 100 μ l of a 10-fold dilution were added
562 to 100 μ l of selective medium in 96-wells microplate (Thermofisher, Nunc). Cultures were
563 grown for 15 hours at 37°C with agitation and A_{600} was measured each 5 minutes using a
564 microplate reader (TECAN). A time of emergence (T_e) considered as time required for each
565 sample to reach $A_{600} = 0.4$ of was calculated. Each value represents the mean of three
566 technical replicates from at least three biological replicates and were compared between
567 strains by a One-way ANOVA followed by Dunnett's multiple comparisons test performed
568 using GraphPad Prism (version 9.3.1 for macOS, GraphPad Software, San Diego, California
569 USA). Negative controls contained only the prey or the predator strain. EAEC 17-2 wild-type
570 strain was used as positive control for W3110 prey lysis.

571 Production in EAEC Δ PAAR Ω Kan of the different derivatives of PAAR protein from pBAD
572 plasmids used for antibacterial competition and fluorescence microscopy observations were
573 controlled by Western Blotting using anti-VSVG antibodies.

574

575 **Protein stability assay.**

576 Overnight cultures of *E. coli* DH5 α transformed with pBAD18-PAAR^{VSVG} and pOK-
577 VgrG^{FLAG} or empty pBAD18 and pOK-VgrG^{FLAG} were diluted 1/100 into 50 ml of LB
578 medium supplemented with the required antibiotics, grown at 37°C to an A_{600} of 0.6 and
579 induced with 0,5 mM IPTG and 0,2% Arabinose. After 1h, spectinomycin (100 μ g/ml) and
580 chloramphenicol (40 μ g/ml) were added. Before and after the induction, at 1 h, 2 h and 3 h
581 after the inhibition of protein synthesis, 2 ml of cultures were collected, centrifuged for 5 min
582 at 6000 \times g and supplemented with 60 or 100 μ l of 4X SDS-PAGE loading dye with β -
583 mercaptoethanol. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose
584 membrane for immunodetection with anti-VSVG, or anti-FLAG or anti-RecA monoclonal
585 antibodies, followed by recognition by secondary antibodies coupled to the Alkaline
586 Phosphatase. Intensity of the bands were measured using BioRad Imager.

587

588 **Computer algorithms.**

589 EAEC PAAR orthologs (EC042_4537; NCBI-protein ID: CBG37359) were retrieved using
590 BlastP analysis against the KEGG genes database and aligned with MultiAlin tool (47, 48).

591

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605 **Author contributions**

606 S.G.B.: conceptualization, formal analysis, investigation, visualization, writing - original
607 draft. N.F.: conceptualization, investigation, writing - review and editing. M.I.: formal
608 analysis, investigation, resources, writing - review and editing. M.B. and EGF: formal
609 analysis, investigation. R.F.: conceptualization, formal analysis, resources, supervision. D.J.:
610 conceptualization, formal analysis, investigation, supervision, visualization, writing - original
611 draft. L.J.: conceptualization, formal analysis, funding acquisition, investigation, resources,
612 supervision, validation, visualization, writing - original draft.

613

614 **Conflict of Interests**

615 The authors declare that they have no conflict of interest.

616

617 **REFERENCES**

618

- 619 **1. Durand E, Cambillau C, Cascales E, Journet L.** 2014. VgrG, Tae, Tle, and beyond: the
620 versatile arsenal of type VI secretion effectors. *Trends Microbiol* 22: 498-507. [https://doi-](https://doi.org/insb.bib.cnrs.fr/10.1016/j.tim.2014.06.004)
621 [org.insb.bib.cnrs.fr/10.1016/j.tim.2014.06.004](https://doi.org/insb.bib.cnrs.fr/10.1016/j.tim.2014.06.004)
- 622 **2. Russell AB, Peterson SB, Mougous JD.** 2014. Type VI secretion system effectors:
623 poisons with a purpose. *Nat Rev Microbiol* 12: 137–48. <https://doi.org/10.1038/nrmicro3185>
- 624 **3. Hernandez RE, Gallegos-Monterrosa R, Coulthurst SJ.** 2020. Type VI secretion system
625 effector proteins: Effective weapons for bacterial competitiveness. *Cellular microbiology* 22:
626 e13241. <https://doi.org/10.1111/cmi.13241>
- 627 **4. Jurénas D, Journet L.** 2021. Activity, delivery, and diversity of Type VI secretion
628 effectors. *Mol Microbiol* 115: 383-394. <https://doi.org/10.1111/mmi.14648>
- 629 **5. Hachani A, Wood TE, Filloux A.** 2016. Type VI secretion and anti-host effectors. *Curr*
630 *Opin Microbiol* 29: 81 – 93. <https://doi.org/10.1016/j.mib.2015.11.006>
- 631 **6. Zoued A, Brunet YR, Durand E, Aschtgen MS, Logger L, Douzi B, Journet L,**
632 **Cambillau C, Cascales E.** 2014. Architecture and assembly of the Type VI secretion system.
633 *Biochim Biophys Acta Mol Cell Res* 1843:1664 – 1673.
634 <https://doi.org/10.1016/j.bbamcr.2014.03.018>
- 635 **7. Coulthurst SJ.** 2013. The Type VI secretion system - a widespread and versatile cell
636 targeting system. *Res Microbiol.* 164:640-54. <https://doi.org/10.1016/j.resmic.2013.03.017>
- 637 **8. Durand E, Nguyen VS, Zoued A, Logger L, Péhau-Arnaudet G, Aschtgen M-S,**
638 **Spinelli S, Desmyter A, Bardiaux B, Dujancourt A, Roussel A, Cambillau C, Cascales**
639 **E; Fronzes R.** 2015. Biogenesis and structure of a type VI secretion membrane core complex.
640 *Nature* 523: 555-560. <https://doi.org/10.1038/nature14667>
- 641 **9. Rapisarda C, Cherrak Y, Kooger R, Schmidt V, Pellarin R, Logger L, Cascales E,**
642 **Pilhofer M, Durand E, Fronzes R.** 2019. In situ and high-resolution cryo- EM structure of a

643 bacterial type VI secretion system membrane complex. *EMBO J* 38: e100886. <https://doi->
644 [org.10.15252/embj.2018100886](https://doi.org/10.15252/embj.2018100886)

645 **10. Basler M, Pilhofer M, Henderson GP, Jensen GJ, Mekalanos JJ.** 2012. Type VI
646 secretion requires a dynamic contractile phage tail-like structure. *Nature* 483: 182 – 186.
647 <https://doi.org/10.1038/nature10846>

648 **11. Brunet YR, Zoued A, Boyer F, Douzi B, Cascales E.** 2015. The type VI secretion
649 TssEFGK-VgrG phage-like baseplate is recruited to the TssJLM membrane complex via
650 multiple contacts and serves as assembly platform for tail tube/sheath polymerization. *PLoS*
651 *Genet* 11: e1005545

652 **12. Taylor NMI, Prokhorov NS, Guerrero-Ferreira RC, Shneider MM, Browning C,**
653 **Goldie KN, Stahlberg H, Leiman PG.** 2016. Structure of the T4 baseplate and its function in
654 triggering sheath contraction. *Nature* 533: 346 – 352

655 **13. Nguyen VS, Logger L, Spinelli S, Legrand P, Huyen Pham TT, Nhung Trinh TT,**
656 **Cherrak Y, Zoued A, Desmyter A, Durand E, Roussel A, Kellenberger C, Cascales E,**
657 **Cambillau C.** 2017. Type VI secretion TssK baseplate protein exhibits structural similarity
658 with phage receptor binding proteins and evolved to bind the membrane complex. *Nat*
659 *Microbiol* 2: 17103. <https://doi.org/10.1038/nmicrobiol.2017.103>

660 **14. Nazarov S, Schneider JP, Brackmann M, Goldie KN, Stahlberg H, Basler M.** 2018.
661 Cryo-EM reconstruction of type VI secretion system baseplate and sheath distal end. *EMBO J*
662 37: e97103. doi: 10.15252/embj.201797103

663 **15. Cherrak Y, Rapisarda C, Pellarin R, Bouvier G, Bardiaux B, Allain F, Malosse C,**
664 **Rey M, Chamot-Rooke J, Cascales E, Fronzes R, Durand E.** 2018. Biogenesis and
665 structure of a type VI secretion baseplate. *Nat Microbiol* 312:1404-1416. doi:
666 10.1038/s41564-018-0260-1

667 **16. Zoued A, Durand E, Brunet YR, Spinelli S, Douzi B, Guzzo M, Flaugnatti N,**
668 **Legrand P, Journet L, Fronzes R, Mignot T, Cambillau C, Cascales E.** 2016. Priming and
669 polymerization of a bacterial contractile tail structure. *Nature* 531:59–63.
670 <http://dx.doi.org/10.1038/nature17182>

671 **17. Brunet YR, Espinosa L, Harchouni S, Mignot T, Cascales E.** 2013. Imaging type VI
672 secretion-mediated bacterial killing. *Cell Rep* 3:36–41.
673 <http://dx.doi.org/10.1016/j.celrep.2012.11.027>

674 **18. Ho BT, Dong TG, Mekalanos JJ.** 2014. A view to a kill: the bacterial type VI secretion
675 system. *Cell Host Microbe*. 15: 9–21. <https://doi.org/10.1016/j.chom.2013.11.008>

676 **19. Cianfanelli FR, Monlezun L, Coulthurst SJ.** 2016. Aim, load, fire: the type VI secretion
677 system, a bacterial nanoweapon. *Trends Microbiol* 24: 51 – 62.
678 <https://doi.org/10.1016/j.tim.2015.10.005>

679 **20. Flaugnatti N, Le TT, Canaan S, Aschtgen MS, Nguyen VS, Blangy S, Kellenberger**
680 **C, Roussel A, Cambillau C, Cascales, E, Journet L.** 2016. A phospholipase A1
681 antibacterial Type VI secretion effector interacts directly with the C-terminal domain of the
682 VgrG spike protein for delivery. *Mol Microbiol* 99:1099-1118.
683 <https://doi.org/10.1111/mmi.13292>

684 **21. Flaugnatti N, Rapisarda C, Rey M, Beauvois SG, Nguyen VA, Canaan S, Durand E,**
685 **Chamot-Rooke J, Cascales E, Fronzes R, Journet L.** 2020. Structural basis for loading and
686 inhibition of a bacterial T6SS phospholipase effector by the VgrG spike. *EMBO J* 39:
687 e104129. <https://doi.org/10.15252/emj.2019104129>

688 **22. Pukatzki S, Ma AT, Revel AT, Sturtevant D, Mekalanos JJ.** 2007. Type VI secretion
689 system translocates a phage tail spike-like protein into target cells where it cross-links actin.
690 *Proc Natl Acad Sci U S A* 104:15508–15513. <http://dx.doi.org/10.1073/pnas.0706532104>

691 **23. Leiman PG, Basler M, Ramagopal UA, Bonanno JB, Sauder JM, Pukatzki S, Burley**
692 **SK, Almo SC, Mekalanos JJ.** 2009. Type VI secretion apparatus and phage tail-associated
693 protein complexes share a common evolutionary origin. *Proc Natl Acad Sci U S A* 106:4154–
694 4159. <http://dx.doi.org/10.1073/pnas.0813360106>

695 **24. Pell LG, Kanelis V, Donaldson LW, Howell PL, Davidson AR.** 2009. The phage
696 lambda major tail protein structure reveals a common evolution for long-tailed phages and the
697 type VI bacterial secretion system. *Proc Natl Acad Sci USA* 106, 4160–4165.
698 <https://doi.org/10.1073/pnas.0900044106>

699 **25. Bönemann G, Pietrosiuk A, Diemand A, Zentgraf H, Mogk A.** 2009. Remodelling of
700 VipA/VipB tubules by ClpV-mediated threading is crucial for type VI protein secretion.
701 *EMBO J* 28, 315–325. <https://doi.org/10.1038/emboj.2008.269>

702 **26. Uchida K, Leiman PG, Arisaka F, Kanamaru S.** 2014. Structure and properties of the
703 C-terminal β -helical domain of VgrG protein from *Escherichia coli* O157. *J Biochem*
704 155:173–182. <http://dx.doi.org/10.1093/jb/mvt109>

705 **27. Spínola-Amilibia M, Davó-Siguero I, Ruiz FM, Santillana E, Medrano FJ, Romero**
706 **A.** 2016. The structure of VgrG1 from *Pseudomonas aeruginosa*, the needle tip of the
707 bacterial type VI secretion system. *Acta Crystallogr D Struct Biol* 72:22–33.
708 <http://dx.doi.org/10.1107/S2059798315021142>

709 **28. Shneider MM, Buth SA, Ho BT, Basler M, Mekalanos JJ, Leiman PG.** 2013. PAAR-
710 repeat proteins sharpen and diversify the type VI secretion system spike. *Nature* 500:350–
711 353. <http://dx.doi.org/10.1038/nature12453>

712 **29. Arisaka F, Kanamaru S, Leiman P, Rossmann MG.** 2003. The tail lysozyme complex
713 of bacteriophage T4. *Int J Biochem Cell Biol* 35:16-21. [https://doi.org/10.1016/S1357-](https://doi.org/10.1016/S1357-2725(02)00098-5)
714 [2725\(02\)00098-5](https://doi.org/10.1016/S1357-2725(02)00098-5)

715 **30. Cianfanelli FR, Alcoforado Diniz J, Guo M, De Cesare V, Trost M, Coulthurst SJ.**
716 2016. VgrG and PAAR Proteins Define Distinct Versions of a Functional Type VI Secretion
717 System. *PLoS Pathog* 12:e1005735. <https://doi.org/10.1371/journal.ppat.1005735>

718 **31. Rigard M, Bröms JE, Mosnier A, Hologne M, Martin A, Lindgren L, Punginelli C,**
719 **Lays C, Walker O, Charbit A, Telouk P, Conlan W, Terradot L, Sjöstedt A, Henry T.**
720 2016. Francisella tularensis IglG Belongs to a Novel Family of PAAR-Like T6SS Proteins
721 and Harbors a Unique N-terminal Extension Required for Virulence. *PLoS Pathog*
722 12:e1005821. <https://doi.org/10.1371/journal.ppat.1005821>

723 **32. Liang X, Pei TT, Li H, Zheng HY, Luo H, Cui Y, Tang MX, Zhao YJ, Xu P, Dong T.**
724 2021. VgrG-dependent effectors and chaperones modulate the assembly of the type VI
725 secretion system. *PLoS Pathog* 17:e1010116. <https://doi.org/10.1371/journal.ppat.1010116>

726 **33. Zheng J, Leung KY.** 2007. Dissection of a type VI secretion system in *Edwardsiella*
727 *tarda*. *Mol Microbiol.* 2007. 66:1192-206. <https://doi.org/10.1111/j.1365-2958.2007.05993.x>

728 **34. Taillefer B, Grandjean MM, Herrou J, Robert D, Mignot T, Sebban-Kreuzer C,**
729 **Cascales E.** 2023. Qualitative and quantitative methods to measure antibacterial activity
730 resulting from bacterial competition. *Bio Protoc.* 13: e4706. doi: 10.21769/BioProtoc.4706

731 **35. Browning C, Shneider MM, Bowman VD, Schwarzer D, Leiman PG.** 2012. Phage
732 pierces the host cell membrane with the iron-loaded spike. *Structure* 20:326-39.
733 <https://doi.org/10.1016/j.str.2011.12.009>

734 **36. Yamashita E, Nakagawa A, Takahashi J, Tsunoda K, Yamada S, Takeda S.** 2011.
735 The host-binding domain of the P2 phage tail spike reveals a trimeric iron-binding structure.
736 *Acta Crystallogr Sect F Struct Biol Cryst Commun* 67:837-41.
737 <https://doi.org/10.1107/S1744309111005999>

738 **37. Buth SA, Menin L, Shneider MM, Engel J, Boudko SP, Leiman PG.** 2015. Structure
739 and Biophysical Properties of a Triple-Stranded Beta-Helix Comprising the Central Spike of
740 Bacteriophage T4. *Viruses* 7:4676-706. <https://doi.org/10.3390/v7082839>

741 **38. Renault M, Zamarreño Beas J, Douzi B, Chabalièr M, Zoued A, Brunet YR,**
742 **Cambillau C, Journet L, Cascales E.** 2018. The gp27-like Hub of VgrG Serves as Adaptor
743 to Promote Hcp Tube Assembly. *J Mol Biol* 430:3143-3156.
744 <https://doi.org/10.1016/j.jmb.2018.07.018>

745 **39. Brunet YR, Bernard CS, Gavioli M, Lloubès R, Cascales E.** 2011. An epigenetic
746 switch involving overlapping fur and DNA methylation optimizes expression of a type VI
747 secretion gene cluster. *PLoS Genet* 7:e1002205. <https://doi.org/10.1371/journal.pgen.1002205>

748 **40. Guzman LM, Belin D, Carson MJ, Beckwith J.** 1995. Tight regulation, modulation, and
749 high-level expression by vectors containing the arabinose PBAD promoter. *J. Bacteriol* 177:
750 4121-30. <https://doi.org/10.1128/jb.177.14.4121-4130.1995>

751 **41. Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in
752 *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U SA* 97: 6640–6645.
753 <https://doi.org/10.1073/pnas.120163297>

754 **42. Aschtgen MS, Bernard CS, de Bentzmann S, Lloubes R, Cascales E.** 2008. SciN is an
755 outer membrane lipoprotein required for type VI secretion in enteroaggregative *Escherichia*
756 *coli*. *J. Bacteriol.* 190: 7523-7531. <https://doi.org/10.1128/JB.00945-08>

757 **43. Bepler T, Morin A, Rapp M, Brasch J, Shapiro L, Noble AJ, Berger B.** 2019.
758 Positive-unlabeled convolutional neural networks for particle picking in cryo-electron
759 micrographs. *Nat Methods* 16:1153-1160. <https://doi.org/10.1038/s41592-019-0575-8>

760 **44. Jumper J, Evans R, Pritzel A. et al.,** 2021. Highly accurate protein structure prediction
761 with AlphaFold. *Nature* 596:583–589. <https://doi.org/10.1038/s41586-021-03819-2>
762 <https://doi.org/10.1038/s41586-021-03819-2>

763 **45. Mirdita, M., Schütze, K., Moriwaki, Y. et al., 2022.** ColabFold: making protein folding
764 accessible to all. *Nat Methods* 19, 679–682. <https://doi.org/10.1038/s41592-022-01488-1>
765 **46. Pettersen EF, Goddard TD, Huang CC, Meng EC, Couch GS, Croll TI, Morris JH,**
766 **Ferrin TE.** 2021. UCSF ChimeraX: Structure visualization for researchers, educators, and
767 developers. *Protein Sci.* 30:70-82. <https://doi.org/10.1002/pro.3943>
768 **47. Corpet F.** 1988. Multiple sequence alignment with hierarchical clustering. *Nucleic Acids*
769 *Res* 16: 10881 – 10890. <https://doi.org/10.1093/nar/16.22.10881>
770 **48. Robert X, Gouet P.** 2014. Deciphering key features in protein structures with the new
771 ENDscript server. *Nucleic Acids Res* 42: W320 –W324. <https://doi.org/10.1093/nar/gku316>

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782 **FIGURE LEGENDS**

783 **Fig. 1. PAAR interacts with the VgrG needle domain and caps the VgrG-Tle1 complex.**

784 **(A)** Schematic representation of the EAEC VgrG1 protein and the truncated variants used for
785 pull-down assays with PAAR. The different domains and their boundaries are indicated –
786 base domain gp27; OB fold domain; β helical domain gp5-C; H - helix; DUF2345 domain;
787 TTR - transthyretin like domain). **(B) Pull-down assays.** Lysates of BL21(DE3) cells co-

788 producing FLAG-tagged PAAR (PAAR^{FL}) and Strep-tag II-tagged VgrG (^SVgrG) or
789 truncated versions of VgrG (^SVgrG ΔTTR, ^SVgrG ΔNeedle) were loaded (L) on a Strep-
790 Tactin column. After washing steps, desthiobiotin-eluted (E) and flow through (FT) fractions
791 were analyzed by SDS-PAGE and Coomassie blue staining (upper panel), immunoblot using
792 anti-StrepII (middle panel) and anti-FLAG (lower panel). **(C) VgrG-PAAR-Tle1 complex**
793 **purification.** SDS-PAGE analysis and Coomassie blue staining (top panel), immunoblot
794 using anti-StrepII (upper middle panel), anti-His (lower middle panel) and anti-FLAG
795 (bottom panel) of the purification steps. Cell lysate (L) co-producing Strep-tag II-VgrG
796 (^SVgrG), Tle1-6xHis (Tle1^H) and PAAR-FLAG (PAAR^{FL}) was loaded onto a StrepTrapHP
797 column. After washing (W) the material was eluted with desthiobiotin directly into a HisTrap
798 column. The imidazole-eluted material (E) was then loaded onto a Superose 6 10/300 gel
799 filtration column (GF). 10 μl of each fraction was loaded onto the gel. The molecular weight
800 markers (in kDa) are indicated on the left and the positions of the different proteins are
801 indicated on the right. **(D) Cryo-EM density maps of VgrG-Tle1 and VgrG-Tle1-PAAR**
802 **complex.** Left: Cryo-EM density maps of VgrG needle bound to Tle1 (in green)
803 corresponding to a (VgrG)₃-(Tle3)₃ tip-to-tip dimer complex. Right: VgrG needle bound to
804 Tle1 in the presence of the PAAR protein (in yellow). In the presence of PAAR, most
805 particles correspond to VgrG needle bound to one or two Tle1 (represented here) and no
806 VgrG needle tip-to-tip dimerization. 2D classes are presented in Fig. S1. **(E) VgrG-Tle1-**
807 **PAAR complex model.** Alphafold2 co-folding model of the needle domain of VgrG (3
808 copies, green, yellow, red) with PAAR (blue) was aligned with the VgrG-Tle1 model
809 (PDB:6SJL) merging on the needle domain. This model was fitted in the cryo-EM density
810 maps (4.2 Å, two Tle1 visible).

811

812 **Fig. 2. PAAR binds a zinc atom involving conserved His and Cys residues.**

813 (A) Alignment of amino acid sequences of PAAR^{EAEc} (EC042_4537), PAAR from *E. coli*
814 CFT073 (c1882) and *Vibrio cholerae* (VCA0105). PAAR motifs are shown in green boxes,
815 His and Cys residues involved in zinc binding characterized in (28) are indicated in black,
816 below the alignment. H14, C41, H46 and C74, the predicted residues involved in metal
817 binding in PAAR^{EAEc} are indicated in red above the alignment. (B) Alphafold2 model of
818 PAAR highlighting His14, Cys41, His46 and Cys74 predicted metal binding residues. PAAR
819 was co-modelled with VgrG needle part and only PAAR is shown here. (C) SDS-PAGE
820 followed by Coomassie blue staining (upper panel) and immunoblot using anti-His-Tag
821 (lower panel) of purified wild type His-SUMO-PAAR proteins and mutated versions (His-SUMO-
822 PAAR^{C41A}, His-SUMO-PAAR^{C74A} and His-SUMO-PAAR^{C41AC74A}). The molecular weight markers
823 (in kDa) are indicated on the left. (D) Zinc content analysis using ICP-OES of purified His-
824 SUMO-PAAR (WT) and cysteine mutants (His-SUMO-PAAR^{C41A}, C41A; His-SUMO-PAAR^{C74A},
825 C74; His-SUMO-PAAR^{C41AC74A}, C41AC74A). Statistical significance was calculated using
826 Ordinary one-way ANOVA followed by Dunnett's multiple comparisons test using GraphPad
827 Prism (GraphPad Software). **** p<0.0001. Each value represents the mean of three
828 technical replicates of two to four different fractions of two (C41A, C74A) to three different
829 purifications preparations (WT, C41AC74A).

830

831 **Fig. 3. PAAR is necessary for T6SS-1 mediated killing.**

832 (A) Antibacterial competition assay by the *Colorimetric method*. The T6SS-1 function of the
833 WT, Δ PAAR Ω and Δ PAAR Ω complemented with PAAR (PAAR⁺) or PAAR Cys and His
834 mutant strains (using pBAD₃₃-PAAR^{VSVG} and corresponding PAAR Cys and His mutant
835 plasmids derivatives) was tested by assessing their ability to kill W3110 *E. coli* K12 bacterial
836 prey. Killing efficiency was monitored by observing degradation of yellow CPRG into purple
837 CPR by free β -galactosidase released from lysed prey cells after being exposed to the predator

838 cells. CPR absorbance ($A_{572\text{nm}}$, upper graph) was measured from the spots (lower panel). The
839 means of three biological replicates is indicated. The error bars represent standard deviation.
840 Statistical significance was calculated using Ordinary one-way ANOVA followed by
841 Dunnett's multiple comparisons test using GraphPad Prism (GraphPad Software). ****
842 $p < 0.0001$.

843 **(B)** Antibacterial competition assay by the *Emergence time method*. The T6SS-1 function of
844 the WT, $\Delta\text{PAAR}\Omega$ and $\Delta\text{PAAR}\Omega$ complemented with PAAR or PAAR-Cys and His-mutant
845 strains was tested by assessing their ability to kill W3110 *E. coli* K12 bacterial prey. Killing
846 efficiency was quantified by measuring the time (in hours) needed for prey cells exposed to
847 predator cells to regrow in selective media to the $A_{600} = 0.4$. Statistical significance was
848 calculated using Ordinary one-way ANOVA followed by Dunnett's multiple comparisons test
849 using GraphPad Prism. **** $p < 0.0001$; *** $p = 0.0009$; ** $p = 0.0092$; * $p = 0.0401$, ns = 0.9711
850 (ΔT6SS vs. $\Delta\text{PAAR}\Omega$), ns = 0.9967 (PAAR^+ vs. H14A).

851

852 **Fig. 4. PAAR is necessary for T6SS assembly.**

853 **(A)** Fluorescence microscopy of EAEC *tssB-sfGFP* strains transformed with the empty vector
854 pBAD₃₃ (*tssB-sfGFP* + pBAD), EAEC *tssB-sfGFP* $\Delta\text{PAAR}\Omega$ Kan transformed with the empty
855 vector pBAD₃₃ (*tssB-sfGFP* $\Delta\text{PAAR}\Omega$ + pBAD) and EAEC *tssB-sfGFP* $\Delta\text{PAAR}\Omega$
856 complemented in trans with pBAD₃₃-PAAR^{VSVG} plasmid (*tssB-sfGFP* $\Delta\text{PAAR}\Omega$ + pBAD-
857 PAAR). Phase contrast images are shown on the left (scale bar = 2 μm). Fluorescence
858 images of TssB-sfGFP (green) and membrane dye FM4-64 (red) are shown on the right.
859 Pictures taken every 30 sec are shown from left to right. White arrows indicate dynamic
860 sheath assembly and/or contraction events. **(B)** Fluorescence microscopy of EAEC *tssB-*
861 *sfGFP* $\Delta\text{PAAR}\Omega$ Kan strain transformed with pBAD₃₃-PAAR^{H14A-VSVG}, pBAD₃₃-PAAR^{C41A-}
862 ^{VSVG}, pBAD₃₃-PAAR^{H46A-VSVG} or pBAD₃₃-PAAR^{C74A-VSVG}. Phase contrast images are shown

863 on the left (scale bar = 2 μ m). Fluorescence images of TssB-sfGFP (green) are shown on the
864 right. Cells are outlined in white, white arrows indicate dynamic sheath assembly and/or
865 contraction events. **(C)** Quantification of the number of extended sheaths per cell from the
866 strains *tssB-sfGFP* + pBAD (B_{sfGFP}), *tssB-sfGFP* $\Delta PAAR\Omega$ + pBAD ($B_{sfGFP}\Delta PAAR$), *tssB-*
867 *sfGFP* $\Delta PAAR\Omega$ + pBAD-PAAR^{VSVG} (PAAR⁺), and *tssB-sfGFP* $\Delta PAAR\Omega$ transformed with
868 pBAD₃₃-PAAR^{H14A-VSVG} (H14A), pBAD₃₃-PAAR^{C41A-VSVG} (C41A), pBAD₃₃-PAAR^{H46A-VSVG}
869 (H46A) or pBAD₃₃-PAAR^{C74A-VSVG} (C74A) analyzed in A and B. The total number of
870 analyzed cells (n) from 3 independent biological replicates is indicated. The error bars
871 represent standard deviation.

872

873 **Fig. 5. PAAR is necessary for TssK but not TssM localization.**

874 **(A)** Fluorescence microscopy analysis of *sfGFP-tssM* and *sfGFP-tssM* $\Delta PAAR\Omega Kan$. Phase
875 contrast images are shown on the left (scale bar = 2 μ m) and fluorescence images of sfGFP
876 (green) are shown on the right. Cells are outlined in white, white arrows indicate fluorescent
877 foci localized at the membrane. **(B)** Quantification of the number of foci per cell from *sfGFP-*
878 *tssM* and *sfGFP-tssM* $\Delta PAAR\Omega Kan$. The total number of analyzed cells (n) from 3
879 independent biological replicates is indicated. The error bars represent standard deviation. **(C)**
880 Fluorescence microscopy analysis of *tssK-sfGFP* and *tssK-sfGFP* $\Delta PAAR\Omega Kan$. Phase
881 contrast images are shown on the left (scale bar = 2 μ m) and fluorescence images of sfGFP
882 (green) are shown on the right. Cells are outlined in white, white arrows indicate fluorescent
883 foci localized at the membrane. **(D)** Quantification of the number of foci per cell from *tssK-*
884 *sfGFP* and *tssK-sfGFP* $\Delta PAAR\Omega Kan$. The total number of analyzed cells (n) from 3
885 independent biological replicates is indicated. The error bars represent standard deviation.

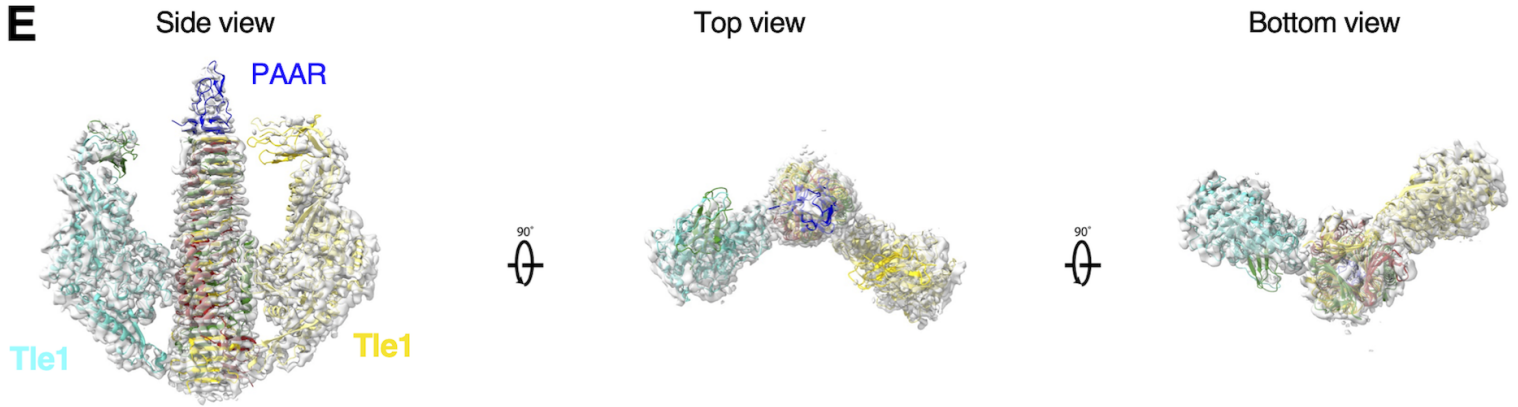
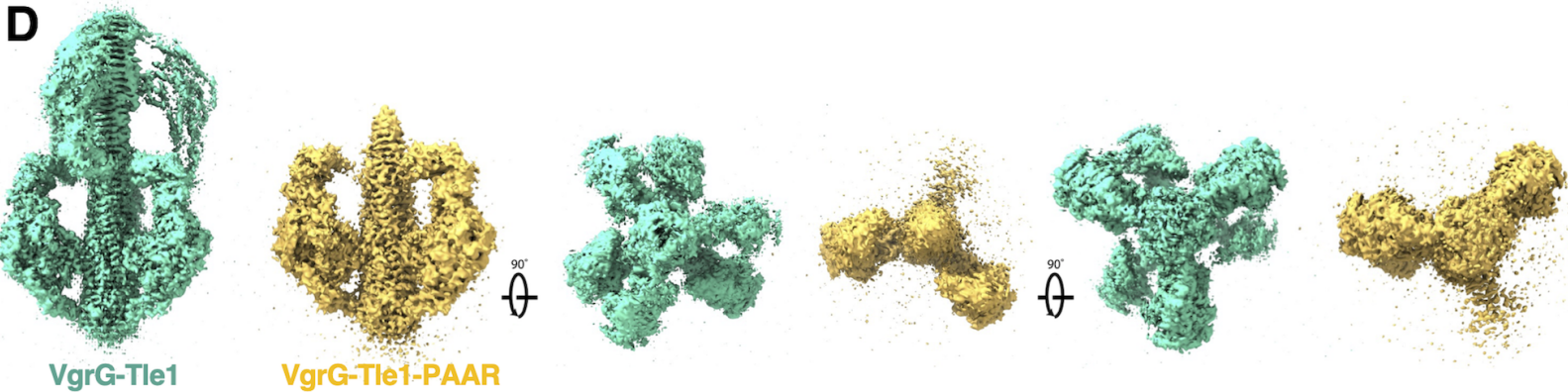
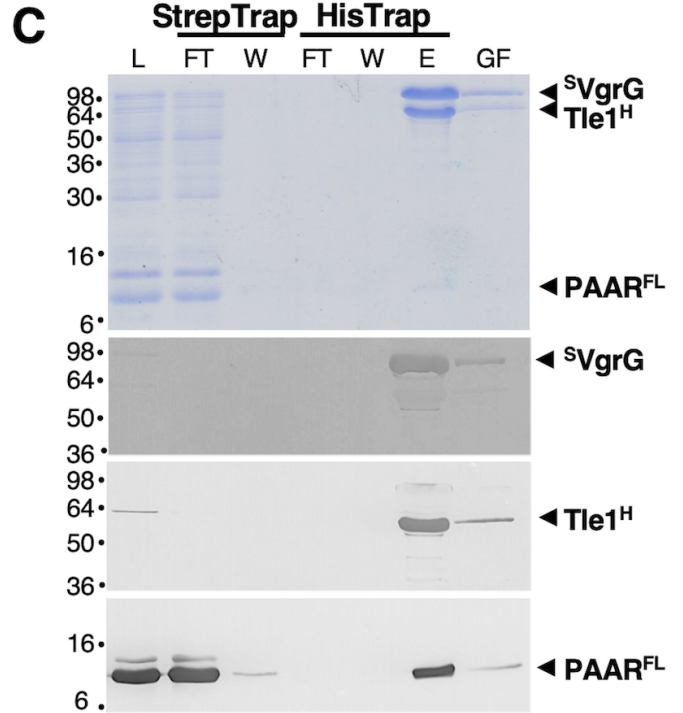
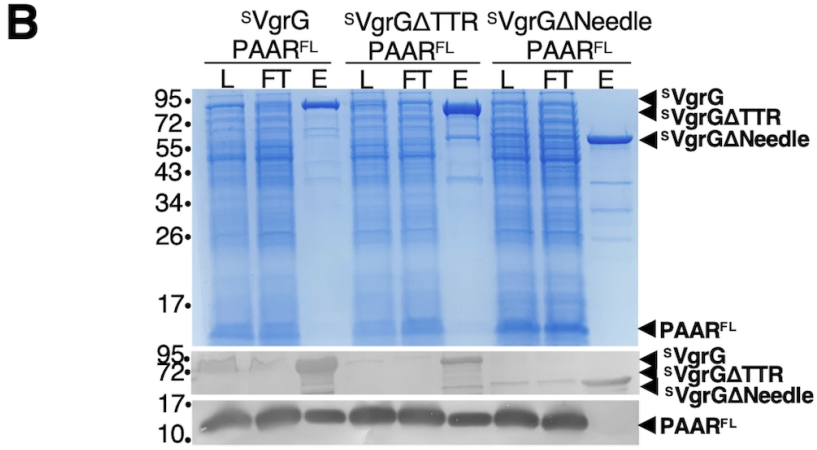
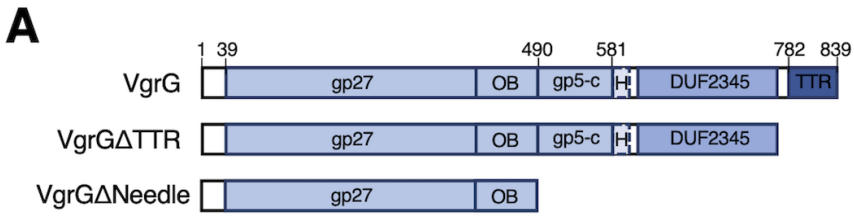
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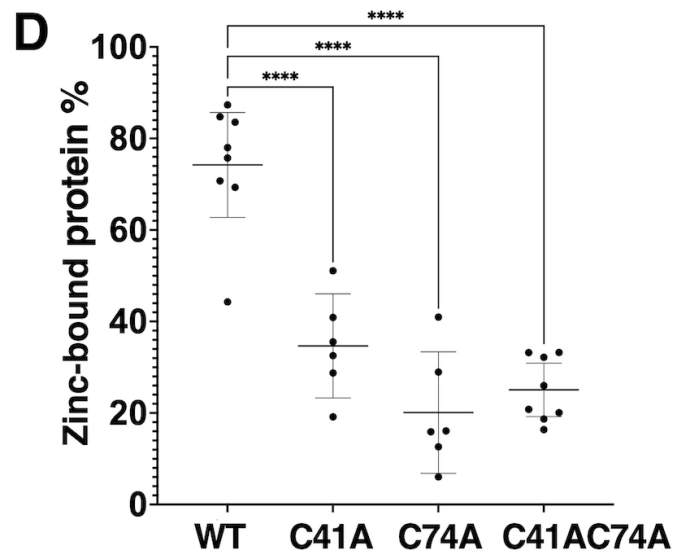
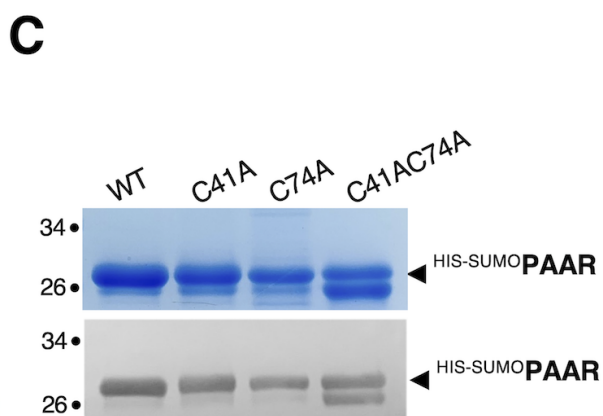
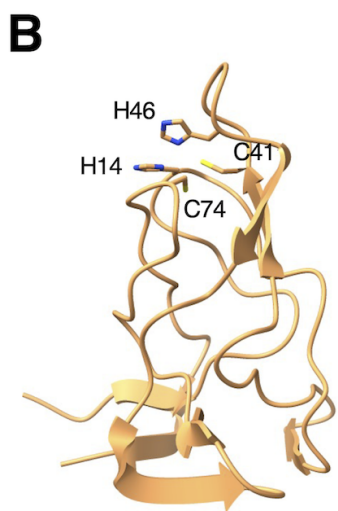
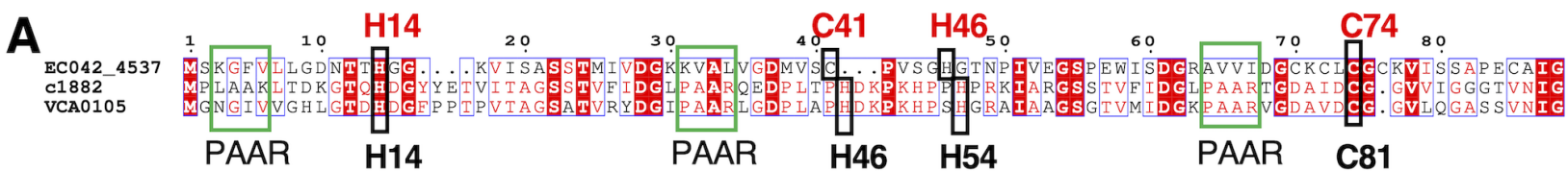
887 **Fig. 6. A conical tip structure consolidated by a metal atom is conserved in different**
888 **phages and bacterial injection systems.**

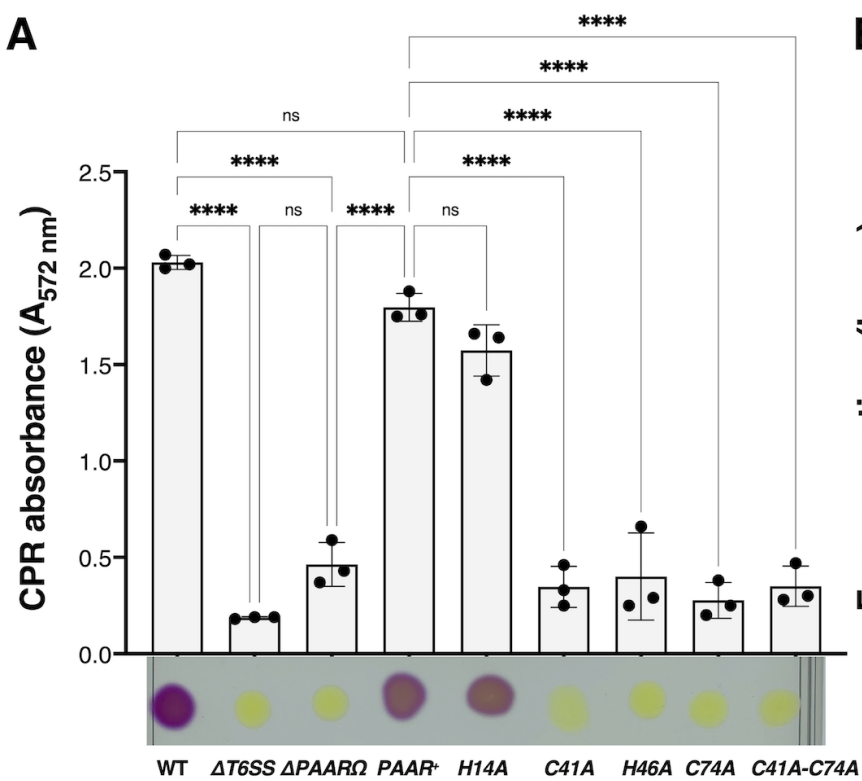
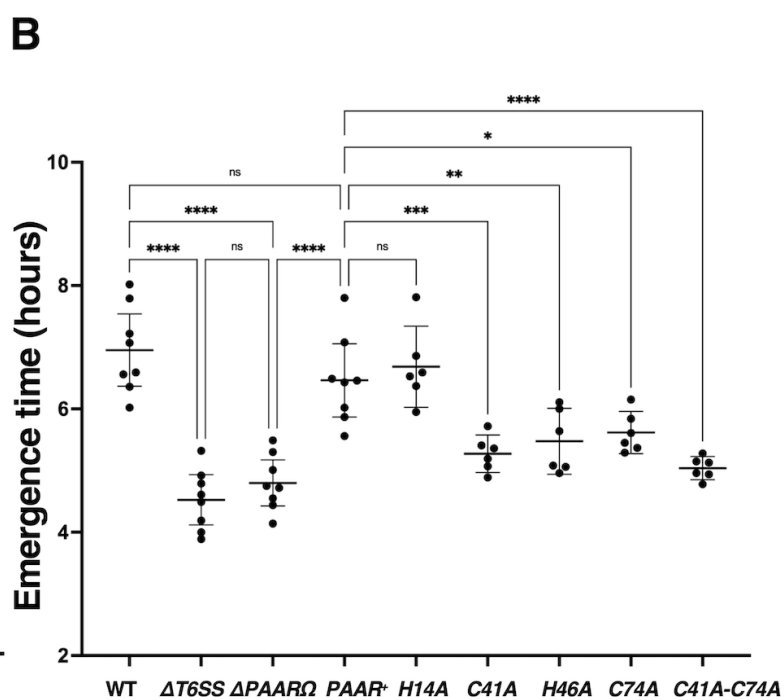
889 Ribbon diagrams of VgrG(gp5C)-PAAR from EAEC, gp5-gp5.4 from T4 phage (PDB:5iv5),
890 gpV from P2 phage (PDB:3aqj), gp138 from Phi92 phage (PDB:3pqi) and gpV from R2
891 pyocin (PDB:4s36). All β -prism spikes are capped and the tip is closed with coordinated
892 metal. Structures are colored by chain.

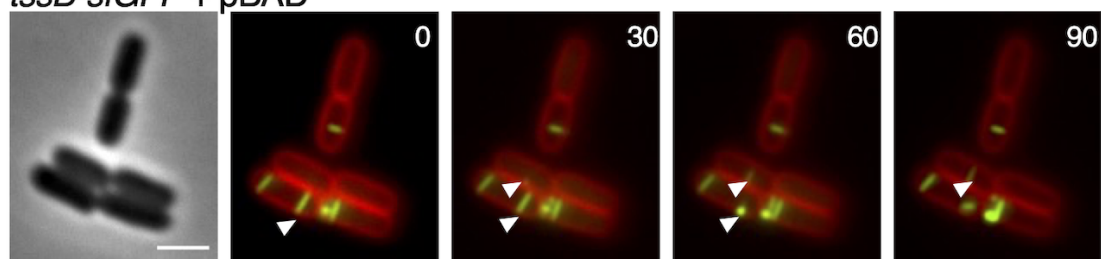
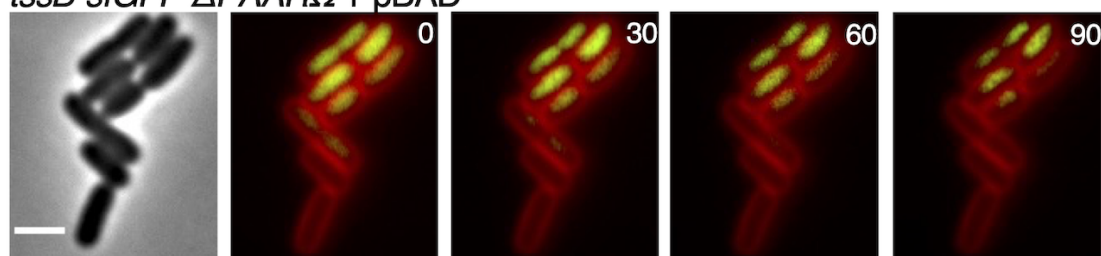
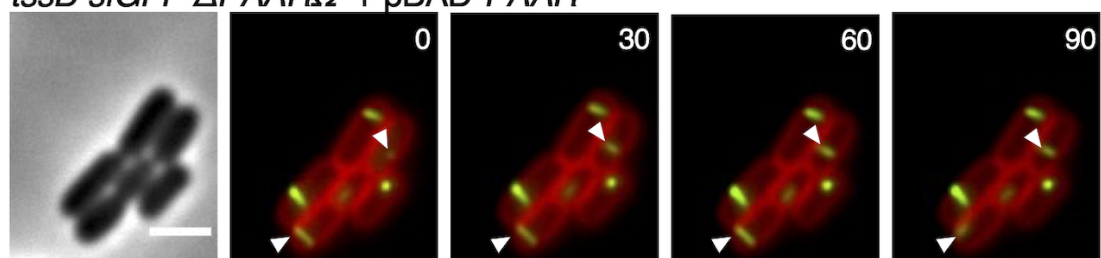
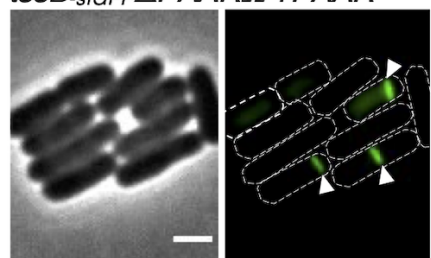
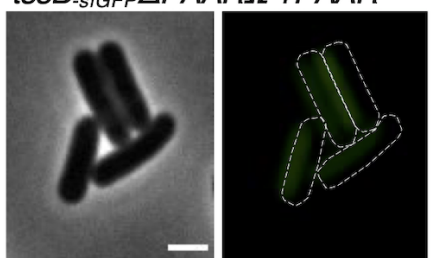
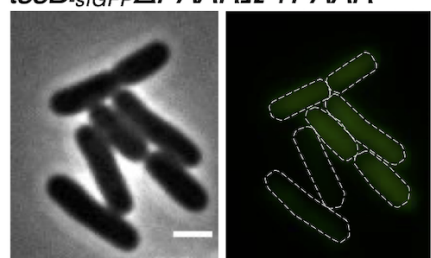
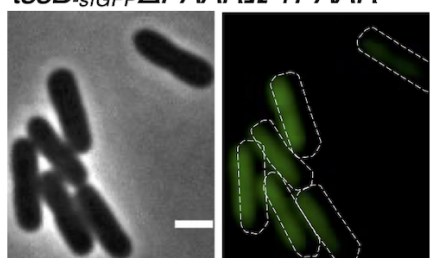
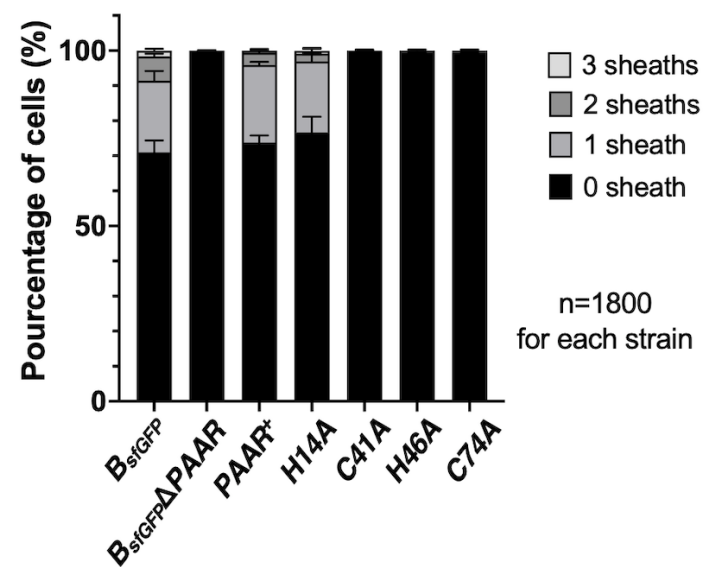
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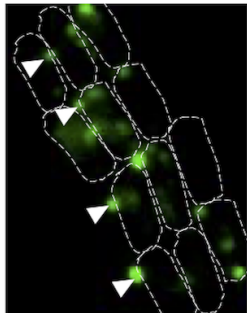
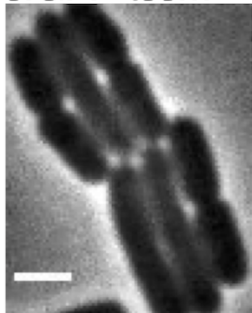
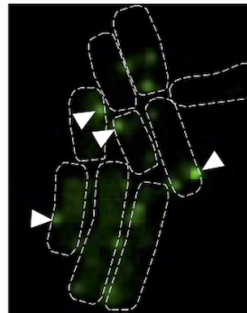
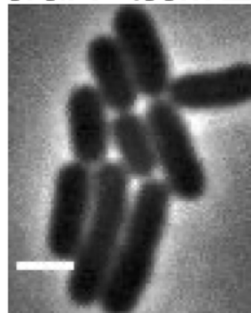
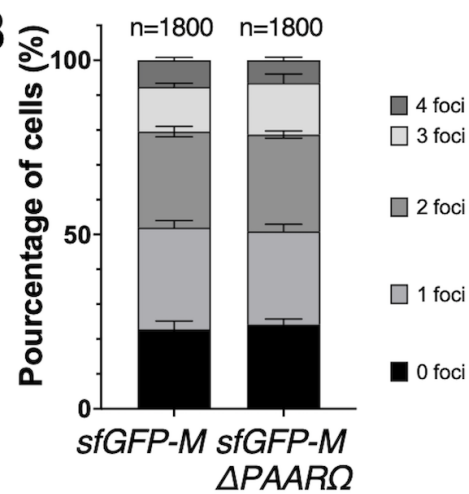
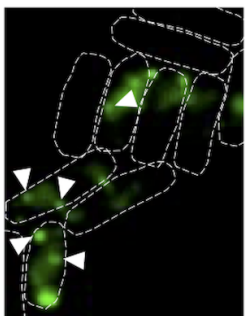
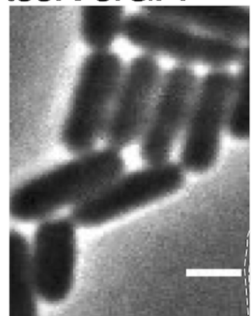
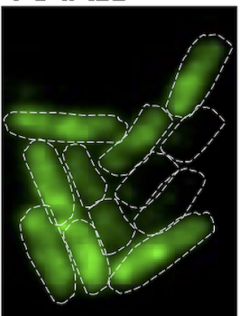
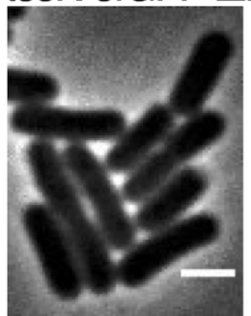
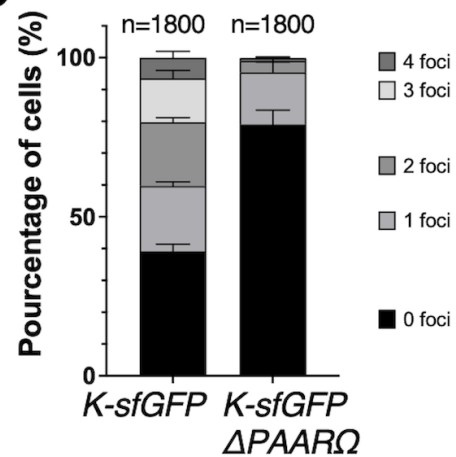
894

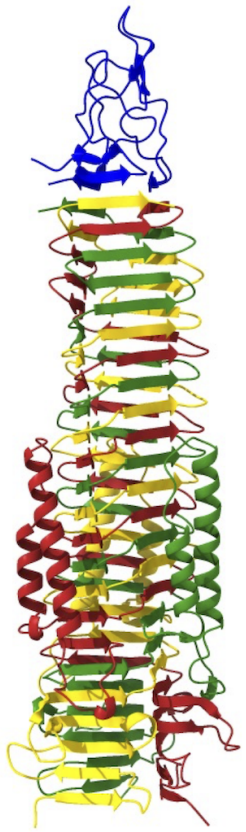




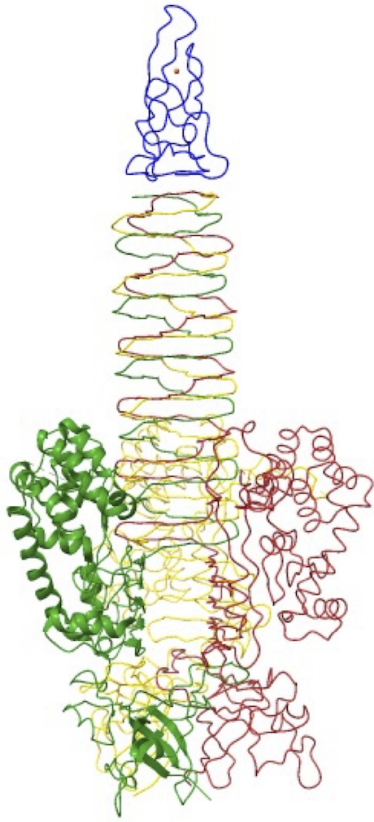
A**B**

A*tssB-sfGFP + pBAD**tssB-sfGFP ΔPAARΩ + pBAD**tssB-sfGFP ΔPAARΩ + pBAD-PAAR***B***tssB-sfGFP ΔPAARΩ + PAAR^{H14A}**tssB-sfGFP ΔPAARΩ + PAAR^{C41A}**tssB-sfGFP ΔPAARΩ + PAAR^{H46A}**tssB-sfGFP ΔPAARΩ + PAAR^{C74A}***C**

A*sfGFP-tssM**sfGFP-tssM ΔPAARΩ***B****C***tssK-sfGFP**tssK-sfGFP ΔPAARΩ***D**



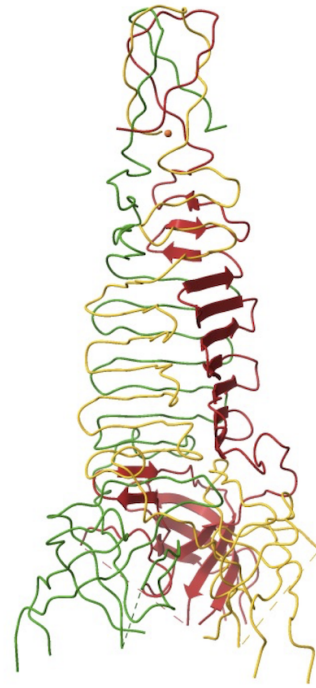
EAEC T6SS
VgrG(gp5C)-PAAR



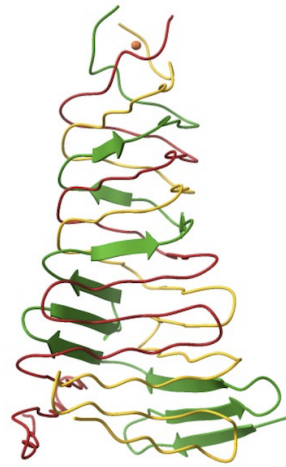
T4 phage
gp5-gp5.4



P2 phage
gpV



Phi92 phage
Gp138



Pyocin
gpV