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Structural basis for loading and inhibition of a bacterial T6 SS phospholipase effector by the VgrG spike

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21 **ABSTRACT**

22 The bacterial Type VI secretion system (T6SS) is a macromolecular machine that
23 injects effectors into prokaryotic and eukaryotic cells. The mode of action of the T6SS is
24 similar to contractile phages: the contraction of a sheath structure pushes a tube topped by a
25 spike into target cells. Effectors are loaded onto the spike or confined into the tube. In
26 enteroaggregative *E. coli*, the Tle1 phospholipase binds the C-terminal extension of the VgrG
27 trimeric spike. Here we purify the VgrG-Tle1 complex and show that a VgrG trimer binds
28 three Tle1 monomers and inhibits their activity. Using covalent cross-linking coupled to high-
29 resolution mass spectrometry we provide information on the sites of contact and further
30 identify the requirement for a Tle1 N-terminal secretion sequence in complex formation.
31 Finally, we report the 2.6-Å resolution cryo-electron microscopy tri-dimensional structure of
32 the (VgrG)₃-(Tle1)₃ complex revealing how the effector binds its cargo, and how VgrG
33 inhibits Tle1 phospholipase activity. The inhibition of Tle1 phospholipase activity once bound
34 to VgrG suggests that Tle1 dissociation from VgrG is required upon delivery.

35

36 *Keywords:* type VI secretion system/bacterial competition/cryo-electron microscopy/ bacterial
37 toxin/protein secretion

38 **INTRODUCTION**

39 The Type VI secretion system (T6SS) is a multiprotein secretion nano-machine used by many
40 Gram-negative bacteria to deliver toxins in a contact-dependent manner directly into target
41 cells. The T6SS can target both eukaryotic and prokaryotic cells, secreting effectors that
42 degrade the peptidoglycan (amidases, glycoside hydrolase), membranes (phospholipases A₁,
43 A₂ and D), or DNA (DNase) (Russell *et al*, 2014; Durand *et al*, 2014; Cianfanelli *et al*, 2016;
44 Hachani *et al*, 2016). Some anti-host effectors interfere with eukaryotic cytoskeleton
45 dynamics (actin, microtubules), leading to phagocytosis inhibition or promoting invasion.
46 (Russell *et al*, 2014; Durand *et al*, 2014; Cianfanelli *et al*, 2016; Hachani *et al*, 2016). The
47 T6SS secretion of anti-bacterial effectors allows bacteria to eliminate competitors, to acquire
48 nutrients, new DNA or metal, thus favoring the colonization of specific niches (Russell *et al*,
49 2014; Durand *et al*, 2014; Cianfanelli *et al*, 2016; Hachani *et al*, 2016).

50 T6SS biogenesis needs at least 14 different proteins. These subunits assemble two sub-
51 complexes: a membrane complex anchored in the envelope (Durand *et al*, 2015; Rapisarda *et*
52 *al*, 2019), on which is docked a platform that controls the assembly of a contractile tail
53 tube/sheath structure (Brunet *et al*, 2015; Logger *et al*, 2016; Taylor *et al*, 2016; Nguyen *et al*,
54 2017; Nazarov *et al*, 2018; Basler *et al*, 2012). The tail tube/sheath complex comprises an
55 inner tube made of Hcp hexamers covered by a sheath and topped by a needle spike
56 constituted of a VgrG trimer and a PAAR-domain protein (Zoued *et al*, 2014; Ho *et al*, 2014).

57 The tail sheath assembles in an extended conformation and its contraction propels the inner
58 tube, the spike and toxins towards target cells. Self-protection is assured by immunity proteins
59 that specifically bind and inhibit their corresponding effectors. A few T6SS effectors are
60 fused to the Hcp, VgrG, or PAAR proteins and are therefore delivered into the recipient cell
61 as domains of syringe components (Pukatzki *et al*, 2006; Blondel *et al*, 2009; Suarez *et al*,
62 2010; Brooks *et al*, 2013; Dong *et al*, 2013; Shneider *et al*, 2013; Ma *et al*, 2017). However,

63 most T6SS effectors are independent polypeptides. Upon T6SS assembly these effectors are
64 loaded within the lumen of the Hcp tube or bind the VgrG/PAAR spike and are then
65 transported as cargo (Durand *et al*, 2014; Cianfanelli *et al*, 2016; Ho *et al*, 2014). Small
66 effectors (< 20 kDa), such as the *Pseudomonas aeruginosa* Tse2 toxin, have been shown to
67 fill the internal hole of Hcp hexamers (Silverman *et al*, 2013) whereas others have been
68 demonstrated to indirectly interact with VgrG. Binding of these effectors is mediated by
69 specific adaptors (Alcoforado Diniz & Coulthurst, 2015; Liang *et al*, 2015; Unterweger *et al*,
70 2015; Bondage *et al*, 2016). These adaptors are not secreted components but are rather
71 required for the secretion of their cognate effectors, suggesting a role in loading effectors to
72 VgrG prior to translocation. A complex constituted of the PAAR-fused Tse6 effector, the
73 EagT6 adaptor, the immunity to Tse6 and EF-Tu was recently visualized by negative stain
74 electron microscopy (EM). It forms a horseshoe-like structure at the tip of the VgrG trimer
75 (Whitney *et al*, 2015). The cryo-EM structure of Tse6-EagT6 in complex with VgrG was
76 further determined (Quentin *et al*, 2018).

77 We recently identified Tle1, a periplasmic-acting phospholipase A₁ (PLA1) antibacterial
78 effector of the enteroaggregative *E. coli* (EAEC) T6SS-1 (Flaunatti *et al*, 2016). We showed
79 that Tle1 is transported into the periplasm of target cells using the VgrG spike protein as
80 carrier. By contrast to other VgrG-dependent effectors, Tle1 interacts directly with VgrG,
81 without the need for a PAAR or adaptor protein. VgrG from EAEC possesses a C-terminal
82 extension that comprises a DUF2345 and a transthyretin-like domain (TTR) responsible for
83 the interaction with Tle1 (Flaunatti *et al*, 2016). Here, we report the purification of the
84 VgrG-Tle1 complex and show that three Tle1 effectors bind to the VgrG trimer. Our data
85 reveal that VgrG inhibits Tle1 PLA1 activity and hence that Tle1 needs to be dissociated from
86 its carrier once in the target cell. Using a combined approach of cross-linking mass
87 spectrometry, deletion studies and functional assays, we identify the motifs involved in VgrG-

88 Tle1 interaction, confirming the importance of the VgrG TTR domain and highlighting an N-
89 terminal interaction motif within Tle1. Finally, we present the cryo-EM structure of Tle1
90 bound to the VgrG needle.

91

92 **RESULTS**

93 **Purification of the VgrG-Tle1 complex reveals that three Tle1 bind to the VgrG trimer**

94 We previously showed that Tle1 from EAEC interacts directly with the C-terminal
95 domain extension of the VgrG spike protein (Flaunatti *et al*, 2016). To gain further insight
96 into the VgrG-Tle1 complex, affinity-tagged VgrG and Tle1 proteins were co-produced in *E.*
97 *coli*. The position of the affinity tag on each protein was rationally chosen to maintain the
98 VgrG-Tle1 interaction: a Strep-tagII was fused to the N-terminus of VgrG (^SVgrG) to
99 maintain the C-terminal Tle1-interacting motif available, and a 6×His tag was fused to the
100 Tle1 C-terminus (Tle1^H), as previous bacterial two-hybrid (BACTH) analysis suggested that
101 T25/T18 fusion at the N-terminus of Tle1 protein impaired the interaction with VgrG
102 (Flaunatti *et al*, 2016). A complex containing both ^SVgrG and Tle1^H was purified to
103 homogeneity using a two-step affinity purification and gel filtration (Fig EV1A). When
104 subjected to size exclusion chromatography analyses, ^SVgrG separated with an apparent mass
105 of ~ 500 kDa (Fig EV1B, black line; Appendix Fig S1), which is higher than the 280-kDa
106 theoretical mass of a VgrG trimer. This could be due to an artefact of the gel filtration
107 technique, the elongated-shape structure of the VgrG β-helix (Leiman *et al*, 2009; Uchida *et*
108 *al*, 2014; Spínola-Amilibia *et al*, 2016), or this species may correspond to a dimer of a VgrG
109 trimer. Once bound to Tle1^H, we observed an increase in the apparent molecular mass (Fig
110 EV1B, blue line). Native-purified Tle1 was previously shown to be monomeric in solution,
111 with an apparent molecular mass of 66 kDa (Flaunatti *et al*, 2016). Taken together, these
112 results suggest that several Tle1 proteins are bound to the VgrG trimer. To provide further

113 information on the stoichiometry of the $^S\text{VgrG-Tle1}^{\text{H}}$ complex, we performed quantitative in-
114 gel SYPRO Ruby staining. This analysis of the purified $^S\text{VgrG-Tle1}^{\text{H}}$ complex gave a 1:0.92
115 molar ratio (Fig EV1C). The approximate 1:1 stoichiometry suggests that three Tle1 effectors
116 are bound to a VgrG trimer.

117

118 **High-resolution cross-linking mass spectrometry (XL-MS) mapping identifies VgrG-** 119 **Tle1 sites of contact**

120 To provide detailed information on the VgrG/Tle1 interaction, the $^S\text{VgrG-Tle1}^{\text{H}}$
121 complex was subjected to cross-linking with the NNP9 cross-linker (Nury *et al*, 2015). NNP9
122 was specifically engineered to carry 2 NHS carbamate reactive groups, which are less prone
123 to hydrolysis than widely used NHS-esters, thus improving cross-linking efficiency and
124 performance compared to commercial ones (Nury *et al*, 2015). Mass spectrometry analysis of
125 cross-linked peptides revealed that VgrG and Tle1 establish numerous contacts (Fig 1A,
126 Dataset EV1). In agreement with previous findings demonstrating that Tle1 binds the VgrG
127 C-terminal TTR extension as well as a second upstream motif (Flaughnatti *et al*, 2016), XL-
128 MS showed that Tle1 binds the VgrG TTR motif and a second motif located in the C-terminal
129 part of the gp27-like domain (Fig 1A). Accordingly, Tle1^{H} no longer co-purified with a
130 $^S\text{VgrG}$ variant lacking the C-terminal 616-841 residues ($^S\text{VgrG}_{\Delta\text{CTD}}$) (Appendix Fig S2A and
131 B). More importantly, several cross-links involving Lys residues in the VgrG TTR domain
132 and at the Tle1 N-terminus (K29, K30) were detected (Fig 1A and Appendix Fig S3A),
133 suggesting that the VgrG TTR binds the Tle1 N-terminus. Interestingly, a sequence alignment
134 of Tle1 orthologs reveals a striking difference at their N-termini. Compared to specialized
135 Tle1 orthologs that are fused to Hcp, PAAR and VgrG proteins, isolated Tle1 proteins bear an
136 N-terminal 26-amino-acid extension (NT) (Fig 1B and Appendix Fig S3B). As deletion of the
137 Tle1 NT region ($\text{Tle1}_{\Delta 1-26}$) did not impact PLA1 specific activity (Appendix Fig S4), this

138 region is not necessary for Tle1 folding and activity. Tle1 NT extension is followed by a Lys-
139 rich linker that precedes the Tle1-domain region (Appendix Fig S3). Substitution of these
140 Lysines by Glutamate residues (K28K29K30-to-E28E29E30) did not impair Tle1
141 antibacterial activity against *E. coli* K12 (Fig 1C), suggesting that the K29 and K30 residues
142 identified by XL-MS are not directly involved in the interaction with VgrG. Rather, the XL-
143 MS data highlight the proximity of the NT and the TTR region in the complex and we
144 hypothesized that the NT region might be an important determinant of a Tle1 secretion signal
145 allowing VgrG recognition and binding. Evidence for the crucial role of this extension was
146 provided by co-immunoprecipitation (Fig 1D) and co-purification (Appendix Fig S2C)
147 assays, which demonstrated that deletion of the Tle1 N-terminal 26 amino-acids strongly
148 affects the interaction with VgrG. To further test the importance of this VgrG interaction
149 motif for the delivery of Tle1, we tested the ability of Tle1 $_{\Delta 1-26}$ to complement a *tle1* knockout
150 mutant in antibacterial competition (Fig 1C). While the production levels of Tle1 and Tle1 $_{\Delta 1-}$
151 $_{26}$ were comparable, only Tle1 restored the killing defect of the *tle1* knockout mutant. Taken
152 together these results showed that Tle1 binds two regions of VgrG including the C-terminal
153 TTR extension, whereas the Tle1 N-terminal 26-amino-acid region is required for VgrG
154 binding and translocation into target cells.

155

156 **Cryo-EM structure of the VgrG-Tle1 complex.** *Architecture of the VgrG spike-Tle1*
157 *complex.* To gain further information on the structure of the VgrG-Tle1 complex, the purified
158 S VgrG-Tle1 H complex was analysed using single particle cryo-EM. The structure of the
159 complex at 2.7-Å resolution overall was obtained by applying no symmetry (Fig EV2A and
160 B). The 2D classes resembled violin bodies and were found in different orientations (Fig
161 EV2A). From the unmasked cryo-EM density we can observe the presence of a D3 symmetry
162 with the dihedral rotational centre found between the two prism-like densities and further

163 strengthened by opposite flexible densities interacting with each other (Fig EV2B and C).
164 This large symmetric complex is 225-Å tall and 150-Å large, and consists of 6 VgrG and 6
165 Tle1 densities as inferred from the gel filtration (Fig EV1B, Fig EV2B and C). This
166 oligomeric state is likely not physiological. The absence of the PAAR capping protein may
167 lead to this tip-to-tip VgrG needle spike interaction. We performed an unmasked refinement
168 to 2.7 Å (Fig EV2B-D) followed by a local refinement on what we will hereby refer to as the
169 physiological complex, consisting of 3 Tle1 and 3 VgrG molecules, and obtained a resolution
170 of 2.6 Å (Fig 2A-C, Fig EV2E). We further locally refined the prism-like density
171 corresponding to VgrG (to 2.3-Å resolution), and one external density belonging to Tle1 (to
172 2.5-Å resolution) (Fig EV2F and G). Thanks to the high resolution of the cryo-EM densities
173 obtained (Fig EV2E-G), we could build *de novo* an atomic model of VgrG amino-acids (aa)
174 491-834 and of the full Tle1 (Fig 2D-H and Fig 3A and B), whose structures were previously
175 unknown.

176 No interpretable cryo-EM density was found for VgrG amino-acids 1-490. This region which
177 is homologous to the gp27-like base followed by the gp5-OB fold-like in the T4 phage,
178 appears to be disordered. Such flexibility may be stabilised by binding of PAAR, and/or by
179 the binding to baseplate components. This region is well conserved amongst the VgrG
180 proteins from different bacterial species and phage families (Leiman *et al*, 2009). We
181 therefore built a model using Phyre2 (Kelley *et al*, 2015), based on the sequence homology
182 (42.5% identity) to the VgrG N-terminus from uropathogenic *E. coli* CFT073, whose
183 structure has been solved by X-ray crystallography (Leiman *et al*, 2009) (Fig 2G).

184 *Structure of the gp5 needle-like domain of VgrG from EAEC.* The atomic model of VgrG (aa
185 491-834) includes the gp5-C-like, DUF2345 and TTR domains predicted by bioinformatic
186 analyses (Flaunatti *et al*, 2016) (Fig 1A and Fig 2G-H). The region from aa 491 to 760
187 assembles a gp5 needle-like structure corresponding to a three-stranded β -prism with a width

188 of 30 Å at the tip and 45 Å at the bottom (Fig 2G-H). The β-prism contains 2 antiparallel β-
189 sheets consisting of 5 and 3 strands respectively at aa 491-549 and 726-747 (Fig EV3A).
190 Unique to the EAEC VgrG (VgrG^{EAEC}) is also the presence of two long helices that break the
191 β-prism at aa 576-622. The rest of the β-prism is composed of parallel β-sheets that intertwine
192 around each other via sharp β-turns (Fig EV3A). There is no cavity at the centre of the β-
193 prism and the structure is maintained by hydrophobic interactions (Fig 2H). The interaction
194 between the monomers of VgrG, as calculated using PISA (Krissinel & Henrick, 2007), is
195 very strong with a mean surface of interaction of 7150 Å².
196 Comparison with the *Pseudomonas aeruginosa* VgrG (VgrG^{PA}) structure and with the phage
197 gp5 needle domain reveals several differences. In VgrG^{PA} (Fig EV3B, PDB: 4MTK), the gp-5
198 needle domain is shorter by 40 Å because it only has a three-strand antiparallel β-sheet at the
199 base of the spike, instead of a 5-strand (in blue, Fig EV3B). In the phage T4 gp5 protein
200 (PDB: 1K28) (Kanamaru *et al*, 2002) there is only one anti-parallel 6-strand β-sheet at the
201 base of the spike and none at the tip (in orange, Fig EV3C). VgrG^{EAEC} is unique also because
202 it has an additional β-sheet TTR domain that binds the effector, (Fig 2G, Fig EV3D). This
203 domain is missing in VgrG^{PA}, but it has been shown that the toxin does not directly bind to
204 VgrG^{PA} but is covalently linked to the PAAR component (Whitney *et al*, 2015) (Fig EV3E).
205 In the T4 phage, gp5 has an additional lysozyme domain (Fig EV3F).
206 *Structure of Tle1 bound to VgrG.* The Tle1 structure bound to VgrG adopts a thin elongated
207 shape, which is 125-Å tall and 50-Å wide on one side and 75 Å on the other (Fig 3A and B).
208 It can be divided into three distinct domains: the finger N-terminal domain (aa 3-33), the
209 phospholipase catalytic module (aa 33-326 and 411-539), and the lid domain (aa 327-410)
210 (Fig 3A, Fig EV4A). Two loops are missing from the density and are most likely disordered:
211 aa 86-94 and aa 133-143. As shown by cross-link MS, deletion analyses and co-purification
212 (Fig 1D and Appendix Fig S2C), the finger domain is responsible for the interaction with the

213 VgrG TTR domain, and consists of a long loop that is highly flexible (Fig 3A-E, Fig EV4B
214 and C). There is a smaller β -sheet composed of strand 17 at the C-terminus and strand 7 and
215 an incomplete Greek key motif between strands 13-16 and 14-15 (Fig 3, Fig EV4A). The
216 catalytic module has the most compact conformation and contains a classical α/β mixed
217 hydrolase fold, composed of one central β -sheet of 6 β -strands (3-2-4-5-6-8) surrounded by
218 α -helices (Fig 3A and B, Fig EV4A). The lid domain mainly comprises one long twisted
219 antiparallel β -sheet, constituted of three strands (11, 12 and 9 and 10). In between strands 11
220 and 12, α -helix 12 (aa 338-395) mediates the interaction with the VgrG base (Fig 3A and B,
221 Fig EV4A).

222 *A new mechanism for T6SS effector loading on the VgrG spike.* In contrast to the VgrG-
223 chaperone-effector in *P. aeruginosa*, where the effector is fused to PAAR and located at the
224 tip of the needle complex (Fig EV3E), the loading of Tle1 proteins on the VgrG spike is
225 independent of PAAR. Moreover, three molecules of Tle1 are loaded on the VgrG trimer, on
226 the sides of the β -prism (Fig 3F-H and Supplementary Fig EV3D). Tle1 binds VgrG in three
227 different regions (bottom, centre and top) and every VgrG chain interacts with each Tle1
228 monomer (Fig 3F-H, and Table 1). The Tle1 catalytic domain interacts with the two α -helices
229 (581-619) of one VgrG (chain A) and at the level of the β -prism with the two other VgrG
230 monomers (chains B and C) (Fig 3F-H). The lid domain of Tle1 (aa 335-405) interacts only
231 with chains B and C at the level of the anti-parallel β -sheet (Fig 3F-H). Finally, the finger
232 domain of Tle1 (aa 3-33) and the loop of the catalytic domain (aa 225-230) between helix 8
233 and strand 5 interact with the TTR domain of VgrG (aa 778-841) (Fig 3C-E). The TTR
234 domain is globular: it contains 2 sheets composed of 3 and 2 anti-parallel β -strands
235 respectively (Fig 3C-E). The finger domain of Tle1 complements the strands of the TTR
236 domain to form a β -barrel (Fig 3C-E). The surface of interaction of VgrG with Tle1 is 1160
237 \AA^2 and has a ΔG of -12.2 kcal/M. The TTR-Tle1 interaction is mediated by several hydrogen

238 bonds and salt bridges (Table 1). While VgrG is very stable, the finger domain and the TTR
239 region are very flexible as shown by the b-factor values (Fig EV4B and C), and the local
240 resolution (Appendix Fig S5) as it is connected to the VgrG spike by a very flexible loop
241 whose density was not resolved in the cryo-EM map.

242

243 **Tle1 binding to VgrG inhibits its phospholipase activity**

244 The Tle1 effector exhibits PLA1 activity *in vitro* (Flaunatti *et al*, 2016). In the cryo-EM
245 structure of VgrG-Tle1, the catalytic module of Tle1 is well defined. It contains the
246 phospholipase catalytic triad in an active conformation as the Ser197 and Asp245 residues are
247 at a <3-Å distance from His310 (Fig 3B). The catalytic triad is located within a cavity that is
248 positively charged at its end, with a hydrophobic entrance (Fig EV5A-B). However,
249 comparison of the cryo-EM structure of Tle1^{EAEc} bound to VgrG to the *P. aeruginosa* Tle1
250 (Tle1^{PA}) crystal structure suggests that Tle1^{EAEc} PLA1 activity is inhibited by VgrG. In the
251 Tle1^{PA} structure, a specific pocket allows binding of the phosphatidylglycerol moiety of the
252 substrate at close proximity to the active site (Fig 4A) (Hu *et al*, 2014). By contrast, in the
253 Tle1^{EAEc} structure, this pocket is partially occluded by the lid domain. Remarkably, the
254 position of the lid domain is constrained by the binding of its tip (helix 389-393) to VgrG (Fig
255 4B). In agreement with this observation, PLA1 activity of the purified ^SVgrG-Tle1^H complex
256 was undetectable compared to the purified isolated Tle1^H protein (Fig 5A), suggesting that
257 Tle1 binding to VgrG inhibits its activity. To confirm this VgrG-mediated inhibition, isolated
258 Tle1^H purified protein was incubated with increasing amounts of purified ^SVgrG. Fig 5B
259 shows that Tle1^H was inhibited by ^SVgrG in a dose-dependent manner, but not with the
260 unrelated lysozyme protein. As a control, we purified ^SVgrG₁₋₄₉₀, a C-terminal truncated
261 variant of VgrG deleted of the gp5 needle-like domain which mediates most of the contacts

262 with Tle1^H (Fig 5C). In contrast with ^SVgrG, incubation of Tle1^H with ^SVgrG₁₋₄₉₀ did not
263 cause Tle1^H inhibition (Fig 5B).

264 Taken together, these results show that the Tle1 phospholipase activity is inhibited by VgrG
265 in the ^SVgrG-Tle1^H complex, and hence that the Tle1 effectors have to be dissociated from
266 VgrG to exhibit toxic PLA1 activity in the target cell.

267

268 **DISCUSSION**

269 In this study, we provide a comprehensive picture of a complex between the T6SS
270 VgrG spike protein and the phospholipase effector it recruits for translocation into target cells.
271 We show that these two proteins form a complex with a 1:1 stoichiometry comprising three
272 Tle1 effectors bound to one VgrG trimer, suggesting that three Tle1 effectors are translocated
273 into the target cell at each T6SS firing event. We further show that Tle1 recruitment to VgrG
274 causes the inhibition of its activity. Bioinformatics, high-resolution mass spectrometry
275 mapping and deletion studies combined with protein-protein interaction and functional assays
276 demonstrated the importance of the N-terminal portion of Tle1 as a critical determinant for
277 the interaction with the VgrG C-terminal TTR extension. Finally, we report the high-
278 resolution cryo-EM structure of the Tle1 effector in complex with the needle/TTR portion of
279 the VgrG spike. This structure confirms the importance of the TTR domain of VgrG for its
280 interaction with the N-terminal finger domain of Tle1, and further highlights two previously
281 unidentified regions of contact at the base and at the centre of the needle. Moreover, we
282 provide a structural explanation for Tle1 inhibition by VgrG.

283

284 **Structural model of the EAEC T6SS baseplate in a pre-firing state**

285 VgrG is not only the spike of the needle but also the hub of the T6SS baseplate (BP)
286 (Taylor *et al*, 2016; Brunet *et al*, 2015). The baseplate is the assembly platform for the tail

287 tube/sheath complex and is docked to the TssJLM membrane complex (Brunet *et al*, 2015;
288 Zoued *et al*, 2016). The structure of the TssKFGE wedge complex of the baseplate was
289 recently determined by cryo-EM (Cherrak *et al*, 2018). The structure of the fully assembled
290 baseplate, including the VgrG hub surrounded by six wedges, was presented, based on the
291 high-resolution wedge structure (Cherrak *et al*, 2018) and the baseplate lower resolution
292 structure (Nazarov *et al*, 2018). We propose here a structural model of the EAEC T6SS
293 baseplate including VgrG-Tle1 (Appendix Fig S6). Interestingly, the baseplate can readily
294 accommodate the three Tle1 effectors (Appendix Fig S6). We propose that the VgrG-Tle1
295 structure represents the "pre-firing complex".

296

297 **A new mode of T6SS substrate loading.**

298 The VgrG-Tle1 complex represents the first example of direct binding of an effector on
299 VgrG, which in general requires the PAAR subunit or an adaptor protein (Shneider *et al*,
300 2013; Alcoforado Diniz & Coulthurst, 2015; Liang *et al*, 2015; Unterweger *et al*, 2015;
301 Bondage *et al*, 2016). Tle1 binds to three different regions of VgrG (bottom, centre and top),
302 and the three Tle1 molecules are located on the side of the needle rather than on its tip. This
303 contrasts to the position of PAAR-fused Tse6 effector/adaptor in the *P. aeruginosa* VgrG tip
304 complex EM structure (Whitney *et al*, 2015; Quentin *et al*, 2018) (Fig EV3B and E). Tle1
305 positioning on the VgrG sides and the multiple attachment zones tightly tether Tle1 to the
306 spike, which will be released by some unknown mechanism in the periplasm of the target cell.
307 The non-covalent nature of the bonds may also allow Tle1 to be separated from the VgrG
308 spike to elicit its function. The oxidative nature of the periplasm or the presence of specific
309 chaperones might provide the source for Tle1 dissociation. Further work will need to be
310 carried out to understand the implication of such flexible interactions in VgrG-Tle1
311 dissociation.

312 The EAEC T6SS-1 cluster also encodes a PAAR protein downstream the Tli1 immunity
313 encoding gene. However, in the VgrG-Tle1 complex characterized here, no PAAR is needed
314 for the three Tle1 proteins to bind to the VgrG trimeric spike. The tip of the needle is free to
315 accommodate a PAAR protein necessary for target cell penetration. In the future, it would be
316 of particular interest to co-purify and image PAAR associated to the VgrG-Tle1 complex.

317

318 In contrast to canonical VgrGs, such as VgrG^{PA}, VgrG^{EAEC} bears a C-terminal extension
319 (CTD) comprising DUF2345 and TTR domains, linked to the gp27/gp5 base by two long
320 helices (Fig 2G). As shown in this study, the DUF2345 domain folds as a β -prism and hence
321 extends the length of the VgrG^{EAEC} gp5-like β -helix. This extension, as well as the TTR
322 domain, corresponds to the major binding regions of Tle1. VgrG^{EAEC} is therefore a fusion
323 between a prototypical VgrG and DUF2345/TTR extensions that serve as internal adaptor
324 domains (Bondage *et al*, 2016; Flaughnatti *et al*, 2016; Wettstadt *et al*, 2019). However, there
325 is an additional contact between Tle1 and the base of gp5-C domain of VgrG. The direct
326 binding of Tle1 on VgrG^{EAEC} therefore represents the first report of a new mode of effector
327 loading on the T6SS spike. Similar modes of binding might be predicted. This organization of
328 VgrG C-terminal extension (*i.e.*, the presence of two long helices followed by the DUF2345
329 and the TTR domains) corresponds to the COG4532 found at the C-terminal domain of a sub-
330 group of VgrG proteins in Proteobacteria (Boyer *et al*, 2009). This group includes proteins
331 such as the VgrG encoded upstream of a putative Tle3 effector in uropathogenic *E. coli*
332 CFT073, or VgrG2a from *P. aeruginosa* encoded upstream the Tle4-like *tlpE* effector (Wood
333 *et al*, 2019) (Appendix Fig S7). The structural organization of these VgrG proteins and their
334 genetic link to phospholipase-encoding genes suggest that these phospholipases might also be
335 directly recruited to the spike extension for delivery.

336

337 **Tle1 inhibition by VgrG**

338 It is noteworthy that the 3' of the *vgrG* gene overlaps with the *tle1* 5' in EAEC (Flaughnatti *et*
339 *al*, 2016), suggesting translational coupling of the VgrG and Tle1 proteins, a mechanism
340 evolved to promote protein stabilization or to prevent protein toxicity. Indeed, our results
341 show that the VgrG-bound Tle1 phospholipase is inactive as the catalytic site of Tle1 is
342 masked by the mode of binding to VgrG. Production of the Tle1 phospholipase may have
343 deleterious effect on the cytoplasmic membrane phospholipids, and VgrG-mediated inhibition
344 could be regarded as a surveillance mechanism to prevent phospholipid degradation in the
345 attacker cell. However, previous results have shown that Tle1 cytoplasmic overproduction has
346 no impact on the growth of *E. coli* K-12 cells (Flaughnatti *et al*, 2016). Another hypothesis is
347 that there is no need to inhibit Tle1 but rather a need to protect the catalytic site and to prevent
348 exposition of the hydrophobic entrance. Nevertheless, Tle1 needs to dissociate from VgrG
349 once in the periplasm of the target cell.

350

351 In conclusion we have identified a novel mechanism of loading and inhibition in the T6SS
352 Tle1 toxin that establishes a new relationship between the spike and its carrier effector.

353

354 **MATERIALS AND METHODS**

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

356 Strains used in this study are listed in Appendix Table S1. *E. coli* DH5 α , BL21(DE3), and
357 W3110 were used for cloning procedures, protein production, and co-immunoprecipitation,
358 respectively. EAEC strain 17-2 and its isogenic derivatives were used for *in vivo* studies.
359 Cells were grown at 37°C with aeration in LB or in *T6SS-1*-inducing medium (SIM; M9
360 minimal medium, 0.2% glycerol, 1 μ g/mL vitamin B1, 100 μ g/mL casaminoacids, 10% LB,
361 supplemented or not with 1.5% bactoagar, ampicillin (100 μ g/mL), kanamycin (50 μ g/mL), or

362 chloramphenicol (30 µg/mL). Gene expression from pBAD (Guzman *et al*, 1995) and pOK12
363 (Vieira & Messing, 1991) derivatives was induced by the addition of 0.02-0.2% of L-
364 arabinose (Sigma-Aldrich) and 100 µM isopropyl-thio-β-D-galactopyrannoside (IPTG,
365 Eurobio), respectively. Gene expression from pETDuet-1 and pRSFDuet-1 (Novagen)
366 derivative vectors was induced by the addition of 1 mM IPTG.

367

368 **Cloning procedures.**

369 Plasmids and primers used in this study are listed in Appendix Table S1. PCRs were
370 performed using a Biometra thermocycler using the Q5 high-fidelity DNA polymerase (New
371 England Biolabs) and EAEC 17-2 chromosomal DNA as a template. Custom oligonucleotides
372 (Sigma-Aldrich) are listed in Appendix Table S1. pRSF-Tle1^H and pET-^SVgrG were
373 constructed by a restriction–ligation procedure. The sequence encoding Tle1 was amplified by
374 PCR using 5-NdeI-Tle1 and 3-FseI-Tle1^H primers introducing NdeI and FseI sites,
375 respectively and cloned into the pRSFDuet-1 (Novagen) multiple cloning site 2 (MCS2) to
376 yield pRSF-Tle1^H. The 3-FseI-Tle1^H primer introduces a sequence encoding a 6×His-tag to
377 allow in-frame fusion of Tle1 with a C-terminal hexa-histidine extension. The sequence
378 encoding VgrG was amplified by PCR using primers 5-BamHI-^SVgrG and 3-HindIII-VgrG
379 introducing BamHI and HindIII sites, respectively and cloned into the pETDuet-1 MCS1 to
380 yield pET-^SVgrG. The 5-BamHI-^SVgrG primer introduces a sequence encoding a Strep-tagII
381 to allow in frame fusion of VgrG with a N-terminal Strep-tagII. The same cloning strategy
382 was used for pET-^SVgrG_{ΔCTD} and pET-^SVgrG₁₋₄₉₀, with the use of primers 5-BamHI-^SVgrG
383 and 3-HindIII-VgrG_{ΔCTD} for pET-^SVgrG_{ΔCTD}, and primers 5-BamHI-^SVgrG and 3-HindIII-
384 VgrG₁₋₄₉₀ for pET-^SVgrG₁₋₄₉₀. For pBAD18-Tle1Δ1-26_{VSVG}, pBAD18-Tle1Δ1-34_{VSVG} and
385 pRSF-Tle1_{Δ1-26}^H constructions, the sequence encoding amino-acids 1 to 26 (or 1 to 34) of Tle1
386 has been deleted from pBAD18-Tle1_{VSVG} (Flaugnatti *et al*, 2016) or pRSF-Tle1^H by Quick-

387 change PCR-based targeted mutagenesis using complementary pairs of oligonucleotides and
388 the Pfu Turbo high-fidelity polymerase (Agilent Technologies). Similarly, the K28-to-E/K29-
389 to-E/K30-to-E substitutions were introduced in the pBAD18-Tle1_{VSVG} plasmid by
390 QuickChange PCR-based targeted mutagenesis. All constructs were verified by restriction
391 analysis and DNA sequencing (Eurofins, MWG).

392

393 **Antibacterial competition assay.**

394 Antibacterial competition assays were performed as described previously (Flaughnatti *et al*,
395 2016), using wild-type *E. coli* W3110 cells transformed with the pUA66-*rrnB* plasmid
396 (Zaslaver *et al*, 2006) as a prey. In addition to conferring resistance to kanamycin, the
397 pUA66-*rrnB* plasmid allows a strong constitutive expression of the green fluorescent protein.
398 The experiments were done in triplicate, with identical results, and the results of a
399 representative experiment is shown here.

400

401 **Co-immunoprecipitation.**

402 W3110 cells producing Tle1, Tle1_{Δ1-26} or VgrG proteins from independent plasmids were
403 subjected to co-immunoprecipitation using ANTI-FLAG[®] M2 affinity gel (Sigma-Aldrich), as
404 described previously (Flaughnatti *et al*, 2016). Anti-FLAG (clone M2, Sigma-Aldrich) or anti-
405 VSV-G (clone P5D4, Sigma-Aldrich) monoclonal antibodies were used for Western-blot
406 analyses.

407

408 **VgrG-Tle1 complex production and co-purification.**

409 The pRSF-Tle1^H and pET-SVgrG plasmids were co-transformed into BL21(DE3). Following
410 growth at 37°C in LB medium to an OD_{600nm} of 0.7, the expression of *tle1* and *vgrG* genes
411 was induced with 1 mM IPTG at 16°C for 16 h. Cell were pelleted, resuspended in lysis

412 buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 100 μ g/mL DNase I, 100
413 μ g/mL lysozyme, cOmplete EDTA-free protease inhibitor cocktail (Sigma) and 10 mM
414 $MgCl_2$) and broken using an Emulsiflex-C5 (Avestin). After centrifugation at $38,500 \times g$ for
415 30 min, the supernatant was loaded onto a 5-mL StrepTrap HP column (GE Healthcare) at
416 4°C and the column was washed with affinity buffer (50 mM Tris-HCl pH 8.0, 150 mM
417 NaCl) at 4°C. The ^{35}S VgrG-Tle1^H complex was then eluted directly into a 5-mL HisTrap HP
418 (GE Healthcare) column with affinity buffer containing 2.5 mM desthiobiotin (IBA). After a
419 washing step in affinity buffer supplemented with 20 mM imidazole, the ^{35}S VgrG-Tle1^H
420 complex was eluted in affinity buffer supplemented with 500 mM imidazole. The fractions
421 corresponding to the peak were pooled and loaded onto a Superose 6 10/300 column (GE
422 Healthcare) equilibrated in 50 mM HEPES pH 7.5, 150 mM NaCl buffer. The ^{35}S VgrG-Tle1^H
423 complex eluted as a single monodisperse peak. The same production conditions and
424 purification protocol were applied for the purification of ^{35}S VgrG, ^{35}S VgrG₁₋₄₉₀, Tle1^H and Tle1 Δ ₁₋₂₆.
425 ₂₆, except that only one affinity column was performed (StrepTrap HP for ^{35}S VgrG and ^{35}S VgrG₁₋₄₉₀.
426 ₄₉₀, HisTrap for Tle1^H and Tle1 Δ ₁₋₂₆) followed by a gel filtration as described above. For Tle1
427 inhibition studies, ^{35}S VgrG and ^{35}S VgrG₁₋₄₉₀ eluted from the StrepTrap column were used directly
428 (without a gel filtration step), due to the tendency of ^{35}S VgrG to precipitate during or after the
429 gel filtration step when purified alone. A representative SDS-PAGE followed by Instant blue
430 staining of the resulting purified proteins is presented in Fig 5C.

431

432 **Sypro-Ruby staining.**

433 Aliquots of 2.5, 5, and 10 μ L of the VgrG-Tle1 complex from two different peak fractions of
434 the gel filtration column were subjected to SDS-PAGE. The gel was then stained with Sypro-
435 Ruby as recommended by the manufacturer (Invitrogen). Protein bands were visualized using
436 a Biorad Chemidoc MP Imaging system, using a 605/50 Filter (excitation wavelength = 488

437 nm, emission wavelength = 605 nm) with 0.5 sec exposition, and the fluorescence intensity
438 was quantified using the Fuji Image software. Intensities were divided by the molecular
439 weight of each protein and the quantification is expressed as the mean +/- standard deviation
440 relative to VgrG bands intensity

441

442 **Cryo-EM sample preparation and data collection**

443 UltraAuFoil Holey gold Film grids (Quantifoil) were glow discharged for 20 s at 2 mA in an
444 ELMO glow discharge cleaning system (Agar Scientific, UK). Four μl of sample at 600
445 $\mu\text{g}/\text{mL}$ were loaded on the glow discharged grid at 100 % humidity and 4°C in a Vitrobot
446 (Thermo Fisher Scientific, USA). Following a 5 s pre-blot the grid was blotted for 4 s and
447 plunged into liquid ethane. Grids were screened on a Talos Arctica electron microscope
448 equipped with a Falcon 3EC camera (Thermo Fisher Scientific, USA), and the final data were
449 collected on a K2-Summit detector through a Gatan Quantum 967 LS energy filter using a
450 20 eV slit width in zero-loss mode (Gatan, Japan) on a Titan Krios transmission electron
451 microscope at 300 kV (Thermo Fisher Scientific, USA). Table 2 summarises the acquisition
452 parameters.

453

454 **Cryo-EM data processing**

455 The 40 frames for each movie collected were aligned for each of the 11,600 movies using
456 MotionCor2, binned by 1.28 with dose weighting ($1.24 \text{ e}^-/\text{\AA}^2/\text{frame}$) and with 5×5 patches
457 applied (Zheng *et al*, 2017). gCTF was used to estimate the CTF parameters (Zhang, 2016).
458 Cryosparc (Punjani *et al*, 2017) template picker selected 1.94 million particles that were
459 extracted with a box-size of 350 pix. After several rounds of 2D classification in cryosparc
460 (Fig EV2A), a heterogeneous ab initio reconstruction with 2 classes, and a heterogeneous
461 refinement (2 classes), 468,438 particles were selected for homogeneous refinement. An

462 initial unmasked refinement using the *ab initio* model from the cryosparc *ab initio* class, gave
463 us a resolution of 2.7 Å with no applied symmetry (Fig EV2B-D). A masked local refinement
464 around the physiological complex yielded a final resolution of 2.6 Å (Fig EV2E). The density
465 corresponding to the Tle1 effector was refined locally with cryosparc to a resolution of 2.5 Å.
466 In Relion, CTF Refine and the Bayesian polishing steps (Zivanov *et al*, 2019) yielded a final
467 resolution of 2.3 Å for the masked VgrG density and was used for model building and real
468 space refinement (Fig EV2F and G). The quality of the maps for the Tle1/TTR densities were
469 worse than those obtained with cryosparc and were thus not used. The resolution for the Tle1
470 and VgrG densities was calculated by cryosparc and Relion postprocess respectively
471 according to the “gold standard” method using 0.143 as the FSC value cut-off (Rosenthal &
472 Henderson, 2003). The local resolution was calculated by cryosparc (Punjani *et al*, 2017) or
473 Relion with a cut-off of 0.5 (Appendix Fig S5A-C).

474 **Model building and validation**

475 Model building proceeded differently for VgrG and Tle1, but for both no homologous protein
476 could be docked in the density and the Phenix autosharpened (Terwilliger *et al*, 2018b) cryo-
477 EM densities were used. For Tle1, a partial initial model based on sequence was built using
478 phenix.map_to_model (Terwilliger *et al*, 2018a). The model was subsequently corrected and
479 extended manually in Coot (Emsley *et al*, 2010). For VgrG, the Phenix automatic model
480 building was not successful. The model was built starting with the only 2 α -helices and using
481 the cryo-EM density to place manually all the amino acid sequence. For the TTR domain that
482 interacts with Tle1, we combined the use of bulky side-chains as guides for model building,
483 secondary structure and residue contact predictions according to the Phyre2 (Kelley *et al*,
484 2015) and the RaptorX (Källberg *et al*, 2012) servers respectively. The VgrG models were
485 both refined using phenix.real_space_refine (Afonine *et al*, 2018) and manual adjustments
486 were done with Coot (Emsley *et al*, 2010). Each model was initially validated using the

487 protocol implemented in Refmac5 (Murshudov *et al*, 2011) and visually (Appendix Fig S8).
488 The FSC map-to-model was calculated with the autosharpened maps (FSC_{sum}). The model
489 was shaken by 0.5 Å and the FSC map-to-model was calculated with one Half map (FSC_{work}).
490 This refined model was then used to calculate the FSC map to model with the other Half map
491 (FSC_{free}) (Appendix Fig S8A-C). The cross-correlation between each amino acid in the model
492 and map and the Molprobit score (Chen *et al*, 2010) were calculated using
493 phenix.real_space_refine (Afonine *et al*, 2018) (Appendix Fig S9, Table 2). Pore radius
494 calculations were carried out using the MOLE server (Berka *et al*, 2012) and the protein
495 interfaces were analysed with PISA (Krissinel & Henrick, 2007). Unless stated otherwise, all
496 of the figures were generated either using Chimera (Pettersen *et al*, 2004), ChimeraX
497 (Goddard *et al*, 2018) and Coot (Emsley *et al*, 2010).

498

499 **Cross-linking mass spectrometry.** Cross-linking of the purified complexes was carried out
500 with freshly prepared NNP9 (10 mM in DMSO) using 10:1 cross-linker/protein molar ratio.
501 Cross-linking was performed at 4°C for 30 min and stopped by the addition of ammonium
502 bicarbonate (AB, final concentration 50 mM) for 15 min at 4°C. The samples were transferred
503 into a molecular filter device (Amicon ultra 0.5 mL with 30 KDa molecular cut-off) and
504 centrifuged at 10,000×g for 5 min. The concentrate was then washed 4 times by
505 concentration-dilution cycle with AB 50 mM to remove the excess of cross-linker. The
506 labelled protein was digested overnight at 37°C by addition of Trypsin Gold, Mass
507 Spectrometry Grade (Promega) at an enzyme:protein ratio of 1:50 (w:w) under 900 rpm
508 shaking. The peptides were recovered through the molecular filtering device by
509 centrifugation. 50 µL of 0.1% (v:v) formic acid were added onto the filter and a second
510 centrifugation was performed to improve the peptide recovery. Cross-linked protein digests
511 were analyzed by nanoLC-MS/MS on an Orbitrap Q Exactive Plus mass spectrometer

512 (Thermo-Scientific). Briefly, peptides were separated on homemade C18 column using a 90
513 min water/acetonitrile gradient. The 10 most intense ions with at least 3 charges were selected
514 for HCD fragmentation (NCE 24). NanoLC-MS/MS data were processed automatically using
515 Mass Spec Studio v2.0 (Sarpe *et al*, 2016) with methionine oxidation as a variable
516 modification and NNP9 modification sites: lysine, serine, threonine, and tyrosine. Mass
517 modifications were set to 314.1127 Da for cross-linked peptides, 288.1335 and 331.1393 for
518 dead-end modifications when NNP9 reacted respectively with water or ammonium molecule.
519 Cross-linked peptides were searched using the parameter file provided in supplemental data
520 (Dataset EV2). MS/MS spectra of all crosslinked candidates were further manually checked
521 to confirm their identification. Experiments were performed in duplicate (using two different
522 complex preparations) and only cross-linked peptides identified in both experiments were
523 considered.

524

525 **Phospholipase A₁ fluorescent assays.**

526 PLA1 activities of Tle1, Tle1 $_{\Delta 1-26}$, VgrG and the VgrG-Tle1 complex were monitored using
527 BODIPYVR dye-labeled phospholipids: PED-A1(N-((6-(2,4-DNP)Amino)Hexanoyl)-
528 (BODIPYVRFL C5)-2-Hexyl-sn-Glycero-3-Phosphoethanolamine (Farber, 2001; Darrow *et*
529 *al*, 2011), as described previously (Flaugnatti *et al*, 2016). Enzyme activities were assayed at
530 room temperature in 50 mM HEPES pH 7.5, 150 mM NaCl for 25 min in a final volume of
531 200 μ L containing 5 μ M of the substrate and 1.18 μ M of Tle1^H, 1.18 μ M of ^SVgrG or 0.393
532 μ M of ^SVgrG-Tle1^H complex (corresponding to 1.18 μ M of Tle1 considering a 3:3
533 stoichiometry) purified proteins (from 1-2 mg/mL stock solutions). *Thermomyces lanuginosus*
534 lipase (TLL, Sigma-Aldrich) was used as a positive control for PLA1 activity. Inhibition
535 assays were performed in desthiobiotin containing buffer (Tris-HCl 50 mM pH 8.0, NaCl 150
536 mM, desthiobiotin 2.5 mM) using ^SVgrG or ^SVgrG₁₋₄₉₀ purified at 1.5-2 mg/mL, after

537 checking that desthiobiotin did not affect Tle1 PLA1 activity. Lysozyme from chicken egg
538 white (Sigma) was used as a negative control. Differences in Tle1 activities between groups
539 were examined by unpaired two-sample Fisher-Pitman permutation test.

540

541 **Computer algorithms.**

542 EAEC Tle1 orthologs (EC042_4534; NCBI Gene Identifier (GI): 284924255) were detected
543 using BlastP analysis against the KEGG genes database and aligned using ClustalW and draw
544 alignment tool in KEGG (Kanehisa *et al*, 2017), or Multialin (Corpet, 1988; Robert & Gouet,
545 2014) Protein domains were identified using Pfam (Sonnhammer *et al*, 1997) or CDart (Geer,
546 2002).

547

548 **Data availability**

549 Electron microscopy maps of VgrG-Tle1, VgrG and Tle1-TTR complexes have been
550 deposited in the Electron Microscopy Databank (EMDB) and the PDB under accession codes
551 EMD-10218 (PDB 6SJL), EMD-10219 (PDB 6SK0) and EMD-10225 (PDB 6SKI)
552 respectively.

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569 **Author contributions**

570 NF, CR, MR, ED, JCR, RF & LJ designed research. NF, CR, MR, SGB, VAN & LJ
571 performed research. MR, SC, JCR, RF & EC provided tools. NF, CR, MR, SGB, ED, SC, EC,
572 RF & LJ analyzed data. NF, CR, RF & LJ wrote the paper with contributions of MR, JCR &
573 EC.

574 **Conflict of Interests**

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

576

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773

775 **FIGURE LEGENDS**

776

777 **Figure 1. The N-terminal extension of Tle1 is required for VgrG-mediated transport**
778 **into target cells.**

779 **A.** Cross-link connectivity map of ^SVgrG-Tle1^H complex. The two proteins are represented as
780 rectangles in which the residues number and known domains are indicated. Straight lines
781 represent inter-molecular cross-links, while dotted lines represent intra-molecular cross-links.
782 Only the cross-links identified in two independent experiments were considered. Raw and
783 cured data are provided in Dataset EV1.

784 **B.** Schematic representation of an alignment of Tle1 orthologs using ClustalW and draw
785 alignment tool in KEGG (Kanehisa *et al*, 2017). Red box, bit score ≥ 200 ; pink box, bit score
786 ≥ 80 -200. Ordered locus names of the identified genes are indicated on the right. Domains are
787 represented in grey boxes (Hcp, PF05638; PAAR (P), PF05488; VgrG, PF05954, COG3501;
788 DUF2345, PF10106). The N-terminal segment found in EAEC Tle1 protein and absent in the
789 others is represented by a blue box.

790 **C.** Antibacterial competition assay. *E. coli* K12 recipient cells (W3110 *gfp*⁺, *kan*^R) were
791 mixed with the indicated EAEC attacker cells (1:4 ratio): WT, Δ T6SS-1 and Δ *tle1-tli1*
792 carrying pBAD18 and pBAD33 vectors, or producing the indicated proteins, and spotted on
793 SIM 0.02 % arabinose agar plates for 4-h at 37°C. The image of the corresponding
794 representative bacterial spots is presented and the number of recovered prey cells is indicated
795 in the upper graph (in log of colony-forming units (cfu)). The black, dark grey and light grey
796 circles indicate values obtained from three different spots, and the average is indicated by the
797 bar. Western blot analysis of the production of Tle1_V and Tle1 _{Δ 1-26 V} is shown in the inset. The
798 experiment was performed in triplicate and a representative result is shown.

799 **D.** Co-immunoprecipitation assay. Lysates from *E. coli* K-12 W3110 cells producing VSVG-
800 tagged Tle1 (Tle1_v) or VSVG-tagged Tle1_{Δ1-26} truncated variant (Tle1_{Δ1-26v}) were mixed with
801 lysates from W3110 cells producing FLAG-tagged VgrG (VgrG_{FL}) or not (-, empty vector)
802 and subjected to immunoprecipitation on anti-FLAG-coupled beads. The mixed soluble
803 lysates (Input) and the immunoprecipitated material (IP) were subjected to 12.5% SDS-PAGE
804 and immunodetected with anti-FLAG (upper panel) and anti-VSVG (lower panel) monoclonal
805 antibodies. Molecular weight markers (in kDa) are indicated on the left. This experiment was
806 performed in triplicate and a representative result is shown.

807

808 **Figure 2. ~~Figure 3~~ Cryo-EM density and pseudoatomic model of the VgrG needle**
809 **(491-834) bound to Tle1.**

810 **A-C.** Autosharpened and masked cryo-EM density (Level 0.728) of the physiological
811 complex in different orientations

812 **D-F.** Ribbon diagram of the VgrG and Tle1 pseudoatomic structure, coloured according to the
813 chain and in different orientations

814 **G.** Full VgrG structure composed of the experimentally-obtained spike (red) and the
815 homology model of the base (dark red)

816 **H.** Structure of the VgrG spike at different planes

817

818 **Figure 3. Tle1 structure and interaction with VgrG.**

819 **A-B.** Ribbon representation of the Tle1 pseudoatomic model. Strands are in pink and helices
820 in light blue. The inset shows the catalytic triad.

821 **C-E.** Ribbon representation of the interaction site between the Tle1 finger domain in yellow
822 green and the VgrG TTR domain in red. The site is shown in different orientations.

823 **F-H.** Molecular surface representation of the interactions between Tle1 chain D and chains A-
824 C of VgrG respectively (**F**, **G** and **H**).

825

826 **Figure 4. Supplementary Figure 10. Comparison of the active site of Tle1.**

827 **A-B.** Surface representation and cross section of Tle1 from *P. aeruginosa* (PDB 4O5P) (**A**)
828 and EAEC - this study (**B**). The active site is shown in red. The substrate entry channel is
829 indicated when present.

830

831 **Figure 5. Inhibition of Tle1 by VgrG.**

832 **A.** Specific phospholipase A₁ (PLA1) activity measurements of Tle1^H, ^SVgrG-Tle1^H complex,
833 and isolated ^SVgrG. *Thermomyces lanuginosus* lipase (TLL) was used as positive standard for
834 PLA1 activity. Mean values and standard deviation from at least three independent assays are
835 shown. Statistical analysis relative to the Tle1 activity is indicated. ****P*<0.001
836 (*P*=0,0001998), unpaired two-sample Fisher-Pitman permutation test.

837 **B.** VgrG inhibition of Tle1^H. Specific phospholipase A₁ (PLA1) activity measurements of
838 purified Tle1^H in the presence of 1:10 molar ratio of lysozyme from chicken egg white (Lyso)
839 and increasing molar ratio (0, 1, 5, and 10) of ^SVgrG or ^SVgrG₁₋₄₉₀. Inhibition experiments
840 with ^SVgrG were repeated three times with independent protein preparations, each measured
841 in triplicate. Inhibition experiments with ^SVgrG₁₋₄₉₀ were repeated twice with two independent
842 protein preparations, each measured in triplicate. Mean values and standard deviation are
843 shown. Statistical analysis relative to the Tle1 activity is indicated. ns, non-significant
844 (*P*>0.05), ***P*<0.01, ****P*<0.001, unpaired two-sample Fisher-Pitman permutation test. (*P*
845 values: Lyso, 0.7006; VgrG 1:1, 0.004946; VgrG 1:5, 1.448e-07; VgrG1:10, 1.448e-07;
846 VgrG₁₋₄₉₀ 1:1, 0.5627; VgrG₁₋₄₉₀ 1:5, 0.2977; VgrG₁₋₄₉₀ 1:10, 0.002631).

847 C. Representative SDS-PAGE / Coomassie blue staining of the Tle1^H, ^SVgrG and ^SVgrG₁₋₄₉₀

848 purified proteins used in **(B)** (5 µg/well).

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INTERACTION	H-BONDS	SALT BRIDGES
A-D	A.K596-D.F257	A.R592-D.E508
	A.K596- D.N260	
	A.Q612- D.D503	
	A.Q605-D.R513	
B-D		B.E513-D.R393
		B.D657-D.K517
C-D	C.G522-D.N390	
	C.E520-D.R393	
TTR-TLE1	G778-S17	D785-K29
	I782- V25	R797-D228
	I782- K23	
	T784- V25	
	S825- H14	
	V826-H14	
	K828-A12	

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Table 1. Interacting amino acids between VgrG and Tle1 as determined by PISA software

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	VgrG-Tle (EMD-10218) (PDB 6SJL)	VgrG (EMD-10219) (PDB 6SK0)	Tle-TTR (EMD-10225) (PDB 6SKI)
Data collection and processing			
Magnification	165,000X	165,000X	165,000X
Voltage (kV)	300	300	300
Electron exposure (e-/Å ²)	53.94	53.94	53.94
Defocus range (µm)	0.5 to 2	0.5 to 2	0.5 to 2
Pixel size (Å)	0.85	0.85	0.85
Spot size	9	9	9
Symmetry imposed	C1	C1	C1
Number of images	11,670	11,670	11,670
Initial particle images (no.)	1,942,084	1,942,084	1,942,084
Final particle images (no.)	468,438	468,438	468,438
Map resolution (Å)	2.6 (2.9)	2.26 (2.7)	2.55 (2.8)
FSC threshold	0.143 (0.5)	0.143 (0.5)	0.143 (0.5)
Map resolution range (Å)	2.2-15	2.15-2.48	2.25-7
Binning	1.28	1.28	1.28
Refinement			
Initial model used (PDB code)	N/A	N/A	N/A
Model resolution (Å)	2.6 (2.7)	2.1 (2.2)	2.6 (2.7)
FSC threshold	0.143 (0.5)	0.143 (0.5)	0.143 (0.5)
Map sharpening <i>B</i> factor (Å ²)	84.99	66.07	75.21
Model composition:			
Number of chains	6	3	2
Non-hydrogen atoms	19460	5890	4434
Protein residues	2537	801	569
Ligands	0	0	0
<i>B</i> factors (Å ²):			
Protein (min/max)	25.27/157.57	34.83/63.05	40.72/92.41
Ligand	N/A	N/A	N/A
R.m.s. deviations:			
Bond lengths (Å)	0.013	0.008	0.004
Bond angles (°)	0.855	0.726	0.713
Validation:			
MolProbity score	2.02	1.70	1.55
Clashscore	12.96	9.23	8.64
Poor rotamers (%)	0.05	0.97	1.29
Ramachandran plot:			
Favoured (%)	94.06	96.69	98.38
Allowed (%)	5.74	3.31	1.62
Disallowed (%)	0.20	0	0

890 **Table 2. Cryo-EM data acquisition and analysis parameters**

892 **EXPANDED VIEW FIGURE LEGENDS**

893

894 **Figure EV1. Purification and biochemical characterization of the VgrG-Tle1 complex**

895 **A.** SDS-PAGE analysis and Coomassie staining (upper panel) or immunoblotting using anti-
896 StrepII (middle panel) and anti-His (lower panel) antibodies of the S VgrG-Tle1^H complex
897 purification steps. Lysate of cells (L) co-producing StrepII-VgrG (S VgrG) and Tle1-6×His
898 (Tle1^H) was loaded on a StrepTrap HP column. After washing (W), the material eluted with
899 desthiobiotin was directed loaded on a HisTrap column. The material eluted with imidazole
900 (E) was pooled and loaded on a Superose 6 10/300 gel filtration (GF) column. Ten microliters
901 of each fraction were loaded on the gel. Molecular weight markers in kDa and the positions of
902 S VgrG and Tle1^H are indicated on the left and right, respectively. FT, flow through.

903 **B.** Size-exclusion chromatography analysis of the purified S VgrG-Tle1^H complex (blue line)
904 and of purified S VgrG alone (Appendix Fig S1) was performed on a Superose 6 column
905 calibrated with 43-, 75-, 158-, 440- and 660-kDa molecular mass markers (dotted lines). The
906 molecular mass of each marker and the position of the peak fractions corresponding to
907 S VgrG-Tle1^H and S VgrG is indicated at the top of each peak.

908 **C.** SYPRO Ruby staining analysis of the S VgrG-Tle1^H complex. The indicated volumes of
909 two different fractions corresponding to the center of the gel filtration peak were subjected to
910 SDS-PAGE and stained with SYPRO Ruby. Fluorescence intensities were divided by the
911 molecular weight of each protein and the quantification is expressed as the mean (+/- SD)
912 relative to VgrG.

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915 **Figure EV2. Data analysis of the VgrG-Tle1 structure**

916 **A.** 2D classes of the VgrG-Tle1 complex representative of all the orientations observed. The
917 number of particles and the resolution reached during classification are indicated in green.
918 **B-C.** Sharpened cryo-EM density of the full complex in two different orientations.
919 **D-G.** Gold standard FSC curves of the Full complex (**D**), the VgrG-Tle1 complex (**E**), VgrG
920 alone (**F**) and Tle1 with the TTR domain (**G**). For (**D**, **E** and **G**) the colours indicate the FSC
921 curves without mask (blue), spherical mask (green), loose mask (grey) and tight mask
922 (orange) applied. For (**F**), the colours indicate the FSC curves that were corrected (blue),
923 unmasked (green), masked (grey) and phase randomised (orange).

924

925 **Figure EV3. VgrG structure and mechanism of effector loading.**

926 **A-C.** Ribbon diagrams of 1 chain of VgrG from (**A**) EAEC, (**B**) *P. aeruginosa* (4MTK), and
927 (**C**) gp5 from the T4 phage (1K28).

928 **D-F.** Ribbon diagrams of the spike complexes with their effector from (**D**) EAEC, (**E**) *P.*
929 *aeruginosa* (PDB 6H3L), and (**F**) the (gp27)₃-(gp5)₃ complex from the T4 phage (1K28). The
930 cryo-EM densities (EMD-0135 for PA) of the VgrG proteins with their effectors are shown
931 with 20% transparency.

932

933 **Figure EV4. Tle1 structure.**

934 **A.** Topology diagram of Tle1 adapted from the output of PDBsum. The helices are in blue
935 and the strands are in pink, as in Fig 3. The catalytic amino acids and the domains relevant to
936 Tle1 structure are highlighted.

937 **B-C.** Ribbon diagram of the pseudoatomic structure of Tle1 and the TTR domain of VgrG
938 coloured according to B-factors. Blue = 34, White = 65 and Red = 96.

939

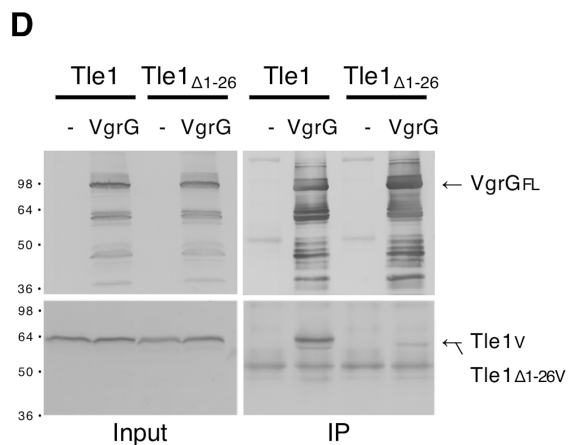
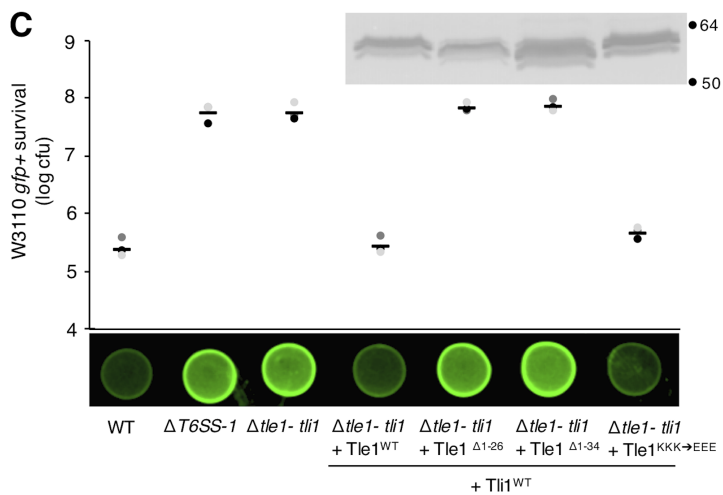
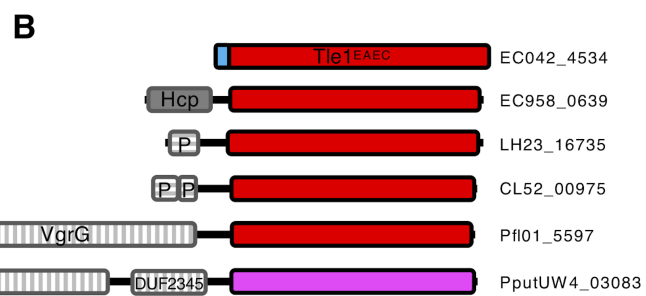
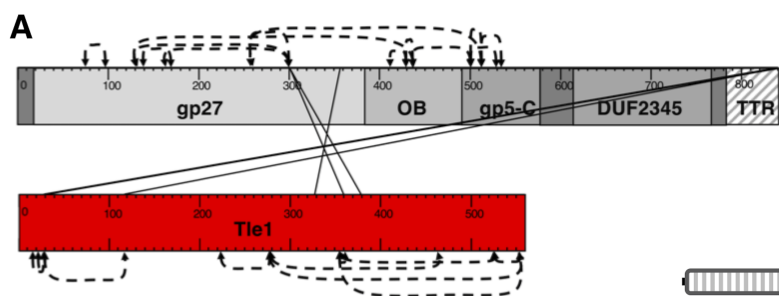
940 **Figure EV5. ~~Supplementary Figure 9.~~ Analysis of the Tle1 active site.**

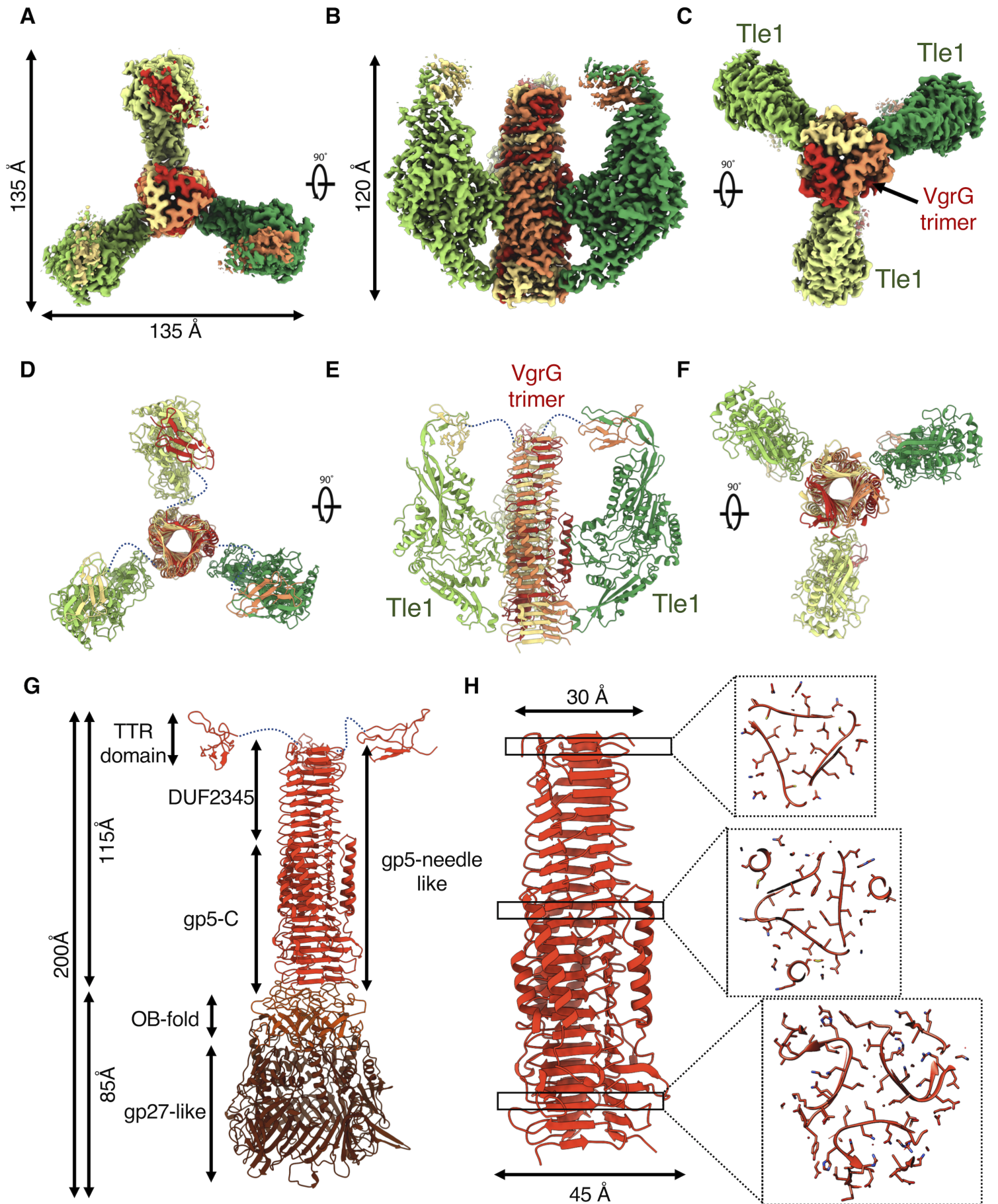
941 **A.** Ribbon diagram of Tle1 with the active site and the pore leading to the catalytic amino
942 acids highlighted.

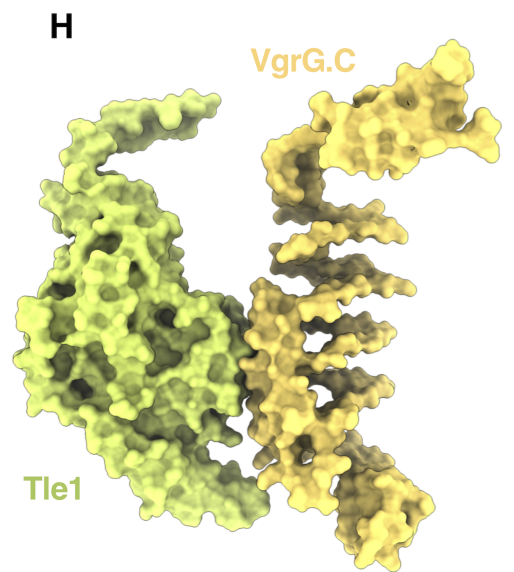
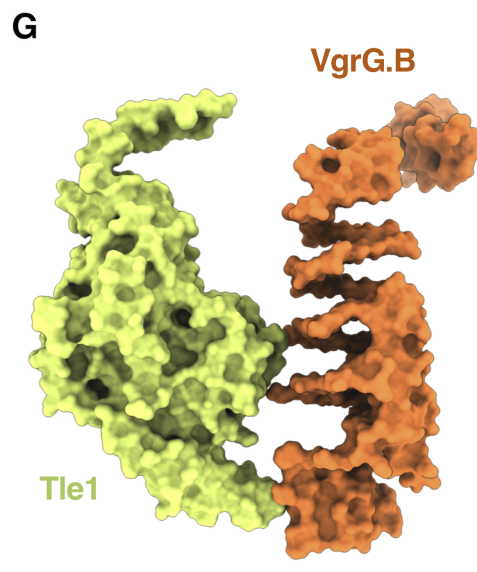
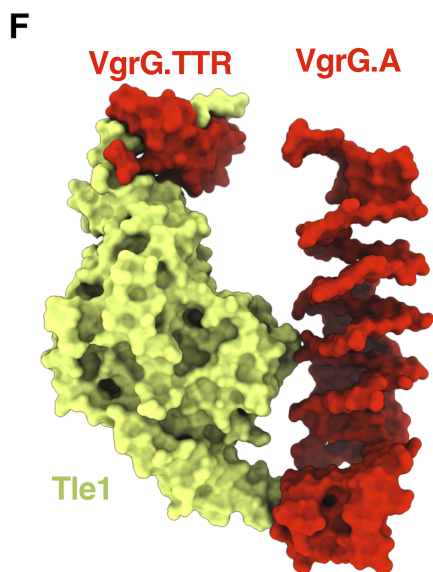
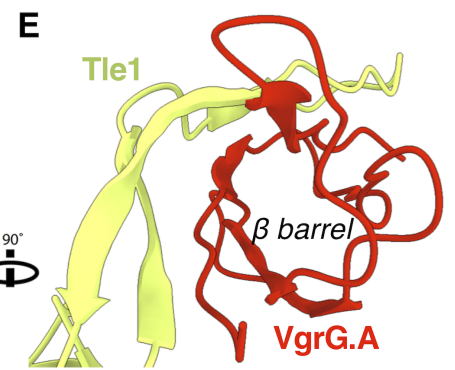
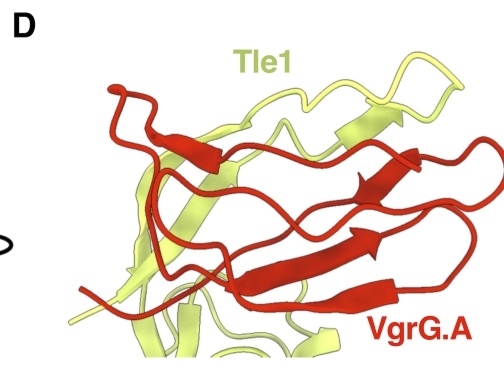
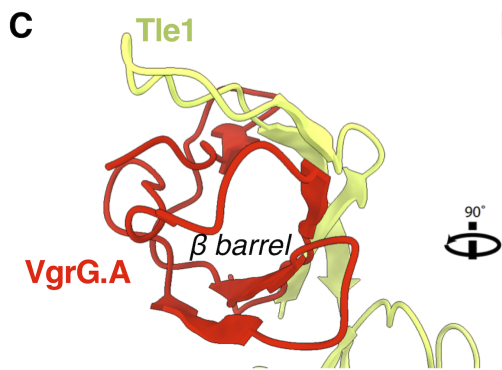
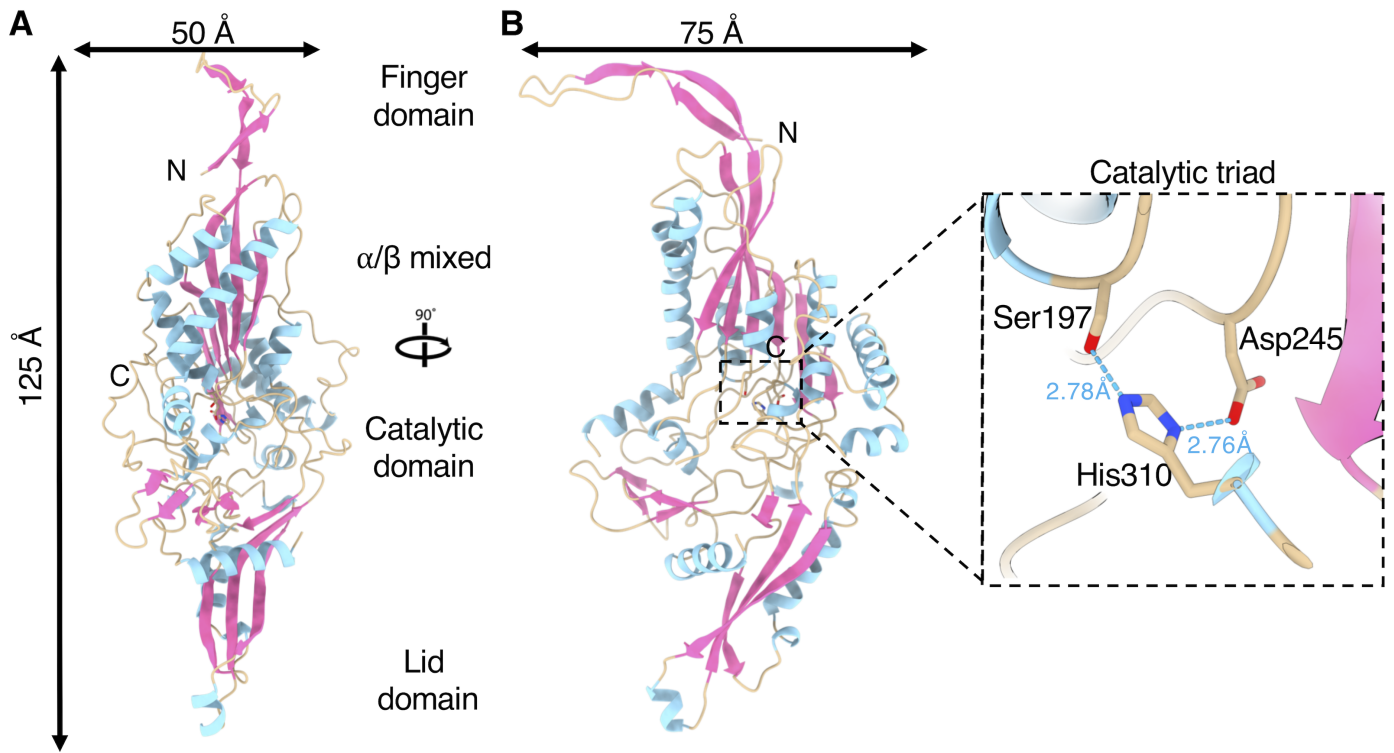
943 **B.** Cross-section of the active site entrance channel of Tle1, coloured according to its charge,
944 as calculated with the APBS server.

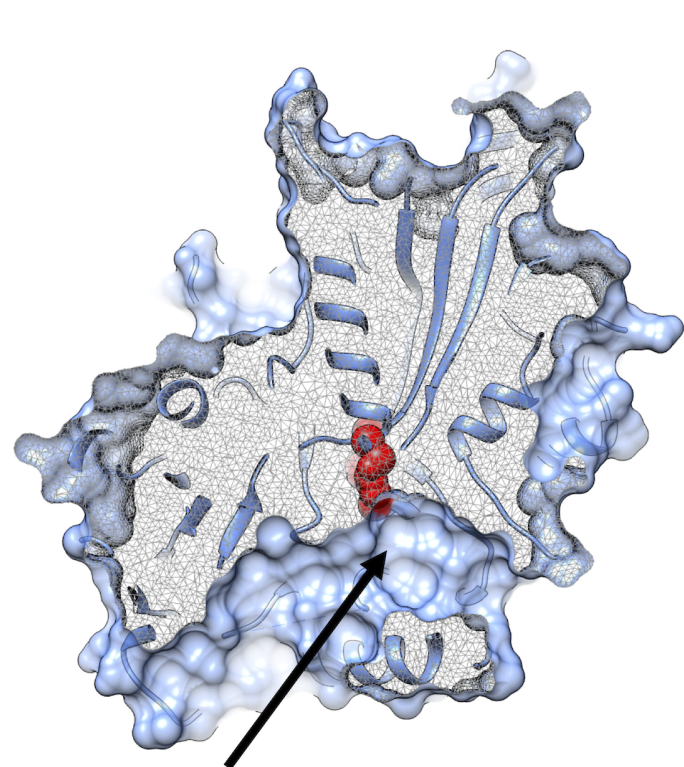
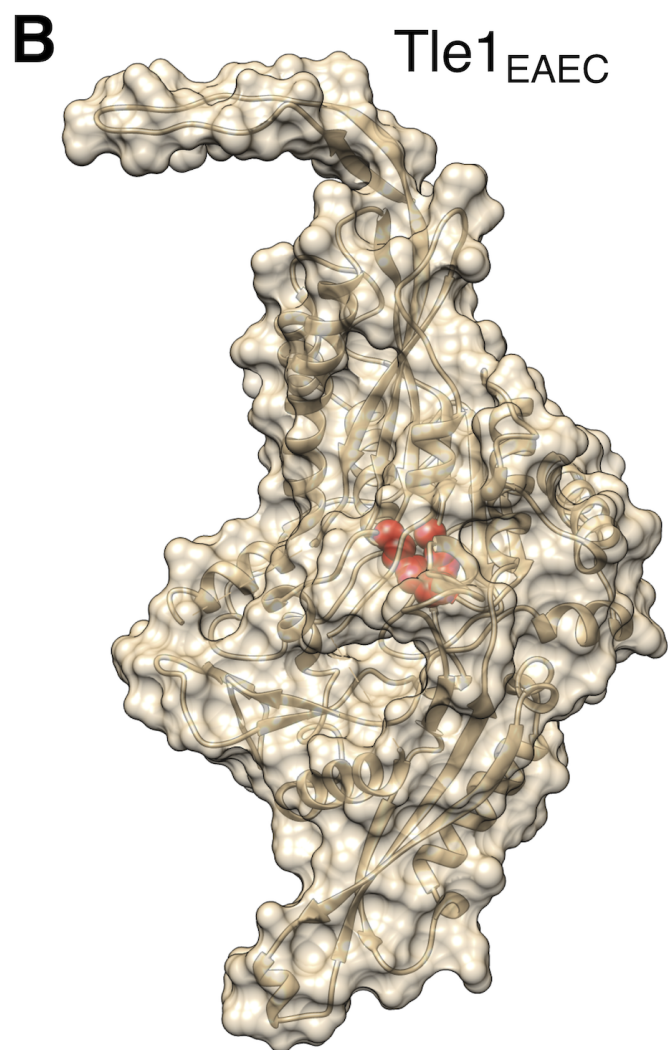
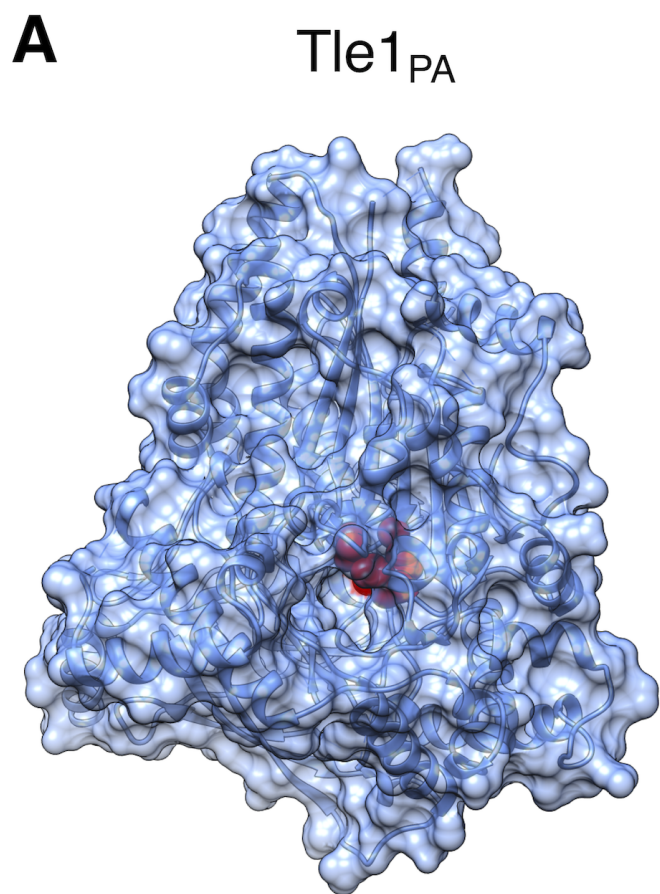
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946

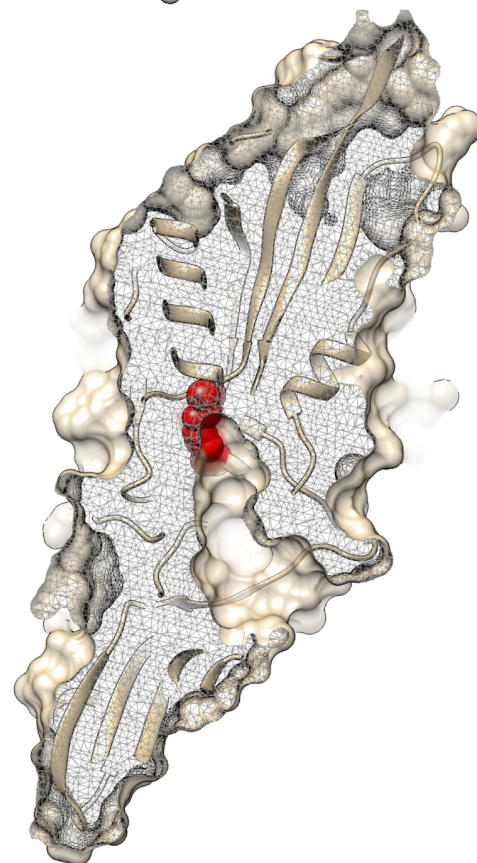


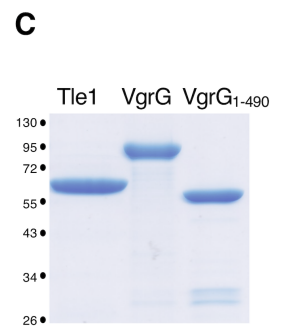
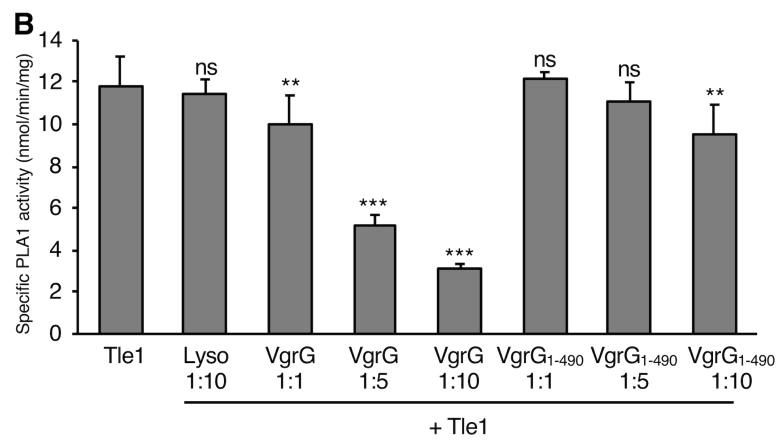
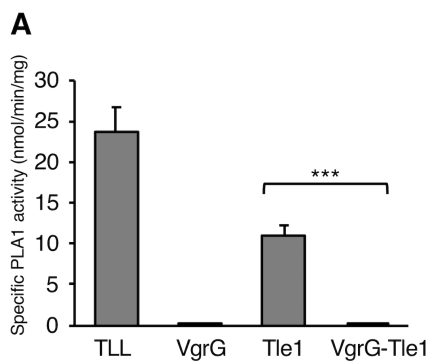


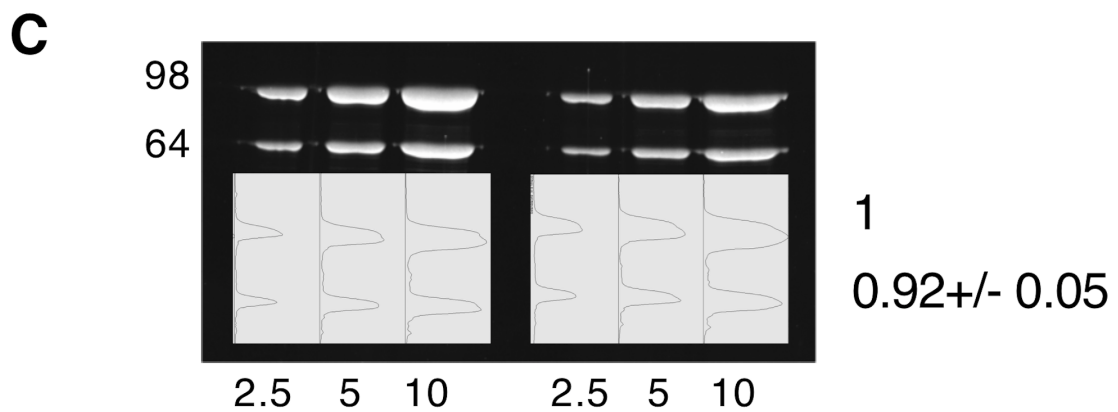
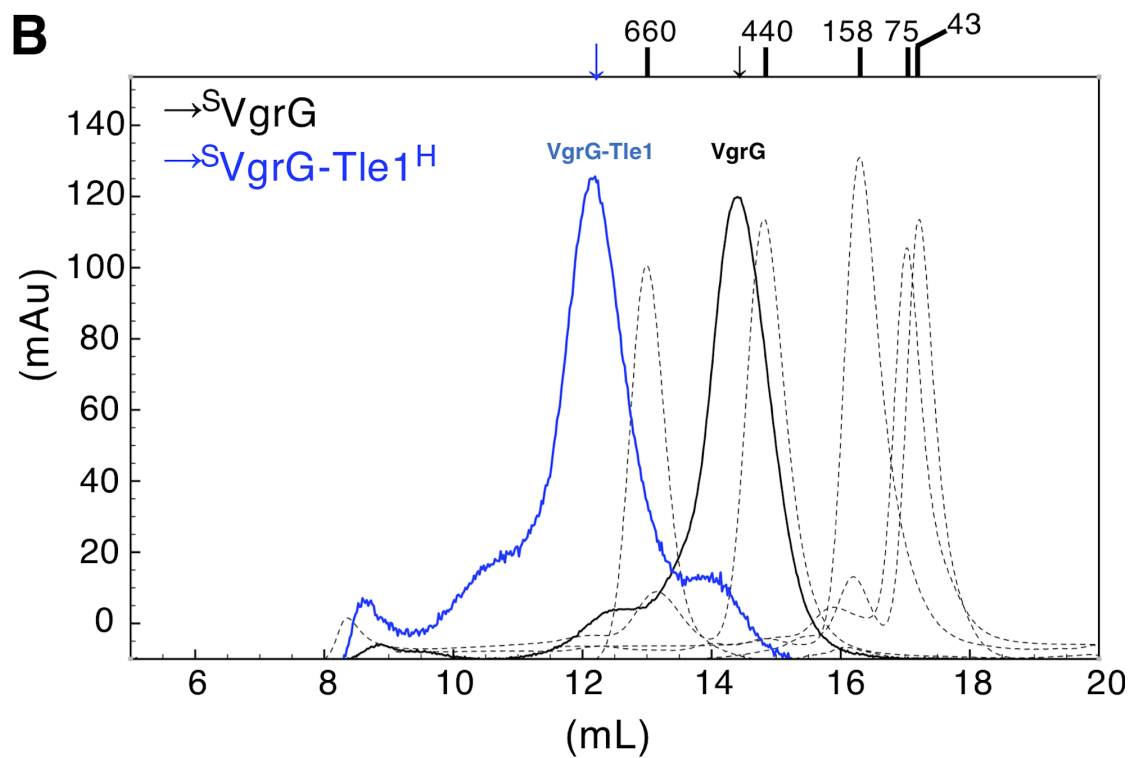
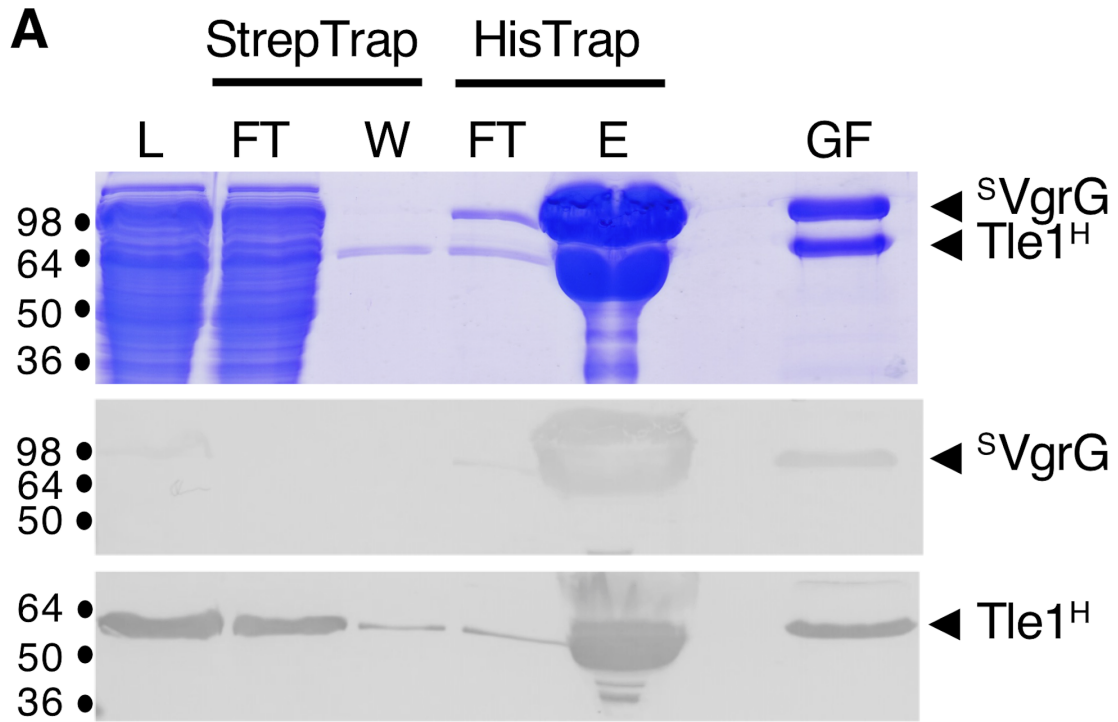


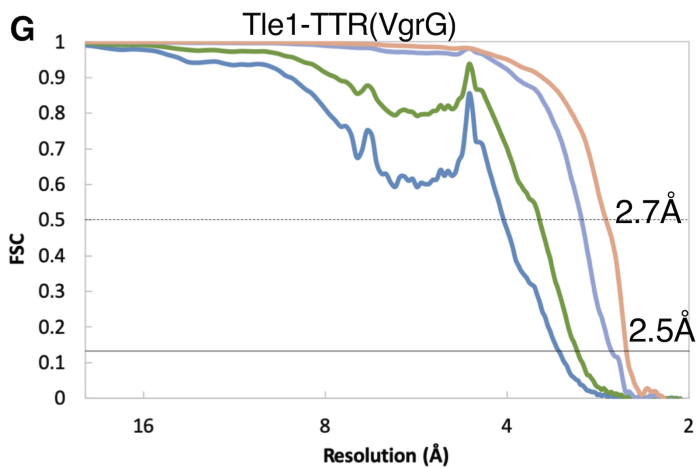
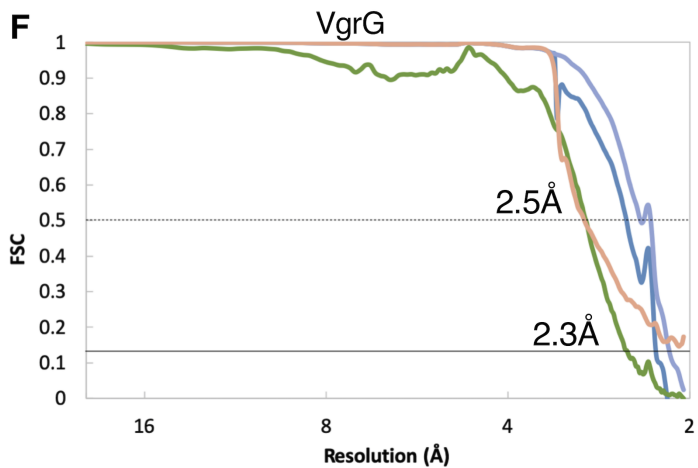
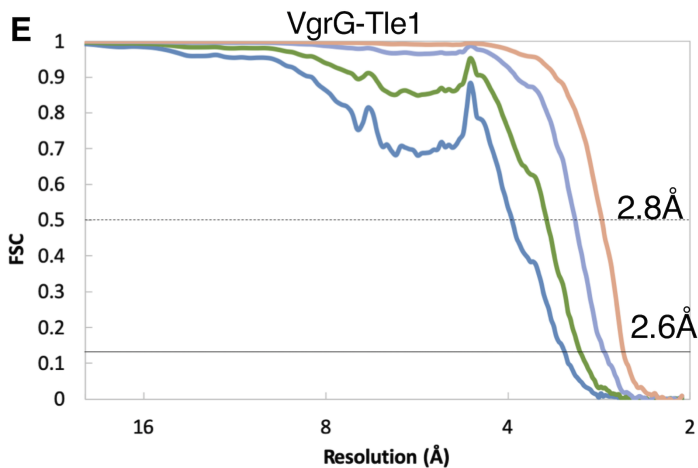
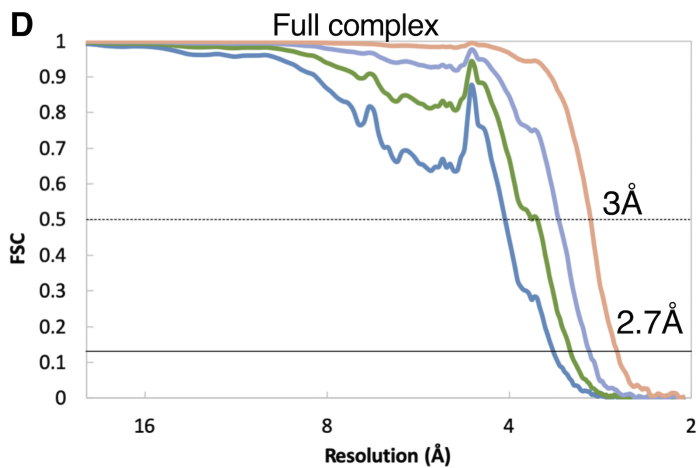
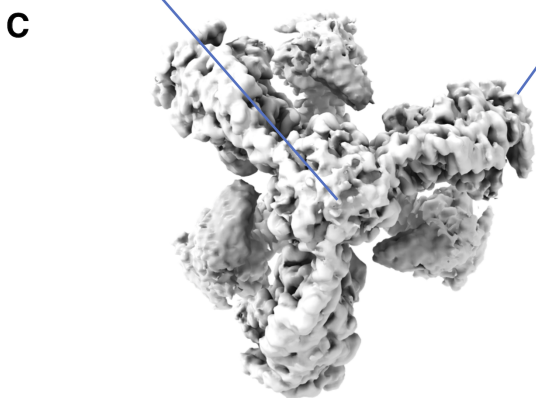
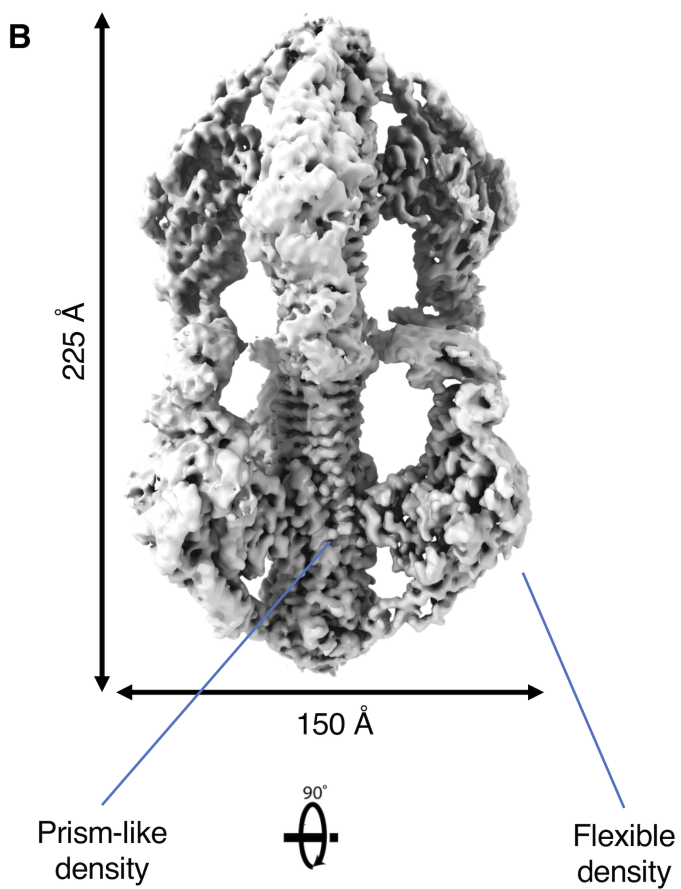
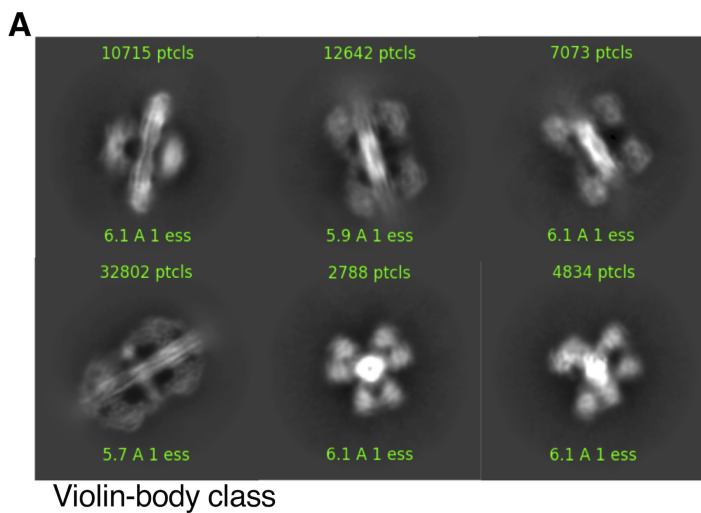


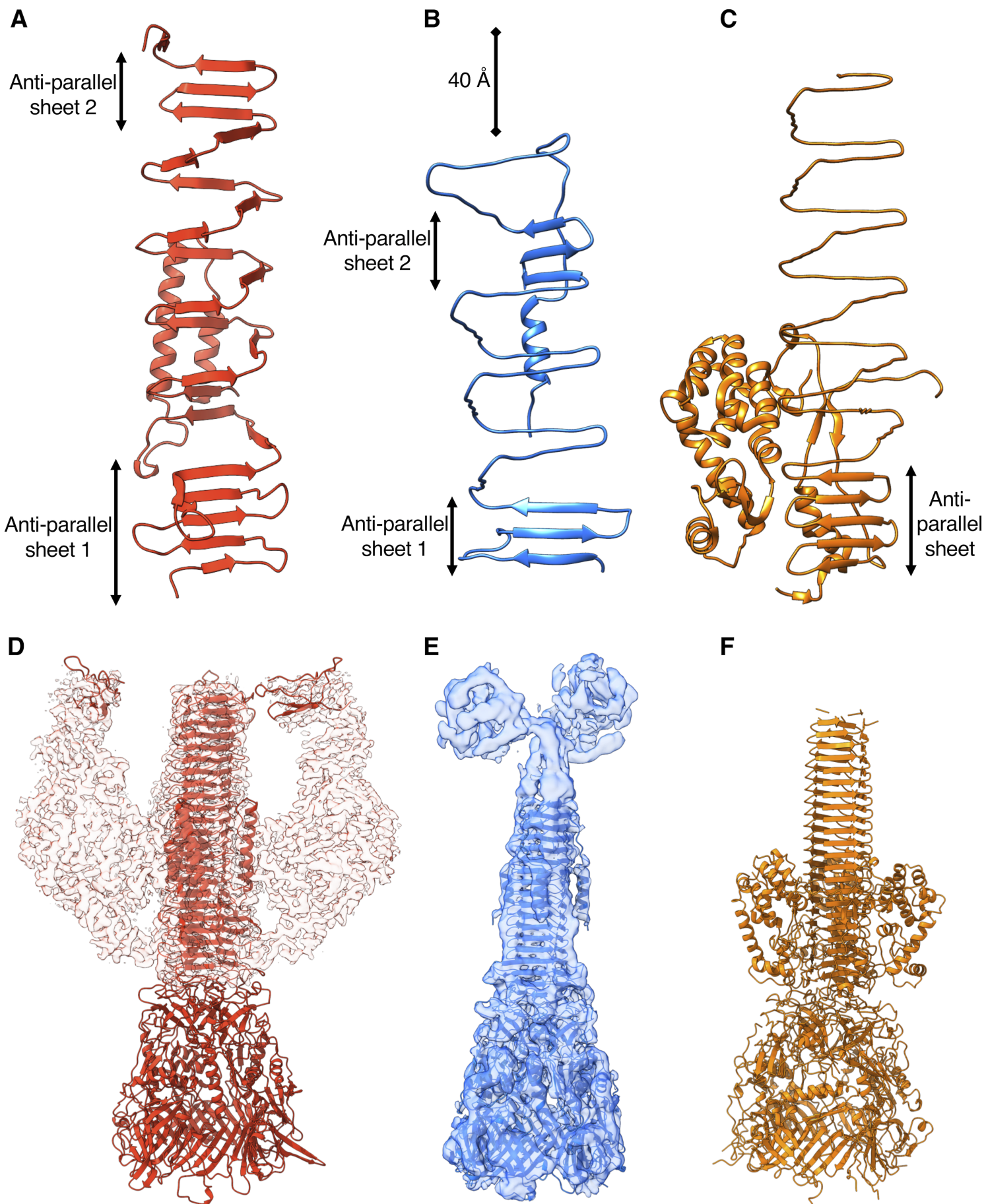
Substrate entry channel

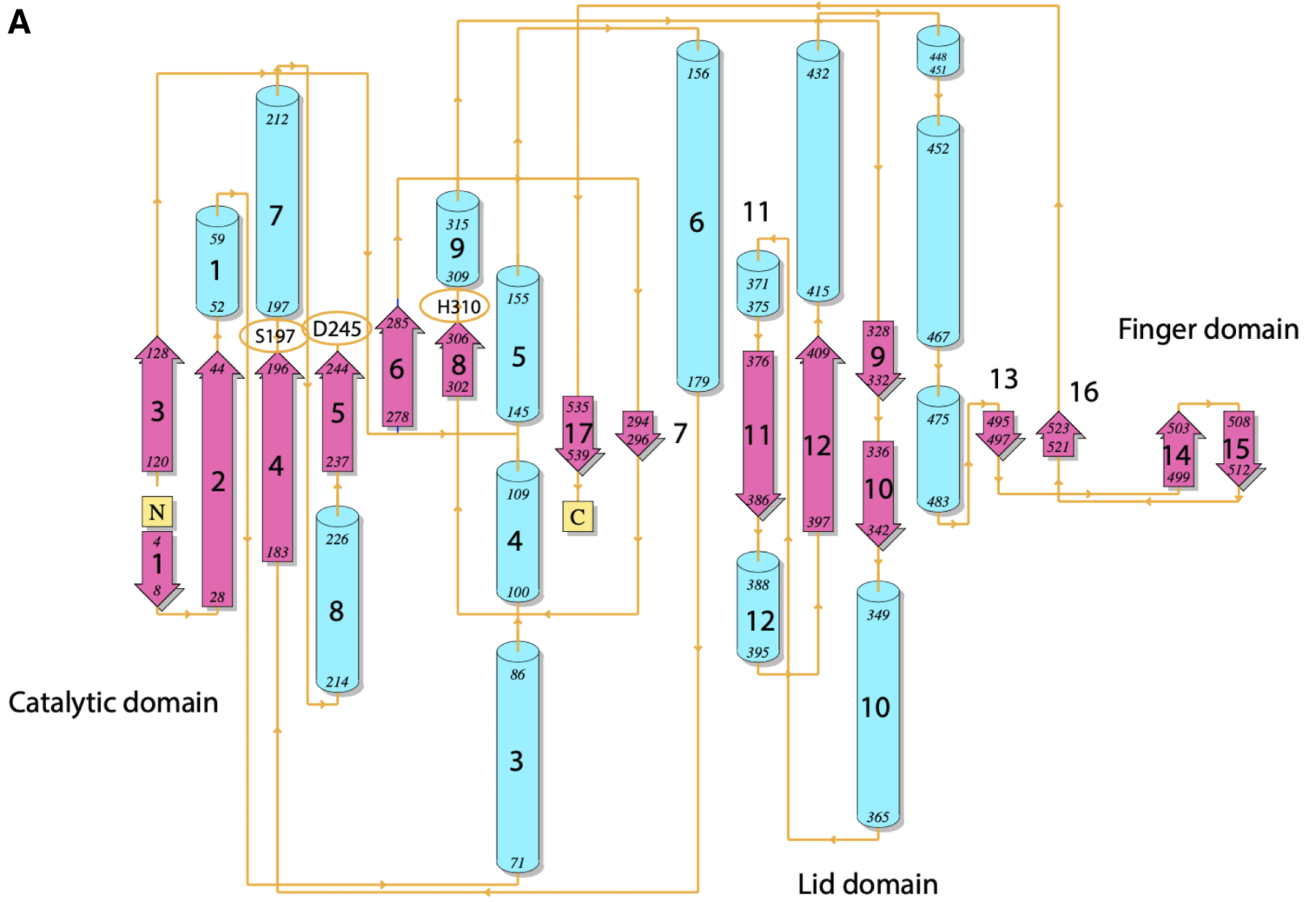






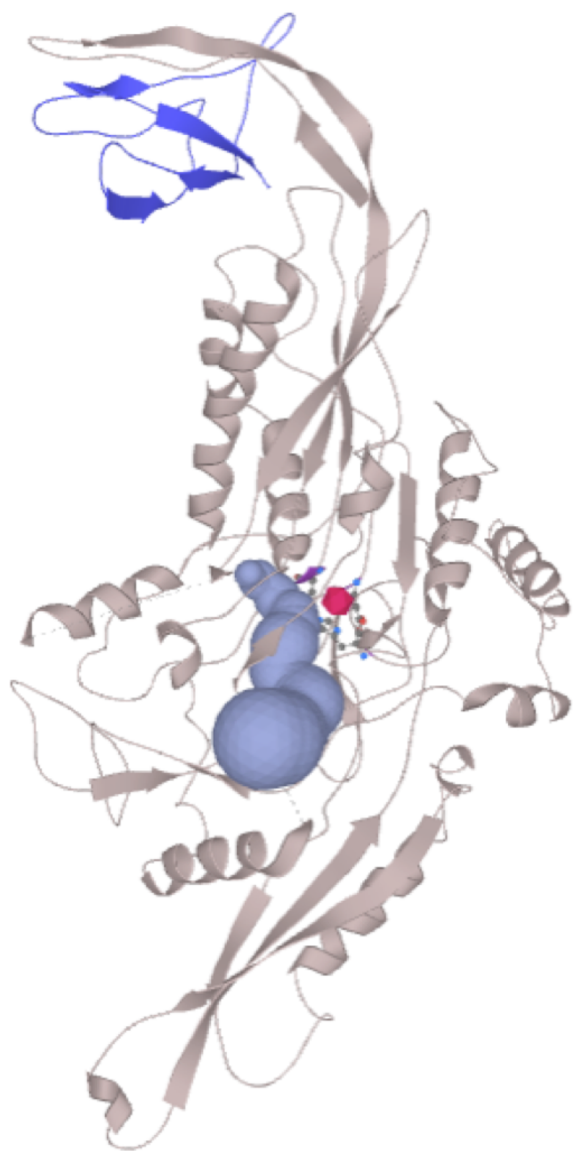




A**B****C**

90°



A**B**