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A Type VI Secretion System trans-kingdom effector is required for the delivery of a novel antibacterial toxin in *Pseudomonas aeruginosa*

Benjamin Berni¹, Chantal Soscia¹, Sarah Djermoun¹, Berengere Ize¹, Sophie Bleves^{1*}

¹Centre National de la Recherche Scientifique (CNRS), France

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Author contribution statement

B.B. and S.B. designed and conceived the experiments. B.B., C.S., and S.D. performed the experiments. S.B. supervised the execution of the experiments. B.B., C.S., B.I. and S.B. analysed and discussed the data. S.B. wrote the paper with contribution from B.B. and reading from B.I.

Keywords

Pseudomonas aeruginosa, antibacterial toxin, trans-kingdom effector, adaptor, T6SS, Chaperone, Virulence, Tle (Type 6 lipase effector), immunity protein

Abstract

Word count: 260

Pseudomonas aeruginosa has evolved multiple strategies to disarm and take advantage of its host. For this purpose this opportunist pathogen has particularly developed protein secretion in the surrounding medium or injection into host cells. Among this, the Type VI Secretion System (T6SS) is utilized to deliver effectors into eukaryotic host as well as target bacteria. It assembles into a contractile bacteriophage tail-like structure that functions like a crossbow, injecting an arrow loaded with effectors into the target cell. The repertoire of T6SS antibacterial effectors of *P. aeruginosa* is remarkably broad to promote environmental adaptation and survival in various bacterial communities, and presumably in the eukaryotic host too.

Here we report the discovery of a novel pair of antibacterial effector and immunity of *P. aeruginosa*, Tle3 and Tli3. Tli3 neutralizes the toxicity of Tle3 in the periplasm to protect from fratricide intoxication. The characterization of the secretion mechanism of Tle3 indicates that it requires a cytoplasmic adaptor, Tla3, to be targeted and loaded onto the VgrG2b spike and thus delivered by the H2-T6SS machinery. Tla3 is different from the other adaptors discovered so far, and defines a novel family among T6SS with a DUF2875.

Interestingly this led us to discover that VgrG2b that we previously characterized as an anti-eukaryotic effector possesses an antibacterial activity as well, as it is toxic towards *Escherichia coli*. Excitingly Tli3 can counteract VgrG2b toxicity. VgrG2b is thus a novel trans-kingdom effector targeting both bacteria and eukaryotes. VgrG2b represents an interesting target for fighting against *P. aeruginosa* in the environment and in the context of host infection.

Contribution to the field

We have previously shown that a T6SS of *P. aeruginosa* (H2-T6SS), facilitates the internalization of the bacterium into non-phagocytic cells (Sana 2012; 2013). We have found that the H2-T6SS effector VgrG2b is responsible for this phenotype by manipulating host microtubule cytoskeleton (Sana, 2015). Here we have discovered that VgrG2b, as a component of the H2-T6SS puncturing device this time, is required for the delivery of an antibacterial effector. We have characterized a novel pair of antibacterial toxin and immunity. We showed that Tle3 is toxic once delivered in the periplasm of prey bacteria and that Tli3 can neutralize it. By characterizing the secretion mechanism of Tle3, we found that it requires Tla3, a cytoplasmic adaptor of a unique family, to be targeted to the VgrG2b spike. Tla3 represents a novel family of T6SS adaptors. Very interestingly we also discovered that the C-terminal extension of VgrG2b is toxic towards *Escherichia coli* making VgrG2b a trans-kingdom effector of *P. aeruginosa*, able to target both bacteria and eukaryotic cells. In conclusion VgrG2b is a fascinating protein, a unique case of trans-kingdom toxin required for the secretion of another antibacterial toxin. To our knowledge this has never been reported in the literature so far.

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Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

Does the study presented in the manuscript involve human or animal subjects: No

Data availability statement

Generated Statement: All datasets generated for this study are included in the manuscript and the supplementary files.

In review

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14 Benjamin Berni¹, Chantal Soccia¹, Sarah Djermoun¹, Bérengère Ize¹ and Sophie Bleves^{1*}
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17 ¹LISM (Laboratoire d'Ingénierie des Systèmes Macromoléculaires-UMR7255), IMM (Institut de
18 Microbiologie de la Méditerranée), Aix-Marseille Univ and CNRS, 31 chemin J. Aiguier, Marseille
19 13402 Cedex 20, France.

20 *Correspondance Pr Sophie Bleves

21 bleves@imm.cnrs.fr
22
23
24
25

26 **Abstract**

27 *Pseudomonas aeruginosa* has evolved multiple strategies to disarm and take advantage
28 of its host. For this purpose this opportunist pathogen has particularly developed protein
29 secretion in the surrounding medium or injection into host cells. Among this, the Type VI
30 Secretion System (T6SS) is utilized to deliver effectors into eukaryotic host as well as target
31 bacteria. It assembles into a contractile bacteriophage tail-like structure that functions like a
32 crossbow, injecting an arrow loaded with effectors into the target cell. The repertoire of T6SS
33 antibacterial effectors of *P. aeruginosa* is remarkably broad to promote environmental
34 adaptation and survival in various bacterial communities, and presumably in the eukaryotic host
35 too.

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37 *P. aeruginosa*, Tle3 and Tli3. Tli3 neutralizes the toxicity of Tle3 in the periplasm to protect
38 from fratricide intoxication. The characterization of the secretion mechanism of Tle3 indicates
39 that it requires a cytoplasmic adaptor, Tla3, to be targeted and loaded onto the VgrG2b spike
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43 anti-eukaryotic effector possesses an antibacterial activity as well, as it is toxic towards
44 *Escherichia coli*. Excitingly Tli3 can counteract VgrG2b toxicity. VgrG2b is thus a novel trans-
45 kingdom effector targeting both bacteria and eukaryotes. VgrG2b represents an interesting
46 target for fighting against *P. aeruginosa* in the environment and in the context of host infection.

47 **Keywords**

48 Virulence, Type VI secretion system, bacterial competition, VgrG, Tle, immunity,
49 adaptor, chaperone
50

51
52

53 **Introduction**

54 *Pseudomonas aeruginosa* is one of the most virulent opportunistic pathogens, being
55 responsible for various diseases such as acute infections of lungs and burned skin that can lead
56 to septicemia more particularly in immunocompromized patients, or broncho-alveolar
57 colonization in Cystic Fibrosis sufferers. *P. aeruginosa* has been classified in 2017 as critical
58 by the WHO in the top three list of antibiotic resistant bacteria (WHO publishes list of bacteria
59 for which new antibiotics are urgently needed). *P. aeruginosa* has developed various
60 pathogenicity strategies among which protein secretion or protein delivery into target cells is
61 key. Indeed this pathogen possesses 5 of the 6 secretion systems so far identified among Gram-
62 negative bacteria, if we exclude the T9SS (Type IX Secretion System) restricted to one phylum,
63 and remarkably in several copies for most of them (Bleves et al., 2010).

64 The T6SS (Type VI Secretion System) was first discovered in the context of eukaryotic
65 host infection (Mougous et al., 2006)(Pukatzki et al., 2006) and later during bacterial
66 competition (Hood et al., 2010), which seems to be its primary function (Cianfanelli et al.,
67 2016b). The T6SS confers a fitness advantage (i) in environmental niches against rival bacteria
68 (inter- and intraspecies competitiveness have been described) and (ii) in the eukaryotic host
69 towards commensal bacteria (Cianfanelli et al., 2016b). Indeed recent studies have highlighted
70 a novel role for T6SS-dependent antibacterial responses in interbacterial competition in the
71 mammalian gut (Sana et al., 2016b)(Hecht et al., 2016), suggesting that T6SSs may be
72 important not only in shaping microbial community composition, but also in governing
73 interactions between the microbiota and invading pathogens. Interestingly, several T6SSs are
74 also known to target both cell type genus such as the T6SS of *Vibrio cholera* (Pukatzki et al.,
75 2006)(MacIntyre et al., 2010) and *P. aeruginosa* (Sana et al., 2012)(Russell et al., 2013). Even
76 more remarkably, three T6SS effectors of *P. aeruginosa*, namely PldA (also called Tle5a), PldB
77 (Tle5b) and TplE (Tle4) (Jiang et al., 2014, 2016) have been called “trans-kingdom effectors”

78 since these toxins can target both prokaryotic and eukaryotic cells (Bleves, 2016). Indeed toxins
79 are usually directed against eukaryotic cells (like AB toxins or RTX pore-forming toxins) or
80 against rival bacteria (like bacteriocins).

81 The T6SS functions as a dynamic contractile phage tail-like structure anchored in the
82 bacterial cell envelope that delivers effector proteins directly into the target cell in a one-step
83 manner. T6SS includes a contractile sheath that cover a nanotube of stacked Hcp topped with
84 a membrane-puncturing spike made of VgrG and PAAR (proline-alanine-alanine-arginine
85 repeat) proteins (Basler, 2015). The sheath can contract and inject the arrow loaded with
86 effectors into the target cell. Characterizing the repertoire of effectors delivered by the T6SS
87 has highlighted a great diversity in terms of effector activities, host cell targets, and mode of
88 recruitment by the T6SS machinery. In brief, there are two broad effector categories:
89 the "specialized" effectors fused to components of the machinery (evolved VgrG, evolved
90 PAAR and evolved Hcp have been described so far) and the "cargo" effectors (Cianfanelli et
91 al., 2016b). The later are addressed to the T6SS machinery by binding directly one of the arrow
92 components (VgrG, PAAR and Hcp) or by being targeted through cytoplasmic adaptor proteins
93 also called chaperones. To date three families of adaptor have been described, the first one
94 harboring a DUF4123 (Liang et al., 2015; Miyata et al., 2013; Salomon et al., 2014; Unterweger
95 et al., 2015), the second a DUF1795 (Alcoforado Diniz and Coulthurst, 2015; Whitney et al.,
96 2015) and the third one a DUF2169 (Bondage et al., 2016). In line with this, many effector-
97 encoding genes are found in close proximity to *vgrG*, *hcp*, *paar* or adaptor genes. Finally to
98 protect themselves from self-intoxication or from antibacterial toxins injected by neighboring
99 sibling cells, bacteria always synthesize immunity proteins, which are encoded by adjacent
100 genes (Benz and Meinhart, 2014).

101 *P. aeruginosa* encodes three distinct T6SS loci, H1- to H3-T6SS. While H1-T6SS has
102 only been involved in antibacterial activity so far (Sana et al., 2016a)(LaCourse et al., 2018),

103 H2-T6SS and H3-T6SS can target both bacterial and eukaryotic cells possessing even as said
104 earlier trans-kingdom effectors (Burkinshaw et al., 2018; Jiang et al., 2014, 2016; Russell et al.,
105 2013; Sana et al., 2012, 2015). We discovered the anti-eukaryotic function of the H2-T6SS
106 machinery that promotes the uptake of *P. aeruginosa* by non-phagocytic cells (Sana et al., 2012).
107 The two phospholipases D mentioned earlier, PldA (Tle5a) and PldB (Tle5b), delivered
108 respectively by H2-T6SS and H3-T6SS machineries, participate in the host kinase pathway
109 highjacking that facilitates further entry of *P. aeruginosa* (Jiang et al., 2014). The evolved
110 VgrG2b effector (Sana et al., 2015) is delivered by H2-T6SS into epithelial cells where it targets
111 the γ -tubulin ring complex, a microtubule-nucleating multiprotein complex to promote a
112 microtubule-dependent internalization of *P. aeruginosa*. Finally TplE (Tle4), which is secreted
113 by the H2-T6SS machinery, promotes autophagy in epithelial cells once localized to the
114 endoplasmic reticulum (Jiang et al., 2016). Interestingly PldA (Tle5a), PldB (Tle5b) and TplE
115 (Tle4) have also been identified as antibacterial phospholipases of the Tle (type VI lipase
116 effectors) family (Russell et al., 2013). They work by affecting membrane integrity of the rival
117 bacteria (Jiang et al., 2014, 2016; Russell et al., 2013). More precisely, PldA degrades the major
118 constituent of bacterial membranes, the phosphatidylethanolamine (Russell et al., 2013).

119 In the present study we have discovered a novel antibacterial toxin, Tle3 and its cognate
120 immunity, Tli3, whose genes are encoded downstream of *vrgG2b*. By characterizing the
121 secretion mechanism of Tle3 by H2-T6SS, we showed that it requires Tla3, a cytoplasmic
122 adaptor of a unique family, to be targeted to the VgrG2b spike. Interestingly we also found that
123 the C-terminal extension of VgrG2b is toxic towards *Escherichia coli* making VgrG2b a new
124 trans-kingdom effector of *P. aeruginosa*.

125

126 **Materials and Methods**

127 **Bacterial strains, growth conditions and plasmid construction**

128 All *P. aeruginosa* and *E. coli* strains used in this study are described in Supplementary
129 Table 1. Briefly, the *E. coli* K-12 DH5 α and CC118 λ Pir were used for cloning procedures. The
130 BL21(DE3)pLysS and BTH101 were used for protein production and BACTH analyses
131 respectively. Strains were grown in LB or in TSB medium (for *P. aeruginosa*) at 37°C or 30°C.
132 Specific growth conditions are specified in the text when necessary. Recombinant plasmids
133 were introduced into *P. aeruginosa* by triparental mating using the conjugative properties of
134 the helper plasmid pRK2013 (Supplementary Table 1). Plasmids were maintained by the
135 addition of ampicillin (50 μ g/mL), kanamycin (50 μ g/mL), chloramphenicol (30 μ g/mL),
136 streptomycin (30 μ g/mL for *E. coli*, 2000 μ g/mL for *P. aeruginosa*) or gentamicin (30 μ g/mL
137 for *E. coli*, 115 μ g/mL for *P. aeruginosa*). Expression of genes from pT7 in BL21(DE3)pLysS
138 was blocked with 0.4% of glucose and induced in exponential phase (OD₆₀₀= 0.4-0.6) for 3
139 hours with 1mM of IPTG. Cloning procedures were described in [26]. The plasmids used and
140 constructed are described in Supplementary Table 1, the list of oligonucleotides (synthesized
141 by Eurogentec or IDT) is given in Supplementary Table 2.

142

143 **Cloning procedures for *P. aeruginosa* mutants**

144 To generate *P. aeruginosa* mutants, 500 bp upstream and 500 bp downstream of the
145 gene to be deleted were amplified by overlapping PCR with Q5 high fidelity DNA polymerase
146 (NEB) using primers listed in Supplementary Table 1. The PCR product was cloned in
147 pKNG101 suicide vector by one-step sequence and ligation-independent cloning (SLIC) (Jeong
148 et al., 2012), which was then sequenced. pKNG101 derivatives, maintained in the *E. coli*
149 CC118 λ pir strain, were mobilized in *P. aeruginosa* strains. The mutants, in which the double
150 recombination events occurred, were confirmed by PCR analysis.

151

152 **Heterologous toxicity assays**

153 *E. coli* BL21(DE3)pLysS containing plasmids producing cytoplasmic or periplasmic
154 targeted proteins were grown overnight at 37°C in LB with 0.4% of glucose. 10 µL drops of
155 bacterial suspensions serially diluted were spotted onto LB agar plates containing 0.1 mM IPTG
156 or 0.4 % glucose and cells were grown for 16 h at 37°C.

157

158 **Bacterial two-hybrid assay**

159 Protein-protein interactions were assessed with the adenylate cyclase-based two-hybrid
160 technique using protocols published previously (Battesti and Bouveret, 2012; Karimova et al.,
161 1998). Briefly, the proteins to be tested were fused to the isolated T18 and T25 catalytic
162 domains of the *Bordetella* adenylate cyclase. After introduction of the two plasmids producing
163 the fusion proteins into the reporter BTH101 strain, plates were incubated at 30°C for 24 h.
164 Three independent colonies for each transformation were inoculated into 600 µl of LB medium
165 supplemented with ampicillin (50 µg/mL), kanamycin (50 µg/mL), and IPTG (0.5 mM). After
166 overnight growth at 30 °C, 5 µl of each culture was spotted onto LB agar plates supplemented
167 with ampicillin, kanamycin, IPTG, and 5-bromo-4-chloro-3-indonyl-D-galactopyrannoside (X-
168 gal, 40µg/mL) and incubated for 16 h at 30 °C.

169

170 **Protein purification by affinity chromatography**

171 *Escherichia coli* BL21(DE3)pLysS cells carrying the pRSFDUET-1 and pETDUET-1
172 derivatives were grown at 37°C in LB to an OD₆₀₀ ~ 0.5 and the expression of the PA0262,
173 PA0261, PA0260 or PA0259 genes was induced with IPTG (1 mM) for 3 h at 37°C. Cells were
174 harvested by centrifugation at 1914 × g for 30 min at 4°C. The cell pellet was resuspended in
175 Tris-HCl 50 mM pH 8.0, NaCl 150 mM, Triton X-100 0.1%, lysozyme 0.5 mg/mL and EDTA
176 1 mM and stored at -80°C. Cells were supplemented with DNase (20 µg/mL), MgCl₂ and
177 phenylmethylsulfonyl fluoride 1mM and cells were lysed by three passages at the Emulsiflex-

178 C5 (Avestin), and lysates were clarified by centrifugation at $16000 \times g$ for 30 min. The
179 supernatant was loaded onto a 5-mL StrepTrap HP (GE Healthcare) column and then washed
180 with 50 mM Tris-HCl pH 8.0, 150 mM NaCl at 4°C. The fusion protein was eluted in the
181 affinity buffer supplemented with 2.5 mM desthiothiotin. Peak fractions were pooled and loaded
182 onto a Superose 200 10/300 column (GE Healthcare) equilibrated in 50 mM Tris-HCl pH 8.0,
183 50 mM NaCl.

184

185 **Fractionation of *P. aeruginosa***

186 Fractionation of cells into spheroplasts (cytoplasm and membranes) and periplasmic
187 fractions were done as previously described (Ize et al., 2014). Proteins corresponding to the
188 cytoplasm and periplasm fractions or to insoluble material were resuspended in loading buffer.

189

190 **Protein secretion**

191 *P. aeruginosa* strains were grown at 25°C in TBS for 24 hours. Cells corresponding to
192 10 units DO₆₀₀ and extracellular medium were separated by centrifugation at 2000 x g for 10
193 min at room temperature. 2/3 of the supernatants were collected and centrifuged at 13 000 x g
194 for 5 min at room temperature. Proteins contained in the supernatant were precipitated with
195 trichloro-acetic acid (TCA, 15%) for 3 h at 4°C. Samples were centrifuged at 13 000 x g for
196 30 min at 4°C, pellets washed with 90% acetone and resuspended in loading buffer.

197

198 **SDS-PAGE and western-blot**

199 Protein samples derived from equivalent amounts of culture (i.e. optical density
200 equivalents) resuspended in loading buffer were boiled and separated by SDS-PAGE. Proteins
201 were then stained by Coomassie-blue or immunodetected as described before (Sana et al., 2015)
202 using primary polyclonal antibodies directed against His6 epitope-tag (Penta His, Qiagen,

203 dilution 1:1000), V5 epitope-tag (Bethyl Laboratories, dilution 1:1000), Strep epitope-tag (IBA
204 StrepMAB Classic, dilution 1:000), DsbA (kindly gifted by K.E. Jaeger – university of
205 Heinrich-Heine, dilution 1:25000), or monoclonal antibodies directed against EF-Tu (Hycult-
206 biotech, dilution 1:20000), XcpY (laboratory collection, dilution 1:5000), TolB (laboratory
207 collection, dilution 1/500). Peroxidase-conjugated anti-Mouse or anti-Rabbit IgGs (Sigma,
208 dilution 1:5000) were used as secondary antibodies. Nitrocellulose membranes were revealed
209 with homemade enhanced chemiluminescence and were scanned using ImageQuant LAS4000
210 analysis software (GE Healthcare Life sciences).

211 Protein samples equivalent to 0.1 OD₆₀₀ units were loaded for whole cell and
212 spheroplasts analysis while protein samples equivalent to 0.2 OD₆₀₀ units were used for
213 cytoplasm or periplasm analysis and protein samples equivalent to 1 OD₆₀₀ units were used for
214 extracellular medium analysis.

216 **Bacterial Competition assays**

217 Intraspecific competition assays between *P. aeruginosa* strains were performed as
218 previously described (Jiang et al., 2014) with modifications. The prey cells carry pJN105 vector
219 (Gm^R) to allow counterselection. Overnight cultures of *P. aeruginosa* attacker and prey cells
220 were mixed in a 5 : 1 (attacker : prey) ratio and harvested by centrifugation at 3724 x g for 5
221 min. The pellet was resuspended in 200 µL of PBS 1X and spotted onto 0.45-µm nitrocellulose
222 membranes overlaid on a 1 % bactoagar plate. After 24 hours of incubation at 37°C, cells were
223 resuspended in 2 mL of PBS 1X, normalized to an OD_{600nm} of 0.5 and 10 µL of bacterial serially
224 diluted (10⁻¹ to 10⁻⁶) were spotted onto selective LB agar plates containing gentamicin (125
225 µg/mL). Significant growth difference of the prey bacteria for each competition assay was
226 computed by one-way ANOVA (Stat Plus) and unpaired Student's Test (Excel).

227

228 **Results**

229 **Tle3 is a novel antibacterial toxin of *P. aeruginosa***

230 The analysis of the *vgrG2b* genetic environment revealed the presence of the PA0260
231 gene encoding a protein with a α/β hydrolase domain (PF00561) and a putative Ser-Asp-His
232 catalytic triad used by various esterase enzymes and has thus been classified in the Tle3 family
233 of antibacterial Tle toxins (Russell et al., 2013) (Fig. 1A). The immunities of Tle proteins,
234 which are lipolytic toxins active in the periplasm of the prey bacterium, are localized in or
235 exposed to the periplasm where they neutralize the cognate toxin (Flaunatti et al., 2016; Jiang
236 et al., 2014, 2016; Russell et al., 2013). The two genes surrounding *tle3*, PA0259 and PA0261,
237 are good candidates as Tle3 immunity. Indeed sequence comparisons of PA0259 and PA0261
238 showed that PA0261 is homologous to Tsi6, the immunity protein of a H1-T6SS effector called
239 Tse6, and PA0259 to TplEi (Tli4), the immunity of TlpE (Tle4), a H2-T6SS effector (Jiang et
240 al., 2016; Lu et al., 2014). We used the SignalP 4.1 server (Nielsen, 2017) to predict the cellular
241 localization of the two immunity candidates. While a Sec signal sequence is predicted at the N-
242 terminal extremity of PA0261, the analysis of the PA0259 sequence did not reveal any.
243 However three upstream ATG can be found in frame with the annotated ATG of PA0259 (Fig.
244 1 sup). The sequence of the proteins synthesized from the two first ATG (ATG₁ et ATG₂)
245 presents then a N-terminal signal peptide, while the protein synthesized from the last codon
246 (ATG₃) does not. Moreover a RBS (Ribosome Binding Sequence) can be found only upstream
247 of ATG₁ and with a significant Kolaskar score that indicates a strong probability to be used as
248 an initiation codon (Kolaskar and Reddy, 1985). Altogether these data tend to indicate an
249 incorrect annotation of the start codon of PA0259 and that ATG₁ should be considered for the
250 initiation of PA0259 translation. Such a correction has been already seen for Tli5 (PA3488) the
251 immunity protein of PldA (Tle5a) of *P. aeruginosa* (Russell et al., 2013). In conclusion of this

252 *in silico* analysis, the two putative immunities may harbor a Sec signal peptide that suggests a
253 periplasmic localization in agreement with Tle3 activity in this compartment.

254 To demonstrate the antibacterial activity of Tle3 and to identify its immunity protein,
255 we developed a heterologous toxicity assay in *E. coli* on the basis that Tle3 should be toxic
256 when produced in the periplasm of *E. coli* and that it should be counteracted by the co-
257 production of its immunity protein. In order to artificially address Tle3 to the periplasm of *E.*
258 *coli* the *tle3* sequence has been cloned in frame with the sequence coding the PelB signal peptide
259 on the pET22b vector under a P_{T7} promoter. PA0259 from ATG₁ and PA0261 were respectively
260 cloned in pRSF-DUET, a higher copy-number vector, to allow a maximal co-expression with
261 *ss-tle3* in the *E. coli* BL21(DE3) pLysS strain. The correct production and localization of all
262 the recombinant proteins in *E. coli* has been verified by western blot after cell fractionation (Fig.
263 2 sup). The results presented in Figure 1B indicate that whereas the cytoplasmic production of
264 Tle3 was not toxic, Tle3 targeting to the periplasm led to *E. coli* killing. Moreover while
265 PA0259 had no effect, the coproduction of PA0261 in the periplasm protected the cells against
266 the toxicity of Tle3. We verified that the sole overproduction of PA0259 and PA0261 was not
267 toxic to *E. coli*. As PA0261 neutralized Tle3 toxicity, we called it “Tli3” for Type VI lipase
268 immunity (Fig. 1A). During this study, we observed that the protection conferred by Tli3
269 coproduction could be sometimes partial and we solved this issue by cloning in tandem *tli3* and
270 *tle3* on the same plasmid like they are organized on *P. aeruginosa* genome (Fig. 1B, line 8).
271 The importance of this genetic link is the proof of the close connection between these two
272 proteins as a pair of toxin-antitoxin.

273

274 **Tli3 (PA0261) is the immunity protein of Tle3**

275 As the immunities bind specifically their effector, which is suggested by the release of
276 Tle3 toxicity by Tli3, we tested the physical interaction between both proteins by co-

277 purification with affinity chromatography (Fig. 2A). A cytoplasmic Strep-tagged version of
278 Tli3 was engineered by fusing the tag to the mature domain of Tli3, lacking its signal peptide.
279 The recombinant protein was coproduced in *E. coli* BL21(DE3) pLysS with the cytoplasmic
280 His₁₀-tagged Tle3. The bacterial lysate was loaded to a StrepTactin matrix (see Materials and
281 Methods section), and Tli3^{C_{Strep}} was eluted with desthio-biotin. The presence of Tle3^{C_{His}} was
282 controlled in the elution fraction with anti-His antibodies. As showed in Fig. 2A, Tle3^{C_{His}} was
283 found in the eluted fraction only upon coproduction with Tli3^{C_{Strep}} (left panel). Indeed when
284 produced alone in *E. coli* Tle3^{C_{His}} was not purified by affinity chromatography (right panel).
285 As expected for an immunity protein, Tli3 directly interacts with Tle3.

286 To go further into Tli3 characterization we chose to determine its cellular localization
287 in *P. aeruginosa*. All the immunity proteins identified so far for Tle proteins are localized in
288 the periplasm or associated to the periplasmic side of the outer membrane (Flaughnatti et al.,
289 2016; Jiang et al., 2014, 2016; Russell et al., 2013) in order to counteract their cognate toxin.
290 A chromosomally encoded Tli3_{V5} translational fusion was engineered in order to specifically
291 immunodetect the protein in *P. aeruginosa* (Supplementary Table 1). After fractionation of *P.*
292 *aeruginosa* (Fig. 2B), Tli3 was readily observed in the same fraction as DsbA that catalyzes
293 intrachain disulfide bond formation as peptides emerge into the periplasm. This indicates a
294 periplasmic localization for Tli3 in *P. aeruginosa* in agreement with the presence of a Sec signal
295 peptide and our working hypothesis suggesting an immunity function.

296

297 **Tle3 protein-protein interaction network**

298 To further characterize Tle3, we performed a bacterial two-hybrid (BACTH) assay with
299 the other gene products of the *vgrG2b* operon hypothesizing that a genetic link could reflect
300 protein-protein interaction. The sequences coding PA0259 and Tli3 (PA0261) after their signal
301 sequences and Tle3 were cloned downstream and upstream the T18 or T25 domains of the

302 *Bordetella adenylate* cyclase. Because of the high molecular weight of VgrG2b and since the
303 interaction of another Tle with a VgrG in entero-aggregative *E. coli* (EAEC) was previously
304 delimited to the C-terminal domain of VgrG (Flaugnatti et al., 2016), we cloned the sequences
305 encoding the C-terminal extension (domains 1, 2 and 3) and a truncated version harboring only
306 the DUF2345 and the transthyretin-like (TTR) domains (domains 1 and 2) of VgrG2b
307 downstream the T18 and T25 domains (Fig. 3A).

308 Unexpectedly the sole interaction revealed by the BACTH assay for Tle3 was with
309 PA0259 (Fig. 3B) since only the T18/T25-Tle3 and T18/T25-PA0259 fusion proteins
310 coproduction activated the expression of the reporter gene. This assay did not confirm the
311 interaction between Tle3 and Tli3 observed by copurification, and did not evidence an
312 interaction with VgrG2b. Interestingly Tle3 and PA0259 did not interact anymore when they
313 were fused upstream the T18 and T25 domains suggesting that both proteins interact via their
314 C-terminal domains (Fig. 3A Sup). To go further in characterizing the interaction between Tle3
315 and PA0259, we constructed truncated variants taking into account their domain organization
316 (Fig. 3 A). We further delimited the interacting domain of Tle3 to its extreme C-terminus (the
317 DUF3274 domain) since only the truncated T18-Tle3D2 construct still interacts with T25-
318 PA0259 (Fig. 3C). We did not find the domain of interaction within PA0259 as none of the
319 DUF2875 domains alone interacts with Tle3 or this may suggest that both of them are required
320 for the interaction (Fig. 3C).

321 Next, we took advantage of all the constructs we made to test other interactions. The
322 BACTH assay also showed that PA0259 interacts with both forms of the VgrG2b C-terminal
323 extension (Fig. 3B). The domain of interaction on VgrG2b is thus at least constituted by the
324 DUF2345 and TTR domains. We also observed that PA0259 at least dimerizes since all the
325 PA0259 constructs interact with each other whatever the orientation of PA0259 (Fig. 3B, 3D,

326 Fig. 3B Sup). VgrG2b and Tli3 (PA0261) are also able to homomultimerize since all the
327 constructs interact with each other (Fig. 3D, Fig. 3B Sup).

328 Taking into account the interactions revealed by the BACTH assay, we propose that the
329 Tle3 toxin can be addressed to the H2-T6SS machinery VgrG2b component via PA0259. We
330 thus named PA0259 “Tla3” for Type VI lipase adaptor protein.

331

332 **Tla3 (PA0259) characterization**

333 To gain insight into the role of Tla3 during Tle3 secretion, we first validated the
334 interactions of Tla3 with Tle3 and VgrG2b by a complementary approach of co-purification by
335 affinity chromatography. Two different tagged versions of Tla3 were engineered by fusing a
336 Strep-tag or a 10His-tag to the mature domain of Tla3 this leading to cytoplasmic tagged Tla3
337 proteins. The recombinant Tla3^{C_{Strep}} was coproduced in *E. coli* BL21(DE3) pLysS with Tle3^{C_{His}},
338 and the Tla3^{C_{His}} with 3 recombinant forms of Strep-tagged VgrG2b consisting in the full-length
339 VgrG2b, or VgrG2b truncated for the extreme C-terminus (deletion of domain 3 in Fig. 3A) or
340 VgrG2b truncated for the extreme C-terminus and the TTR domain (deletion of domains 2 and
341 3 in Fig. 3A). We initially tried with His-tagged VgrG2b but a problem of protein instability
342 led us to shift for Strep-tagged VgrG2b. The bacterial lysates were loaded on a StrepTactin
343 matrix, and Tla3^{C_{Strep}} or the three recombinant VgrG2b^{Strep} were eluted with desthio-biotin. The
344 presence of Tle3^{C_{His}} and of Tla3^{C_{His}} was visualized in the elution fractions with anti-His
345 antibodies. As shown in Fig. 4A and 2A right panel, Tle3^{C_{His}} was only found in the eluted
346 fraction upon coproduction with Tla3^{C_{Strep}}. We observed that Tla3^{C_{His}} is copurified only upon
347 coproduction with the full length VgrG2b (Fig. 4B) or the VgrG2b truncated for the extreme
348 C-terminus (Fig. 4C). Indeed when produced alone (Fig. 4B) or with VgrG2b truncated for the
349 extreme C-terminus and the TTR domain (Fig. 4D), Tla3^{C_{His}} was not purified by affinity
350 chromatography. Since VgrG2b truncated for the extreme C-terminus still copurified with Tla3

351 we can exclude that this domain is required for the interaction. This is in line with the BACTH
352 assay that showed an interaction between Tla3 and a truncated VgrG2b consisting in only the
353 DUF2345 and TTR domains (domains 1 and 2, Fig. 3A). Moreover, the deletion of the TTR
354 domain affecting the copurification, one can conclude that this domain is key for the interaction.
355 Taken together these data confirmed a direct interaction of Tla3 with Tle3 on one side and with
356 VgrG2b on the other side. By taking into account the BACTH data and the copurification with
357 two truncated forms of VgrG2b, the domain of interaction of VgrG2b with Tla3 can be
358 delimited to the TTR domain.

359 We then analyzed the cellular localization of Tla3 in *P. aeruginosa* that, according to
360 its interactions with Tle3 and VgrG2b, should be cytoplasmic. As for Tli3, we engineered a
361 chromosomally encoded Tla3_{V5} translational fusion (Supplementary Table 1). Tla3 was indeed
362 immunodetected in the cytoplasmic fraction (Fig. 5A). One could note that in contrast with our
363 first hypothesis suggesting an incorrect start codon for *tla3* and our observation of the
364 recombinant protein in the periplasm of *E. coli* (Fig. 1 Sup), Tla3 was totally absent from the
365 periplasmic fraction of *P. aeruginosa*. To strengthen this result, each putative ATG was
366 individually mutated on the chromosome of the PAO1 strain encoding the Tla3_{V5} translational
367 fusion (Fig. 5B). In agreement with its cytoplasmic localization in *P. aeruginosa*, Tla3 was only
368 produced if the fourth ATG was intact. Accordingly the Tla3 protein synthesized from this ATG
369 is not predicted to possess a N-terminal signal peptide (Fig. 1 Sup). In conclusion Tla3 is a
370 cytoplasmic protein synthesized from the annotated translation start (pseudomonas.com) and
371 this localization is in agreement with a role in the targeting of the toxin to the secretion
372 machinery.

373 Next, we asked whether Tla3 is specific for Tle3 or if it can be required for the secretion
374 of other substrates of the H2-T6SS machinery. Since Hcp secretion is the hallmark of a
375 functional secretion system, we studied the secretion of Hcp2b whose gene is upstream *vgrG2b*

376 (Fig. 1A). Like Allsopp and colleagues (2017) we deleted the *rsmA* gene to enable Hcp2b
377 production and thus secretion by a PAO1 strain encoding a Hcp2b_{His} translational fusion (Fig.
378 5C, compare line 1 and 2). RsmA is a posttranscriptional regulator known to repress all three
379 T6SS clusters of *P. aeruginosa* (Allsopp et al., 2017). This results in a massive secretion since
380 Hcp2b_{His} can be observed in the extracellular protein samples by Coomassie-blue staining (Fig.
381 5C, lower panel). While Hcp2b_{His} secretion was abolished in a *rsmA clpV2* mutant, a H2-T6SS
382 mutant, we observed that Hcp2b_{His} is still secreted in the absence of Tla3 (Fig. 5C, compare
383 line 3 with line 4), suggesting that Tla3 is specific for the secretion of Tle3 but not for other
384 H2-T6SS proteins. In line with this specific adaptor-toxin pair, Tla3^{C_{Strep}} did not copurify TplE
385 (Tle4), another antibacterial phospholipase delivered by the H2-T6SS machinery (Fig. 5D).

386 Finally as the interaction with a VgrG can suggest that Tla3 is itself a T6SS effector, we
387 studied whether Tla3 is secreted by *P. aeruginosa*. To this end the *rsmA* gene was deleted from
388 the PAO1 strain encoding a Tla3_{V5} translational fusion. Whereas Tla3_{V5} was better produced
389 upon *rsmA* deletion (Fig. 5E), this did not lead to immunodetection of Tla3_{V5} in the extracellular
390 medium although Hcp2b was readily observed by Coomassie-blue staining of the same samples.
391 Tla3 is thus not an effector *per se*.

392 In conclusion the cytoplasmic localization of Tla3 in *P. aeruginosa* is appropriate with
393 a recruiting role of Tle3 to the H2-T6SS machinery through an interaction with the TTR domain
394 of VgrG2b. Tla3 is not co-secreted with Tle3. Tla3 role seems specific to Tle3 since it is not
395 required for a functional H2-T6SS machinery and does not interact with TplE (Tle4), another
396 H2-T6SS effector.

397

398 **Tle3 secretion mechanism by the H2-T6SS machinery of *P. aeruginosa***

399 In order to study the antibacterial role and the secretion of Tle3 by *P. aeruginosa*, we
400 performed intra-species bacterial competition assays between *P. aeruginosa* strains. This

401 consists in studying the survival of prey bacteria lacking the *tli3* immunity gene (by CFU
402 counting of antibiotic resistant bacteria) co-cultivated 24 hours at 37°C on plate with various
403 attackers. As immunity genes are essential genes (protection from fratricide intoxication), both
404 toxin and immunity genes have been deleted in order to construct a viable $\Delta tli3\Delta tle3$ mutant
405 strain (Supplementary Table 1). The figure 6 first confirms the antibacterial function of Tle3
406 and the immunity role of Tli3 since the growth of the immunity mutant was affected by the WT
407 strain because it cannot resist Tle3 toxicity, while the $\Delta tle3$ mutant had no effect (Fig. 6 compare
408 line 1 with line 2). The use of the $\Delta clpV2$ strain, a H2-T6SS mutant and of $\Delta vgrG2b$ and $\Delta tla3$
409 mutants allowed to demonstrate that Tle3 is delivered to prey bacteria through the H2-T6SS
410 machinery and confirms that VgrG2b and Tla3 participate in Tle3 targeting to the H2-T6SS
411 machinery (Fig. 6 compare line 1 with lines 4, 5 and 6). Indeed $\Delta clpV2$, $\Delta vgrG2b$ and $\Delta tla3$
412 mutants had no effect on the immunity mutant growth since they cannot deliver Tle3 into prey
413 bacteria. The complementation *in cis* of *tle3* and *tla3* deletions (the mutants have been
414 constructed for this study) restored a WT phenotype (Fig. 6 compare line 2 with line 3, and line
415 6 with line 7) this demonstrating no polar effect on downstream genes. Furthermore the
416 introduction of a wild-type copy of *tli3* at the *attB* site on *P. aeruginosa* chromosome of the
417 $\Delta tli3\Delta tle3$ mutant restores wild-type competition capacity to this strain (Fig. 4A Sup). This
418 confirms that the absence of the immunity was responsible of the phenotypes observed for the
419 $\Delta tli3\Delta tle3$ strain (Fig. 6). Finally, the $\Delta tla3$ mutant has been used as a prey and was not affected
420 by the WT strain or any of the mutants excluding definitively a role of immunity as proposed
421 by its annotation (Pseudomonas.com) and confirming its adaptor function (Fig. 4B Sup).

422 Taken together these results demonstrated that Tle3 is an effective H2-T6SS-dependent
423 antibacterial toxin loaded onto the VgrG2b puncturing device via Tla3 and neutralized by Tli3
424 in resistant prey bacteria.

425

426 **VgrG2b is a trans-kingdom toxin**

427 A putative neutral zinc metallopeptidase domain has been predicted at the extreme C-terminus
428 of VgrG2b by Pukatzki and colleagues (Pukatzki et al., 2007) (Fig. 3A). This motif (Prosite
429 PS00142, PFAM04298) consists in a metal-binding consensus motif HExxH, the two histidine
430 residues being ligands of the catalytic Zn^{2+} and the glutamic acid residue involved in
431 nucleophilic attack. As an effector with a protease activity can target both eukaryotic and
432 bacterial proteins, we searched for an antibacterial activity of the VgrG2b C-terminal extension.
433 To do this we performed the same heterologous toxicity assay in *E. coli* as with Tle3. As shown
434 in figure 7, whereas the production of VgrG2b_{Cter} in the cytoplasm did not impact *E. coli* growth,
435 its periplasmic production killed *E. coli* (Fig. 7, compare line 2 and 5). Moreover substitution
436 of the histidine in position 935 and of the glutamic acid 936 for an alanine relieves VgrG2b_{Cter}
437 toxicity this showing that VgrG2b is a novel antibacterial protease active in the periplasm.

438 Finally because of their genetic proximity, we tested whether Tli3 can be the immunity
439 of VgrG2b_{Cter}. Indeed the coproduction of Tli3 counteracts VgrG2b_{Cter} toxicity whether it is
440 coproduced from a second plasmid (Fig. 7, line 6) or from the same transcript (Fig. 7, line 7).
441 In conclusion Tli3 serves two toxins and protects also from the antibacterial activity of VgrG2b.

442

443 **Discussion**

444 Here we report the existence of a novel pair of antibacterial effector and immunity of
445 the H2-T6SS of *P. aeruginosa*, Tle3 (PA0260) and Tli3 (PA0261), and we propose a
446 chronology of Tle3 secretion process that includes a cytoplasmic adaptor protein, Tla3 (PA0259)
447 to load the toxin onto the VgrG2b spike (a model is proposed in Fig. 8). Through heterologous
448 toxicity assay and bacterial competition, we show that Tle3 was toxic once delivered in the
449 periplasm of prey bacteria and that Tli3 can neutralize the toxin in this compartment.

450 Interestingly this led us to discover that VgrG2b that we previously recognized as an anti-
451 eukaryotic effector possesses an antibacterial activity as well.

452 The VgrG-recruitment of cargo effectors has been previously evidenced for several
453 antibacterial effectors among them two toxins of the Tle family, TseL (Tle2) of *V. cholerae*
454 (Dong et al., 2013; Liang et al., 2015; Unterweger et al., 2015) and Tle1 of EAEC (Flaughnatti
455 et al., 2016). In both cases the *tle* genes were just downstream the *vgrG* genes like the
456 organization of *vgrG2b* and *tle3* of *P. aeruginosa*. TseL and Tle1 have been shown to directly
457 bind a dedicated VgrG, VgrG3 and VgrG1 respectively (Dong et al., 2013; Flaughnatti et al.,
458 2016). The domain of interaction within EAEC VgrG1 has been delimited to the TTR domain
459 and may also include the DUF2345, both of which are present in the *P. aeruginosa* VgrG2b. In
460 line with this, and despite Tle3 requiring an adaptor to be targeted to VgrG2b, we have shown
461 that Tla3 interacts with the TTR domain of VgrG2b. Taken together these data demonstrate that
462 TTR domains of VgrGs are involved in recruitment and transport of Tle effectors, directly or
463 through adaptor. Likewise C-terminal extensions of VgrG1 and VgrG2 of *Agrobacterium*
464 *tumefaciens* were identified as specifically required for the delivery of each cognate DNase
465 toxins, named Tde1 and Tde2 respectively (Bondage et al., 2016). C-terminal domains of
466 VgrGs can thus be considered more generally as specificity determinants for T6SS effector
467 loading and transport.

468 Interestingly TseL of *V. cholerae* requires also Tap-1 (Tec) as an adaptor protein to be
469 delivered to another VgrG, called VgrG1 (Liang et al., 2015; Unterweger et al., 2015), showing
470 that a sole toxin can be targeted directly and indirectly to two different VgrG proteins. Tap-1
471 (Tec) belongs to the DUF4123 family of adaptor proteins that contains also VasW of *V.*
472 *cholerae* (Miyata et al., 2013) and several uncharacterized gene products linked to effector
473 genes with a MIX (marker for type VI effectors) motif in *Proteus mirabilis* or *B. thailandensis*
474 for instance (Salomon et al., 2014). Interestingly TecT, a DUF4123 adaptor of *P. aeruginosa*,

475 has been shown to require a co-adaptor, called co-TecT, to deliver the TseT effector to the
476 PAAR4 protein (Burkinshaw et al., 2018). This is the first example of an adaptor-co-adaptor
477 module. Taken together these data suggest a conserved role for DUF4123 adaptors in the
478 recruitment of a number of T6SS effectors. Remarkably Tla3 of *P. aeruginosa* does not belong
479 to the DUF4123 adaptor family, or to that of the two other unrelated families, the DUF1795
480 adaptor family, reported with EagR (effector-associated gene with Rhs) in *Serratia marcescens*
481 (Alcoforado Diniz and Coulthurst, 2015) or EagT6 in *P. aeruginosa* (Whitney et al., 2015), and
482 the DUF2169 adaptor family reported with Atu3641 in *A. tumefaciens* (Bondage et al., 2016).
483 Nor Tla3 is a PAAR protein, the last class of effector targeting mode to a VgrG (Bondage et
484 al., 2016; Burkinshaw et al., 2018; Cianfanelli et al., 2016a; Shneider et al., 2013; Whitney et
485 al., 2014). Instead we find that Tla3 harbors two DUF2875 domains (Fig. 3A) that are both
486 required for the interaction with the toxin. Moreover genes coding DUF2875 containing
487 proteins can be find at the vicinity of *tle*, *tli*, *vgrG* or PAAR genes, but they are restricted to α
488 and β proteobacteria (Fig. Sup 5). We thus hypothesize that DUF2875 might assist in T6SS-
489 mediated effector delivery. Like the three other adaptor families (DUF4123, DUF1795,
490 DUF2169), we have observed that Tla3 is not required for the H2-T6SS functionality since the
491 $\Delta tla3$ mutant still secrete Hcp2b and can compete with a WT strain, and that Tla3 is specific
492 for the Tle3 toxin since it did not interact with another H2-T6SS effector, TplE (Tle4). Finally
493 as we did not detect Tla3 secretion under constitutive H2-T6SS condition, we propose that Tla3
494 hands over Tle3 to VgrG2b in the cytoplasm prior to its loading to the baseplate and further
495 recruitment to the central Hcp tube in preparation (Fig. 8). Overall, the existence of various
496 modes of effector recruitment, further refined with adaptors, likely explains how the T6SS is
497 able to deliver numerous and structurally diverse proteins.

498 Five families of Tle, Tle1-5, have been identified among Gram-negative bacteria
499 (Russell et al., 2013) and four Tle have been studied in *P. aeruginosa* so far. Our demonstration

500 of the activity of Tle3 in the periplasm is consistent with the observations that the heterologous
501 periplasmic production of PldA (Tle5a) (Jiang et al., 2014; Russell et al., 2013), PldB (Tle5b)
502 (Jiang et al., 2014), Tle1 (Hu et al., 2014) and TplE (Tle4) (Jiang et al., 2016) is toxic. The
503 reason of the periplasmic activity of Tle proteins is still unclear although several hypothesis
504 have been proposed (Flaughnatti et al., 2016), the most likely being an activation of the toxin in
505 this compartment. Very recently this has nicely been exemplified with the hijacking of DsbA
506 in the target cells of *Serratia macescens* for the activation of incoming effectors (Mariano et al.,
507 2018). No member of the Tle3 family has yet been enzymatically characterized. Our attempts
508 to efficiently purify Tle3 from *E. coli* or from *P. aeruginosa* have been unsuccessful, even if
509 we have noticed that the presence of the Tla3 adaptor stabilized Tle3, it still formed inclusion
510 bodies. In the future we will decipher the enzymatic activity of Tle3, which is presumably active
511 on membrane phospholipids as our preliminary data of thin-layer chromatography tend to show.

512 The periplasmic activity of Tle toxins is counteracted by the synthesis of a cognate
513 immunity protein that is usually a periplasmic soluble protein, as we showed for Tli3 in *P.*
514 *aeruginosa*, or a membrane-anchored lipoprotein (Russell et al., 2013). Interestingly the genetic
515 organization of the *tli3* gene upstream of the *tle3* gene observed in *E. coli*, *K. pneumoniae*, *B.*
516 *cenocepacia* or *R. solanacearum* (Russell et al., 2013) is conserved in *P. aeruginosa*. The fact
517 that the two genes are co-transcribed (the immunity being the first) is key for the protection
518 against toxicity. Indeed we have observed systematic protection against the periplasmic toxicity
519 of Tle3 in *E. coli* when the two genes were expressed from the same promoter under the same
520 plasmid whereas it was not as efficient when the genes were on two plasmids. This genetic link
521 reinforces the connection within the toxin/immunity pair. This has previously been noticed with
522 a T7SS antibacterial toxin and its immunity in *Staphylococcus aureus* (Cao et al., 2016). Other
523 immunities of Tle characterized so far have been shown to inhibit the action of the effector by
524 direct protein-protein contacts (Flaughnatti et al., 2016; Jiang et al., 2014; Russell et al., 2013).

525 Our copurification assay in *E. coli* demonstrates a direct interaction between Tle3 and Tli3 that
526 was already suggested with the release of Tle3 toxicity upon coproduction of Tli3 in the
527 periplasm. A crystal structure of the *P. aeruginosa* TplE (Tle4) effector in complex with its
528 immunity protein TplEI (Tli4) revealed that the immunity uses a grasp mechanism to prevent
529 the interfacial activation of the toxin (Lu et al., 2014).

530 Like two other H2-T6SS related orphan *vgrG* loci, the *vgrG4b* cluster encoding PldA
531 (Tle5a) and the *vgrG2a* encoding TplE (Tle4), we show here that the *vgrG2b* cluster has both
532 antibacterial activities (through Tle3 and VgrG2b) and anti-eukaryotic (through VgrG2b; (Sana
533 et al., 2015)). Interestingly VgrG2b is thus (i) a structural component of the H2-T6SS
534 puncturing device since our bacterial competition showed its requirement for Tle3 delivery, (ii)
535 an anti-eukaryotic effector through an interaction with the microtubule nucleating complex
536 (Sana et al., 2015) and (iii) an antibacterial effector as suggested by our toxicity assay in *E. coli*.
537 We have shown that two conserved residues of the putative metallopeptidase motif (an histidine
538 and a glutamic acid) are essential for the VgrG2b antibacterial activity. This is consistent with
539 an antibacterial protease activity for VgrG2b that will be, to our knowledge, the first case in the
540 T6SS effector literature. The discovery of the VgrG2b immunity is even more exciting since
541 we observed that Tli3 also relieves VgrG2b_{Cter} toxicity. To our knowledge, Tli3 is the first
542 example of an T6SS immunity serving two different proteins. But one can cite the chaperones
543 of the type III secretion translocators that form a pore in the eukaryotic target cell membrane
544 (Neyt and Cornelis, 1999). Indeed the chaperone SycD from *Yersinia enterocolitica* and its
545 homologues from other Gram negative bacteria bind the two translocators to prevent their
546 premature interaction in the inner membrane and avoid further toxicity.

547

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557

558 **Author contributions statement**

559 B.B. and S.B. designed and conceived the experiments. B.B., C.S., and S.D. performed
560 the experiments. S.B. supervised the execution of the experiments. B.B., C.S., B.I. and S.B.
561 analyzed and discussed the data. S.B. wrote the paper with contribution from B.B. and reading
562 from B.I.

563

564 **Conflict of Interest Statement**

565 The authors declare no conflict of interest.

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In review

711 **Figure legends**

712 **Fig. 1: *vgrG2b* island organization (A)** The genes are labeled with the given name (i.e., *hcp2b*)
 713 and are indicated by their annotation number (e.g., PA0263). The Sec signal peptide of Tli3
 714 (PA0261) and the two DU2875 of Tla3 (PA0259) are represented with striped boxes. **The Tle3**
 715 **periplasmic toxicity is counteracted by Tli3 (PA0261) (B).** Serial dilutions (from non-diluted
 716 to 10^{-7}) of normalized cultures of *E. coli* BL21(DE3)pLysS producing the wild-type Tle3 in
 717 the cytoplasm, called Tle3^c (from pVT1, a pETDuet-1 derivate) or in the periplasm, called Tle3^p
 718 (from pSBC81, a pET22b(+) derivate yielding a fusion of Tle3 with a Sec signal peptide) were
 719 spotted on LB agar plates supplemented (left panel) with 0.4% glucose or (right panel) with
 720 0.1mM IPTG. Glucose and IPTG allow respectively repression and induction of the gene
 721 encoding the T7 RNA polymerase. When indicated Tli3 (PA0261) or Tla3 (PA0259) were
 722 produced in the periplasm from pVT8, pSBC107 and pVT9 respectively, pRSFDuet-1 derivatives.
 723 Line 1: pET22b(+) and pRSFDuet-1, line 2: pVT1 and pRSFDuet-1, line 3: pSBC81 and
 724 pRSFDuet-1, line 4: pSBC81 and pVT8, line 5: pSBC81 and pVT9, line 6: pVT8 and
 725 pET22b(+), line 7: pVT9 and pET22b(+) line 8: pSBC107 is a pRSFDuet-1 derivate producing
 726 Tli3 (PA0261) and Tle3^p from the same transcript.

727

728 **Fig. 2 : Tli3 (PA0261) interacts with Tle3 (A)** Copurification assay on StrepTactin column of
 729 Tli3cSTREP with Tle3c10HIS produced in *E. coli* BL21(DE3)pLysS produced from pBB26
 730 and pVT1 respectively. The unbound (U) and eluted (E) fractions were collected and subjected
 731 to SDS-PAGE (10.5 %) and Western blot analyses using anti-His antibody (Upper) and anti-
 732 streptavidin antibody (Lower). The position of the proteins and the molecular mass markers (in
 733 kDa) are indicated. **Tli3 (PA0261) is a periplasmic protein in *P. aeruginosa* (B)** Cells of *P.*
 734 *aeruginosa* PAO1 tli3V5 were subjected to fractionation and immunoblotting using antibodies
 735 directed against the V5 tag, XcpY and DsbA. XcpY and DsbA were used as membrane and

736 periplasmic controls respectively. T: whole cell, C: cytoplasm, Mb: total membrane, P:
737 periplasm. The position of the proteins and the molecular mass markers (in kDa) are indicated.

738

739 **Fig. 3: Tla3 (PA0259) interacts with the Tle3 toxin and with VgrG2b. Domain**
740 **organization of VgrG2b, Tle3 and Tla3 (PA0259) (A).** The first 581 residues of VgrG2b
741 carry the VgrG domain homologous to gp27 and gp5 phage tail proteins and consisting of three
742 sub-domains. This is followed by the C-terminal extension, composed of a conserved domain
743 of uncharacterized proteins (DUF2345, PF10106) (2bCt1), a TTR (transthyretin-like region)
744 (2bCt2), and a putative zinc-dependent metallopeptidase pattern (LFIHEMTHVW signature,
745 PS00142 (2bCt3). Tle3 architecture consists of an α β hydrolase fold domain (Tle3_{D1}) followed
746 by a DUF3274 (Tle3_{D2}), Tla3 of a tandem of DUF2875 (Tla3_{D1} and Tla3_{D2}). **Bacterial two-**
747 **hybrid assay (B, C and D).** BTH101 reporter cells producing the indicated proteins or domains
748 fused to the T18 or T25 domain of the *Bordetella* adenylate cyclase were spotted on X-gal
749 indicator plates. The blue color of the colony reflects the interaction between the two proteins.
750 TolB and Pal are two proteins known to interact but unrelated to the T6SS. The experiment was
751 performed in triplicate and a representative result is shown.

752

753 **Fig. 4: Tla3 interaction with Tle3 (A) and VgrG2b (B-D)** Copurification assay on
754 StrepTactin column of Tla3^c_{STREP} with Tle3^c_{His} produced *in E. coli* BL21(DE3)pLysS from
755 pBB27 and pVT1 respectively (A) or of Tla3^c_{His} with VgrG2b^{FL}_{STREP} (B) or VgrG2b^{FLD3}_{STREP}
756 (C) or VgrG2b^{FLD2-3}_{STREP} (D) produced from pBB28 and pBB25 (B) or pBB41 (C) or pBB42 (D).
757 The unbound (U) and eluted (E) fractions were collected and subjected to SDS-PAGE (10.5%)
758 and Western blot analysis using anti-His antibody (Upper) and anti-streptavidin antibody
759 (Lower). The dashed line separates lanes from non-adjacent part of the same gel. The position
760 of the proteins and the molecular mass markers (in kDa) are indicated.

761

762 **Fig. 5: Tla3(PA0259) is a cytoplasmic protein of *P. aeruginosa* (A)** Cells of *P. aeruginosa*
 763 PAO1 *tla3_{V5}* were subjected to fractionation and immunoblotting using antibodies directed
 764 against the V5 tag, XcpY and DsbA. XcpY and DsbA were used as membrane and periplasmic
 765 controls respectively. T: whole cell, C: cytoplasm, Mb: total membrane, P: periplasm. **The**
 766 **annotated ATG drives the initiation of translation of *tla3* in *P. aeruginosa* (B).**
 767 Immunodetection of Tla3_{V5} with anti-V5 antibodies produced in a WT background or in strains
 768 in which one of the four predicted ATG in *tla3* have been substituted in ATA. The number
 769 followed by a star indicates which ATG from Fig. 1Supp., ATG₄ corresponding to the annotated
 770 ATG. **Tla3 is not required for Hcp2b secretion (C).** Immunodetection of Hcp2b_{6His} with anti-
 771 His antibodies produced in a WT background (line 1) or in strains deleted for *rsmA* (lines 2 to
 772 4) and *clpV2* (line 3) or *tla3* (line 4). The strains were grown at 25°C for 24 h and total bacteria
 773 were separated from extracellular medium. Anti-EF-Tu is used as a lysis control. The
 774 extracellular medium proteins were also stained with Coomassie-blue. **Tla3 doesn't interact**
 775 **with Tle4 (D).** Copurification assay on StrepTactin column of Tla3^c_{STREP} with Tle4_{His} produced
 776 in *E. coli* BL21(DE3)pLysS from pBB27 and pVT3 respectively. Legend as in Fig. 4. **Tla3 is**
 777 **not secreted (E).** Immunodetection of Tla3_{V5} with anti-HV5 antibodies produced in a WT
 778 background (line 1) or in strain deleted for *rsmA* (line 2). The strains were grown at 25°C for
 779 24 h and total bacteria were separated from extracellular medium. Anti-EF-Tu is used as a lysis
 780 control. The extracellular medium proteins were also stained with Coomassie-blue. (A-E) The
 781 position of the proteins and the molecular mass markers (in kDa) are indicated.

782 **Fig. 6: *P. aeruginosa* growth competition.** The *P. aeruginosa* prey strain ($\Delta tli3\Delta tle3$) was
 783 incubated with various *P. aeruginosa* attacker strains as indicated in the figure for 24 h at 37°C.
 784 The number of recovered prey bacteria is indicated in CFU/ml. “comp” stand for *cis*
 785 complementation of the corresponding mutation with a wild-type copy inserted at the *attB* site

786 on *P. aeruginosa* chromosome. Error bars represent \pm SEM (n = 3); ***p < 0.001, ns not
787 significant.

788

789 **Fig. 7: The protease domain of VgrG2b is required for its antibacterial activity.** Serial
790 dilutions (from non-diluted to 10^{-5}) of normalized cultures of *E. coli* BL21(DE3)pLys
791 producing the wild-type C-terminal domain of VgrG2b in the cytoplasm, called CterVgrG2b^C
792 (from pBB43, a pETDuet-1 derivate, line 5) or in the periplasm, called CterVgrG2b^P (from
793 pBB44, a pET22b(+) derivate yielding a fusion of the C-terminal domain with a Sec signal
794 peptide, line 2) or two variants CterVgr2b^P_{H935A} (from pBB45, line 3) and CterVgr2b^P_{E936A}
795 (from pBB46, line 4) were spotted on LB agar plates supplemented (left panel) with 0.4%
796 glucose or (right panel) with 0.1mM IPTG. In line 6, the CterVgrG2b^P (from pBB44) was
797 coproduced with Tli3 (from pVT8) and in line 7, the CterVgrG2b^P and Tli3 were coproduced
798 from the same transcript (from pSBC110). Glucose and IPTG allow respectively repression and
799 induction of the gene encoding the T7 RNA polymerase.

800

801 **Fig. 8: Working model: Tle3 targeting to the H2-T6SS machinery (left panel)** Tle3 (in pink)
802 is taken in charge in the cytoplasm by Tla3 (in orange) that binds VgrG2b (green). Upon Hcp2b
803 (in dark grey) assembly into the growing sheath (light grey), the VgrG2b loaded with the Tle3
804 is placed at the tip of the Hcp arrow. **Tle3 and VgrG2b_{Cter} delivery into bacteria (right panel).**
805 The sheath contraction in the cytoplasm propels the Hcp arrow towards the target bacterium.
806 Tle3 associated with this expelled structure is thus translocated into target cells, as well as
807 VgrG2b_{Cter} (green circle). The prey is killed (Prey^S) except if it has the Tli3 immunity (in blue)
808 in the periplasm (Prey^R). The mechanism of resistance to VgrG2b_{Cter} is still unknown. OM:
809 outer membrane, PG: peptidoglycan, IM: inner membrane, BP: base plate, MC: membrane
810 complex. Prey^S: sensitive prey, Prey^R: resistant prey.

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In review

Figure 1

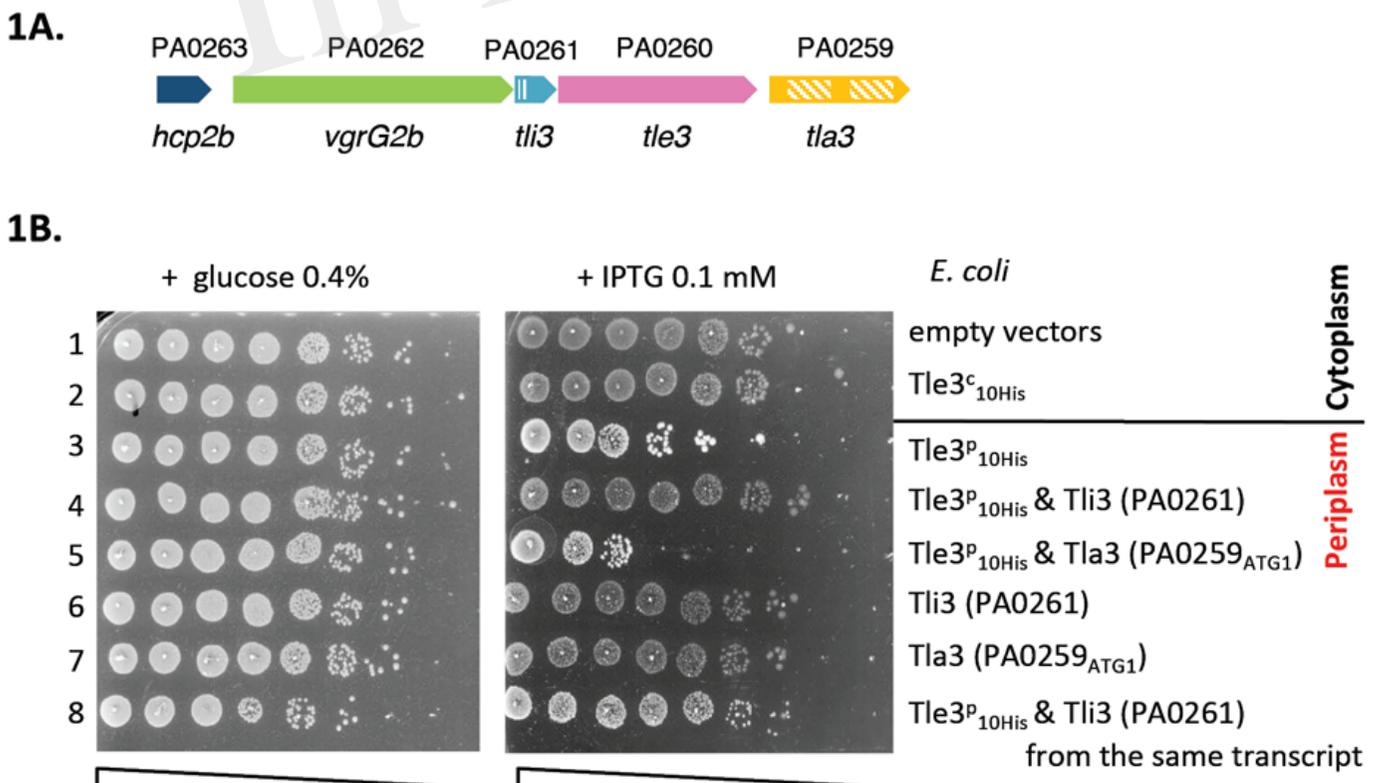


Figure 2

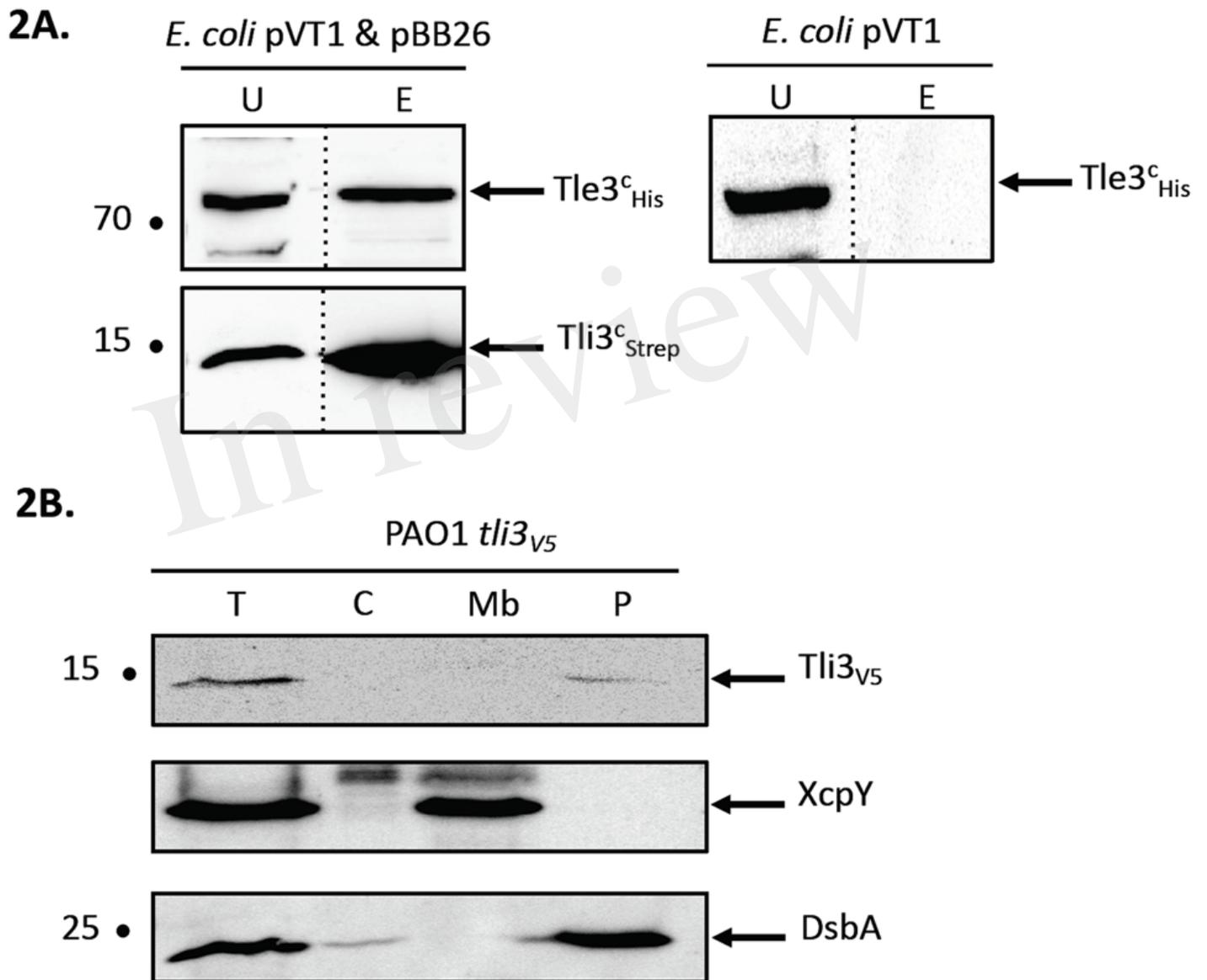


Figure 3

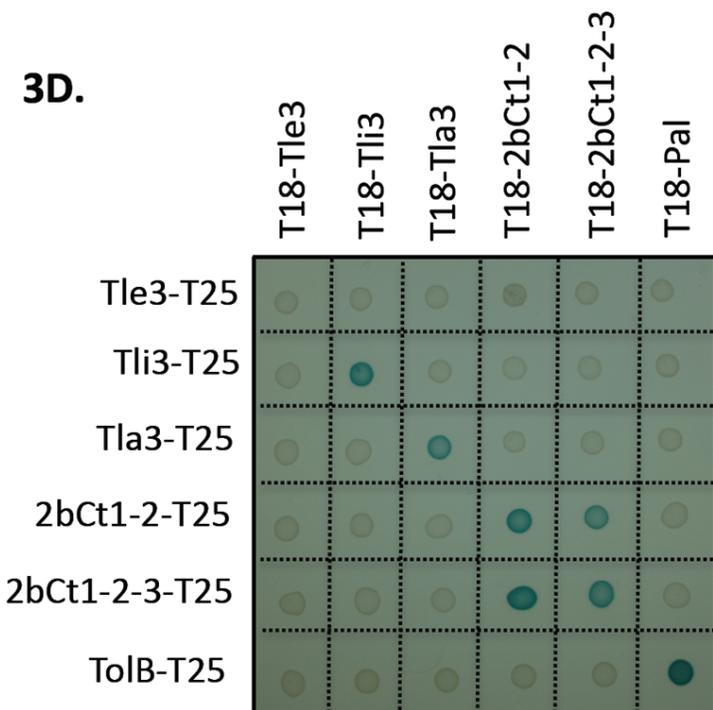
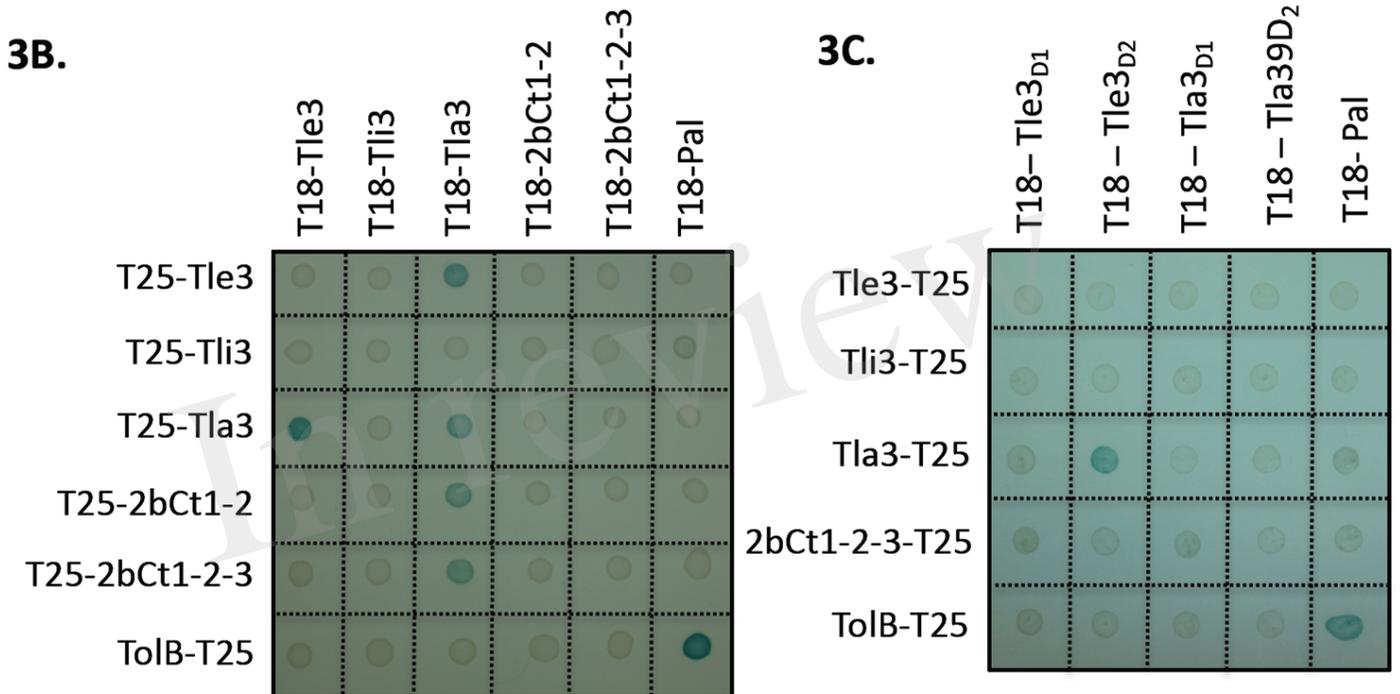
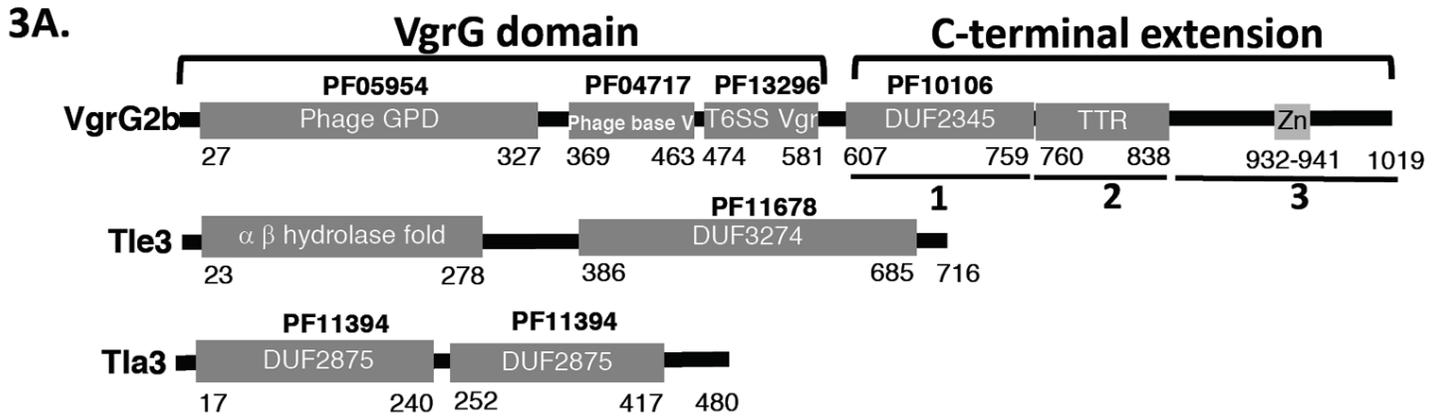
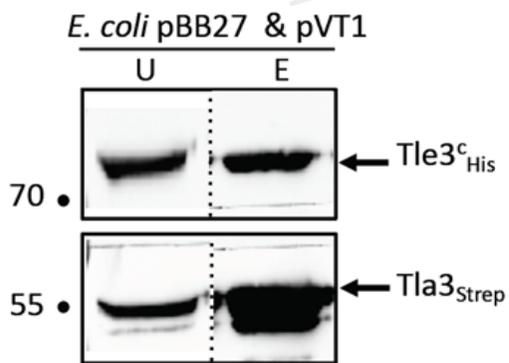
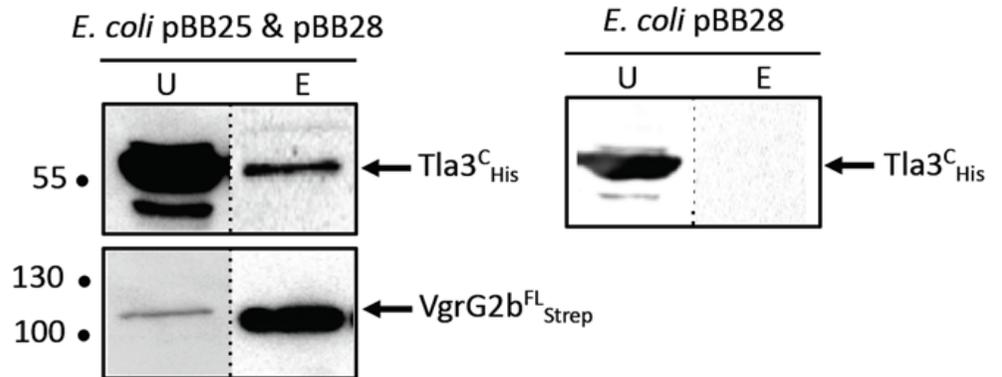


Figure 4

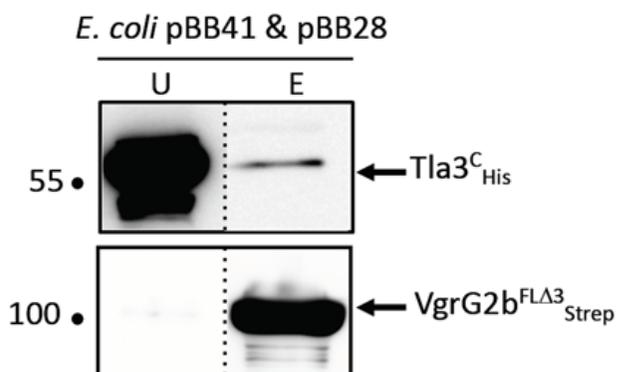
4A.



4B.



4C.



4D.

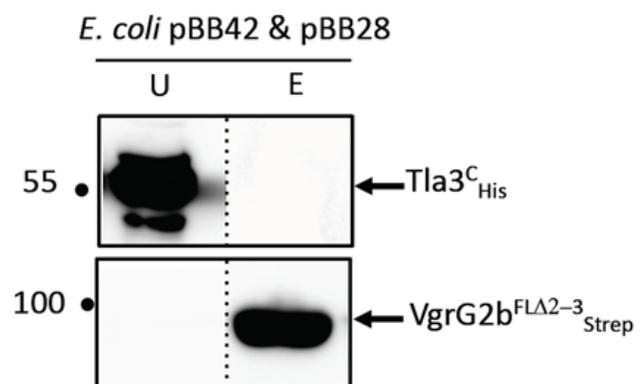


Figure 5

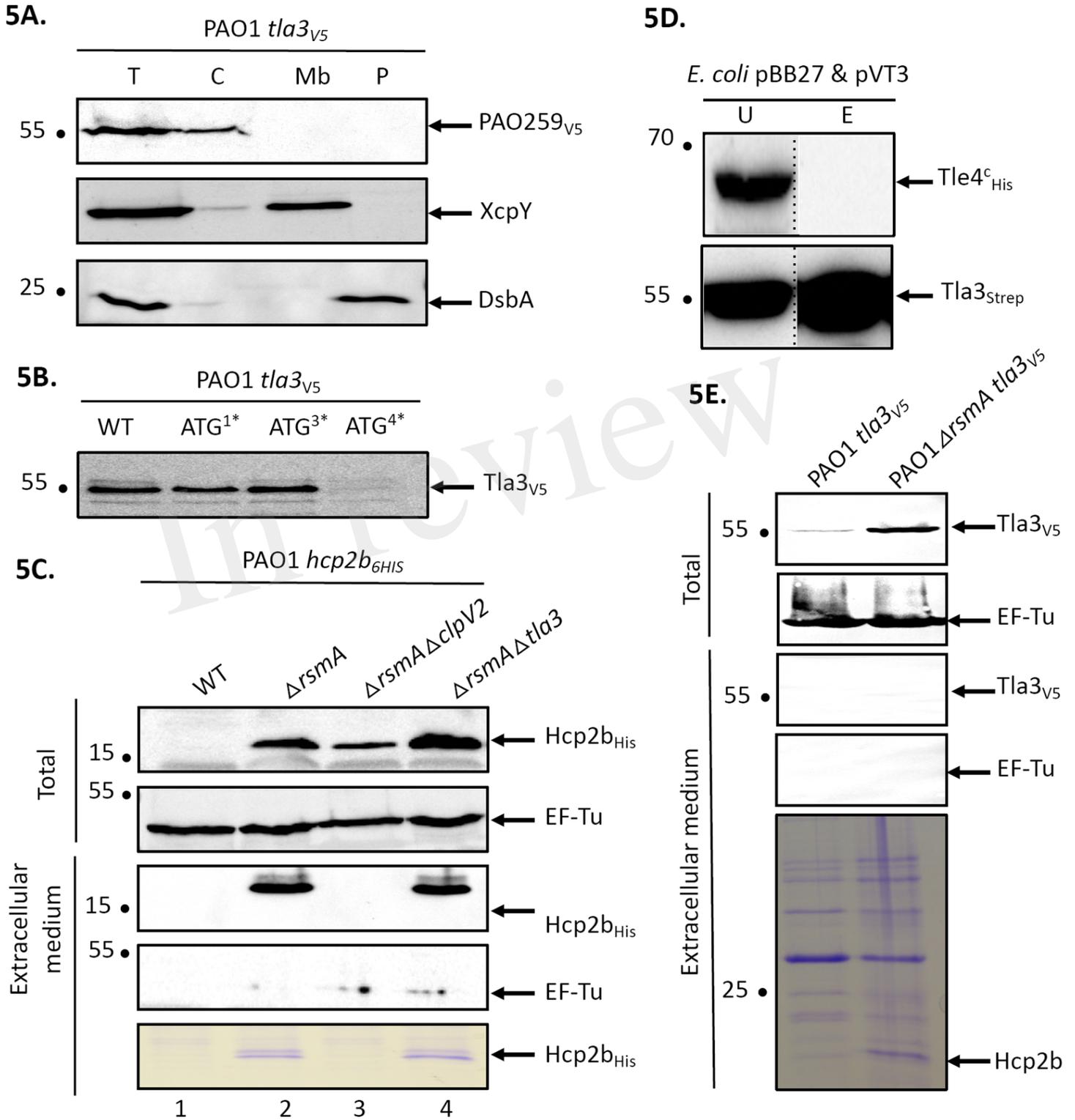


Figure 6

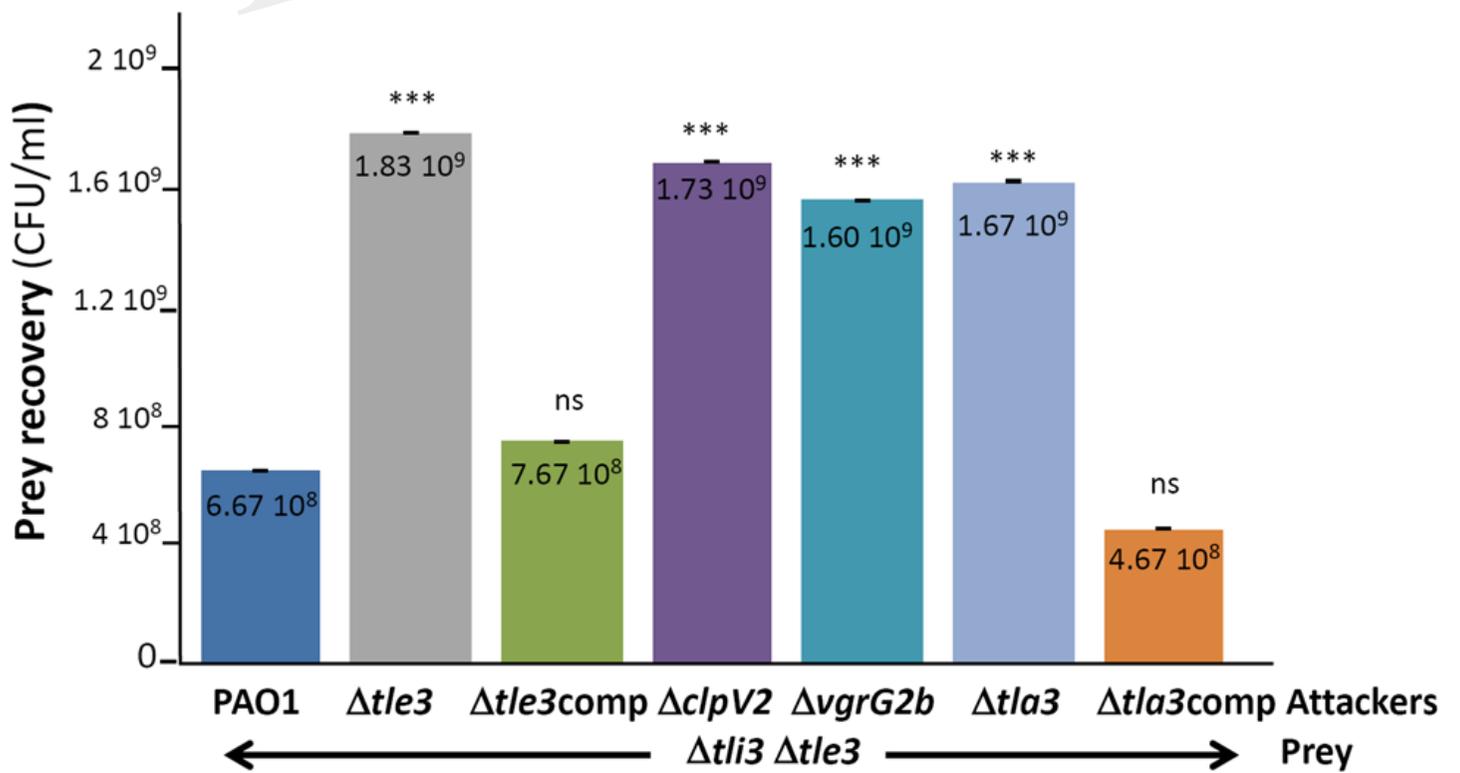


Figure 7

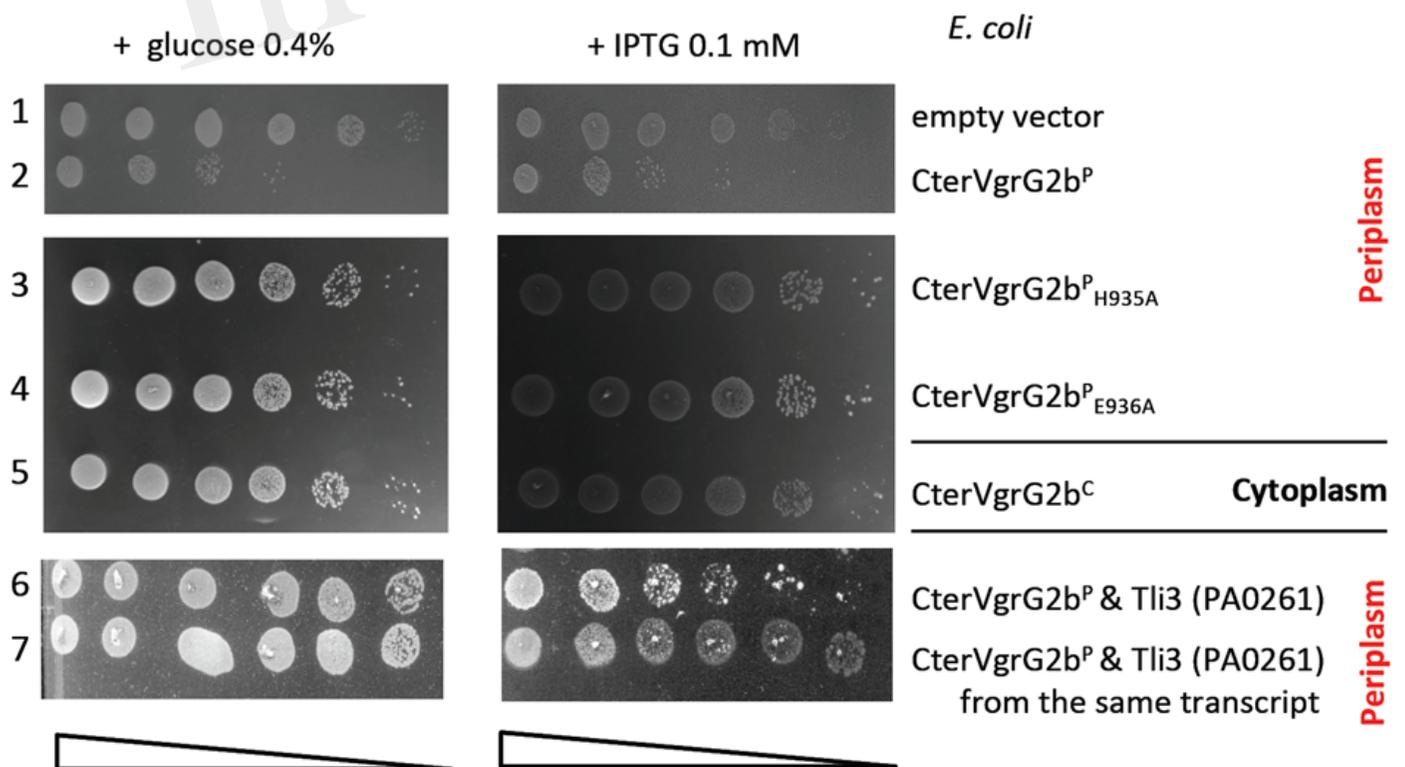


Figure 8

