

1           **Antibacterial T6SS effectors with a VRR-Nuc domain induce target cell**  
2           **death via DNA Double-Strand Breaks**

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16           Running title: The antibacterial SPI-22 T6SS uses VRR-Nuc-containing effectors.

17

18

19           **Abstract**

20           The T6SS (Type VI secretion System) secretes antibacterial effectors into target  
21 competitors. *Salmonella* spp. encode five phylogenetically distinct T6SSs. Here we  
22 characterize the function of the SPI-22 T6SS of *S. bongori*, showing that it has  
23 antibacterial activity. We identify a group of antibacterial T6SS effectors (TseV1-4)  
24 containing an N-terminal PAAR-like domain and a C-terminal VRR-Nuc domain  
25 encoded next to cognate immunity proteins that contain the DUF3396 domain (TsiV1-4).  
26 TseV2 and TseV3 are toxic when expressed in *Escherichia coli* and bacterial competition  
27 assays confirm that TseV2 and TseV3 are secreted by the SPI-22 T6SS. Phylogenetic  
28 analysis reveals that TseV1-4 are evolutionarily related to enzymes involved in DNA  
29 repair. TseV2 and TseV3 maintained the ability to bind DNA, but instead cause specific  
30 DNA double-strand breaks and induce the SOS response in target cells. The crystal  
31 structure of the TseV3:TsiV3 complex reveals that the immunity protein likely blocks the  
32 effector interaction with the DNA substrate. These results expand our knowledge on the  
33 function of *Salmonella* pathogenicity islands, the evolution of toxins used in biological  
34 conflicts, and the endogenous mechanism regulating the activity of these toxins.

35   **Keywords:** DUF3396/Effectors/SPI-22/T6SS/VRR-Nuc.

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37           **Introduction**

38           Bacteria use a series of antagonistic mechanisms to counteract competitors. These  
39 processes either require physical contact between attacker and target cells or function in  
40 a contact-independent manner via soluble molecules secreted into the medium (Peterson  
41 *et al*, 2020). The type VI secretion system (T6SS) is a multi-protein contractile  
42 nanomachine evolutionarily related to bacteriophages (Leiman *et al*, 2009). This system  
43 is widespread in Gram-negative bacteria and secretes toxic effectors into target cells in a  
44 contact-dependent manner (Coulthurst, 2019). The T6SS is composed of three major  
45 complexes: the membrane complex, the baseplate and the tail (Nguyen *et al*, 2018). The  
46 tail has a spear-like shape and is propelled against target cells upon a contraction event  
47 (Wang *et al*, 2017; Salih *et al*, 2018). The tail tube is composed of hexameric rings of  
48 Hcp (hemolysin co-regulated protein) capped with a spike composed of a trimer of VgrG  
49 (valine-glycine repeat protein G) and a PAAR protein (proline-alanine-alanine-arginine  
50 repeats) (Mougous *et al*, 2006; Shneider *et al*, 2013; Renault *et al*, 2018). The effectors  
51 secreted via T6SSs associate with Hcp, VgrG or PAAR either directly or indirectly via  
52 adaptor proteins (cargo effectors). In addition, so-called evolved effectors are fused to the  
53 C-terminus of Hcp, VgrG or PAAR (Cianfanelli *et al*, 2016; Jana & Salomon, 2019).  
54 Several isoforms of VgrG, Hcp and PAAR proteins can be encoded in the same bacterial  
55 genome, usually outside of the T6SS structural gene cluster (and are thus named orphan  
56 proteins). These Hcp, VgrG and PAAR proteins can assemble in different combinations  
57 to secrete specific subsets of effectors (Hachani *et al*, 2014; Bondage *et al*, 2016).

58           T6SSs effectors can target eukaryotic cells, prokaryotic cells or contribute to the  
59 acquisition of micronutrients (Coulthurst, 2019). The variety of targets is related to the  
60 diversity of biochemical activities of T6SS effectors, which can be nucleases,  
61 peptidoglycan hydrolases, lipases, NADases, pore-forming proteins or enzymes that post-

62 translationally modify target proteins (Jurėnas & Journet, 2021). Antibacterial effectors  
63 with nuclease activity are among the most potent weapons used by an attacker to  
64 intoxicate target cells. Several T6SS effectors with nuclease activity have been reported  
65 including, *Dickeya dadantii* RhsA-CT and RhsB-CT (Koskiniemi *et al.*, 2013);  
66 *Agrobacterium tumefaciens* Tde1 and Tde2 (Ma *et al.*, 2014); *Pseudomonas aeruginosa*  
67 PA0099 (Hachani *et al.*, 2014), TseT (Burkinshaw *et al.*, 2018) and Tse7 (Pissaridou,  
68 2018); *Serratia marcescens* Rhs2 (Alcoforado Diniz & Coulthurst, 2015); *Escherichia*  
69 *coli* Hcp-ET1, -ET3 and -ET4 (Ma *et al.*, 2017a), and Rhs-CT3, -CT4, -CT5, -CT6, -CT7  
70 and -CT8 (Ma *et al.*, 2017b); *Acinetobacter baumannii* Rhs2-CT (Fitzsimons *et al.*, 2018);  
71 *Vibrio parahaemolyticus* PoNe (Jana *et al.*, 2019); *Aeromonas dhakensis* TseI (Pei *et al.*,  
72 2021); and *Burkholderia gladioli* TseTBg (Yadav *et al.*, 2021).

73 The majority of the nuclease domains mentioned above have been previously  
74 predicted by a seminal *in silico* study using comparative genomics (Zhang *et al.*, 2012).  
75 Among those characterized are Ntox15 (PF15604) (Ma *et al.*, 2014); Ntox30 (PF15532),  
76 Ntox34 (PF15606) and Ntox44 (PF15607) (Ma *et al.*, 2017a); Tox-REase-1 (Jana *et al.*,  
77 2019); Tox-REase-3 (PF15647) (Ma *et al.*, 2017a); Tox-REase-5 (PF15648) (Burkinshaw  
78 *et al.*, 2018; Yadav *et al.*, 2021); Tox-GHH2 (PF15635) (Hachani *et al.*, 2014; Pissaridou,  
79 2018); HNH (PF01844) (Koskiniemi *et al.*, 2013; Alcoforado Diniz & Coulthurst, 2015;  
80 Ma *et al.*, 2017b); Tox-JAB-2 (Ma *et al.*, 2017a); AHH (PF14412) (Ma *et al.*, 2017a;  
81 Fitzsimons *et al.*, 2018); and Tox-HNH-EHHH (PF15657) (Pei *et al.*, 2021).

82 In *Salmonella* species, T6SSs are encoded in five distinct *Salmonella*  
83 pathogenicity islands (SPI-6, SPI-19, SPI-20, SPI-21 and SPI-22) acquired by different  
84 horizontal gene transfer events (Blondel *et al.*, 2009; Bao *et al.*, 2019). The *S. enterica*  
85 serovar Typhimurium SPI-6 T6SS is involved in competition with the host microbiota  
86 and gut colonization (Pezoa *et al.*, 2014; Brunet *et al.*, 2015; Sana *et al.*, 2016; Sibinelli-

87 Sousa *et al*, 2020); whereas the SPI-19 T6SS of *S. Gallinarium* is involved in survival  
88 within macrophages (Blondel *et al*, 2013; Schroll *et al*, 2019). So far, only two T6SS  
89 effectors have been characterized in *Salmonella* spp., both targeting peptidoglycan: Tae4  
90 (type VI amidase effector 4) is a gamma-glutamyl-D,L-endopeptidases that cleaves  
91 between D-iGlu<sup>2</sup> and mDAP<sup>3</sup> within the same peptide stem (Russell *et al*, 2012; Benz *et*  
92 *al*, 2013; Zhang *et al*, 2013); and Tlde1 (type VI L,D-transpeptidase effector 1), which  
93 exhibits both L,D-carboxypeptidase and L,D-transpeptidase D-amino acid exchange  
94 activity, cleaving between mDAP<sup>3</sup> and D-Ala<sup>4</sup> of the acceptor tetrapeptide stem or  
95 replacing the D-Ala<sup>4</sup> by a noncanonical D-amino acid, respectively (Sibinelli-Sousa *et*  
96 *al.*, 2020).

97       Herein we report the characterization of the SPI-22 T6SS of *S. bongori*, which  
98 displays antibacterial activity. We characterize a group of antibacterial effectors secreted  
99 by this system that contain a VRR-Nuc (virus-type replication-repair nuclease) domain  
100 (Kinch *et al*, 2005; Iyer *et al*, 2006), named type VI effector VRR-Nuc 1-4 (TseV1-4).  
101 These effectors are encoded next to DUF3396-containing proteins, which function as  
102 immunity proteins (TsiV1-4) that are specific to each effector. Phylogenetic analysis  
103 revealed that TseVs effectors form a clade together with other antibacterial effectors  
104 belonging to the PD-(D/E)xK phosphodiesterase superfamily. This toxic clade is  
105 phylogenetically related to enzymes containing the VRR-Nuc domain conventionally  
106 involved in DNA repair and metabolism. We confirm that TseV2 and TseV3 display  
107 DNase activity, inducing the SOS response with resultant target cell death via DNA  
108 double-strand breaks. Our crystal structure of the TseV3:TsiV3 complex reveals that the  
109 immunity protein likely impairs effector toxicity by interacting with and occluding its  
110 DNA-binding site. Our results provide mechanistic knowledge about a new group of  
111 antibacterial toxins that co-opt the VRR-Nuc domain for a previously undescribed role in

112 bacterial antagonism, and further reveal the mode of neutralization via specific immunity  
113 protein complexation.

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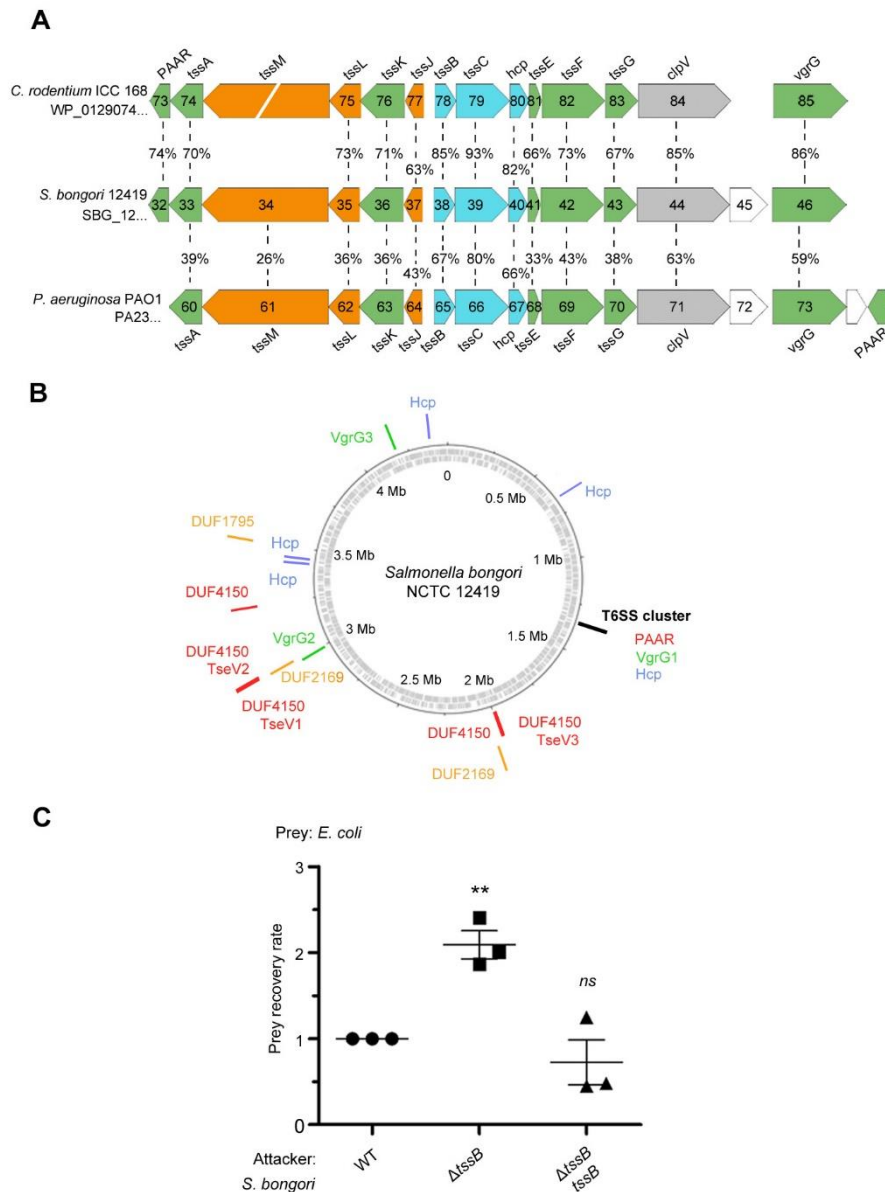
## 115 **Results**

### 116 **The SPI-22 T6SS of *S. bongori* has antibacterial activity**

117 The SPI-22 T6SS of *S. bongori* is phylogenetically related to the HSI-III (Hcp  
118 secretion island III) T6SS of *Pseudomonas aeruginosa* (amino acid similarity ranging  
119 from 26-80%), and the CTS2 (*Citrobacter rodentium* T6SS cluster 2) of *C. rodentium*  
120 (amino acid similarity ranging from 63-93%) (Petty *et al*, 2010; Fookes *et al*, 2011) (Fig.  
121 1A). Besides the structural T6SS components encoded within SPI-22, the genome of *S.*  
122 *bongori* NCTC 12419 encodes several orphan proteins comprising two VgrGs  
123 (SBG\_2715, SBG\_3770), four Hcps (SBG\_0599, SBG\_3120, SBG\_3143, SBG\_3925),  
124 three DUF4150/PAAR-like proteins (SBG\_1846, SBG\_2718, SBG\_2955), two adaptors  
125 containing DUF2169 (SBG\_1847, SBG\_2721), and one adaptor with DUF1795  
126 (SBG\_3173) (Fig. 1B).

127 To analyze whether *S. bongori* SPI-22 T6SS has *bona fide* antibacterial activity,  
128 we performed bacterial competition assays using the wild-type (WT) and T6SS null  
129 mutant ( $\Delta tssB$ /SBG\_1238) strains as attacker cells, and *Escherichia coli* K12 W3110 as  
130 prey. Results showed that the prey recovery rate was higher when co-incubation was  
131 performed with  $\Delta tssB$  compared to the WT (Fig. 1C). In addition, competition with a  
132  $\Delta tssB$  strain complemented with a plasmid expressing TssB restored the WT phenotype  
133 (Fig. 1C). These results show that the SPI-22 T6SS of *S. bongori* is active in the  
134 conditions tested and contributes to interbacterial antagonism, thus priming investigation  
135 to further characterize this activity.

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138 **Fig. 1. The *S. bongori* SPI-22 encodes an antibacterial T6SS.** (A) Comparison between the SPI-22 T6SS  
 139 of *S. bongori* with the systems of *C. rodentium* and *P. aeruginosa*. The T6SS proteins forming the three  
 140 subcomplexes are in colors: membrane components (orange), sheath and inner tube (blue), and baseplate  
 141 and spike components (green). (B) Representation of the circular genome of *S. bongori* with T6SS  
 142 components highlighted: the structural cluster is marked by a black line; VgrG proteins are represented by  
 143 green lines; Hcps are in blue; adaptor proteins are in orange; and PAAR or PAAR-like proteins are in red.  
 144 TseV1, TseV2 and TseV3 fused to PAAR-like domain are also in red. (C) Bacterial competition assays  
 145 between *S. bongori* WT,  $\Delta tssB$  and  $\Delta tssB$  complemented with pFPV25.1 *tssB* against *E. coli* in LB-agar  
 146 incubated for 24 h. The prey recovery rate was calculated by dividing the colony-forming unit (CFU) counts  
 147 of the output by the input. Data represent the mean  $\pm$  SD of three independent experiments performed in  
 148 duplicate and were analyzed through comparison with WT that were normalized to 1. One-way ANOVA  
 149 followed by Dunnett's multiple comparison test. \*\*p < 0.01 and ns not significant.

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151 **TseV2 and TseV3 are antibacterial SPI-22 T6SS effectors**

152 After verifying that the SPI-22 T6SS has antibacterial activity, we set out to  
153 identify the effectors contributing to the antagonistic effect. Initially, we performed *in*  
154 *silico* analysis using Bastion6 (Wang *et al.*, 2018) to evaluate several candidates (10 genes  
155 up- and downstream of all T6SS components) (Fig. 1B) for their probability of being a  
156 T6SS effector (cutoff score  $\geq 0.5$ ) (data not shown). Two candidates called our attention:  
157 SBG\_2718 (TseV1) and SBG\_2723 (TseV2), which contain an N-terminal PAAR-like  
158 domain and a C-terminal VRR-Nuc domain (Fig. 2A) (Kinch *et al.*, 2005; Iyer *et al.*,  
159 2006). Both putative effectors are encoded next to pairs of genes encoding DUF3396-  
160 containing proteins that resemble putative immunity proteins: SBG\_2719/TsiV1.1 and  
161 SBG\_2720/TsiV1.2, and SBG\_2724/TsiV2.1 and SBG\_2725/TsiV2.2 (Fig. 2A).  
162 Additional BLASTP searches in the genome of *S. bongori* identified two extra VRR-Nuc-  
163 containing proteins (SBG\_1841/TseV3 and SBG\_1828/TseV4), but only one of them  
164 encoding an N-terminal PAAR-like domain (SBG\_1841). Similarly, SBG\_1828 and  
165 SBG\_1841 are encoded upstream of a DUF3396-containing protein (SBG\_1829/TsiV4  
166 and SBG\_1842/TsiV3) (Fig. 2A).

167 To analyze whether these proteins comprise four effector-immunity pairs, we  
168 cloned these genes into compatible vectors under the control of different promoters. To  
169 evaluate the toxicity of TseV1-4 upon expression in *E. coli*, the C-terminal regions of  
170 TseV1-3 and the full-length TseV4 were cloned into the pBRA vector under the control  
171 of the P<sub>BAD</sub> promoter (inducible by L-arabinose and repressed by D-glucose). The  
172 putative immunity proteins were cloned into the pEXT22 vector under the control of the  
173 P<sub>TAC</sub> promoter, which is inducible by isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG). *E.*  
174 *coli* strains carrying different combinations of pBRA and pEXT22 were serially diluted  
175 and spotted onto LB agar plates containing either 0.2% D-glucose or 0.2% L-arabinose



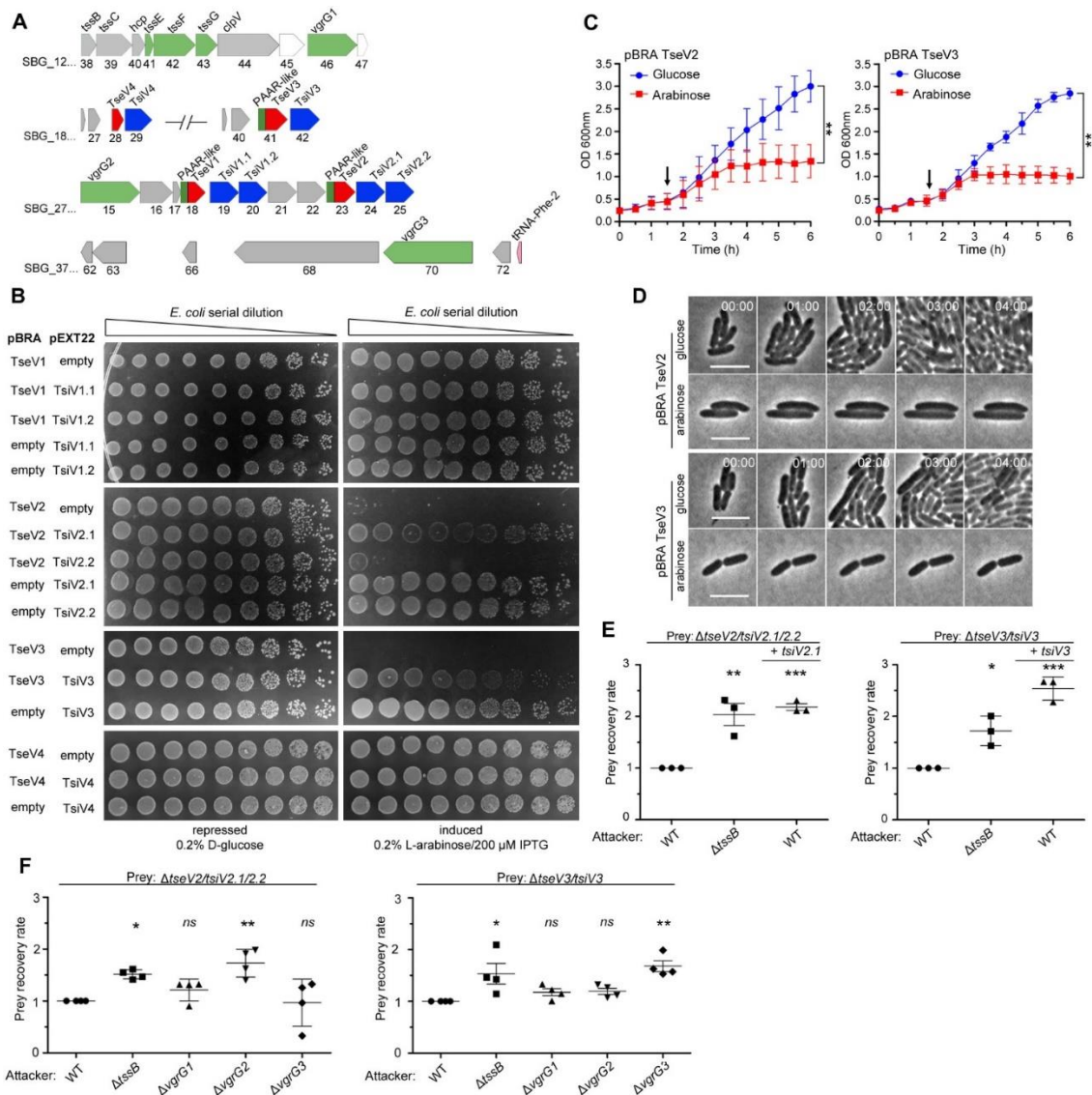
176 plus 200  $\mu$ M IPTG (Fig. 2B). Results showed that TseV2 and TseV3 are toxic in the  
177 cytoplasm of *E. coli*, whereas TseV1 and TseV4 do not confer toxicity (Fig. 2B). Co-  
178 expression of TseV2 with either TsiV2.1 or TsiV2.2 revealed that only the first immunity  
179 protein neutralizes TseV2 toxicity (Fig. 2B). Similarly, the toxic effect of TseV3 can be  
180 neutralized by co-expression with TsiV3 (Fig. 2B). Co-expression of TseV2 and TseV3  
181 with all combinations of immunity proteins (TsiV1.1, TsiV1.2, TsiV2.1, TsiV2.2, TsiV3  
182 and TsiV4) revealed that the effectors are neutralized only by the specific cognate  
183 immunity protein (Fig. S1). The effect of TseV2 and TseV3 on cell growth was also  
184 analyzed in liquid media by measuring the OD<sub>600nm</sub> of *E. coli* carrying pBRA TseV2 or  
185 TseV3 (Fig. 2C). Under these conditions, bacteria grew normally in media containing D-  
186 glucose; but once L-arabinose was added, the culture stopped growing, and the OD<sub>600nm</sub>  
187 stabilized (Fig. 2C).

188 We performed time-lapse microscopy to evaluate growth and morphology of  
189 individual *E. coli* cells harboring pBRA TseV2 or TseV3. Bacteria grew normally when  
190 incubated in LB agar pads containing 0.2% D-glucose (repressed) over a time frame of 8  
191 h (Fig. 2D, Movies S1, S3). However, in the presence of 0.2% L-arabinose (induced)  
192 bacteria did not grow and remained mostly morphologically unaltered – displaying a  
193 modest increase in cell length (Fig. 2D, Movies S2, S4).

194 To verify whether TseV2 and TseV3 are SPI-22 T6SS substrates, we performed  
195 bacterial competition assays using *S. bongori* WT and  $\Delta$ tssB (attacker) versus *S. bongori*  
196 lacking either TsiV2.1/2.2 ( $\Delta$ tseV2/tsiV2.1/2.2) or TsiV3 ( $\Delta$ tseV3/tsiV3) as prey (Fig. 2E).  
197 Results demonstrated that the prey recovery rate was higher when prey cells were co-  
198 incubated with  $\Delta$ tssB compared to WT (Fig. 2E). Complementation of preys with a  
199 plasmid encoding either TsiV2.1 or TsiV3 increased the prey recovery rate, showing that

200 prey became immune to the TseV2 and TseV3-induced toxicity (Fig. 2E). These results  
201 confirm that TseV2 and TseV3 are antibacterial effectors secreted by the SPI-22 T6SS.

202 As TseV2 and TseV3 contain an N-terminal PAAR-like domain, which interacts  
203 with VgrG during T6SS assembly and effector secretion (Shneider *et al.*, 2013), we  
204 decided to determine which of the three VgrG proteins encoded in *S. bongori* genome  
205 (Fig. 1B, 2A) were responsible for the secretion of TseV2 and TseV3. To shed light on  
206 this matter, we performed bacterial competition assays using *S. bongori* WT,  $\Delta tssB$ ,  
207  $\Delta vgrG1$  (SBG\_1246),  $\Delta vgrG2$  (SBG\_2715) or  $\Delta vgrG3$  (SBG\_3770) (attacker) versus  
208  $\Delta tseV2/tsiV2.1/2.2$  or  $\Delta tseV3/tsiV3$  (prey) (Fig. 2F). The prey recovery rate of  
209  $\Delta tseV2/tsiV2.1/2.2$  increased when this strain was co-incubated with  $\Delta vgrG2$ , suggesting  
210 that VgrG2 is responsible for secreting TseV2 into target cells (Fig. 2F). Conversely, the  
211 prey recovery rate of  $\Delta tseV3/tsiV3$  increased when this strain was co-incubated with  
212  $\Delta vgrG3$ , suggesting that VgrG3 is responsible for secreting TseV3 into target cells (Fig.  
213 2F). VgrG2 and VgrG3 are 96.9% identical in their N-terminal region (VgrG2<sub>1-565</sub> and  
214 VgrG3<sub>1-545</sub>) but display a distinct C-terminal domain with only 26% identity (VgrG2<sub>566-</sub>  
215 <sub>709</sub> and VgrG3<sub>546-728</sub>) (Fig. S2), thus suggesting that this region is responsible for cargo  
216 selection (Liang *et al.*, 2021). Together, these results show that each effector has its own  
217 mechanism of secretion, which is dependent on distinct VgrGs.



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**Fig. 2. TseV2 and TseV3 are antibacterial SPI-22 T6SS effectors.** (A) Scheme of the genomic region containing VgrGs and TseV/TsiV effector/immunity pairs. VRR-Nuc domain (red), PAAR-like domain (dark green), VgrG (light green), and DUF3396-containing immunities (blue). (B) *E. coli* toxicity assay. Serial dilutions of *E. coli* containing pBRA and pEXT22 constructs, as indicated, spotted onto LB agar plates, and grown for 20 h. Images are representative of three independent experiments. (C) Growth curve of *E. coli* harboring pBRA TseV2 or TseV3 before and after toxin induction by the addition of 0.2% L-arabinose (arrow). Results represent the mean  $\pm$  SD of three independent experiments performed in duplicate.  $**p < 0.01$  (Student's *t* test). (D) Time-lapse microscopy of *E. coli* carrying either pBRA TseV2 or pBRA TseV3 grown on LB-agar pads containing either 0.2% D-glucose (repressed) or 0.2% L-arabinose (induced). Scale bar: 5  $\mu$ m. Timestamps in hh:mm. (E) Bacterial competition assay using *S. bongori* WT,  $\Delta$ *tssB* and  $\Delta$ *tssB* complemented with pFPV25.1 *tssB* against *S. bongori*  $\Delta$ *tseV2*/*tsiV2.1*/*tsiV2.2* or  $\Delta$ *tseV3*/*tsiV3* complemented or not with pFPV25.1 *tsiV2.1* or pFPV25.1 *tsiV3*. Strains were co-incubated for 20 h ( $\Delta$ *tseV2*/*tsiV2.1*/*tsiV2.2*) or 6 h ( $\Delta$ *tseV3*/*tsiV3*) prior to measuring CFU counts. The prey recovery rate was calculated by dividing the CFU of the output by the input. Data represent the mean  $\pm$  SD of three independent experiments performed in duplicate and were analyzed through comparison with WT that were

234 normalized to 1. One-way ANOVA followed by Dunnett's multiple comparison test. \* $p < 0.05$ , \*\* $p < 0.01$   
235 and \*\*\* $p < 0.001$ . (F) Bacterial competition assay using *S. bongori* WT,  $\Delta tssB$ ,  $\Delta vgrG1$ ,  $\Delta vgrG2$  or  
236  $\Delta vgrG3$  against *S. bongori*  $\Delta tseV2/tseV2.1/tseV2.2$  or  $\Delta tseV3/tseV3$ . Strains were co-incubated for 20 h prior  
237 to measuring CFU counts. Prey recovery rate was calculated as in (E). Data represent the mean  $\pm$  SD of  
238 four independent experiments performed in duplicate. One-way ANOVA followed by Dunnett's multiple  
239 comparison test. \* $p < 0.05$ , \*\* $p < 0.01$  and *ns* (not significant).

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## 241 **VRR-Nuc-containing effectors are evolutionarily related to Holliday junction** 242 **resolvases and enzymes involved in DNA repair.**

243 TseV2 and TseV3 contain a VRR-Nuc domain at their C-terminus, which was  
244 initially annotated as DUF994 (Kinch *et al.*, 2005) and later renamed VRR-Nuc due to its  
245 association with enzymes linked to DNA metabolism (Iyer *et al.*, 2006). VRR-Nuc-  
246 containing proteins are found in a wide range of organisms, including bacteria,  
247 bacteriophages, fungi, and eukaryotes (Iyer *et al.*, 2006). Proteins containing this domain  
248 comprise a family (PF08774) belonging to the PD-(D/E)xK superfamily, which  
249 constitutes a large and functionally diverse group containing representatives involved in  
250 DNA replication (Holliday junction resolvases), restriction-modification, repair, and  
251 tRNA-intron splicing (Steczkiewicz *et al.*, 2012). Members of this superfamily exhibit  
252 low sequence similarity but display a common fold in its enzymatic core (with  
253  $\alpha_1\beta_1\beta_2\beta_3\alpha_2\beta_4$  topology), which contains conserved residues (Asp, Glu, Lys) responsible  
254 for catalysis (Steczkiewicz *et al.*, 2012).

255 To gain insight into the molecular function of TseV2 and TseV3 and understand  
256 their phylogenetic relationship, we used TseV1, TseV2 and TseV3 (TseV4 is 79.1%  
257 identical to TseV3 and was not used) amino acid sequences as queries in JackHMMER  
258 searches (Potter *et al.*, 2018) for four iterations on the NCBI nr database (November 4th,  
259 2021) to fetch a total of 2254 sequences with significant similarity (inclusion threshold  
260  $\leq 10^{-9}$  and reporting threshold  $\leq 10^{-6}$ ). Additional JackHMMER searches were performed  
261 using selected VRR-Nuc-containing proteins as queries (Bce1019, PmgM, T1p21,

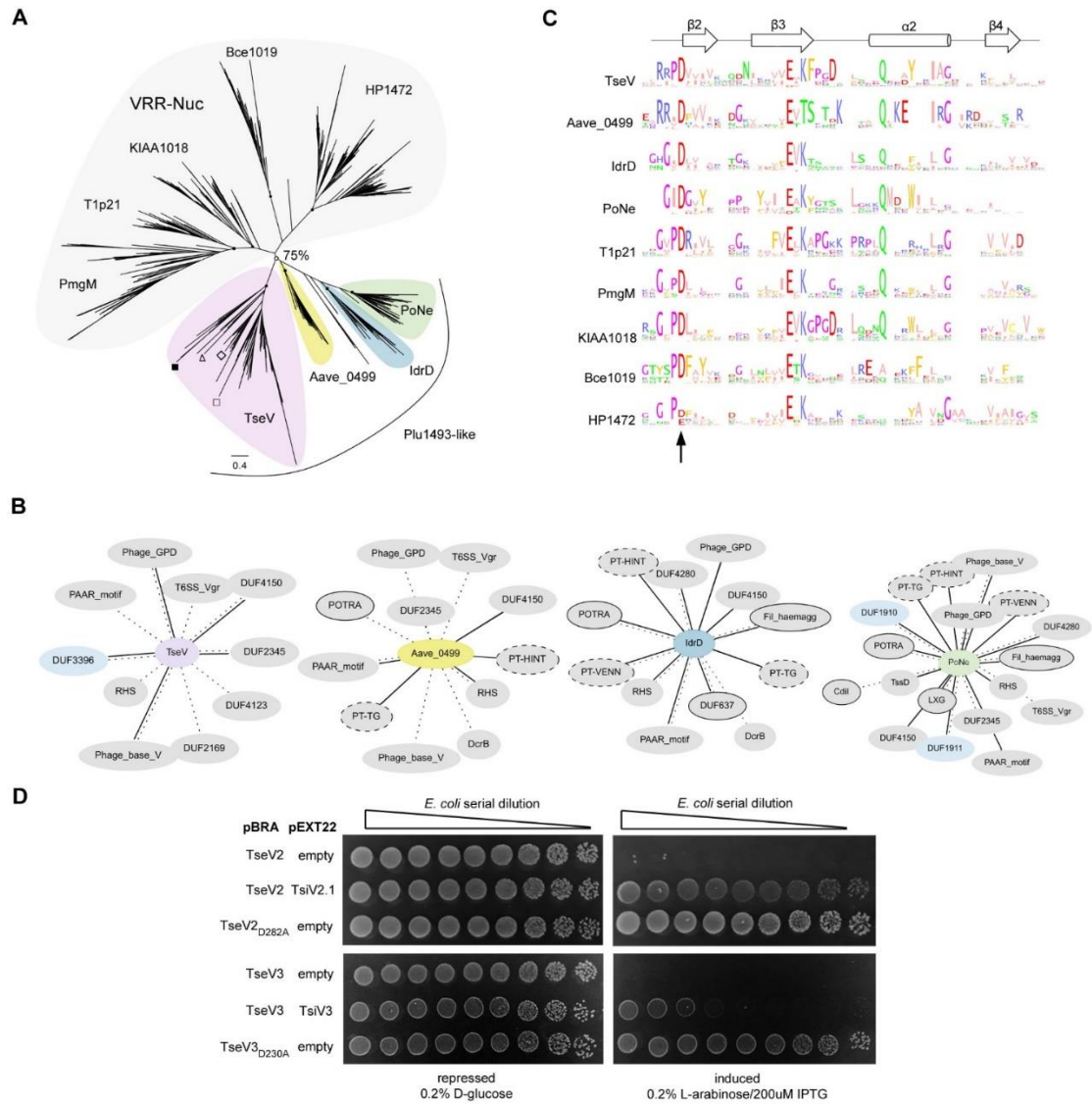
262 KIAA1018, HP1472 and Plu1493) (Iyer *et al.*, 2006), and recently reported *bona fide* or  
263 putative T6SS effectors that also belong to the PD-(D/E)xK superfamily: TseT  
264 (Burkinshaw *et al.*, 2018); PoNe (Jana *et al.*, 2019); IdrD-CT (Sirias *et al.*, 2020); TseTBg  
265 (Yadav *et al.*, 2021); Aave\_0499 (Pei *et al.*, 2021); and TseV<sup>PA</sup> (Wang *et al.*, 2021). A  
266 total of 39159 sequences were collected. For each JackHMMER dataset, we produced  
267 alignments with representatives from clusters formed by sequences displaying 80%  
268 coverage and 50-70% identity. These alignments were manually inspected, and  
269 divergent/truncated sequences were removed. We observed that the  $\beta_2\beta_3\alpha_2\beta_4$  region of  
270 the enzymatic core was more conserved so we used this region for a new multiple  
271 sequence alignment to build a phylogenetic tree using maximum likelihood (Fig. 3A).

272         The resulting tree is composed of 9 main clades, with 5 of these clades comprising  
273 PmgM, T1p21, KIAA1018, Bce1019 and HP1472 that reproduce the classification  
274 proposed by Iyer *et al.* (2006) in which each of these clades constitutes a subfamily of the  
275 VRR-Nuc family (Fig. 3A, grey; Table S1). Bce1019 subfamily contains the  
276 endonuclease I from Bacteriophage T7 (PDB 1M0D) (Hadden *et al.*, 2002) and the  
277 transposon Tn7 encoded nuclease protein TnsA from *E. coli* (PDB 1F1Z) (Hickman *et al.*,  
278 2000) (PDB 1T0F) (Ronning *et al.*, 2004). The PmgM subfamily contains a nuclease with  
279 the same name from phage P1 (Iyer *et al.*, 2006). The T1p21 subfamily contains proteins  
280 encoded upstream of helicases (Iyer *et al.*, 2006). The KIAA1018 group includes the  
281 human Fanconi anemia-associated nuclease 1 (FAN1) (PDB 4REA) (PDB 4RIA) and its  
282 bacterial homolog *Pa*FAN1 (PDB 4R89), which are involved in DNA repair (Gwon *et al.*,  
283 2014; Wang *et al.*, 2014; Zhao *et al.*, 2014). Curiously, antibacterial T6SS effectors formed  
284 4 groups (Fig. 3A, colors) in which TseV2 and TseV3 clustered with Plu1493 (Iyer *et al.*,  
285 2006) and TseV<sup>PA</sup> (Wang *et al.*, 2021), whereas homologs of Aave\_0499 (Pei *et al.*,  
286 2021), IdrD (Sirias *et al.*, 2020) and PoNe (Jana *et al.*, 2019) formed separated clades

287 (Fig. 3A, colors; Table S1). These results indicate that TseV proteins are members of the  
288 Plu1493 subfamily (Iyer *et al.*, 2006). Conversely, homologs of TseT were too divergent  
289 to be grouped in the phylogenetic tree and impaired its reproducibility, thus indicating  
290 that they probably have a distinct evolutionary origin (Fig. S3; Table S1).

291 All T6SS effectors (TseVs, Aave\_0499, IdrD and PoNe), except for TseT  
292 homologs, formed a clade with a bootstrap value higher than 75% (Fig. 3A, colors). The  
293 genomic context of TseV/Plu1493 homologs is different from the other VRR-Nuc family  
294 members (Table S2). While most of VRR-Nuc members (PmgM, T1p21, KIAA1018,  
295 Bce1019 and HP1472) are encoded next to genes involved in DNA metabolism, the gene  
296 neighborhood of antibacterial T6SS effectors (TseVs, Aave\_0499, IdrD and PoNe) is  
297 enriched in proteins encoding components of the T6SS apparatus, adaptors, and immunity  
298 proteins (Fig. 3B; Table S2). In addition, we observed proteins containing domains of  
299 other secretion systems involved in biological conflicts, such as CdiB and POTRA  
300 (T5SS) and LXG (T7SS) domains (Fig. 3B; Table S2). Therefore, based on genomic  
301 context and biological function, we propose to name Plu1493-like subfamily the group  
302 formed by the clades containing TseVs, Aave\_0499, IdrD and PoNe (Fig. 3A, colors).

303 Multiple amino acid sequence alignments from each clade revealed the conserved  
304 residues characteristic of the PD-(D/E)xK superfamily (Fig. 3C), which comprise the  
305 aspartic acid (D), glutamic acid (E) and lysine (K) that are part of the catalytic site  
306 responsible for hydrolyzing phosphodiester bonds (Steczkiewicz *et al.*, 2012). Using this  
307 information as a guide, substitution of the conserved aspartic acid for alanine in TseV2  
308 and TseV3 (TseV<sub>D282A</sub> and TseV<sub>D230A</sub>) abrogated toxicity in *E. coli* (Fig. 3D). These  
309 results confirm that the enzymatic activity of the VRR-Nuc domain is essential for  
310 toxicity.



311

312 **Fig. 3 VRR-Nuc-containing effectors are evolutionarily related to enzymes involved in DNA**

313 **metabolism.** (A) Maximum likelihood phylogenetic tree of VRR-Nuc family members (Bce1019, PmgM,

314 T1p21, KIAA1018, HP1472, Plu1493) (Iyer *et al.*, 2006) and recently reported bona fide or putative T6SS

315 effectors belonging to the PD-(D/E)xK superfamily (TseT, PoNe, IdrD-CT, TseTBg, Aave\_0499, TseV<sup>PA</sup>).

316 In the TseV clade (pink) the localization of TseV1 (□), TseV2 (Δ), TseV3 (■), and Plu1493 (◇) are marked.

317 (B) Contextual network representation of domains and the genomic context of proteins belonging to

318 Plu1493-like group (TseV, Aave\_0499, IdrD, PoNe). Each circle represents a domain, which is either fused

319 to (solid line) or encoded up- or downstream (dashed line) of the gene of interest (center). Borderless gray

320 circles represent domains related to T6SS; bordered gray circles denote domains associated with a different

321 bacterial secretion system; dashed nodes indicate pre-toxin domains; and light blue circles represent

322 immunity proteins. (C) Sequence logo from the conserved  $\beta_2\beta_3\alpha_2\beta_4$  of the PD-(D/E)xK enzymatic core

323 from all clades shown in (A). The arrow indicates conserved aspartic acid that was mutated in (D). (D) *E.*

324 *coli* toxicity assay. Serial dilution of *E. coli* containing pBRA and pEXT22 constructs, as indicated, spotted

325 onto LB agar plates, and grown for 20 h. Images are representative of three independent experiments.

326

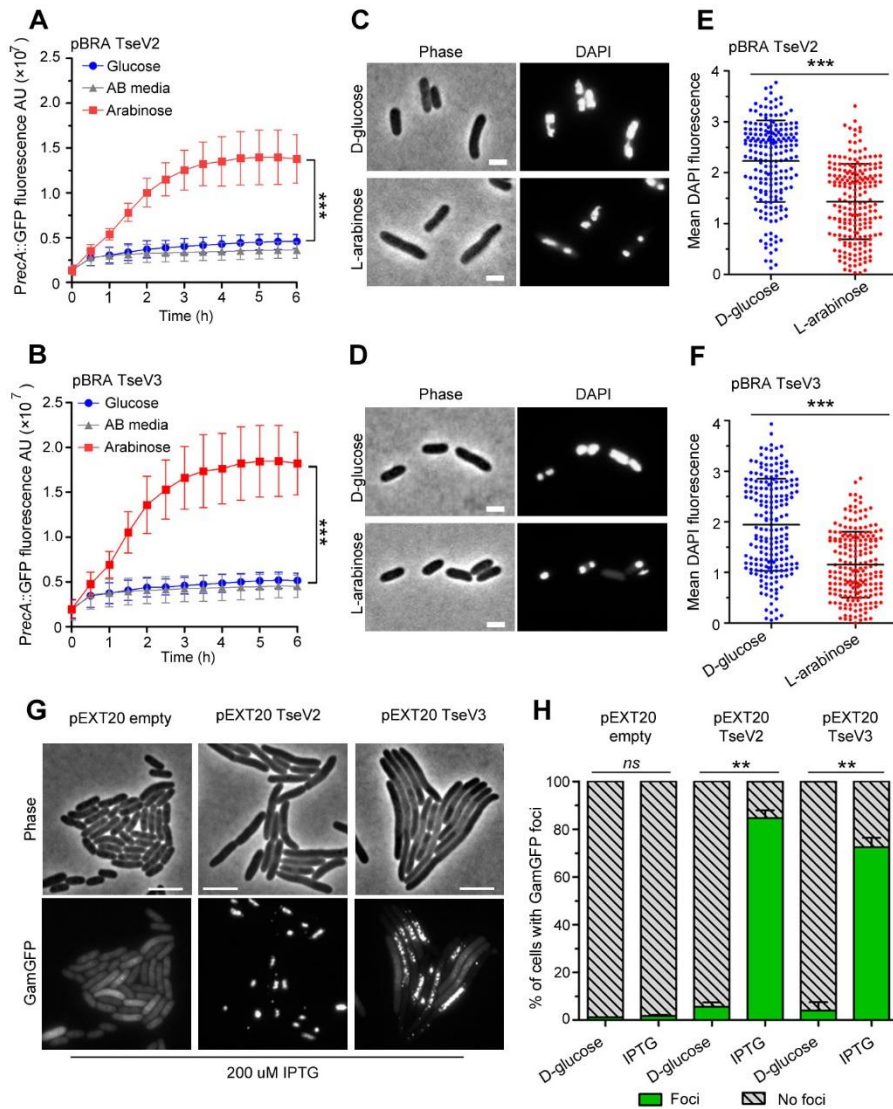
327 **TseV2 and TseV3 induce cell death via DNA double-strand breaks**

328 We set out to determine whether TseV2 and TseV3 could cause DNA damage by  
329 analyzing the activation of the SOS response - a stress response mechanism induced by  
330 the activation of RecA (recombinase protein A) in response to DNA damage (Walker,  
331 1996). *E. coli* harboring the reporter plasmid pSC101- $P_{recA}::GFP$  (Ronen *et al*, 2002),  
332 which carries the green fluorescent protein (GFP) under the control of the  $P_{recA}$  promoter,  
333 was co-transformed with either pBRA TseV2 or TseV3 and grown in AB media  
334 containing either 0.2% D-glucose or 0.2% L-arabinose (Fig. 4A and 4B). We observed  
335 an increase in GFP fluorescence when the expression of TseV2 or TseV3 was induced  
336 with L-arabinose, indicating the activation of the SOS response (Fig. 4A and 4B). To  
337 further assess the impact of TseV2 and TseV3 on bacterial chromosome stability, we used  
338 DAPI (4',6-diamidino-2-phenylindole) to stain *E. coli* cells after inducing the expression  
339 of TseV2 or TseV3 for 1 h and evaluate nucleoid integrity by measuring the mean DAPI  
340 fluorescence per cell (Fig. 4C-F). Cells expressing TseV2 or TseV3 revealed  
341 smaller/degraded nucleoids and displayed reduced DAPI fluorescence (Fig. 4C-F), which  
342 indicates DNA degradation.

343 VRR-Nuc-containing enzymes have been shown to degrade 5' flap single-strand  
344 DNA (Gwon *et al.*, 2014) and Holliday junctions (a four-way junction in which two DNA  
345 double-strands are held together) (Pennell *et al*, 2014). Expression of TseV2 and TseV3  
346 in *E. coli* followed by genomic and plasmid DNA extraction failed to reveal any  
347 significant difference in DNA integrity between induced and repressed conditions after  
348 visualization via electrophoresis in agarose gels (data not shown). To evaluate whether  
349 TseV2 and TseV3 could induce a small number of DNA double-strand breaks that were  
350 not detected by electrophoresis, we used the reporter strain *E. coli* SMR14354 encoding  
351 a chromosomal GFP fused to the Gam protein from bacteriophage Mu (GamGFP) under



352 the control of the  $P_{tet}$  promoter (induced by tetracycline) (Shee *et al.*, 2013). The Gam  
353 protein binds with high affinity and specificity to DNA double-strand ends, thus inducing  
354 the formation of GFP foci at specific sites (Shee *et al.*, 2013). *E. coli* SMR14354 carrying  
355 an empty pEXT20 plasmid or encoding either TseV2 or TseV3 were grown with 0.2%  
356 D-glucose (repressed) or with 200  $\mu$ M IPTG (induced) and examined by fluorescence  
357 microscopy (Fig. 4G and 4H). Cells carrying an empty plasmid revealed an even  
358 distribution of GamGFP in the cytoplasm, with only a few foci representing spontaneous  
359 double-strand breaks (Fig. 4G and 4H). Conversely, *E. coli* expressing either TseV2 or  
360 TseV3 revealed several intense GFP foci in more than 80% and 75% of cells, respectively  
361 (Fig. 4G and 4H). Interestingly, the expression of TseV2 leads to the formation of fewer  
362 intense foci per cell, whereas the expression of TseV3 induces the development of several  
363 less intense foci per cell (Fig. 4G and 4H), suggesting that TseV3 might cleave DNA at  
364 more sites than TseV2. Together, these results reveal that TseV2 and TseV3 cause target  
365 cell death via specific DNA double-strand breaks.



366  
 367 **Fig. 4. TseVs induce target cell death via DNA double-strand breaks.** (A-B) Activation of the SOS  
 368 response was analyzed using *E. coli* cells harboring the reporter plasmid pSC101-*P<sub>recA</sub>::GFP* and pBRA  
 369 TseV2 (A) or pBRA TseV3 (B), which were grown in AB defined media with D-glucose or L-arabinose.  
 370 Data is the mean  $\pm$  SD of three independent experiments. \*\*\* $p < 0.001$  (Student's *t* test). (C-D) Bright-field  
 371 and DAPI images of *E. coli* cells carrying pBRA TseV2 (C) or pBRA TseV3 (D) grown in the presence of  
 372 D-glucose (repressed) or L-arabinose (induced). Results are representative images of three independent  
 373 experiments. (E-F) Quantification of the mean DAPI fluorescence per cell of 200 cells. Data correspond to  
 374 the mean  $\pm$  SD of a representative experiment. Scale bar 2  $\mu$ m. \*\*\* $p < 0.001$  (Student's *t* test). (G)  
 375 Representative bright-field and GFP images of *E. coli* co-expressing GamGFP and pEXT20 TseV2 or  
 376 pEXT20 TseV3. Double-strand breaks appear as foci of GamGFP. Images are representatives of three  
 377 independent experiments. Scale bar: 5  $\mu$ m. (H) Quantification of the GamGFP foci shown in (G). Data are  
 378 shown as the mean  $\pm$  SD of the three independent experiments. \*\* $p < 0.01$  (Student's *t* test).  
 379

380 **TsiV3 interacts with the putative DNA-binding site of TseV3 to neutralize**  
 381 **toxicity**

382 To obtain information about the inhibitory mechanism of TsiV3, we decided to  
383 analyze whether this protein could directly interact with TseV3. Recombinant proteins  
384 were expressed, and the purified complex was analyzed using size exclusion  
385 chromatography coupled to multiple-angle light scattering (SEC-MALS) (Fig. S4). The  
386 MALS calculated average mass for the complex was  $66.4 \pm 3.3$  kDa, which is close to  
387 the sum of the theoretical values of their monomers: 26.8 kDa and 37.4 kDa for 6xHis-  
388 TseV3 and TsiV3, respectively. SDS-PAGE analysis of the mixture confirmed the  
389 presence of 6xHis-TseV3 and TsiV3 (Fig. S4). These results reveal that TseV3 and TsiV  
390 form a 1:1 heterodimeric complex.

391 We were able to obtain crystals of the TseV3:TsiV3 complex, which belong to  
392 space group  $P2_11$  and diffracted to a moderate resolution of 4 Å (Table S3). Matthews  
393 coefficient analysis indicated that two TseV3:TsiV3 complexes would be the most likely  
394 composition in the asymmetric crystal unit. We used AlphaFold (Jumper *et al*, 2021)  
395 models of TseV3<sub>132-281</sub> and TsiV3<sub>10-327</sub> for molecular replacement using Phaser (McCoy  
396 *et al*, 2007), which was able to place two copies of each monomer in the asymmetric unit  
397 with a final LLG (log-likelihood gain) of 486.87 and TFZ (translation function Z-score)  
398 of 12.4 - with both heterodimeric complexes adopting the same pose (our docked model  
399 is available using accession code ma-oyho8 at modelarchive.org). Therefore, the  
400 molecular replacement solution using the AlphaFold models most likely represents the  
401 correct relative orientation of the two subunits in the TseV3:TsiV3 complex (Fig. 5A).  
402 Given the relatively low resolution of the X-ray diffraction data, we chose not to refine  
403 these models against the processed dataset; however, our molecular replacement solution  
404 using the AlphaFold models was confirmed by identical placement using experimental  
405 PDB homologs taken from the DALI search described below - both TsiV and TsiT can  
406 be successfully utilized as search models for our experimental data, producing TFZ scores

407 of 8.3 and 9.2, respectively. Attempts to co-fold the TseV3 and TsiV3 complex with  
408 AlphaFold did not result in the extensive interface we observe in our experimentally  
409 docked single models, thus confirming the requirement for data-derived docking.

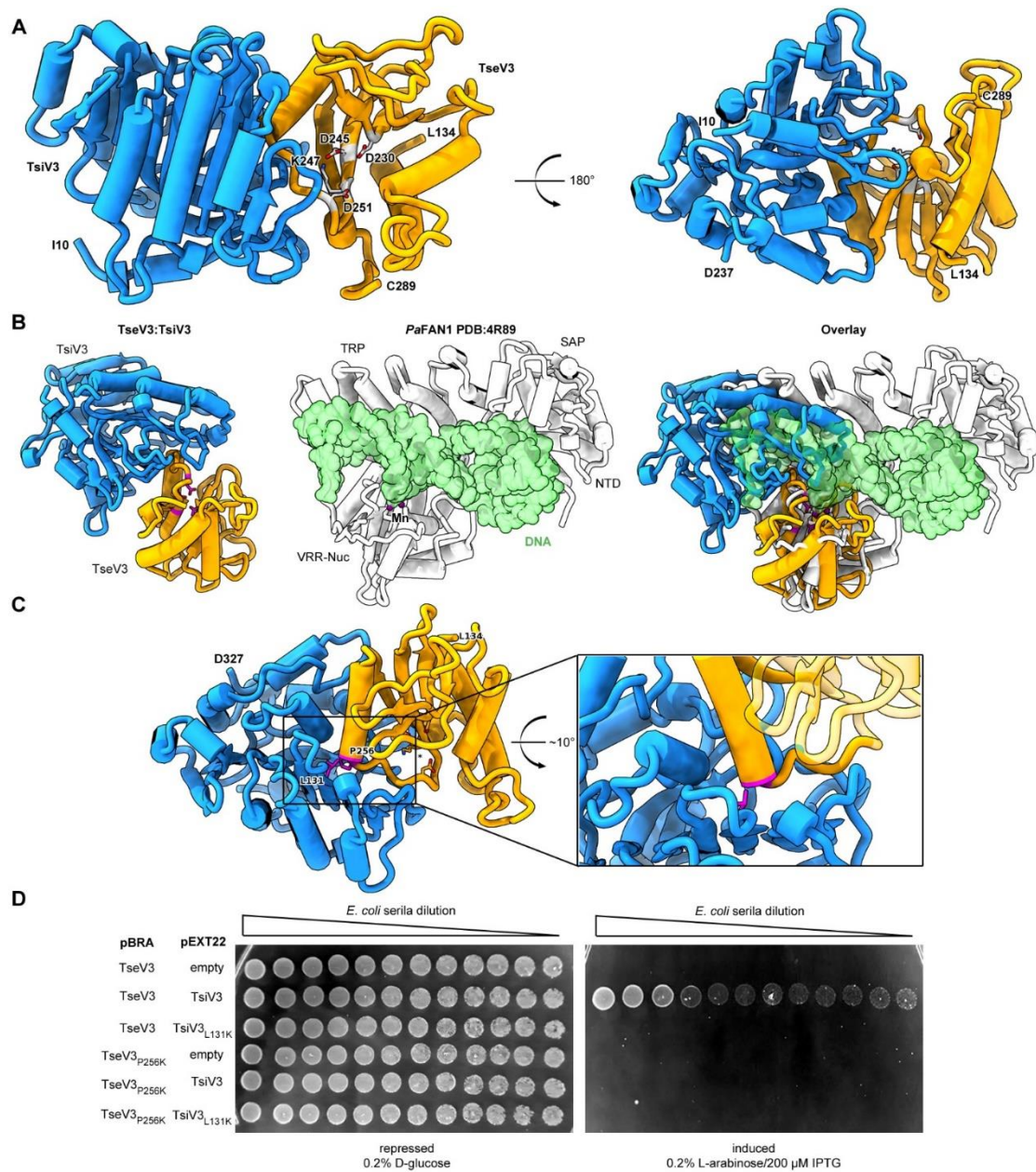
410 TsiV3 possesses a central  $\beta$ -sheet that is flanked by  $\alpha$ -helices on one side and  
411 exposed on the other (Fig. 5A). This exposed  $\beta$ -sheet surface of TsiV3 binds to an  $\alpha$ - $\beta$   
412 element of TseV3 that flanks its putative active site (composed of residues D<sub>230</sub>, D<sub>245</sub>,  
413 K<sub>247</sub>) (Fig. 5A). In this configuration, the TseV3-interacting  $\alpha$ -helix, which corresponds  
414 to the  $\alpha_2$  of the classical PD-(D/E)xK  $\alpha_1\beta_1\beta_2\beta_3\alpha_2\beta_4$  core topology, projects its N-terminus  
415 towards the TsiV3  $\beta$ -sheet (Fig. 5A).

416 Searches for structures similar to TseV3<sub>132-281</sub> and TsiV3<sub>10-327</sub> using the DALI  
417 server (Holm, 2020) revealed matches to proteins of related function: (a top Z-score of  
418 33.5 for the immunity protein PA0821 PDB:7DRG and TsiV3; and a top Z-score of 3.8  
419 between VRR-Nuc protein psNUC PDB:4QBL and TseV3). The modest RMSD (Root-  
420 Mean-Square Deviation) for the C $\alpha$  positions in TseV3 and other VRR-Nuc enzymes  
421 indicates that TseV3 represents a variant of the VRR-Nuc family fold. Nevertheless, the  
422 PD-(D/E)xK consensus catalytic residues are identifiable as the modified sequence  
423 MD<sub>230</sub>IX<sub>n</sub>D<sub>245</sub>VK<sub>247</sub> in TseV3. These residues are found in positions commensurate with  
424 active nucleases of the PD-(D/E)xK superfamily. Accordingly, superimposition of TseV3  
425 with the well-characterized VRR-Nuc member *Pa*FAN1 (PDB 4R89) (Gwon *et al.*, 2014)  
426 (clade KIAA1018 in Fig. 3A) matches residues D<sub>230</sub>, D<sub>245</sub> and K<sub>247</sub> of the former with  
427 residues D<sub>507</sub>, E<sub>522</sub>, and K<sub>524</sub> of the latter (Fig. 5B).

428 *Pa*FAN1 (Gwon *et al.*, 2014) is the bacterial homolog of the human FAN1  
429 (MacKay *et al.*, 2010), which is involved in the repair of DNA damage such as interstrand  
430 cross-links (ICLs) (Gwon *et al.*, 2014; Jin *et al.*, 2018). Human FAN1 is a structure-  
431 selective nuclease consisting of four domains: ubiquitin-binding zinc (UBZ); SAF-A/B,

432 Acinus and PIAS (SAP); tetratricopeptide repeat (TPR); and VRR-Nuc (Iyer *et al.*, 2006).  
433 Conversely, *PaFAN1* lacks the UBZ domain and contains an uncharacterized N-terminal  
434 domain (NTD), followed by SAP, TPR and VRR-Nuc (Iyer *et al.*, 2006; Gwon *et al.*,  
435 2014). The structure of *PaFAN1* has been solved in complex with a 5' flap DNA substrate  
436 (PDB 4R89) (Fig. 5B, middle). As the catalytic residues of TseV3 align with those of  
437 *PaFAN1*, we used the structure of the latter as a guide to analyze the likely mechanism  
438 by which TsiV3 may neutralize TseV3 activity. Comparison between the *PaFAN1*:DNA  
439 and TseV3:TsiV3 complexes reveals that the path of the DNA substrate is potentially  
440 incompatible with the presence of TsiV3 (Fig. 5B). Hence, assuming the mode of  
441 substrate recognition is similar between *PaFAN1* and TseV3, this result suggests that the  
442 binding of TsiV3 sterically blocks the toxin active site. In our model of the complex,  
443 TsiV3 occlusion of the TseV3 active site would be enabled by TsiV3 loop, which projects  
444 into the putative TseV3 DNA-binding pocket (Fig. 5A, B).

445 To validate the accuracy of our TseV3:TsiV3 structural model, we designed point  
446 mutations to disrupt the interaction surface between the two proteins without perturbing  
447 the active site or its ability to bind DNA. Thus, residue P256 at the N-terminus of  $\alpha_2$  of  
448 TseV3 was replaced by lysine (TseV3<sub>P256K</sub>), and residue L131 in the central  $\beta$ -sheet of  
449 TsiV3 was also replaced by lysine (TsiV3<sub>L131K</sub>) (Fig. 5C). Native and mutated versions  
450 of the effector and immunity protein were co-transformed in *E. coli* to analyze toxicity  
451 (Fig. 5D). Results revealed that TsiV3<sub>L131K</sub> was unable to neutralize TseV3 toxicity. In  
452 addition, the mutated TseV3<sub>P256K</sub> maintained its enzymatic activity, displaying toxicity in  
453 *E. coli*; however, this mutant was not neutralized by co-expression with the native  
454 immunity protein (TsiV3) (Fig. 5D). Together, these results reinforce the accuracy of our  
455 model, which is comprised of both experimental constraints and theoretical model  
456 components.



457

458 **Fig. 5. The effector-immunity complex reveals that TsiV3 blocks TseV3 substrate-binding site. (A)**

459 Constrained model of the TseV3:TsiV3 heterodimer with two different views: TsiV3 in blue (I<sub>10</sub>-D<sub>327</sub>) and

460 TseV3 in orange (L<sub>134</sub>-C<sub>289</sub>). Models are labeled to assist interpretation. PD-(D/E)xK superfamily

461 conserved residues of TseV3 (D<sub>230</sub>, D<sub>245</sub>, and K<sub>247</sub>) are shown in stick form and colored light grey,

462 confirming that they converge to form a putative consensus active site. (B) Superimposition of the

463 TseV3:TsiV3 coordinates with those of the PaFAN1:DNA complex (PDB 4R89). PaFAN1 protein in

464 white, DNA duplex in green, and catalytic Mn<sup>2+</sup> are depicted as purple spheres. The overlay (right)

465 is presented in the same orientation as the individual complexes: TseV3:TsiV3 (left, catalytic residues in

466 magenta) and PaFAN1:DNA (middle). (C) Prediction of interface-compromising mutants in the

467 TseV3:TsiV3 heterodimer. TsiV3 (blue) and TseV3 (orange) with putative active sites labeled with asterisk.

468 Residues L<sub>131</sub> of TsiV3 and P<sub>256</sub> of TseV3 (both in stick form, magenta) form the closest point of contact

469 in the heterodimer and are at the center of a hydrophobic-rich interface. (D) *E. coli* toxicity assay using

470 cells carrying plasmids with wild-type or point mutations in TsiV3 (L<sub>131</sub>K) or TseV3 (P<sub>256</sub>K) as a potential  
471 means to destabilize the TseV3:TsiV3 complex interaction.  
472

## 473 Discussion

474 Bacterial antagonistic strategies targeting nucleic acids are very effective as these  
475 components are critical for life. In this study, we characterized a group of effectors  
476 containing the VRR-Nuc domain. This domain has not previously been reported to be  
477 used in biological conflicts (Zhang *et al.*, 2012), but recently was suggested to work as a  
478 T6SS effector due to its localization next to a PAAR protein in *P. aeruginosa* (Wang *et al.*, 2021) – for consistency we have decided to keep the name TseV for this group of  
479 effectors. Proteins containing the VRR-Nuc domain comprise a family (Iyer *et al.*, 2006)  
480 belonging to the PD-(D/E)xK superfamily, which contain a conserved enzymatic core  
481 composed by  $\alpha_1\beta_1\beta_2\beta_3\alpha_2\beta_4$  (Steczkiewicz *et al.*, 2012). The conserved catalytic residues  
482 (D, E, K) are located in the central  $\beta_2\beta_3$ -sheet, while the  $\alpha_1$ -helix is associated with the  
483 formation of the active site and  $\alpha_2$ -helix with substrate binding (Steczkiewicz *et al.*, 2012).  
484 Curiously, *S. bongori* encodes four TseV homologs: TseV2 and TseV3 are toxic in *E.*  
485 *coli*, whereas TseV1 and TseV4 are not toxic (Fig. 2). Based on what is known about the  
486 catalytic mechanism of PD-(D/E)xK nucleases, we hypothesize that the lack of  $\alpha_2$ - and  
487  $\alpha_1$ -helix in TseV1 and TseV4, respectively, might explain the lack of toxicity (Fig. S5).  
488 Another curiosity is the presence of two homologs of the DUF3396 immunity genes  
489 downstream of both TseV2 and TseV1 (Fig. 2A). Such genomic organization is also  
490 conserved in other bacterial species like *Photorhabdus thracensis* (VY86\_01065,  
491 VY86\_01040), *Photorhabdus asymbiotica* (PAU\_03539, PAU\_03660)  
492 (Enterobacterales), *Marinobacter nauticus* (MARHY2492) (Pseudomonadales) and  
493 *Herbaspirillum huttiense* (E2K99\_00955) (Burkholderiales). The fact that only one  
494 immunity protein (TsiV2.1) can neutralize the effector (TseV2) makes us wonder about  
495

496 the role of the additional immunity protein gene - and why such genomic context is  
497 conserved. One possibility is that the extra immunity protein could regulate the effector  
498 at the transcriptional level as has been reported for the immunity protein TsiTBg known  
499 to regulate a different PD-(D/E)xK effector (TseTBg) (Yadav *et al.*, 2021).

500 The complexity of the PD-(D/E)xK superfamily and the rapid evolution of  
501 polymorphic toxins makes it difficult to categorize antibacterial effectors belonging to  
502 this group. However, our phylogenetic analysis was able to confidently group VRR-Nuc-  
503 containing effectors in one clade (TseV) and show that this group is different from the  
504 clades formed by the homologs of additional T6SS effectors (Aave\_0499, IdrD, PoNe)  
505 (Fig. 3). Although proteins belonging to clades Aave\_0499, IdrD, PoNe are not  
506 recognized by the Pfam model of VRR-Nuc, these proteins share similar genetic  
507 architectures concerning domain fusions and gene vicinity (Fig. 3B); therefore, we  
508 decided to call this larger group Plu1493-like to respect the original nomenclature  
509 proposed by Iyer *et al.* (2006).

510 The enzymatic activity of proteins belonging to the PD-(D/E)xK superfamily is  
511 quite diverse, but we were able to narrow down the possibilities and reveal that TseV  
512 effectors intoxicate target cells by inducing DNA double-strand breaks. Despite killing  
513 cells by the same mechanism, *E. coli* intoxicated by TseV2 and TseV3 display slight  
514 differences in phenotype. Due to the difference in GamGFP phenotype (Fig. 4G) and the  
515 number of resistant colonies obtained after ectopic expression of TseV2 and TseV3 in *E.*  
516 *coli* (Fig. S1), we hypothesize that the former cleaves DNA at fewer sites compared to  
517 the latter. It remains to be defined whether these effectors target a specific nucleotide  
518 sequence or recognize some conformational DNA structure. Unfortunately, whole  
519 genome sequencing of 20 resistant *E. coli* clones expressing the toxin (Fig. S1) failed to



520 uncover any evident mutation that could be attributed to the resistance phenotype (data  
521 not shown).

522 T6SSs effector-immunity complexes are related to type II toxin-antitoxin (TA)  
523 systems, which play several roles in bacterial physiology ranging from genomic  
524 stabilization and abortive phage infection to stress modulation and antibiotic persistence  
525 (Fraikin *et al.*, 2020). Most T6SS immunity proteins described to date bind to effectors to  
526 regulate their enzymatic activity (Benz *et al.*, 2012; Benz *et al.*, 2013; Dong *et al.*, 2013;  
527 Li *et al.*, 2013; Lu *et al.*, 2014; Robb *et al.*, 2016). An exception is Tri1 (type VI secretion  
528 ADP-ribosyltransferase immunity 1) from *Serratia proteamaculans*, which exhibits two  
529 modes of inhibition: active site occlusion and enzymatic removal of a post-translational  
530 modification (Ting *et al.*, 2018). The neutralization mechanism of TsiT, which counteracts  
531 the PD-(D/E)xK effector TseT from *P. aeruginosa*, was also proposed to be different:  
532 TsiT interferes with the effector oligomerization state and hinders its nuclease activity  
533 (Wen *et al.*, 2021). Our structural model of the TseV3:TsiV3 complex revealed that TsiV3  
534  $\beta$ -sheet binds to the  $\alpha_2$ -helix of TseV3, which is involved in DNA binding in other PD-  
535 (D/E)xK members (Steczkiewicz *et al.*, 2012). In addition, the superposition of the  
536 TseV3:TsiV3 complex with the structure of PaFAN1 bound to DNA confirms the  
537 hypothesis that TsiV3 likely occludes the substrate-binding site of TseV3.

538 It is estimated that *E. coli* and *Salmonella* diverged millions of years ago (Fookes  
539 *et al.*, 2011). *Salmonella*-specific functions are encoded by genes located in prophages  
540 and specific SPIs. Several characteristics of *S. bongori* suggest that this species may lie  
541 somewhere between *E. coli* and *S. enterica* during evolution (Christensen *et al.*, 1998;  
542 Fookes *et al.*, 2011). Analyzing the synteny between *S. Typhimurium* 14028s and *S.*  
543 *bongori* NCTC 12419, we observed that the SPI-2 T3SS of *S. enterica* is localized in the  
544 equivalent genomic loci of the SPI-22 T6SS of *S. bongori* (Fig. S6). The SPI-2 T3SS of

545 *S. enterica* is well characterized for its importance during the intracellular stage of the  
546 infection cycle, allowing bacteria to manipulate host cellular functions and replicate  
547 (Hensel *et al.*, 1995; Jennings *et al.*, 2017). Whether the SPI-22 T6SS of *S. bongori* also  
548 works against eukaryotic cells remains to be established.

549 The genome of *S. bongori* NCTC 12419 encodes a large repertoire of orphan Hcp,  
550 VgrG and PAAR-like proteins (Fig. 1B) that are responsible for diversifying the array of  
551 effectors secreted by the SPI-22 T6SS. Conversely, the genome of *S. Typhimurium*  
552 14028s that encodes a phylogenetically unrelated SPI-6 T6SS involved in competition  
553 with species of the gut microbiota (Sana *et al.*, 2016; Sabinelli-Sousa *et al.*, 2020) displays  
554 a restricted repertoire of orphan T6SS genes composed of only two Hcps (STM14\_3785  
555 and STM14\_5414) (Blondel *et al.*, 2009). These observations suggest that the former  
556 bacterium may use its SPI-22 T6SS to target a greater variety of competitors, while the  
557 latter uses its SPI-6 T6SS to target a restricted number of species.

558 Here we add to the known diversity of antibacterial weapons, placing the VRR-  
559 Nuc family within the remit of T6SS effectors. Knowledge about the phylogeny and  
560 mechanism of action of this group of effectors will be important in interpreting its usage  
561 in other bacterial species, including the requirements of neutralization by very specific  
562 immunity pairings.

563

## 564 **Material and Methods**

### 565 **Bacterial strains and growth conditions**

566 A list of bacterial strains used in this work can be found in Table S4. Strains were  
567 grown at 37°C in Lysogeny Broth (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract)  
568 under agitation. Cultures were supplemented with antibiotics in the following

569 concentration when necessary: 50 µg/mL kanamycin, 100 µg/mL ampicillin, and 50  
570 µg/mL streptomycin.

### 571 **Cloning and mutagenesis**

572 Putative effectors SBG\_1828, SBG\_1841, SBG\_2718 and SBG\_2723 were  
573 amplified by PCR and cloned into pBRA vector under the control of P<sub>BAD</sub> promoter  
574 (Souza *et al*, 2015). Immunity proteins SBG\_1828, SBG\_1842, SBG\_2719, SBG\_2720,  
575 SBG\_2724 and SBG\_2725 were cloned into pEXT22 under the control of P<sub>TAC</sub> promoter  
576 (Dykhhoorn *et al*, 1996). TseV2 and TseV3 were cloned in the pEX20 vector under the  
577 control of P<sub>TAC</sub> promoter (Dykhhoorn *et al.*, 1996) for GamGFP assays. For  
578 complementation, SBG\_1238 (TssB), SBG\_1842 (TsiV3) and SBG\_2724 (TsiV2.1)  
579 were cloned into pFPV25.1 by replacing the GFP mut3 coding region for the genes of  
580 interest (Valdivia & Falkow, 1996). Point mutations were created using QuikChange II  
581 XL Site-Directed Mutagenesis Kit (Agilent Technologies) and pBRA TseV2 and pBRA  
582 TseV3 plasmids as templates. *S. bongori* mutant strains were constructed by λ-Red  
583 recombination engineering using a one-step inactivation procedure (Datsenko & Wanner,  
584 2000). All constructs were confirmed by sequencing.

### 585 **Interbacterial competition assay**

586 Bacterial competition assays were performed using *S. bongori* (WT,  $\Delta$ tssB,  
587  $\Delta$ tseV2/tsiV2.1/tsiV2.2 and  $\Delta$ tseV3/tsiV3) as attackers, and *E. coli* K-12 W3110 carrying  
588 pFPV25.1 Amp<sup>R</sup> as prey. Overnight cultures of the attacker and prey cells were sub-  
589 cultured in LB (1:30) until reaching OD<sub>600nm</sub> 1.6, then adjusted to OD<sub>600nm</sub> 0.4 and mixed  
590 in a 10:1 ratio (attacker:prey), 5 µL of the mixture were spotted onto 0.22 µm  
591 nitrocellulose membranes (1 × 1 cm) and incubated on LB agar (1.5%) at 37°C for the  
592 indicated periods. Membranes containing the bacterial mixture were placed on 1.5 mL  
593 tubes containing 1 mL of LB, homogenized by vortex, serially diluted, and plated on

594 selective plates containing antibiotics. The prey recovery rate was calculated by dividing  
595 the CFU (colony forming units) counts of the output by the CFU of the input.

### 596 ***E. coli* toxicity assays**

597 Overnight cultures of *E. coli* DH5 $\alpha$  (LB with 0.2% glucose) carrying effectors (in  
598 pBRA) and immunity proteins (in pEXT22) were adjusted to OD<sub>600nm</sub> 1, serially diluted  
599 in LB (1:4) and 5  $\mu$ L were spotted onto LB agar plates containing either 0.2% D-glucose  
600 or 0.2% L-arabinose plus 200  $\mu$ M IPTG – both supplemented with streptomycin and  
601 kanamycin - and incubated at 37°C for 20 h. For growth curves, overnight cultures of *E.*  
602 *coli* carrying pBRA TseV2 or TseV3 were inoculated in LB (1:50) with 0.2% D-glucose  
603 and grown at 37°C (180 rpm) for 1.5 h. Next, media was replaced with either fresh warm  
604 LB containing 0.2% D-glucose or 0.2% L-arabinose.

### 605 **Microscopy Time-lapse studies**

606 For time-lapse microscopy, LB agar (1.5%) pads were prepared by cutting a  
607 rectangular piece out of a double-sided adhesive tape, which was taped onto a microscopy  
608 slide as described previously (Bayer-Santos *et al*, 2019). *E. coli* DH5 $\alpha$  harboring pBRA  
609 TseV2 or TseV3 were sub-cultured in LB (1:50) with 0.2% D-glucose until reaching  
610 OD<sub>600nm</sub> 0.4-0.6 and adjusted to OD<sub>600nm</sub> 1.0. Cultures were spotted onto LB agar pads  
611 supplemented either with 0.2% D-glucose or 0.2% L-arabinose plus antibiotics. Images  
612 were acquired every 15 min for 16 h using a Leica DMI-8 epifluorescent microscope  
613 fitted with a DFC365 FX camera (Leica) and Plan-Apochromat 63x oil objective (HC PL  
614 APO 63x/1.4 Oil ph3 objective Leica). Images were analyzed using FIJI software  
615 (Schindelin *et al*, 2012).

### 616 **Bioinformatic analysis**

617 Iterative profile searches using JackHMMER (Eddy, 2011) with a cutoff e-value  
618 of 10<sup>-6</sup> and a maximum of four iterations were performed to search a non-redundant (nr)

619 protein database from the National Center for Biotechnology Information (NCBI) (Sayers  
620 *et al*, 2019). Similarity-based clustering of proteins was carried out using MMseqs  
621 software (Steinegger & Söding, 2017). Sequence alignments were produced with MAFFT  
622 local-pair algorithm (Kato & Standley, 2013), and non-informative columns were  
623 removed with trimAl software (Capella-Gutiérrez *et al*, 2009). Approximately-  
624 maximum-likelihood phylogenetic trees were built using FastTree 2 (Price *et al*, 2010).  
625 Sequence logos were generated using Jalview (Waterhouse *et al*, 2009). HMM models  
626 were produced for each sequence alignment and compared against each other with the  
627 HH-suite package (Steinegger *et al*, 2019). Proteins were annotated using the HHMER  
628 package (Eddy, 2011) or HHPRED software (Söding *et al*, 2005) and Pfam (Bateman *et*  
629 *al*, 2004), PDB (Berman *et al*, 2007) or Scope (Fox *et al*, 2014) databases. An in-house  
630 Python script was used to collect the gene neighborhoods based on information  
631 downloaded from the complete genomes and nucleotide sections of the NCBI database  
632 (Sayers *et al.*, 2019).

633 TseV1-4 sequence alignments were produced with MAFFT local-pair algorithm  
634 (Kato & Standley, 2013) and analyzed in AilView (Larsson, 2014) to separate the  
635 regions of interest. Sequence logos were produced using the Jalview (Waterhouse *et al.*,  
636 2009). Protein structure predictions were performed with ColabFold (Mirdita *et al*, 2021)  
637 and AlphaFold (Jumper *et al.*, 2021), and visualization was performed using Pymol  
638 (DeLano, 2002).

639 The genome of *S. bongori* NCTC 12419 and *S. Typhimurium* NCTC 14028s were  
640 retrieved from the NCBI database and aligned with BLASTn (Camacho *et al*, 2009). The  
641 alignment was analyzed using Artemis Comparison Tool (ATC) (Carver *et al*, 2005). The  
642 genome map was constructed using the ATC plug-in DNAPlotter (Carver *et al*, 2009).

643 **SOS response assays**

644 Overnight cultures of *E. coli* DH5 $\alpha$  harboring the reporter plasmid pSC101-  
645 P<sub>recA</sub>::GFP (Ronen *et al.*, 2002) and pBRA TseV2 or TseV3 were sub-cultured (1:50) in  
646 LB with 0.2% D-glucose and grown at 37°C until OD<sub>600nm</sub> 0.4-0.6. Bacteria were  
647 harvested and resuspended in AB defined media (0.2% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.6% Na<sub>2</sub>HPO<sub>4</sub>, 0.3%  
648 KH<sub>2</sub>PO<sub>4</sub>, 0.3% NaCl, 0.1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 3  $\mu$ M FeCl<sub>3</sub>) supplemented with 0.2%  
649 sucrose, 0.2% casamino acids, 10  $\mu$ g/mL thiamine and 25  $\mu$ g/mL uracil (Bayer-Santos *et*  
650 *al.*, 2019). Cells (OD<sub>600nm</sub> 1.0) were placed in a black 96 well plate with clear bottom  
651 (Costar) with 0.2% D-glucose or 0.2% L-arabinose to a final volume of 200  $\mu$ L. GFP  
652 fluorescence was monitored in a plate reader SpectraMax Paradigm Molecular Devices  
653 for 6 h at 30°C.

#### 654 **DAPI staining**

655 *E. coli* DH5 $\alpha$  carrying pBRA TseV2 and TseV3 were sub-cultured in LB with  
656 0.2% D-glucose (1:50) and grown at 37°C (180 rpm) until OD<sub>600nm</sub> 0.4-0.6. Cells were  
657 harvested and resuspended in new media with 0.2% D-glucose or 0.2% L-arabinose and  
658 growth for an additional 1 h. Bacteria were fixed with 4% paraformaldehyde for 15 min  
659 on ice, washed in phosphate buffer saline (PBS) and stained with DAPI (3  $\mu$ g/mL) for 15  
660 min at room temperature. Samples were washed once with PBS before transferring 1  $\mu$ L  
661 of each culture to a 1.5% PBS-agarose pad for visualization. Images were acquired in  
662 Leica DMi-8 epifluorescent microscope fitted with a DFC365 FX camera (Leica) and  
663 Plan-Apochromat 63x and 100x oil objectives (HC PL APO 63x and 100x/1.4 Oil ph3  
664 objectives Leica). Images were analyzed using FIJI software (Schindelin *et al.*, 2012). To  
665 assess DNA integrity, the mean pixel fluorescence per cell was manually measured from  
666 200 bacteria from different fields from each experiment. The cell area was determined  
667 using the bright field, and the mean pixel fluorescence per cell was measured in the DAPI  
668 channel subtracting the background.

669 **DNA double-strand break microscopy**

670 *E. coli* SMR14354 containing a chromosomal GamGFP under the control of P<sub>tet</sub>  
671 promoter (Shee *et al.*, 2013) and harboring an empty pEXT20 or encoding TseV2 or  
672 TseV3 were sub-cultured in LB (1:100) with 0.2% D-glucose grown for 1.5 h at 37°C  
673 (180 rpm) before the induction of GamGFP with 50 µg/mL tetracycline for 2 h. Bacteria  
674 were resuspended in new media with either 0.2% D-glucose or 200 µM IPTG and grown  
675 for 1 h. One microliter of each culture was spotted onto a 1.5% LB-agarose pad. Images  
676 were acquired in a Leica DMI-8 epifluorescent microscope fitted with a DFC365 FX  
677 camera (Leica) and Plan-Apochromat 100x oil objective (HC PL APO 100x/1.4 Oil ph3  
678 objective Leica). Images were analyzed using FIJI software (Schindelin *et al.*, 2012). At  
679 least 400 bacteria from each experiment were quantified.

680 **Protein expression and purification**

681 *E. coli* SHuffle cells carrying pRSFDuet 6xHis-TseV3-TsiV3 were grown in LB  
682 supplemented with kanamycin (30°C, 180 rpm) until OD<sub>600nm</sub> 0.4-0.6. Expression was  
683 induced with 0.5 mM IPTG followed by incubation at 16°C for 16 h. Cells were harvested  
684 via centrifugation at 9000 g for 15 min, and pellets were resuspended in buffer A (50 mM  
685 Tris-HCl pH 7.5, 200 mM NaCl, 5 mM imidazole) and lysed at 4°C using an Avestin  
686 EmulsiFlex-C3 homogenizer. The lysate was collected and centrifuged (48000 g) for 1 h  
687 at 4 °C. The supernatant was loaded onto a 5 ml HisTrap HP cobalt column (GE  
688 Healthcare) equilibrated in buffer A. The column was washed with 10 column volumes  
689 (CV) of buffer A before running an elution gradient of 0-50% buffer B (50 mM Tris-HCl  
690 pH 7.5, 200 mM NaCl, 500 mM imidazole) over 10 CV, followed by a final 10 CV wash  
691 with 100% buffer B. The presence of TseV3-TsiV3 was confirmed by SDS-PAGE of  
692 eluted fractions. TseV3-TsiV3 was concentrated using a Vivaspin® spin-concentrator

693 and further purified by size-exclusion chromatography on a Superdex 200 26/60 column  
694 (GE Healthcare) equilibrated in 50 mM Tris-HCl pH 7.5, 150 mM NaCl.

695 SEC-MALS analyses were used to determine the molar mass of the TseV3-TsiV3  
696 complex (concentration 3.2 mg/mL). Protein samples (400  $\mu$ L injection volume) were  
697 separated using a Superdex 200 10/300 column (GE Healthcare) equilibrated with buffer  
698 (50 mM Tris-HCl pH 7.5, 20 mM NaCl) coupled to a miniDAWN TREOS multi-angle  
699 light scattering system and an Optilab rEX refractive index detector. Data analysis was  
700 performed using the Astra Software package version 7.1 (Wyatt TechnologyCorp).  
701 Molecular mass was calculated assuming a refractive index increment  $dn/dc = 0.185$   
702 mL/g (Wen *et al.*, 1996). Fractions were analyzed in SDS-PAGE to confirm protein  
703 molecular weight.

#### 704 **Crystallography and structure determination**

705 TseV3-TsiV3 was concentrated to 18 mg/mL and crystallized in 0.1 M HEPES pH  
706 7.5 and 30 % v/v PEG Smear Low (12.5% v/v PEG 400, 12.5% v/v PEG 500,  
707 monomethylether, 12.5% v/v PEG 600, 12.5% v/v PEG 1000). The crystals were  
708 cryoprotected in the mother liquor supplemented with 20% ethylene glycol and  
709 subsequently cryo-cooled in liquid nitrogen. X-ray diffraction data were collected at  
710 Diamond Light Source on beamline i04, and initial data processing was performed using  
711 the xia2-dials pipeline (Winter, 2010; Winter *et al.*, 2018). The data were phased by  
712 molecular replacement in Phaser (McCoy *et al.*, 2007) using AlphaFold (Jumper *et al.*,  
713 2021) models of TseV3<sub>134-289</sub> and TsiV3<sub>10-327</sub>, which were trimmed to include only the  
714 high-confidence regions and omit the N-terminal DUF4150 domain of TseV3.

#### 715 **Quantification and statistical analyses**



716 Statistical test, number of events, mean values and standard deviations are  
717 reported in each figure legend accordingly. Statistical analyses were performed using  
718 GraphPad Prism5 software and significance is determined by the value of  $p < 0.05$ .

719

## 720 **Acknowledgements**

721 We are grateful to Cristiane Rodrigues Guzzo for sharing reagents and equipment,  
722 and Alexandre Bruni Cardoso for allowing access to the fluorescence microscope. We  
723 thank members of the LEEP and EBS groups for scientific discussions. Crystallography  
724 analyses were performed at Diamond Light Source. This work was supported by São  
725 Paulo Research Foundation (FAPESP) grants to RFS (2016/09047-8), CSF (2017/17303-  
726 7) and EBS (2017/02178-2). AL is supported by Wellcome Trust (209437/Z/17/Z).  
727 FAPESP fellowships were awarded to JTH (2018/25316-4), DESL (2019/22715-8), GGN  
728 (2021/03400-6), EEL (2019/12234-2), GSC (2020/15389-4), and EBS (2018/04553-8).  
729 LM is supported by a MIBTP studentship.

730

## 731 **Author contributions**

732 JTH, DESL and EBS outlined the study. JTH, DESL, EEL, GSC, and EBS  
733 performed experiments and analyzed data. LM and AL performed protein crystallography  
734 and analyzed data. GGN and RFS contributed with bioinformatic analyses. JTH, DESL,  
735 GGN, CSF, RFdS, RSG, AL and EBS contributed to the scientific discussions. JTH and  
736 EBS wrote the manuscript with input from other authors. All authors revised and  
737 approved the manuscript.

738

## 739 **Conflict of interest**

740 The authors declare no conflict of interest.

741 **References**

- 742 Alcoforado Diniz J, Coulthurst SJ (2015) Intraspecies competition in *Serratia marcescens* is mediated by  
743 type VI-secreted Rhs effectors and a conserved effector-associated accessory protein. *Journal of*  
744 *bacteriology* 197: 2350-2360
- 745 Bao H, Zhao J-H, Zhu S, Wang S, Zhang J, Wang X-Y, Hua B, Liu C, Liu H, Liu S-L (2019) Genetic  
746 diversity and evolutionary features of type VI secretion systems in *Salmonella*. *Future microbiology*  
747 14: 139-154
- 748 Bateman A, Coin L, Durbin R, Finn RD, Hollich V, Griffiths-Jones S, Khanna A, Marshall M, Moxon S,  
749 Sonnhammer EL (2004) The Pfam protein families database. *Nucleic acids research* 32: D138-D141
- 750 Bayer-Santos E, Cenens W, Matsuyama BY, Oka GU, Di Sessa G, Mininel IDV, Alves TL, Farah CS  
751 (2019) The opportunistic pathogen *Stenotrophomonas maltophilia* utilizes a type IV secretion system  
752 for interbacterial killing. *PLoS pathogens* 15: e1007651
- 753 Benz J, Reinstein J, Meinhart A (2013) Structural insights into the effector–immunity system Tae4/Tai4  
754 from *Salmonella typhimurium*. *PLoS One* 8: e67362
- 755 Benz J, Sendlmeier C, Barends TR, Meinhart A (2012) Structural insights into the effector–immunity  
756 system Tse1/Tsi1 from *Pseudomonas aeruginosa*. *PLoS One* 7: e40453
- 757 Berman H, Henrick K, Nakamura H, Markley JL (2007) The worldwide Protein Data Bank (wwPDB):  
758 ensuring a single, uniform archive of PDB data. *Nucleic acids research* 35: D301-D303
- 759 Blondel CJ, Jiménez JC, Contreras I, Santiviago CA (2009) Comparative genomic analysis uncovers 3  
760 novel loci encoding type six secretion systems differentially distributed in *Salmonella* serotypes. *BMC*  
761 *genomics* 10: 1-17
- 762 Blondel CJ, Jiménez JC, Leiva LE, Álvarez SA, Pinto BI, Contreras F, Pezoa D, Santiviago CA, Contreras  
763 I (2013) The type VI secretion system encoded in *Salmonella* pathogenicity island 19 is required for  
764 *Salmonella enterica* serotype Gallinarum survival within infected macrophages. *Infection and immunity*  
765 81: 1207-1220
- 766 Bondage DD, Lin J-S, Ma L-S, Kuo C-H, Lai E-M (2016) VgrG C terminus confers the type VI effector  
767 transport specificity and is required for binding with PAAR and adaptor–effector complex. *Proceedings*  
768 *of the National Academy of Sciences* 113: E3931-E3940
- 769 Brunet YR, Khodr A, Logger L, Aussel L, Mignot T, Rimsky S, Cascales E (2015) H-NS silencing of the  
770 *Salmonella* pathogenicity island 6-encoded type VI secretion system limits *Salmonella enterica* serovar  
771 Typhimurium interbacterial killing. *Infection and immunity* 83: 2738-2750
- 772 Burkinshaw BJ, Liang X, Wong M, Le AN, Lam L, Dong TG (2018) A type VI secretion system effector  
773 delivery mechanism dependent on PAAR and a chaperone–co-chaperone complex. *Nature*  
774 *microbiology* 3: 632-640
- 775 Camacho C, Coulouris G, Avagyan V, Ma N, Papadopoulos J, Bealer K, Madden TL (2009) BLAST+:  
776 architecture and applications. *BMC bioinformatics* 10: 1-9
- 777 Capella-Gutiérrez S, Silla-Martínez JM, Gabaldón T (2009) trimAl: a tool for automated alignment  
778 trimming in large-scale phylogenetic analyses. *Bioinformatics* 25: 1972-1973
- 779 Carver T, Thomson N, Bleasby A, Berriman M, Parkhill J (2009) DNAPlotter: circular and linear  
780 interactive genome visualization. *Bioinformatics* 25: 119-120
- 781 Carver TJ, Rutherford KM, Berriman M, Rajandream M-A, Barrell BG, Parkhill J (2005) ACT: the Artemis  
782 comparison tool. *Bioinformatics* 21: 3422-3423
- 783 Christensen H, Nordentoft S, Olsen JE (1998) NOTE: Phylogenetic relationships of *Salmonella* based on  
784 rRNA sequences. *International Journal of Systematic and Evolutionary Microbiology* 48: 605-610
- 785 Cianfanelli FR, Monlezun L, Coulthurst SJ (2016) Aim, load, fire: the type VI secretion system, a bacterial  
786 nanoweapon. *Trends in microbiology* 24: 51-62
- 787 Coulthurst S (2019) The Type VI secretion system: a versatile bacterial weapon. *Microbiology* 165: 503-  
788 515

- 789 Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12  
790 using PCR products. *Proceedings of the National Academy of Sciences* 97: 6640-6645
- 791 DeLano WL (2002) Pymol: An open-source molecular graphics tool. *CCP4 Newsletter on protein*  
792 *crystallography* 40: 82-92
- 793 Dong C, Zhang H, Gao Z-Q, Wang W-J, She Z, Liu G-F, Shen Y-Q, Su X-D, Dong Y-H (2013) Structural  
794 insights into the inhibition of type VI effector Tae3 by its immunity protein Tai3. *Biochemical Journal*  
795 454: 59-68
- 796 Dykxhoorn DM, Pierre RS, Linn T (1996) A set of compatible tac promoter expression vectors. *Gene* 177:  
797 133-136
- 798 Eddy SR (2011) Accelerated profile HMM searches. *PLoS computational biology* 7: e1002195
- 799 Fitzsimons TC, Lewis JM, Wright A, Kleifeld O, Schittenhelm RB, Powell D, Harper M, Boyce JD (2018)  
800 Identification of novel *Acinetobacter baumannii* type VI secretion system antibacterial effector and  
801 immunity pairs. *Infection and immunity* 86: e00297-00218
- 802 Fookes M, Schroeder GN, Langridge GC, Blondel CJ, Mammina C, Connor TR, Seth-Smith H, Vernikos  
803 GS, Robinson KS, Sanders M (2011) *Salmonella bongori* provides insights into the evolution of the  
804 *Salmonellae*. *PLoS pathogens* 7: e1002191
- 805 Fox NK, Brenner SE, Chandonia J-M (2014) SCOPe: Structural Classification of Proteins—extended,  
806 integrating SCOP and ASTRAL data and classification of new structures. *Nucleic acids research* 42:  
807 D304-D309
- 808 Fraikin N, Goormaghtigh F, Van Melderen L (2020) Type II toxin-antitoxin systems: evolution and  
809 revolutions. *Journal of bacteriology* 202: e00763-00719
- 810 Gwon GH, Kim Y, Liu Y, Watson AT, Jo A, Etheridge TJ, Yuan F, Zhang Y, Kim Y, Carr AM (2014)  
811 Crystal structure of a Fanconi anemia-associated nuclease homolog bound to 5' flap DNA: basis of  
812 interstrand cross-link repair by FAN1. *Genes & development* 28: 2276-2290
- 813 Hachani A, Allsopp LP, Oduko Y, Filloux A (2014) The VgrG proteins are “a la carte” delivery systems  
814 for bacterial type VI effectors. *Journal of Biological Chemistry* 289: 17872-17884
- 815 Hadden JM, Déclais AC, Phillips SE, Lilley DM (2002) Metal ions bound at the active site of the junction-  
816 resolving enzyme T7 endonuclease I. *The EMBO journal* 21: 3505-3515
- 817 Hensel M, Shea JE, Gleeson C, Jones MD, Dalton E, Holden DW (1995) Simultaneous identification of  
818 bacterial virulence genes by negative selection. *Science* 269: 400-403
- 819 Hickman AB, Li Y, Mathew SV, May EW, Craig NL, Dyda F (2000) Unexpected structural diversity in  
820 DNA recombination: the restriction endonuclease connection. *Molecular cell* 5: 1025-1034
- 821 Holm L (2020) Using Dali for protein structure comparison. In: *Structural Bioinformatics*, pp. 29-42.  
822 Springer:
- 823 Iyer LM, Babu M, Aravind L (2006) The HIRAN domain and recruitment of chromatin remodeling and  
824 repair activities to damaged DNA. *Cell Cycle* 5: 775-782
- 825 Jana B, Fridman CM, Bosis E, Salomon D (2019) A modular effector with a DNase domain and a marker  
826 for T6SS substrates. *Nature communications* 10: 1-12
- 827 Jana B, Salomon D (2019) Type VI secretion system: a modular toolkit for bacterial dominance. *Future*  
828 *microbiology* 14: 1451-1463
- 829 Jennings E, Thurston TL, Holden DW (2017) *Salmonella* SPI-2 type III secretion system effectors:  
830 molecular mechanisms and physiological consequences. *Cell Host & Microbe* 22: 217-231
- 831 Jin H, Roy U, Lee G, Schärer OD, Cho Y (2018) Structural mechanism of DNA interstrand cross-link  
832 unhooking by the bacterial FAN1 nuclease. *Journal of Biological Chemistry* 293: 6482-6496
- 833 Jumper J, Evans R, Pritzel A, Green T, Figurnov M, Ronneberger O, Tunyasuvunakool K, Bates R, Židek  
834 A, Potapenko A (2021) Highly accurate protein structure prediction with AlphaFold. *Nature* 596: 583-  
835 589
- 836 Jurėnas D, Journet L (2021) Activity, delivery, and diversity of Type VI secretion effectors. *Molecular*  
837 *Microbiology* 115: 383-394

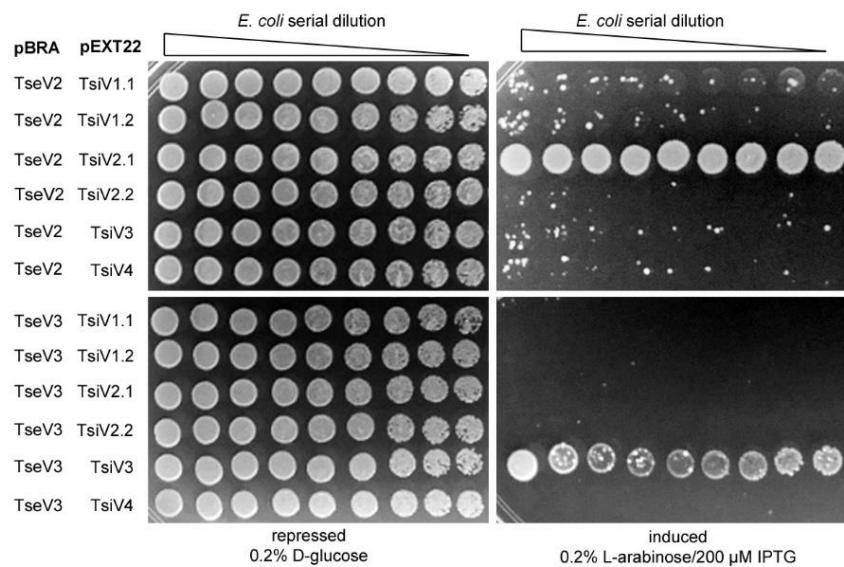
- 838 Katoh K, Standley DM (2013) MAFFT multiple sequence alignment software version 7: improvements in  
839 performance and usability. *Molecular biology and evolution* 30: 772-780
- 840 Kinch LN, Ginalski K, Rychlewski L, Grishin NV (2005) Identification of novel restriction endonuclease-  
841 like fold families among hypothetical proteins. *Nucleic acids research* 33: 3598-3605
- 842 Koskiniemi S, Lamoureux JG, Nikolakakis KC, de Roodenbeke CtK, Kaplan MD, Low DA, Hayes CS  
843 (2013) Rhs proteins from diverse bacteria mediate intercellular competition. *Proceedings of the*  
844 *National Academy of Sciences* 110: 7032-7037
- 845 Larsson A (2014) AliView: a fast and lightweight alignment viewer and editor for large datasets.  
846 *Bioinformatics* 30: 3276-3278
- 847 Leiman PG, Basler M, Ramagopal UA, Bonanno JB, Sauder JM, Pukatzki S, Burley SK, Almo SC,  
848 Mekalanos JJ (2009) Type VI secretion apparatus and phage tail-associated protein complexes share a  
849 common evolutionary origin. *Proceedings of the National Academy of Sciences* 106: 4154-4159
- 850 Li L, Zhang W, Liu Q, Gao Y, Gao Y, Wang Y, Wang DZ, Li Z, Wang T (2013) Structural Insights on the  
851 bacteriolytic and self-protection mechanism of muramidase effector Tse3 in *Pseudomonas aeruginosa*.  
852 *Journal of Biological Chemistry* 288: 30607-30613
- 853 Liang X, Pei T-T, Li H, Zheng H-Y, Luo H, Cui Y, Tang M-X, Zhao Y-J, Xu P, Dong T (2021) VgrG-  
854 dependent effectors and chaperones modulate the assembly of the type VI secretion system. *PLoS*  
855 *pathogens* 17: e1010116
- 856 Lu D, Zheng Y, Liao N, Wei L, Xu B, Liu X, Liu J (2014) The structural basis of the Tle4–Tli4 complex  
857 reveals the self-protection mechanism of H2-T6SS in *Pseudomonas aeruginosa*. *Acta Crystallographica*  
858 *Section D: Biological Crystallography* 70: 3233-3243
- 859 Ma J, Pan Z, Huang J, Sun M, Lu C, Yao H (2017a) The Hcp proteins fused with diverse extended-toxin  
860 domains represent a novel pattern of antibacterial effectors in type VI secretion systems. *Virulence* 8:  
861 1189-1202
- 862 Ma J, Sun M, Dong W, Pan Z, Lu C, Yao H (2017b) PAAR-Rhs proteins harbor various C-terminal toxins  
863 to diversify the antibacterial pathways of type VI secretion systems. *Environmental microbiology* 19:  
864 345-360
- 865 Ma L-S, Hachani A, Lin J-S, Filloux A, Lai E-M (2014) *Agrobacterium tumefaciens* deploys a superfamily  
866 of type VI secretion DNase effectors as weapons for interbacterial competition in planta. *Cell host &*  
867 *microbe* 16: 94-104
- 868 MacKay C, Déclais A-C, Lundin C, Agostinho A, Deans AJ, MacArtney TJ, Hofmann K, Gartner A, West  
869 SC, Helleday T (2010) Identification of KIAA1018/FAN1, a DNA repair nuclease recruited to DNA  
870 damage by monoubiquitinated FANCD2. *Cell* 142: 65-76
- 871 McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ (2007) Phaser  
872 crystallographic software. *Journal of applied crystallography* 40: 658-674
- 873 Mirdita M, Schütze K, Moriwaki Y, Heo L, Ovchinnikov S, Steinegger M (2021) ColabFold-Making  
874 protein folding accessible to all.
- 875 Mougous JD, Cuff ME, Raunser S, Shen A, Zhou M, Gifford CA, Goodman AL, Joachimiak G, Ordoñez  
876 CL, Lory S (2006) A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus.  
877 *Science* 312: 1526-1530
- 878 Nguyen VS, Douzi B, Durand E, Roussel A, Cascales E, Cambillau C (2018) Towards a complete structural  
879 deciphering of Type VI secretion system. *Current Opinion in Structural Biology* 49: 77-84
- 880 Pei T-T, Kan Y, Wang Z-H, Tang M-X, Li H, Yan S, Cui Y, Zheng H-Y, Luo H, Dong T (2021) Breaching  
881 the cell-envelope barriers of gram-positive and fungal microbes by a type VI secretion system in  
882 *Acidovorax citrulli*. *bioRxiv*
- 883 Pennell S, Déclais A-C, Li J, Haire LF, Berg W, Saldanha JW, Taylor IA, Rouse J, Lilley DM, Smerdon  
884 SJ (2014) FAN1 activity on asymmetric repair intermediates is mediated by an atypical monomeric  
885 virus-type replication-repair nuclease domain. *Cell reports* 8: 84-93
- 886 Peterson SB, Bertolli SK, Mougous JD (2020) The central role of interbacterial antagonism in bacterial life.  
887 *Current Biology* 30: R1203-R1214

- 888 Petty NK, Bulgin R, Crepin VF, Cerdeño-Tárraga AM, Schroeder GN, Quail MA, Lennard N, Corton C,  
889 Barron A, Clark L (2010) The *Citrobacter rodentium* genome sequence reveals convergent evolution  
890 with human pathogenic *Escherichia coli*. *Journal of bacteriology* 192: 525-538
- 891 Pezoa D, Blondel CJ, Silva CA, Yang H-J, Andrews-Polymenis H, Santiviago CA, Contreras I (2014) Only  
892 one of the two type VI secretion systems encoded in the *Salmonella enterica* serotype Dublin genome  
893 is involved in colonization of the avian and murine hosts. *Veterinary research* 45: 1-9
- 894 Pissaridou P, 2018. *Pseudomonas Aeruginosa* Type Six Secretion System (T6SS): The Tse7 DNase  
895 Effector and Post-translational Regulation of the H1-T6SS. Imperial College London.
- 896 Potter SC, Luciani A, Eddy SR, Park Y, Lopez R, Finn RD (2018) HMMER web server: 2018 update.  
897 *Nucleic acids research* 46: W200-W204
- 898 Price MN, Dehal PS, Arkin AP (2010) FastTree 2—approximately maximum-likelihood trees for large  
899 alignments. *PLoS one* 5: e9490
- 900 Renault MG, Beas JZ, Douzi B, Chabalier M, Zoued A, Brunet YR, Cambillau C, Journet L, Cascales E  
901 (2018) The gp27-like hub of VgrG serves as adaptor to promote Hcp tube assembly. *Journal of*  
902 *molecular biology* 430: 3143-3156
- 903 Robb CS, Robb M, Nano FE, Boraston AB (2016) The structure of the toxin and type six secretion system  
904 substrate Tse2 in complex with its immunity protein. *Structure* 24: 277-284
- 905 Ronen M, Rosenberg R, Shraiman BI, Alon U (2002) Assigning numbers to the arrows: parameterizing a  
906 gene regulation network by using accurate expression kinetics. *Proceedings of the national academy of*  
907 *sciences* 99: 10555-10560
- 908 Ronning DR, Li Y, Perez ZN, Ross PD, Hickman AB, Craig NL, Dyda F (2004) The carboxy-terminal  
909 portion of TnsC activates the Tn7 transposase through a specific interaction with TnsA. *The EMBO*  
910 *journal* 23: 2972-2981
- 911 Russell AB, Singh P, Brittnacher M, Bui NK, Hood RD, Carl MA, Agnello DM, Schwarz S, Goodlett DR,  
912 Vollmer W (2012) A widespread bacterial type VI secretion effector superfamily identified using a  
913 heuristic approach. *Cell host & microbe* 11: 538-549
- 914 Salih O, He S, Planamente S, Stach L, MacDonald JT, Manoli E, Scheres SH, Filloux A, Freemont PS  
915 (2018) Atomic structure of type VI contractile sheath from *Pseudomonas aeruginosa*. *Structure* 26: 329-  
916 336. e323
- 917 Sana TG, Flaugnatti N, Lugo KA, Lam LH, Jacobson A, Baylot V, Durand E, Journet L, Cascales E,  
918 Monack DM (2016) *Salmonella Typhimurium* utilizes a T6SS-mediated antibacterial weapon to  
919 establish in the host gut. *Proceedings of the National Academy of Sciences* 113: E5044-E5051
- 920 Sayers EW, Agarwala R, Bolton EE, Brister JR, Canese K, Clark K, Connor R, Fiorini N, Funk K, Hefferon  
921 T (2019) Database resources of the national center for biotechnology information. *Nucleic acids*  
922 *research* 47: D23
- 923 Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C,  
924 Saalfeld S, Schmid B (2012) Fiji: an open-source platform for biological-image analysis. *Nature*  
925 *methods* 9: 676-682
- 926 Schroll C, Huang K, Ahmed S, Kristensen BM, Pors SE, Jelsbak L, Lemire S, Thomsen LE, Christensen  
927 JP, Jensen PR (2019) The SPI-19 encoded type-six secretion-systems (T6SS) of *Salmonella enterica*  
928 serovars Gallinarum and Dublin play different roles during infection. *Veterinary microbiology* 230: 23-  
929 31
- 930 Shee C, Cox BD, Gu F, Luengas EM, Joshi MC, Chiu L-Y, Magnan D, Halliday JA, Frisch RL, Gibson JL  
931 (2013) Engineered proteins detect spontaneous DNA breakage in human and bacterial cells. *Elife* 2:  
932 e01222
- 933 Shneider MM, Buth SA, Ho BT, Basler M, Mekalanos JJ, Leiman PG (2013) PAAR-repeat proteins sharpen  
934 and diversify the type VI secretion system spike. *Nature* 500: 350-353
- 935 Sibinelli-Sousa S, Hespanhol JT, Nicastro GG, Matsuyama BY, Mesnage S, Patel A, de Souza RF, Guzzo  
936 CR, Bayer-Santos E (2020) A family of T6SS antibacterial effectors related to I, d-transpeptidases  
937 targets the peptidoglycan. *Cell reports* 31: 107813

- 938 Sirias D, Utter DR, Gibbs KA (2020) A family of contact-dependent nuclease effectors contain an  
939 exchangeable, species-identifying domain. *bioRxiv*
- 940 Söding J, Biegert A, Lupas AN (2005) The HHpred interactive server for protein homology detection and  
941 structure prediction. *Nucleic acids research* 33: W244-W248
- 942 Souza DP, Oka GU, Alvarez-Martinez CE, Bisson-Filho AW, Dunger G, Hobeika L, Cavalcante NS,  
943 Alegria MC, Barbosa LR, Salinas RK (2015) Bacterial killing via a type IV secretion system. *Nature*  
944 *communications* 6: 1-9
- 945 Steczkiewicz K, Muszewska A, Knizewski L, Rychlewski L, Ginalski K (2012) Sequence, structure and  
946 functional diversity of PD-(D/E) XK phosphodiesterase superfamily. *Nucleic acids research* 40: 7016-  
947 7045
- 948 Steinegger M, Meier M, Mirdita M, Vöhringer H, Haunsberger SJ, Söding J (2019) HH-suite3 for fast  
949 remote homology detection and deep protein annotation. *BMC bioinformatics* 20: 1-15
- 950 Steinegger M, Söding J (2017) MMseqs2 enables sensitive protein sequence searching for the analysis of  
951 massive data sets. *Nature biotechnology* 35: 1026-1028
- 952 Ting S-Y, Bosch DE, Mangiameli SM, Radey MC, Huang S, Park Y-J, Kelly KA, Filip SK, Goo YA, Eng  
953 JK (2018) Bifunctional immunity proteins protect bacteria against FtsZ-targeting ADP-ribosylating  
954 toxins. *Cell* 175: 1380-1392. e1314
- 955 Valdivia RH, Falkow S (1996) Bacterial genetics by flow cytometry: rapid isolation of *Salmonella*  
956 typhimurium acid-inducible promoters by differential fluorescence induction. *Molecular microbiology*  
957 22: 367-378
- 958 Walker GC, 1996. The SOS response of *Escherichia coli*. *Escherichia coli and Salmonella: cellular and*  
959 *molecular biology*.
- 960 Wang J, Brackmann M, Castano-Diez D, Kudryashev M, Goldie KN, Maier T, Stahlberg H, Basler M  
961 (2017) Cryo-EM structure of the extended type VI secretion system sheath-tube complex. *Nature*  
962 *microbiology* 2: 1507-1512
- 963 Wang J, Yang B, Leier A, Marquez-Lago TT, Hayashida M, Rucker A, Zhang Y, Akutsu T, Chou K-C,  
964 Strugnell RA (2018) Bastion6: a bioinformatics approach for accurate prediction of type VI secreted  
965 effectors. *Bioinformatics* 34: 2546-2555
- 966 Wang R, Persky NS, Yoo B, Ouerfelli O, Smogorzewska A, Elledge SJ, Pavletich NP (2014) Mechanism  
967 of DNA interstrand cross-link processing by repair nuclease FAN1. *Science* 346: 1127-1130
- 968 Wang S, Geng Z, Zhang H, She Z, Dong Y (2021) The *Pseudomonas aeruginosa* PAAR2 cluster encodes  
969 a putative VRR-NUC domain-containing effector. *The FEBS Journal*
- 970 Waterhouse AM, Procter JB, Martin DM, Clamp M, Barton GJ (2009) Jalview Version 2—a multiple  
971 sequence alignment editor and analysis workbench. *Bioinformatics* 25: 1189-1191
- 972 Wen H, Liu G, Geng Z, Zhang H, Li Y, She Z, Dong Y (2021) Structure and SAXS studies unveiled a  
973 novel inhibition mechanism of the *Pseudomonas aeruginosa* T6SS TseT-TsiT complex. *International*  
974 *Journal of Biological Macromolecules* 188: 450-459
- 975 Wen J, Arakawa T, Philo JS (1996) Size-exclusion chromatography with on-line light-scattering,  
976 absorbance, and refractive index detectors for studying proteins and their interactions. *Analytical*  
977 *biochemistry* 240: 155-166
- 978 Winter G (2010) xia2: an expert system for macromolecular crystallography data reduction. *Journal of*  
979 *applied crystallography* 43: 186-190
- 980 Winter G, Waterman DG, Parkhurst JM, Brewster AS, Gildea RJ, Gerstel M, Fuentes-Montero L, Vollmar  
981 M, Michels-Clark T, Young ID (2018) DIALS: implementation and evaluation of a new integration  
982 package. *Acta Crystallographica Section D* 74: 85-97
- 983 Yadav SK, Magotra A, Ghosh S, Krishnan A, Pradhan A, Kumar R, Das J, Sharma M, Jha G (2021)  
984 Immunity proteins of dual nuclease T6SS effectors function as transcriptional repressors. *EMBO reports*  
985 22: e51857

- 986 Zhang D, de Souza RF, Anantharaman V, Iyer LM, Aravind L (2012) Polymorphic toxin systems:  
987 comprehensive characterization of trafficking modes, processing, mechanisms of action, immunity and  
988 ecology using comparative genomics. *Biology direct* 7: 1-76
- 989 Zhang H, Gao Z-Q, Wang W-J, Liu G-F, Xu J-H, Su X-D, Dong Y-H (2013) Structure of the Type VI  
990 Effector-Immunity Complex (Tae4-Tai4) Provides Novel Insights into the Inhibition Mechanism of the  
991 Effector by Its Immunity Protein\*[S]. *Journal of Biological Chemistry* 288: 5928-5939
- 992 Zhao Q, Xue X, Longerich S, Sung P, Xiong Y (2014) Structural insights into 5' flap DNA unwinding and  
993 incision by the human FAN1 dimer. *Nature communications* 5: 1-9
- 994

995 **Supplementary Figures**



- 996 **Fig. S1.** Toxicity assay in *E. coli* co-transformed with pBRA TseV2 or pBRA TseV3 and the six different  
997 immunity proteins. Only one specific immunity could abrogate the toxic effect. Images are representative  
998 of three independent experiments.  
999  
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1001

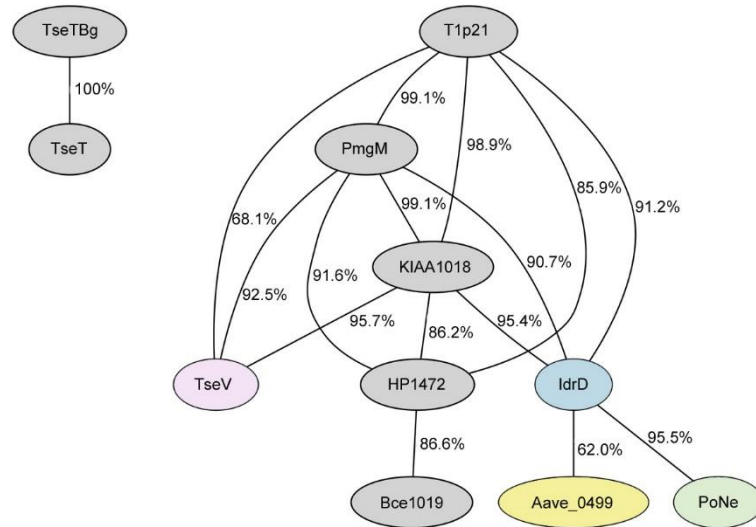
1002

**Fig. S2.** (A) Amino acid sequence alignment of VgrG1, VgrG2 and VgrG3. (B) Amino acid sequence alignment of VgrG2 and VgrG3. Amino acids are color-coded according to their properties.

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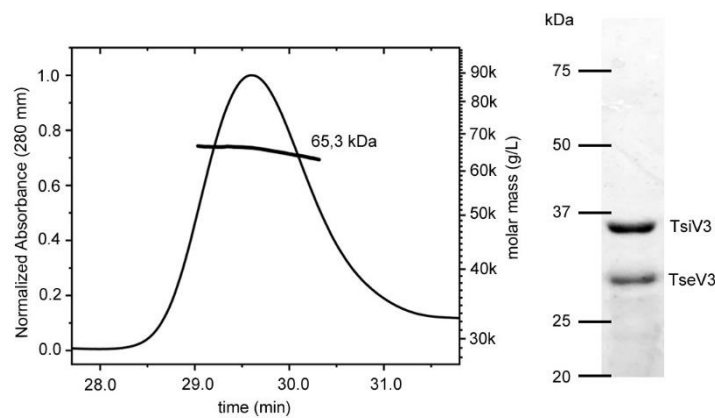




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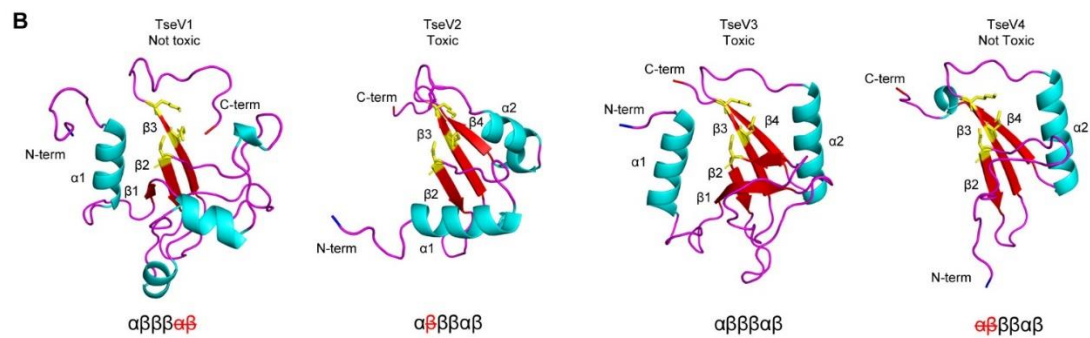
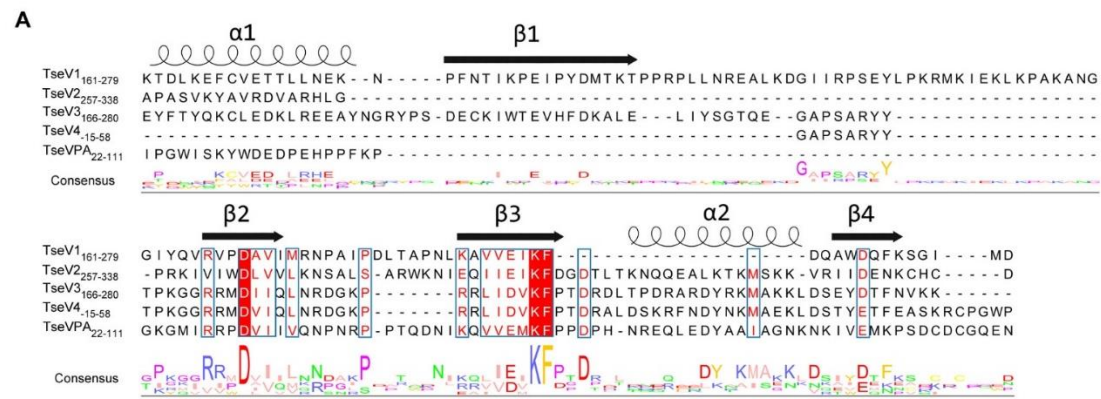
1006 **Fig. S3.** Comparison of the HMM (Hidden Markov Model) from each clade shown in Fig. 3A. All clades  
1007 present enough similarity to be clustered together, while the homologs of *P. aeruginosa* TseT and *B.*  
1008 *gladioli* TseTBg differ from the other models.

1009



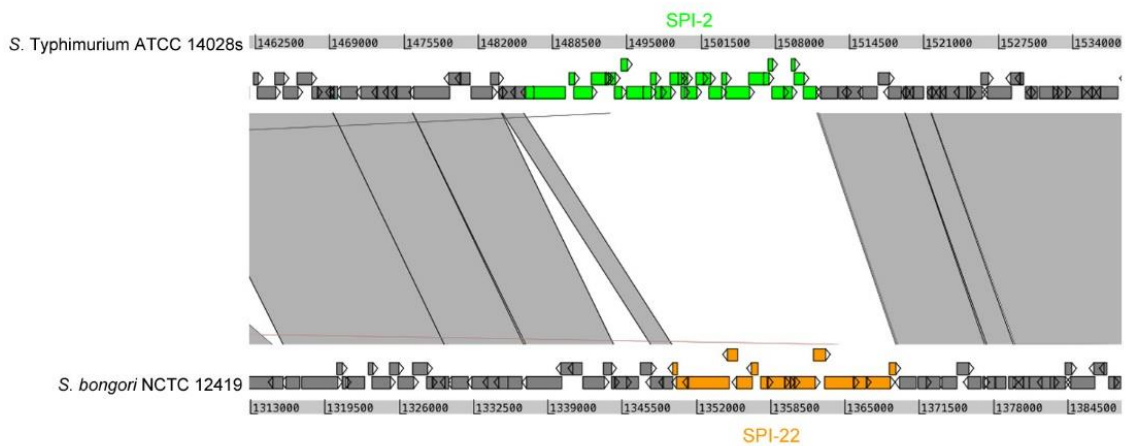
1010

1011 **Fig. S4.** SEC-MALS analysis shows the formation of a stable complex between TseV3:TsiV3. The line  
1012 corresponds to the calculated molecular mass. Right panel: SDS-PAGE showing the apparent molecular  
1013 mass of proteins eluted from SEC-MALS peak.



1014

1015 **Fig. S5.** (A) Manual amino acid sequence alignment of TseV1-4 and *P. aeruginosa* TseV (PA0822) based  
1016 on secondary structures. The secondary structures are indicated above the alignments with  $\alpha$ -helixes  
1017 represented by spirals and  $\beta$ -sheets by arrows. The conserved catalytic residues are highlighted in red with  
1018 the logo underneath the alignments. TseV4 contains another start codon located upstream of the annotated  
1019 one. (B) TseV1-4 structures predicted by the AlphaFold (Jumper *et al.*, 2021). Underneath is the conserved  
1020 PD-(D/E)xK enzymatic core with the absent structures marked in dashed red.  
1021



1022

1023 **Fig. S6.** Genomic alignment comparing *S. Typhimurium* and *S. bongori*. The regions encoding *S.*  
1024 *Typhimurium* SPI-2 T3SS and *S. bongori* SPI-22 T6SS are in focus.  
1025

1026 **Supplementary Legends**

1027

1028 **Table S1A-J.** List of all homologs collected by JackHMMER searches and used to build the  
1029 phylogenetic tree shown in Fig. 3A.

1030

1031 **Table S2A-J.** List of genes surrounding representative sequences from each VRR-Nuc subfamily.

1032

1033 **Table S3.** Crystallographic statistics of the TseV3:TsiV3 complex.

1034

1035 **Table S4.** Strains, plasmids and primers used in the study.

1036

1037 **Movie S1.** Time-lapse microscopy of *E. coli* harboring pBRA TseV2 growing in media  
1038 supplemented with 0.2% D-glucose. Timestamp in hh:mm. Scale bar: 5  $\mu$ m. Arrows indicate selected  
1039 bacteria shown in Fig. 2D.

1040

1041 **Movie S2.** Time-lapse microscopy of *E. coli* harboring pBRA TseV2 growing in media  
1042 supplemented with 0.2% L-arabinose. Timestamp in hh:mm. Scale bar: 5  $\mu$ m. Arrows indicate selected  
1043 bacteria shown in Fig. 2D.

1044

1045 **Movie S3.** Time-lapse microscopy of *E. coli* harboring pBRA TseV3 growing in media  
1046 supplemented with 0.2% D-glucose. Timestamp in hh:mm. Scale bar: 5  $\mu$ m. Arrows indicate selected  
1047 bacteria shown in Fig. 2D.

1048

1049 **Movie S4.** Time-lapse microscopy of *E. coli* harboring pBRA TseV3 growing in media  
1050 supplemented with 0.2% L-arabinose. Timestamp in hh:mm. Scale bar: 5  $\mu$ m. Arrows indicate selected  
1051 bacteria shown in Fig. 2D.