

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.

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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-iGlu² and mDAP³ 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 mDAP³ and D-Ala⁴ of the acceptor tetrapeptide stem or
95 replacing the D-Ala⁴ 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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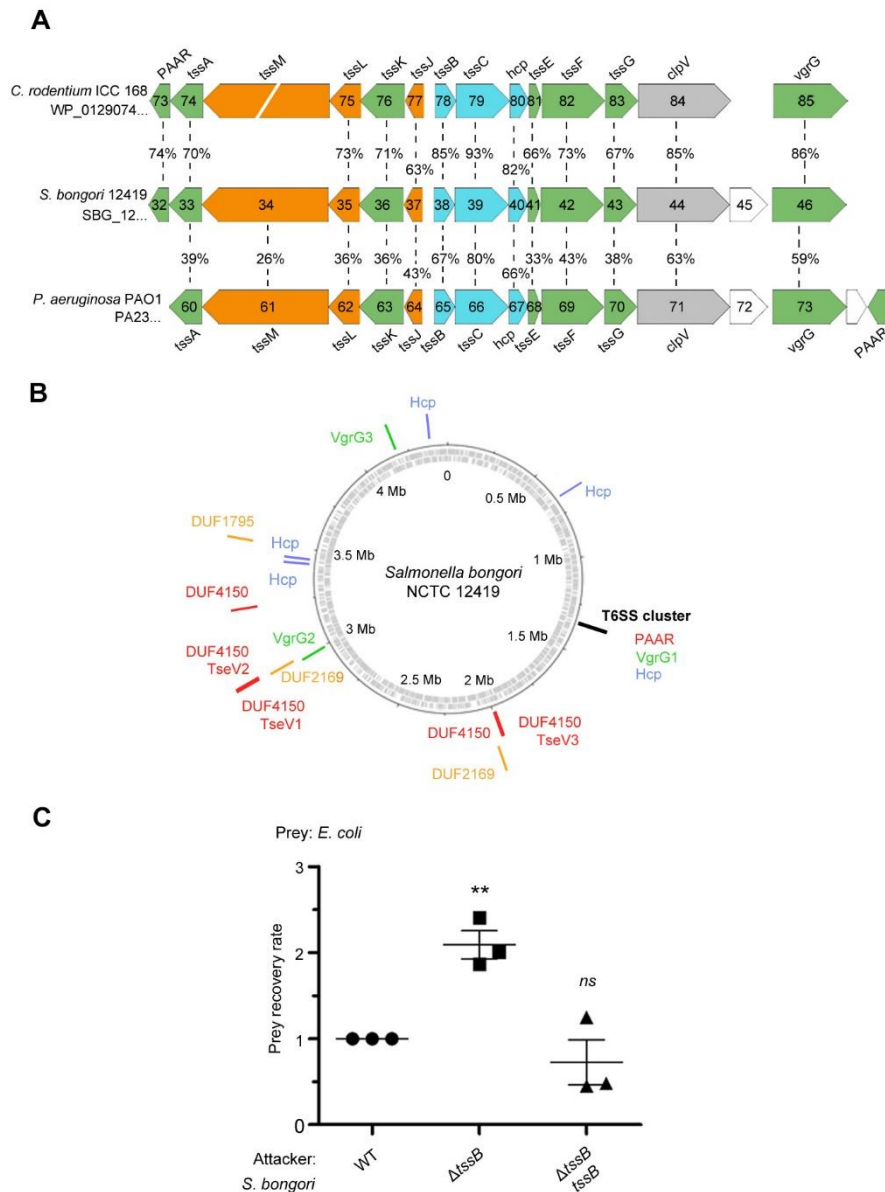
115 **Results**

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 ≥ 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_{BAD} 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_{TAC} promoter, which is inducible by isopropyl β -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

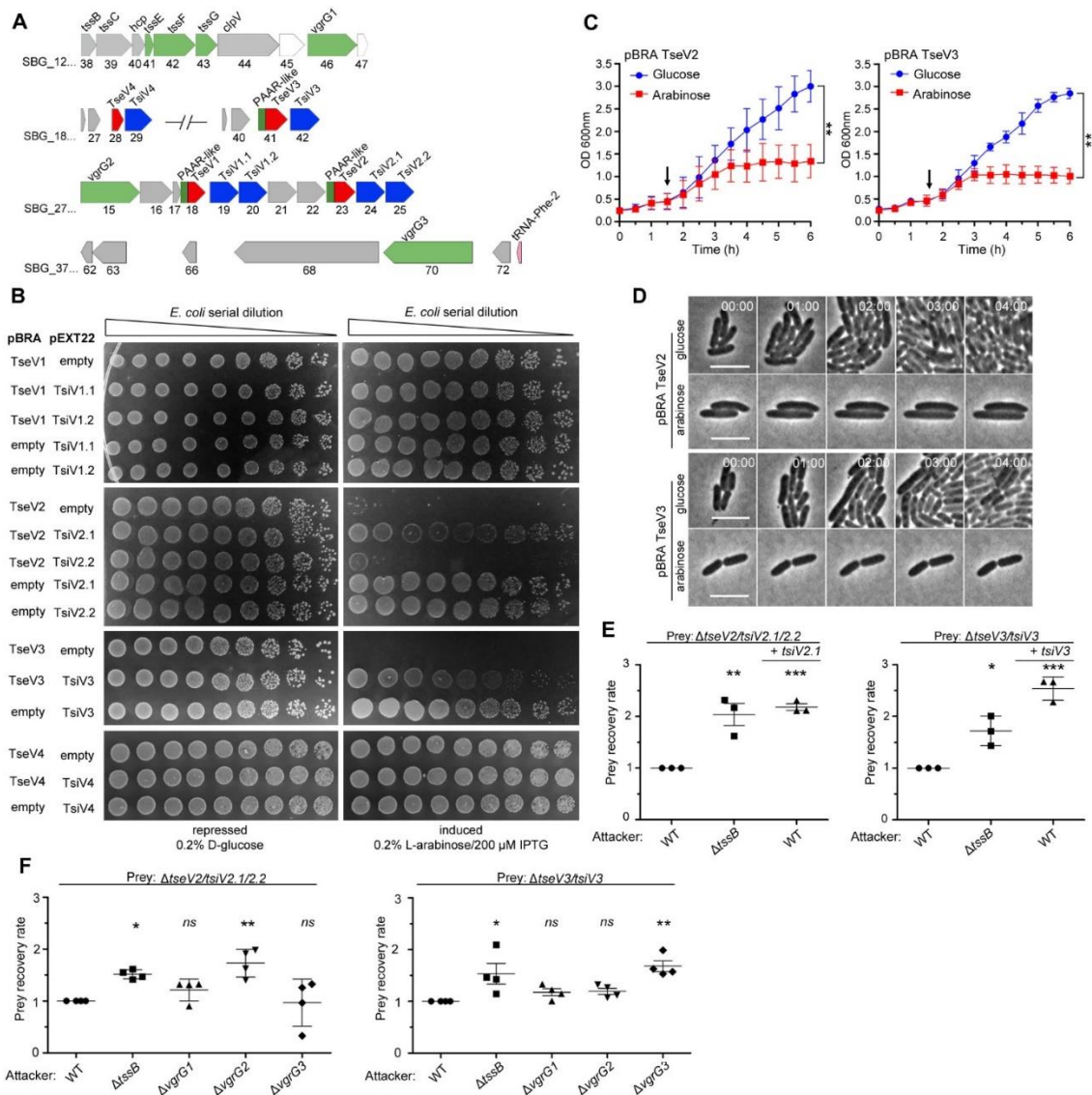
176 plus 200 μ M IPTG (Fig. 2B). Results showed that TseV2 and TseV3 are toxic in the
177 cytoplasm of *E. coli*, whereas TseV1 and TseV4 do not confer toxicity (Fig. 2B). Co-
178 expression of TseV2 with either TsiV2.1 or TsiV2.2 revealed that only the first immunity
179 protein neutralizes TseV2 toxicity (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_{600nm} 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_{600nm}
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 Δ tssB (attacker) versus *S. bongori*
196 lacking either TsiV2.1/2.2 (Δ tseV2/tsiV2.1/2.2) or TsiV3 (Δ tseV3/tsiV3) as prey (Fig. 2E).
197 Results demonstrated that the prey recovery rate was higher when prey cells were co-
198 incubated with Δ 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 (VgrG2₁₋₅₆₅ and
214 VgrG3₁₋₅₄₅) but display a distinct C-terminal domain with only 26% identity (VgrG2₅₆₆₋
215 ₇₀₉ and VgrG3₅₄₆₋₇₂₈) (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 μ 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,

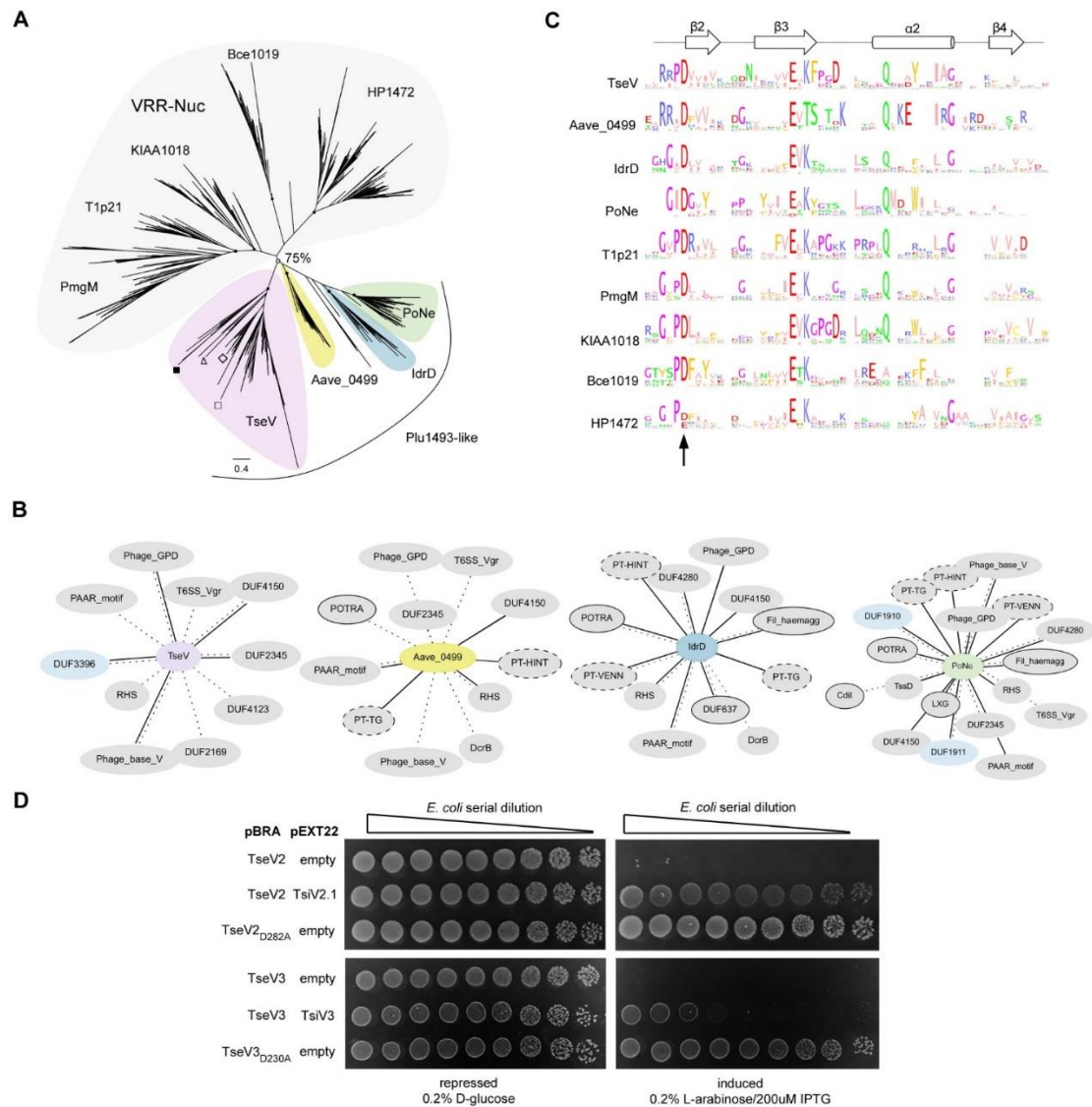
262 KIAA1018, HP1472 and Plu1493) (Iyer *et al.*, 2006), and recently reported *bona fide* or
263 putative T6SS effectors that also belong to the PD-(D/E)xK superfamily: TseT
264 (Burkinshaw *et al.*, 2018); PoNe (Jana *et al.*, 2019); IdrD-CT (Sirias *et al.*, 2020); TseTBg
265 (Yadav *et al.*, 2021); Aave_0499 (Pei *et al.*, 2021); and TseV^{PA} (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^{PA} (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_{D282A} and TseV_{D230A}) 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^{PA}).

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.

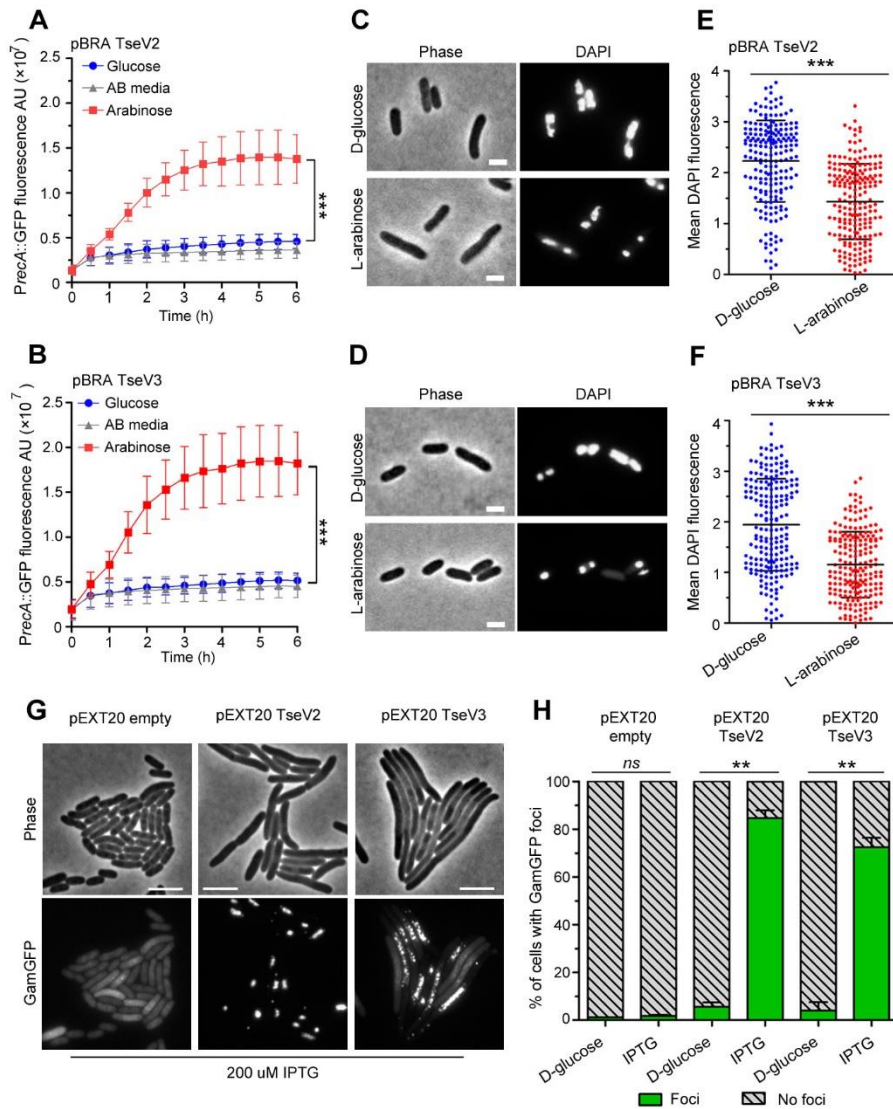
326

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 μ 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_{recA}::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 μ 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 μ 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 ± 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 TseV3₁₃₂₋₂₈₁ and TsiV3₁₀₋₃₂₇ 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.

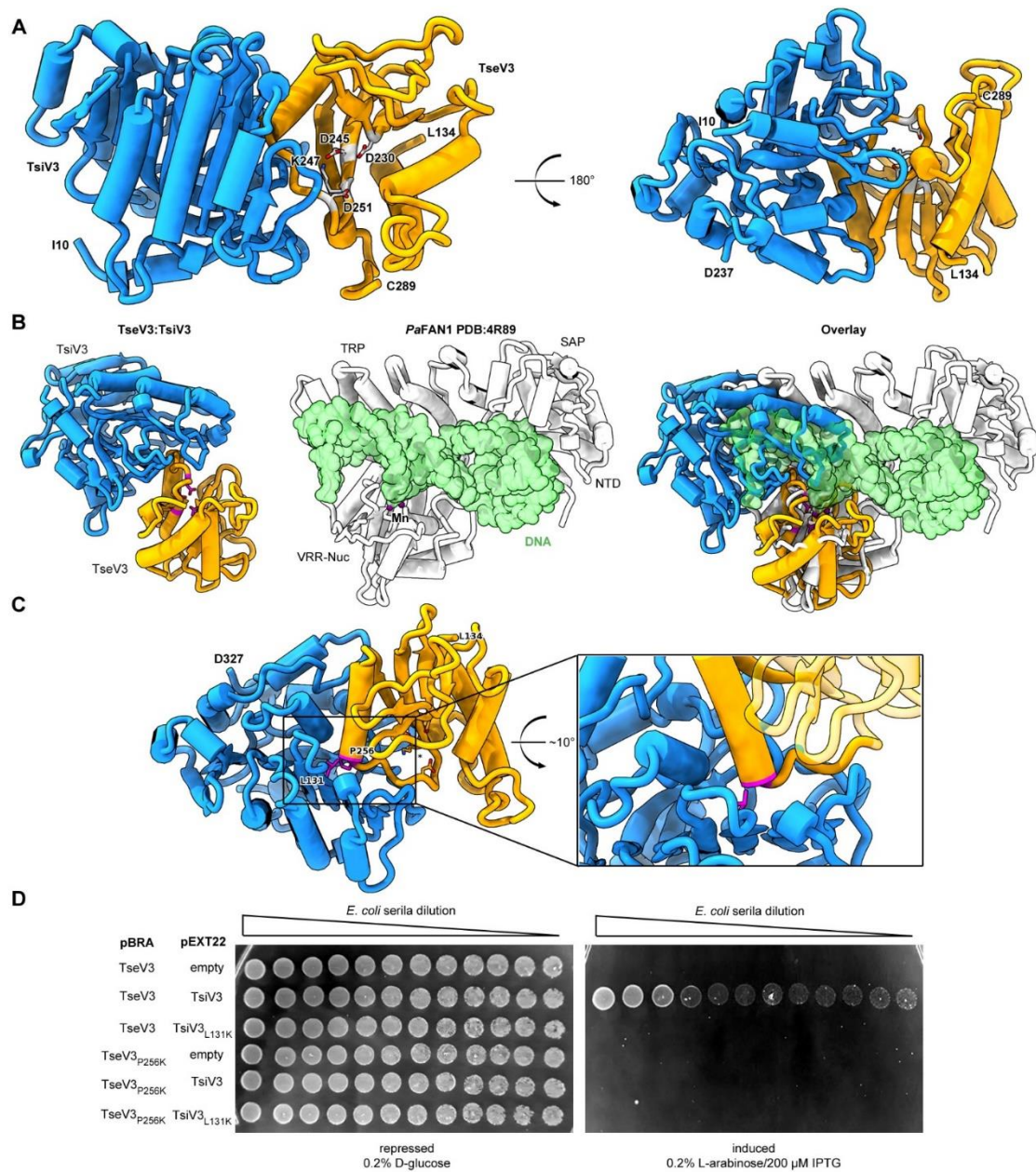
410 TsiV3 possesses a central β -sheet that is flanked by α -helices on one side and
411 exposed on the other (Fig. 5A). This exposed β -sheet surface of TsiV3 binds to an α - β
412 element of TseV3 that flanks its putative active site (composed of residues D₂₃₀, D₂₄₅,
413 K₂₄₇) (Fig. 5A). In this configuration, the TseV3-interacting α -helix, which corresponds
414 to the α_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 β -sheet (Fig. 5A).

416 Searches for structures similar to TseV3₁₃₂₋₂₈₁ and TsiV3₁₀₋₃₂₇ 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 α 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 MD₂₃₀IX_nD₂₄₅VK₂₄₇ 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 D₂₃₀, D₂₄₅ and K₂₄₇ of the former with
427 residues D₅₀₇, E₅₂₂, and K₅₂₄ 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 α_2 of
448 TseV3 was replaced by lysine (TseV3_{P256K}), and residue L131 in the central β -sheet of
449 TsiV3 was also replaced by lysine (TsiV3_{L131K}) (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_{L131K} was unable to neutralize TseV3 toxicity. In
452 addition, the mutated TseV3_{P256K} 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 (I₁₀-D₃₂₇) and

460 TseV3 in orange (L₁₃₄-C₂₈₉). Models are labeled to assist interpretation. PD-(D/E)xK superfamily

461 conserved residues of TseV3 (D₂₃₀, D₂₄₅, and K₂₄₇) 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 Mn²⁺ 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 L₁₃₁ of TsiV3 and P₂₅₆ 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 (L₁₃₁K) or TseV3 (P₂₅₆K) 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.*,
479 2021) – for consistency we have decided to keep the name TseV for this group of
480 effectors. Proteins containing the VRR-Nuc domain comprise a family (Iyer *et al.*, 2006)
481 belonging to the PD-(D/E)xK superfamily, which contain a conserved enzymatic core
482 composed by $\alpha_1\beta_1\beta_2\beta_3\alpha_2\beta_4$ (Steczkiewicz *et al.*, 2012). The conserved catalytic residues
483 (D, E, K) are located in the central $\beta_2\beta_3$ -sheet, while the α_1 -helix is associated with the
484 formation of the active site and α_2 -helix with substrate binding (Steczkiewicz *et al.*, 2012).
485 Curiously, *S. bongori* encodes four TseV homologs: TseV2 and TseV3 are toxic in *E.*
486 *coli*, whereas TseV1 and TseV4 are not toxic (Fig. 2). Based on what is known about the
487 catalytic mechanism of PD-(D/E)xK nucleases, we hypothesize that the lack of α_2 - and
488 α_1 -helix in TseV1 and TseV4, respectively, might explain the lack of toxicity (Fig. S5).
489 Another curiosity is the presence of two homologs of the DUF3396 immunity genes
490 downstream of both TseV2 and TseV1 (Fig. 2A). Such genomic organization is also
491 conserved in other bacterial species like *Photorhabdus thracensis* (VY86_01065,
492 VY86_01040), *Photorhabdus asymbiotica* (PAU_03539, PAU_03660)
493 (Enterobacterales), *Marinobacter nauticus* (MARHY2492) (Pseudomonadales) and
494 *Herbaspirillum huttiense* (E2K99_00955) (Burkholderiales). The fact that only one
495 immunity protein (TsiV2.1) can neutralize the effector (TseV2) makes us wonder about

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 β -sheet binds to the α_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_{BAD} 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_{TAC} promoter
576 (Dykhhoorn *et al*, 1996). TseV2 and TseV3 were cloned in the pEX20 vector under the
577 control of P_{TAC} 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, Δ tssB,
587 Δ tseV2/tsiV2.1/tsiV2.2 and Δ tseV3/tsiV3) as attackers, and *E. coli* K-12 W3110 carrying
588 pFPV25.1 Amp^R as prey. Overnight cultures of the attacker and prey cells were sub-
589 cultured in LB (1:30) until reaching OD_{600nm} 1.6, then adjusted to OD_{600nm} 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 α (LB with 0.2% glucose) carrying effectors (in
598 pBRA) and immunity proteins (in pEXT22) were adjusted to OD_{600nm} 1, serially diluted
599 in LB (1:4) and 5 μ L were spotted onto LB agar plates containing either 0.2% D-glucose
600 or 0.2% L-arabinose plus 200 μ 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 α harboring pBRA
609 TseV2 or TseV3 were sub-cultured in LB (1:50) with 0.2% D-glucose until reaching
610 OD_{600nm} 0.4-0.6 and adjusted to OD_{600nm} 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 10⁻⁶ 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 α harboring the reporter plasmid pSC101-
645 P_{recA}::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_{600nm} 0.4-0.6. Bacteria were
647 harvested and resuspended in AB defined media (0.2% (NH₄)₂SO₄, 0.6% Na₂HPO₄, 0.3%
648 KH₂PO₄, 0.3% NaCl, 0.1 mM CaCl₂, 1 mM MgCl₂, 3 μ M FeCl₃) supplemented with 0.2%
649 sucrose, 0.2% casamino acids, 10 μ g/mL thiamine and 25 μ g/mL uracil (Bayer-Santos *et*
650 *al.*, 2019). Cells (OD_{600nm} 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 μ 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 α 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_{600nm} 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 μ g/mL) for 15
660 min at room temperature. Samples were washed once with PBS before transferring 1 μ 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_{tet}
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 ng/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_{600nm} 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 μ 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 TseV3₁₃₄₋₂₈₉ and TsiV3₁₀₋₃₂₇, 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

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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.

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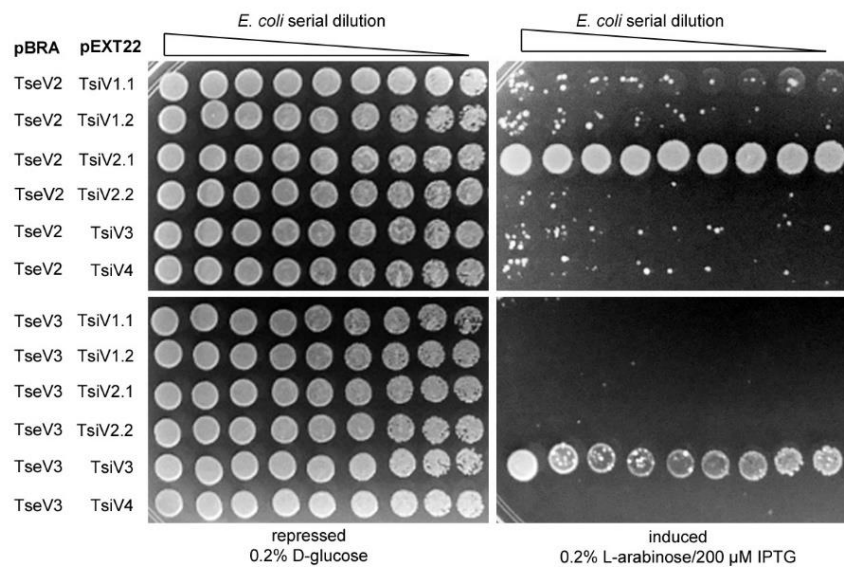
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993 incision by the human FAN1 dimer. *Nature communications* 5: 1-9
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995 Supplementary Figures



- 996
997 **Fig. S1.** Toxicity assay in *E. coli* co-transformed with pBRA TseV2 or pBRA TseV3 and the six different
998 immunity proteins. Only one specific immunity could abrogate the toxic effect. Images are representative
999 of three independent experiments.
- 1000



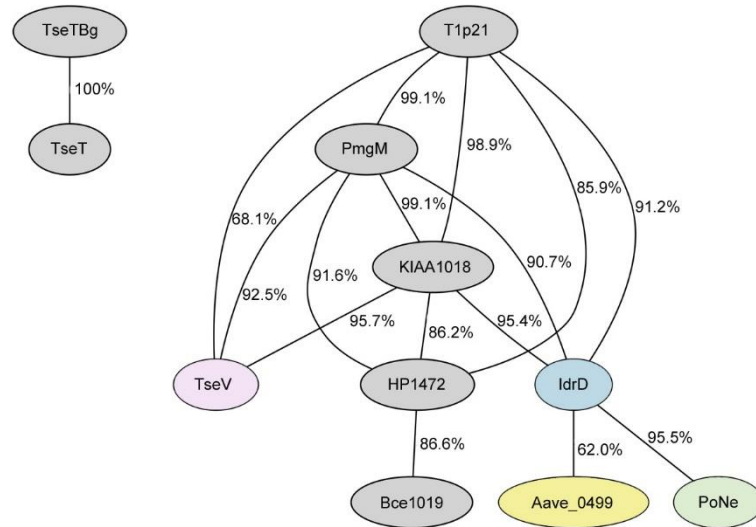
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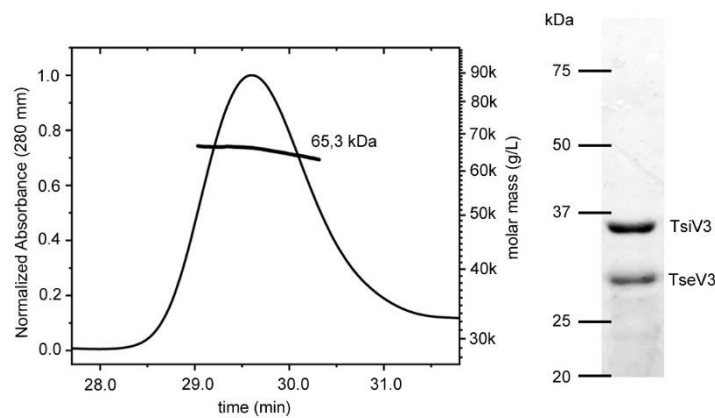
Fig. S2. (A) Amino acid sequence alignment of VgrG1, VgrG2 and VgrG3. (B) Amino acid sequence alignment of VgrG2 and VgrG3. Amino acids are color-coded according to their properties.



1005

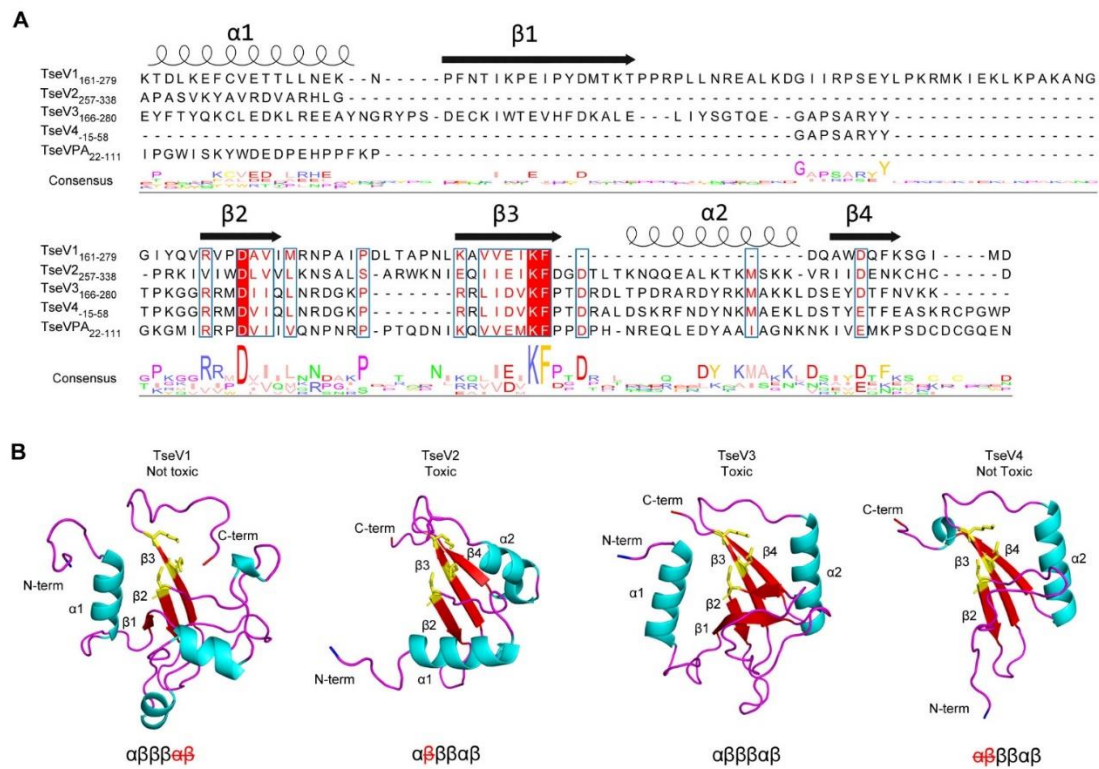
1006 **Fig. S3.** Comparison of the HMM (Hidden Markov Model) from each clade shown in Fig. 3A. All clades
1007 present enough similarity to be clustered together, while the homologs of *P. aeruginosa* TseT and *B.*
1008 *gladioli* TseTBg differ from the other models.

1009



1010

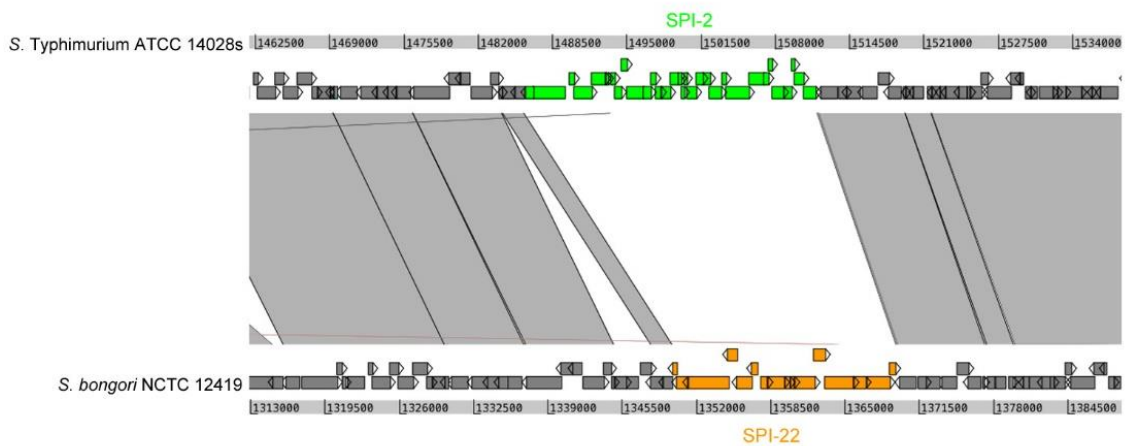
1011 **Fig. S4.** SEC-MALS analysis shows the formation of a stable complex between TseV3:TsiV3. The line
1012 corresponds to the calculated molecular mass. Right panel: SDS-PAGE showing the apparent molecular
1013 mass of proteins eluted from SEC-MALS peak.



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 α -helixes
1017 represented by spirals and β -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 μ 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 μ 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 μ 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 μ m. Arrows indicate selected
1051 bacteria shown in Fig. 2D.