

1 **Formylglycine-generating enzyme-like proteins constitute a novel family of widespread type**
2 **VI secretion system immunity proteins**

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4 Juvenal Lopez¹, Nguyen-Hung Le¹, Ki Hwan Moon^{1,2}, Dor Salomon³, Eran Bosis^{4,#} and Mario F.
5 Feldman^{1,#}

6 ¹Department of Molecular Microbiology, Washington University in St. Louis, School of Medicine,
7 St. Louis, MO, USA.

8 ²Current address: Division of Convergence on Marine Science, Korea Maritime and Ocean
9 University, Busan, Korea 49112.

10 ³Department of Clinical Microbiology and Immunology, Sackler Faculty of Medicine, Tel Aviv
11 University, Tel Aviv, Israel

12 ⁴Department of Biotechnology Engineering, ORT Braude College of Engineering, Karmiel, Israel

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14 Running Head: FGE-like T6SS immunity proteins are widespread

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16 #Address correspondence to Eran Bosis, boasis@braude.ac.il; Mario F. Feldman,
17 mariofeldman@wustl.edu

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20 **Abstract**

21 Competition is a critical aspect of bacterial life, as it enables niche establishment and
22 facilitates the acquisition of essential nutrients. Warfare between Gram-negative bacteria is largely
23 mediated by the type VI secretion system (T6SS), a dynamic nanoweapon that delivers toxic
24 effector proteins from an attacking cell to adjacent bacteria in a contact-dependent manner.
25 Effector-encoding bacteria prevent self-intoxication and kin cell killing by the expression of
26 immunity proteins, which prevent effector toxicity by specifically binding their cognate effector
27 and occluding its active site. In this study, we investigate Tsi3, a previously uncharacterized T6SS
28 immunity protein present in multiple strains of the human pathogen *Acinetobacter baumannii*. We
29 show that Tsi3 is the cognate immunity protein of the antibacterial effector of unknown function
30 Tse3. Our bioinformatic analyses indicate that Tsi3 homologs are widespread among Gram-
31 negative bacteria, often encoded within T6SS effector-immunity modules. Surprisingly, we found
32 that Tsi3 homologs possess a characteristic formylglycine-generating enzyme (FGE) domain,
33 which is present in various enzymatic proteins. Our data shows that Tsi3-mediated immunity is
34 dependent on Tse3-Tsi3 protein-protein interactions and that Tsi3 homologs from various bacteria
35 do not protect against Tse3-dependent bacterial killing. Thus, we conclude that Tsi3 homologs are
36 unlikely to be functional enzymes. Collectively, our work identifies FGE domain-containing
37 proteins as important mediators of immunity against T6SS attacks and indicates that the FGE
38 domain can be co-opted as a scaffold in multiple proteins to carry out diverse functions.

39

40 **Importance**

41 Despite the wealth of knowledge on the diversity of biochemical activities carried out by
42 T6SS effectors, comparably little is known about the various strategies bacteria employ to prevent

43 susceptibility to T6SS-dependent bacterial killing. Our work establishes a novel family of T6SS
44 immunity proteins with a characteristic FGE domain. This domain is present in enzymatic proteins
45 with various catalytic activities. Our characterization of Tsi3 expands the known functions carried
46 out by FGE-like proteins to include defense during T6SS-mediated bacterial warfare. Moreover,
47 it highlights the evolution of FGE domain-containing proteins to carry out diverse biological
48 functions.

49

50 **Introduction**

51 Bacteria employ a variety of secretion systems to adapt to and thrive in the diverse
52 environments they inhabit (1). The type VI secretion system (T6SS) of Gram-negative bacteria is
53 an especially versatile tool implicated in various functions, including bacterial antagonism,
54 horizontal gene transfer, metal ion acquisition, virulence, immune evasion and anti-fungal
55 competition (2–9). This delivery device is composed of a cytosolic, membrane-anchored
56 contractile phage tail-like complex that extends the width of the cell. When the sheath of the tail
57 complex contracts, it propels a spiked tube structure, composed of Hcp hexameric rings topped
58 with a VgrG trimer and a PAAR protein, from the attacking cell (predator) to an adjacent
59 eukaryotic or prokaryotic target cell (prey). Effectors destined to secretion via the T6SS associate
60 with the expelled Hcp-VgrG-PAAR structure via non-covalent interactions (named cargo
61 effectors) or C-terminal translational fusions (named specialized or evolved effectors) (10).

62 The versatility of the T6SS is due to the diverse arsenal of effectors that is secreted by this
63 system (11). Most T6SS effectors characterized to date mediate contact-dependent interbacterial
64 competition, with the earliest enzymatic activities identified being peptidoglycan hydrolases,
65 nucleases and phospholipases (12, 13). Non-enzymatic pore-forming effectors were also promptly

66 recognized as important mediators of bacterial killing (14). More recently, the use of modern,
67 integrative methodologies has expanded the known repertoire of antibacterial T6SS effectors to
68 include NAD(P)⁺ hydrolases, ADP-ribosyl transferases, (p)ppApp synthetases, cytidine
69 deaminases and bifunctional L,D-carboxypeptidase/L,D-transpeptidase or lytic
70 transglycosylase/endopeptidase enzymes (15–22). Despite the wealth of information regarding
71 T6SS effector function, comparably little is known about the diversity of strategies involved in
72 preventing T6SS-dependent cell death (23, 24).

73 Mechanisms of broad protection against T6SS attacks are only beginning to be uncovered.
74 In general, these mechanisms include modifying the T6SS effector target, preventing direct cell-
75 to-cell contact with the bacterial predator or mounting an appropriate stress response upon
76 perceiving an attack (25–30). Nonetheless, the most extensively studied defense mechanism
77 against T6SS attacks is the expression of immunity proteins, which are commonly encoded in an
78 operon with genes coding for a T6SS effector, and occasionally also a VgrG, PAAR or Hcp protein
79 (31). Classically, immunity proteins prevent effector toxicity by specifically binding to their
80 cognate effector and occluding its active site (32, 33). In this model, the specificity between each
81 effector-immunity protein (E-I) pair largely limits the biological role of immunity proteins to
82 mostly preventing kin cell killing, as immunity against non-kin effectors is dependent on the
83 accumulation of non-kin immunity proteins (31, 34, 35).

84 Recent characterization of the immunity protein Tri1 from *Serratia proteamaculans*
85 revealed a paradigm-shifting dual mechanism of immunity against T6SS attacks (15). First,
86 similarly to all other T6SS immunity proteins reported to date, Tri1 prevents intoxication by the
87 ADP-ribosyltransferase effector Tre1 via a mechanism of direct E-I protein-protein interactions.
88 Additionally, however, Tri1 is a functional ADP-ribosylhydrolase capable of preventing cell

89 toxicity by enzymatically removing Tre1-mediated ADP-ribose modifications off of the essential
90 bacterial tubulin-like protein FtsZ (15). Importantly, this enzymatic mechanism of immunity is
91 independent of E-I interactions, thus enabling diverse Tri1 homologs to protect against non-
92 cognate ADP-ribosyltransferase effectors (15). Despite the clear advantage of enzymatic immunity
93 proteins over canonical immunity proteins (i.e., broad T6SS effector protection), no additional
94 enzymatically active T6SS immunity proteins have been described.

95 In this study, we investigate Tsi3, a previously uncharacterized immunity protein present
96 in multiple strains of the human pathogen *Acinetobacter baumannii*. Our bioinformatic analyses
97 indicate that Tsi3 homologs are widespread among Gram-negative bacteria encoding T6SSs.
98 Surprisingly, Tsi3 homologs are predicted to structurally resemble formylglycine-generating
99 enzymes (FGEs), which are implicated in the post-translational modification of sulfatases. Here,
100 we employ genetic and biochemical approaches to investigate the hypothesis that Tsi3 homologs
101 represent a novel family of enzymatic T6SS immunity proteins.

102

103 **Results**

104 **Tse3 and Tsi3 are an E-I pair.** Previously, we showed that Tse3 from Ab17978
105 (*ACX60_11695*, accession number WP_001070510.1) is a potent antibacterial T6SS effector of
106 unknown function capable of killing *Escherichia coli* and *S. marcescens* (36, 37). Given the
107 genetic proximity to *tse3*, we hypothesized that gene *ACX60_11690* (accession number
108 WP_032046197.1, hereafter “*t si3*”) coded for the immunity protein of Tse3 (Fig. 1a). Interestingly,
109 *t si3* is encoded in the opposite strand compared to *tse3*, which is an unusual genetic arrangement
110 compared with other E-I pairs. To determine whether Tsi3 confers protection from Tse3
111 intoxication, we incubated wild-type (WT) Ab17978 with Ab17978 Δ 3, a mutant strain lacking the

112 entire *vgrG3-tse3-tsi3* gene cluster (Fig. 1a), and measured Ab17978Δ3 survival after 3.5 hours.
113 We found that Ab17978Δ3 was susceptible to killing by WT Ab17978 (Fig. 1b). Importantly,
114 expression of *tsi3* in the Ab17978Δ3 background (*tsi3*+) prevented killing by the WT strain (Fig.
115 1b).

116 Next, we employed a Far Western blot assay to determine whether Tsi3 physically interacts
117 with Tse3 (Fig. S1). Briefly, cell lysates of *E. coli* overexpressing FLAG-tagged Tsi3 (Tsi3-FLAG)
118 or a vector control were separated by SDS-PAGE and transferred onto a nitrocellulose membrane.
119 Transferred proteins were then renatured in-membrane and incubated with purified 6xHis-tagged
120 Tse3 (Tse3-His). After several washes, Tsi3-FLAG and Tse3-His were detected by
121 immunoblotting. In this assay, a direct protein-protein interaction between Tsi3 and Tse3 is
122 detected as a merge signal (FLAG and His, respectively) at the location of the nitrocellulose
123 membrane corresponding to Tsi3. Because the nitrocellulose membrane also contains soluble
124 proteins from *E. coli*, the absence of a Tse3-His signal elsewhere on the membrane is indicative of
125 the specificity of the Tse3-Tsi3 interaction. Our Far Western blot assay revealed that Tse3 bound
126 specifically to Tsi3 (Fig. 1c). Together, these results indicate that Tsi3 is the cognate immunity
127 protein of the antibacterial effector Tse3.

128 **Tsi3 homologs are widespread and genetically associated with T6SS genes.** We
129 employed a bioinformatics approach (38–40) to determine the prevalence of the Tse3-Tsi3 E-I pair
130 among diverse bacteria. Using RPS-Blast, we searched a local RefSeq database for homologs of
131 Tsi3. We identified 3,937 Tsi3 homologs (E-value < 10⁻⁵⁰) in 8,779 bacterial strains, including
132 alpha-, beta-, gamma- and deltaproteobacteria (Fig. S2 and Dataset S1). Importantly, the vast
133 majority of Tsi3-encoding bacteria (~98.2%) possess a T6SS (Dataset S2). In total, we identified
134 26,803 occurrences of Tsi3 homologs in bacterial genomes. In ~84.4% of cases, Tsi3 homologs

135 were found in groups of two or more adjacently; the majority were in groups of 4 Tsi3 homologs
136 (Fig. 2a-b and Dataset S3). Alignment of Tsi3 homologs using BLASTP revealed that in most
137 cases, adjacently encoded Tsi3 homologs differed from each other, possibly to provide immunity
138 against Tse3-like effectors delivered by non-kin bacterial predators (Fig. 2c and Dataset S3).
139 Furthermore, we found that Tsi3 homologs are commonly encoded on the strand opposite to a *tse3*
140 homolog, which is an unusual configuration for a T6SS E-I pair (Fig. 2a). Finally, genes enriched
141 in the neighborhood of *tsi3* include those coding for T6SS structural proteins (e.g., VgrG, Hcp and
142 PAAR), adaptor/chaperone proteins (DUF4123) and transposases, among others (Dataset S4). Our
143 bioinformatic analyses expand on previous work by the Basler group, whose work revealed similar
144 insights (41). Together, our results indicate that Tsi3 homologs are widespread and are often
145 encoded within E-I modules of bacteria encoding T6SSs.

146 **Tsi3 homologs possess a characteristic formylglycine-generating enzyme (FGE)**
147 **domain.** Surprisingly, domain prediction servers identified Tsi3 homologs as possessing an FGE
148 domain (previously referred to as DUF323) (Dataset S1) (42). Besides being present in bona fide
149 FGEs, the FGE domain is also found in non-FGE enzymes of diverse function (42), leading us to
150 hypothesize that Tsi3 homologs function as enzymatic immunity proteins. Consistent with our
151 hypothesis, Phyre2 identified enzymes PvdO, FGE and EgtB as the top three unique structural
152 homologs of Tsi3 from Ab17978 (abTsi3), each with 100% confidence (Table 1). To identify
153 putative catalytic residues of Tsi3, we modelled abTsi3 based on the known structures of PvdO
154 from *Pseudomonas aeruginosa* (paPvdO), FGE from *Streptomyces coelicolor* (scFGE) or EgtB
155 from *Mycobacterium thermophilum* (mtEgtB).

156 PvdO is a putative oxidoreductase involved in the biosynthesis of the siderophore
157 pyoverdine (43). Although the catalytic activity of PvdO has not been demonstrated *in vitro*,

158 previous work identified a glutamate residue as essential for pyoverdine biosynthesis (43, 44). We
159 found that abTsi3 has a glutamate residue in the equivalent position (E273) (Fig. 3a). However,
160 this residue is not conserved among Tsi3 homologs (Fig. S3), making it unlikely to be a catalytic
161 residue in Tsi3.

162 FGE is widespread among eukaryotic and prokaryotic organisms. FGE post-translationally
163 activates sulfatases, which catalyze the hydrolysis of sulfate esters from various substrates, thereby
164 carrying out diverse roles in hormone biosynthesis, nutrient acquisition, and host-pathogen
165 interactions (45–47). Specifically, FGE converts sulfatase cysteines or serines within a conserved
166 [C/S]-X-P-X-R motif to formylglycine (48–50). This oxygen-dependent reaction relies on two
167 active site cysteines to coordinate a copper ion, which in turn binds the FGE substrate and primes
168 it for reaction with oxygen (51–53). Notably, we found that Tsi3 homologs lack the catalytic
169 cysteines essential to FGE function (Fig. 3b and Fig. S3), indicating that Tsi3 homologs are
170 unlikely to be functional FGEs.

171 EgtB is a nonheme iron-dependent sulfoxide synthase involved in the biosynthesis of
172 ergothioneine (54, 55). mtEgtB consists of an N-terminal DinB_2 domain and a C-terminal FGE
173 domain, and the active site is composed of residues within both domains. Specifically, the FGE
174 domain contains a metal-binding histidine triad, while the DinB_2 domain contains a catalytic
175 tyrosine residue. The DinB_2 domain is absent in Tsi3 homologs. Thus, not surprisingly, we found
176 that the catalytic tyrosine residue of mtEgtB is absent in abTsi3 (Fig. 3c). Moreover, no histidine
177 triad was identified in the Tsi3 consensus sequence (Fig. S3). We conclude that Tsi3 homologs are
178 FGE domain-containing proteins but are unlikely to be functional PvdO, FGE or EgtB enzymes.

179 **Non-cognate Tsi3 homologs do not provide protection against toxicity by Tse3 from**
180 **Ab17978.** Considering that FGE-like proteins are implicated in a wide range of activities, it

181 remains possible that Tsi3 homologs are enzymes with an unknown catalytic activity. Based on
182 the only report of an enzymatic mechanism of T6SS immunity (15), we premised that if Tsi3
183 homologs are enzymes with the same catalytic activity, non-cognate Tsi3 homologs should prevent
184 intoxication by Tse3 from Ab17978 (abTse3). To this end, we expressed Tsi3 homologs from *A.*
185 *baylyi* (ayTsi3) or *Klebsiella pneumoniae* (kpTsi3), representing 69% and 38% identity to abTsi3
186 (76% and 46% identity within the FGE domain), respectively, in *E. coli* and determined *E. coli*
187 survival following co-incubation with Ab17978. Because strain Ab17978 encodes multiple
188 antibacterial T6SS effectors, which could mask bacterial killing due to Tse3 alone, we employed
189 strains Ab17978 Δ vgrG2,vgrG4 (hereafter Ab17978 $tse3^+$) and Ab17978 Δ vgrG2,vgrG4 Δ tse3
190 (hereafter Ab17978 $tse3^-$) as predators (36). We have previously shown that T6SS-mediated
191 bacterial killing by strain Ab17978 $tse3^+$ is dependent on Tse3, while the isogenic mutant strain,
192 Ab17978 $tse3^-$, is unable to kill *E. coli* (36). In our assay, we also included the T6SS mutant strain,
193 Ab17978 Δ tssM, as a negative control for bacterial killing (Fig. 4b and Fig. S4). We found that
194 unlike abTsi3, expression of ayTsi3 or kpTsi3 did not prevent *E. coli* killing by Ab17978 $tse3^+$
195 (Fig. 4b). These results indicate that Tsi3 homologs do not provide cross-protection against non-
196 cognate T6SS effectors.

197 **Disruption of Tse3-Tsi3 interaction prevents immunity to effector toxicity.** Our
198 previous results are consistent with the current paradigm that immunity proteins function by
199 specifically binding to their cognate effector. Thus, we hypothesized that Tsi3 variants incapable
200 of binding Tse3 will be unable to provide immunity to Tse3. To test this hypothesis, we employed
201 our Far Western blot assay (Fig. S1) to screen several point mutants of abTsi3 for variants unable
202 to bind abTse3 while retaining a relatively high expression level in Ab17978 Δ 3 (Fig. S5). We
203 found that mutant N194I was unable to bind Tse3, whereas mutant E236A bound Tse3 efficiently,

204 albeit at levels lower than WT Tsi3 (Fig. 5a). In this assay, ayTsi3, which does not protect against
205 abTse3 (Fig. 4b), serves as a negative control. Next, we tested whether the aforementioned Tsi3
206 variants prevent Ab17978Δ3 killing by WT Ab17978. Unlike WT Tsi3 and E236A, expression of
207 N194I failed to protect Ab17978Δ3 from killing by the WT strain, suggesting that residue N194 is
208 important for Tsi3 function. Notably, it is unlikely that residue N194 is a catalytic residue involved
209 in a hypothetical enzymatic activity, as mutant N194A binds Tse3 and prevents Tse3-mediated
210 toxicity (Fig. 5). Together, our results demonstrate that Tsi3-mediated immunity correlates with
211 the ability to bind Tse3, suggesting a mechanism of immunity dependent on Tse3-Tsi3 protein-
212 protein interactions.

213 **Tsi3 homologs constitute a family of FGE-like T6SS immunity proteins.** Our previous
214 results indicate that although Tsi3 homologs possess an FGE domain, they are unlikely to be
215 functional enzymes. To better understand the relationship between Tsi3 homologs and FGE
216 domain-containing proteins, we constructed a phylogenetic tree of Tsi3 homologs and 40
217 representatives of the FGE family (PF03781) (Fig. 6 and Table S1). This group of proteins includes
218 FGEs, oxygenases, serine/threonine kinases, enhancer-binding proteins (e.g., XylR),
219 uncharacterized proteins implicated in nitrite reduction (e.g., NirV), putative oxidoreductases (e.g.,
220 PvdO), and sulfoxide synthases (e.g., EgtB) (42). Consistent with our previous results, we found
221 that Tsi3 homologs form a clade that is distinct from that of previously characterized FGE domain-
222 containing proteins (Fig. 6). We propose that Tsi3 homologs constitute a family of FGE domain-
223 containing proteins specialized for mediating immunity to T6SS effectors.

224

225 **Discussion**

226 Since their discovery in 2010, T6SS immunity proteins were shown to prevent toxicity by
227 interacting with their cognate effector (56). Further studies into diverse E-I pairs served to solidify
228 this model into a paradigm for immunity protein defense (32, 57, 58). However, it was recently
229 shown that immunity proteins can also protect potential prey via an enzymatic mechanism of
230 immunity that is independent of E-I protein-protein interactions (15). This finding prompted us to
231 investigate the possibility that Tsi3 homologs could represent a second family of enzymatic T6SS
232 immunity proteins, since they possess a characteristic FGE domain, which is common in enzymes
233 of various functions, including PvdO, FGE and EgtB. In this work, we report that Tsi3 homologs
234 are widespread among T6SS-encoding Gram-negative bacteria, often encoded in gene clusters
235 containing *vgrG* and *tse3* homologs. Using Ab17978 as our model organism, we experimentally
236 determined that Tse3 and Tsi3 are in fact a cognate E-I pair and that they interact with each other.
237 Surprisingly, although they contain a FGE domain, our structural modeling, phenotypic
238 experiments and biochemical assays suggest that Tsi3 homologs are unlikely to be functional
239 enzymes.

240 Although we cannot entirely rule out that Tsi3 homologs are enzymes, we found that Tsi3
241 homologs do not prevent intoxication by non-cognate Tse3-like effectors and that an abTsi3
242 mutant that lacks the ability to bind abTse3 is unable to provide immunity. Thus, our data suggest
243 that the mechanism of immunity mediated by Tsi3 is dependent on the binding of the immunity
244 protein to its cognate effector. Consistent with this proposed mechanism of Tsi3-mediated
245 immunity, we found that in most cases, Tsi3 homologs are encoded within arrays containing more
246 than one non-identical *tsi3* gene. It is well established that polymorphism between effector and
247 immunity proteins underlies T6SS-dependent antagonistic bacterial interactions (59–61). Thus, the
248 accumulation of distinct Tsi3 homologs could constitute a strategy to prevent toxicity from Tse3-

249 like effectors of non-kin bacteria, as has been proposed for other E-I pairs in which multiple copies
250 of immunity proteins are encoded (35, 62). For *Vibrio cholerae*, it was suggested that arrays of
251 T6SS immunity genes are likely established through a combination of homologous and homology-
252 facilitated illegitimate recombination (35). Further work is necessary to demonstrate the functional
253 relevance of encoding multiple T6SS immunity genes and to provide a mechanistic understanding
254 of the establishment of immunity gene arrays in diverse bacteria.

255 The striking relatedness of Tsi3 homologs to FGE domain-containing proteins suggests
256 that these proteins likely share a common ancestor. Crystal structures from several FGE homologs
257 indicate that the FGE domain adopts a unique “FGE fold” with low secondary structure (<20% of
258 each β -sheets and α -helices) (49, 50, 52, 53, 63, 64). The FGE fold is also found in the X-ray
259 crystal structures of non-FGE enzymes, including the putative oxidoreductase PvdO and the
260 sulfoxide synthase EgtB. Our characterization of Tsi3 provides further evidence that the FGE
261 domain can be co-opted as a scaffold in multiple proteins to carry out diverse functions. Our work
262 expands the known functions carried out by FGE-like proteins to include defense during T6SS-
263 dependent bacterial warfare. In addition, our findings point to the FGE domain as a potential
264 marker to facilitate the identification of uncharacterized T6SS immunity proteins.

265 In sum, our work establishes Tsi3 homologs as a novel family of FGE-like immunity
266 proteins. Future structural characterization of Tsi3 homologs in complex with their cognate
267 effector will provide valuable information regarding the nature of the Tse3-Tsi3 interaction and
268 may elucidate the biochemical role of the antibacterial effector Tse3.

269

270 **Materials and Methods**

271 **Bacterial strains and growth conditions.** All strains, plasmids and primers used in this
272 study are listed in Table S2. Strains were grown in Luria-Bertani (LB) broth at 37°C with shaking.
273 Antibiotics were added to the media when appropriate (see below).

274 **Generation of strain Ab17978Δ3.** Construction of the kanamycin cassette-marked mutant
275 strain Ab17978Δ3::Km was reported previously (65). To remove the KanR cassette,
276 electrocompetent Ab17978Δ3::Km was transformed with plasmid pAT03 (66), which encodes the
277 FLP recombinase. Transformants were plated on LB agar containing 2 mM IPTG supplemented
278 with carbenicillin (200 μg/mL). Removal of the kanamycin resistant cassette was confirmed by
279 PCR and sequencing.

280 **Generation of expression plasmids for Ab17978Δ3 and *E. coli*.** The pWH1266-based
281 (67) construct for abTsi3-His expression was generated by linearizing pWH-*vgrGi*-6xHis (68) by
282 PCR, amplifying *tsi3* from genomic DNA of Ab17978, then ligating both PCR products by In-
283 Fusion (Takara Bio, Mountain View, CA), according to the manufacturer's instructions. Point
284 mutants were generated using the QuikChange II site-directed mutagenesis kit (Agilent
285 Technologies, Santa Clara, CA), according to the manufacturer's instructions. The specific alanine
286 mutations made to Tsi3 were selected to represent a wide sample of charged and polar residues
287 conserved among Tsi3 homologs. Mutations N194I and R287C were selected because mutations
288 in equivalent residues of human FGE were shown to disrupt FGE function (49). Ab17978Δ3
289 transformants were selected for using 15 μg/mL tetracycline. These strains were employed as prey
290 in bacterial killing assays.

291 The pBAVMCS-based (69) constructs for abTsi3-His expression was generated by PCR
292 amplification of the abTsi3 gene and restriction cloning into BamHI/PstI sites. Due to homology
293 between the two *tsi3* homologs encoded by *A. baylyi* ADP1, we first amplified a segment of DNA

294 containing both *tsi3* homologs. Then, we digested that fragment with EcoRI to separate both
295 homologs. The segment containing gene ACIAD3113 was then amplified, digested, and ligated
296 into pBAVMCS at KpnI/SalI sites (ACIAD3113 has internal BamHI/PstI sites). The gene
297 encoding the *tsi3* homolog from *K. pneumoniae* NTUH-K2044 was obtained as a geneblock from
298 Integrated DNA Technologies (IDT), Coralville, Iowa. This geneblock served as a template for
299 PCR amplification, restriction enzyme digestion, and ligation into the BamHI/PstI sites. The
300 specific *A. baylyi* ADP1 and *K. pneumoniae* NTUH-K2044 Tsi3 homologs selected for this study
301 have the highest percentage identity to abTsi3 in their respective E-I module. *E. coli* Rosetta 2
302 transformants were selected for using 50 µg/mL kanamycin and 12.5 µg/mL chloramphenicol.
303 These strains were employed as prey in bacterial killing assays.

304 abTse3-His was cloned into vector pET28a by In-Fusion (Takara Bio). *E. coli* Rosetta 2
305 transformants were selected for using 30 µg/mL kanamycin and 12.5 µg/mL chloramphenicol.
306 abTsi3-FLAG and ayTsi3-FLAG were cloned into vector pETDUET at sites NdeI/KpnI. Point
307 mutants of abTsi3 were generated as described above. *E. coli* Rosetta 2 transformants were selected
308 for using 200 µg/mL ampicillin and 12.5 µg/mL chloramphenicol. These strains were used to
309 perform Far Western blot assays probing for the interaction between Tse3 and Tsi3 (see below).

310 All constructs were verified by PCR and sequencing.

311 **Bacterial killing assays.** Overnight cultures of predator and prey strains were washed three
312 times in fresh LB and normalized to an OD600 of 1. Predator and prey strains were then mixed at
313 the appropriate ratio (1:1 WT Ab17978:Ab17978Δ3 or 1:10 WT Ab17978:*E. coli* Rosetta 2,
314 respectively), spotted onto dry LB-agar plates, and incubated for 3.5 h at 37°C. Spots were then
315 resuspended in 1 mL LB and serially diluted onto dry LB-agar plates supplemented with antibiotics

316 appropriate to select for surviving prey. Plates were incubated overnight at 37°C and CFUs were
317 quantified thereafter.

318 **Tse3 purification.** Overnight cultures of *E. coli* Rosetta 2 cells harboring pET28a-Tse3-
319 6xHis were diluted 1:100 into autoinduction media ZYM-5052 (70) and grown for 16 h at 30°C
320 with the appropriate antibiotics. Cells were harvested by centrifugation, resuspended in
321 resuspension buffer (50 mM Tris, 300 mM NaCl, 25 mM imidazole, pH 8) containing EDTA-free
322 protease inhibitor tablets, and lysed by three passages through a cell disruptor at 35 kpsi. Cell
323 lysates were clarified by centrifugation and loaded onto a Ni-NTA agarose column (Gold Bio, St.
324 Louis, MO) equilibrated with resuspension buffer. The column was then washed with resuspension
325 buffer and wash buffer (50 mM Tris, 300 mM NaCl, 50 mM imidazole, pH 8), and immobilized
326 Tse3 was eluted with elution buffer (50 mM Tris, 300 mM NaCl, 300 mM imidazole, pH 8).
327 Purified Tse3 was concentrated, buffer exchanged into 50 mM Tris, 150 mM NaCl, pH 8, and
328 concentrated once more to a final concentration of ~ 1 mg/mL.

329 **Induction of FLAG-tagged abTsi3 variants and ayTsi3.** Overnight cultures of *E. coli*
330 Rosetta 2 cells expressing C-terminal FLAG-tagged Tsi3 variants/homologs were diluted to an
331 OD₆₀₀ of 0.05 in fresh LB containing the appropriate antibiotics. The cultures were then grown
332 at 37°C (shaking) until mid-exponential phase and induced with 1 mM IPTG for 4 h at 30°C. Next,
333 cells were harvested by centrifugation and resuspended in resuspension buffer.

334 **Far Western blot assay and immunoblotting.** The interaction between Tse3 and Tsi3
335 variants was determined by Far Western blot using a previously published protocol with few
336 modifications (71). First, resuspended *E. coli* Rosetta 2 cells expressing C-terminal FLAG-tagged
337 Tsi3 or variants thereof (see above) were treated with concentrated lysis buffer to a final
338 concentration of 1% triton X-100, 100 µg/mL lysozyme, 100 µg/mL DNaseI, 10 mM MgCl₂, 10

339 mM CaCl₂ and EDTA-protease inhibitor tablet. Cells were lysed by sonication and clarified lysates
340 were loaded onto a 12% SDS-PAGE gel (in duplicate) and ran at 150 V. Proteins were then
341 transferred at room temperature (RT) onto a nitrocellulose membrane using a semi-dry transfer
342 cell (Biorad) (20 V, 35 min) and cold transfer buffer (10% methanol, 0.1% SDS, 25 mM Tris, 200
343 mM glycine). One membrane was treated with Ponceau S stain to visualize all cell lysate proteins,
344 and the other membrane was used for Far Western blot analysis.

345 For the Far Western blot, transferred proteins were denatured and renatured in the
346 nitrocellulose membrane by treatment with decreasing concentrations of guanidine-HCl then
347 incubated overnight at 4°C without guanidine-HCl, as previously described (71); however, instead
348 of 2% milk for each treatment, we used Odyssey Blocking Buffer (TBS) (LI-COR, Lincoln, NE)
349 at up to 50% by volume. The membrane containing renatured proteins was then blocked with
350 Odyssey Blocking Buffer (TBS) at room temperature for 1 h, treated with purified Tse3-His in
351 “protein-binding buffer” (71) at 1 µg/mL and incubated overnight at 4°C. Unbound Tse3-His was
352 removed by washing with Tris-buffered saline–Tween (TBST). Proteins were then detected with
353 monoclonal mouse anti-FLAG M2 (1:1000; Sigma-Aldrich, St. Louis, MO) and polyclonal rabbit
354 anti-6xHis (1:2,000; Invitrogen, Waltham, MA) as well as IRDye-conjugated anti-mouse and anti-
355 rabbit secondary antibodies (both at 1:15,000; LI-COR Biosciences). Blots were visualized with
356 an Odyssey CLx imaging system (LI-COR Biosciences). Quantification was done using Image
357 Studio 5.2

358 ([https://www.licor.com/bio/help/imagestudio5/index.html#Introduction_help.html%3FTocPath%](https://www.licor.com/bio/help/imagestudio5/index.html#Introduction_help.html%3FTocPath%3D%20)
359 [3D](#) [2](#)), as previously described (72). The data are presented as relative merge signal, where
360 the His/FLAG value corresponding to WT Tsi3 is defined as 1.

361 **Structural modeling of abTsi3.** Structural homologs of abTsi3 were identified using
362 Phyre2 (73). Three independent structural models of abTsi3 were generated using I-TASSER (74)
363 based on the known X-ray crystal structures of paPvdO (PDB: 5HHA), scFGE (PDB: 6MUJ) and
364 mtEgtB (PDB: 4X8E). The FGE substrate was extracted from PDB: 2AIK. All structures were
365 visualized using the PyMOL Molecular Graphics System, Version 2.3.4 (Schrödinger, LLC).

366 **Identification of Tsi3 homologs using PSI-Blast.** Identification of Tsi3 homologs was
367 performed as described previously for other proteins (38–40). First, the PSSM of Tsi3 was
368 constructed using the amino acid sequence of Tsi3 from Ab17978 (WP_032046197.1). Five
369 iterations of PSI-BLAST (75) against the reference protein database were performed. In each
370 iteration, a maximum of 500 hits with an e-value threshold of 10^{-6} were used. RPS-BLAST (75)
371 was then used to identify Tsi3 homologs in a local RefSeq database (downloaded April 17th, 2021).
372 E-value threshold was set to 10^{-50} .

373 The sequences of proteins located in the neighborhood of Tsi3 homologs were analyzed;
374 conserved domains were identified (see below), signal peptides and cleavage sites were predicted
375 using SignalP 5.0 (76), and transmembrane topology and cleavage sites were predicted using
376 Phobius (77). Identification of conserved domains enriched in Tsi3 genomic neighborhoods was
377 performed as described before (78).

378 The neighborhood of Tsi3 homologs was scanned both upstream and downstream for the
379 existence of adjacently encoded Tsi3 homologs. Two Tsi3 homologs were counted as adjacently
380 encoded proteins if not more than one unrelated protein was identified in between the proteins.
381 Tsi3 homologs were aligned using BLASTP and average percent identity of the Tsi3 homolog to
382 the adjacently encoded Tsi3 homologs was calculated.

383 **Illustration of conserved residues in Tsi3 homologs.** Tsi3 homologs were aligned using
384 Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/) (79). Aligned columns missing from
385 Ab17978 Tsi3 (WP_032046197.1) were removed from the alignment. WebLogo was created using
386 the WebLogo 3 server (weblogo.threeplusone.com/) (80).

387 **Identification of conserved domains and additional domains.** The Conserved Domain
388 Database (CDD) version 3.19 and related information were downloaded from NCBI (81). RPS-
389 BLAST was employed to identify conserved domains and the output was processed using the Post-
390 RPS-BLAST Processing Utility v0.1. The expect value threshold was set to 10^{-5} .

391 **Identification of T6SS core components in bacterial genomes.** RPS-BLAST was
392 employed to identify the T6SS core components in bacterial genomes containing Tsi3 homologs,
393 as described before (39). Briefly, the proteins were aligned against 11 COGs that were shown to
394 specifically predict T6SS (COG3516, COG3517, COG3157, COG3521, COG3522, COG3455,
395 COG3523, COG3518, COG3519, COG3520 and COG3515) (82). Bacterial genomes encoding at
396 least nine T6SS core components were regarded as harboring T6SS.

397 **Construction of the phylogenetic tree of bacterial genomes containing Tsi3 homologs.**
398 The DNA sequences of *rpoB* coding for DNA-directed RNA polymerase subunit beta were
399 retrieved for bacterial genomes containing Tsi3 homologs. Sequences were clustered using CD-
400 HIT (83) with a sequence identity threshold of 0.99. Representative sequences from the identified
401 clusters were aligned using MAFFT v7 FFT-NS-i (84, 85). The evolutionary history was inferred
402 using the neighbor-joining method (86) with the Jukes-Cantor substitution model (JC69). The
403 analysis involved 474 nucleotide sequences and 3,174 conserved sites. Evolutionary analyses were
404 conducted using the MAFFT server (<https://mafft.cbrc.jp/alignment/server/>), and the tree was
405 visualized using iTOL (87).

406 **Construction of the phylogenetic tree of the representatives of the FGE family.** A list
407 of 40 representative FGE homologs is shown in Table S1. The list was built based on the FGE
408 homologs identified in the original paper describing the FGE family (42) as well as the seed
409 proteins that were used to define the FGE-sulfatase Pfam family (PF03781)
410 (<https://pfam.xfam.org/family/PF03781>). The protein sequences were retrieved from NCBI and
411 trimmed according to the conserved domain. The proteins were aligned using MUSCLE (88). The
412 evolutionary history was inferred using the Neighbor-Joining method (86). The analysis involved
413 40 amino acid sequences and 136 conserved positions (95% site coverage). Evolutionary analyses
414 were conducted in MEGA7 (89) and the tree was visualized using iTOL (87).

415

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422

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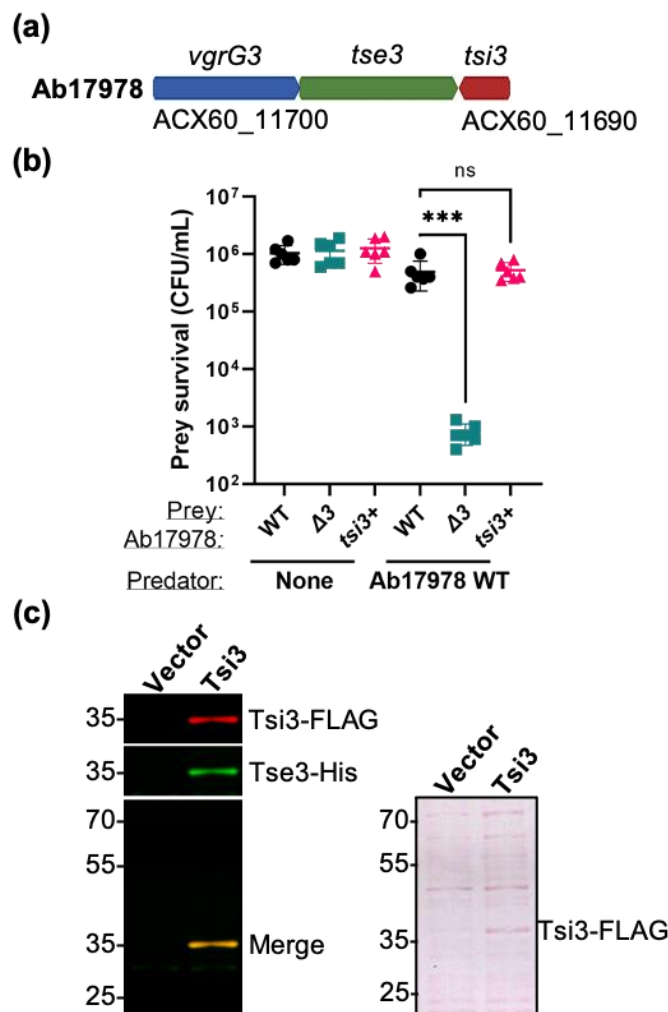
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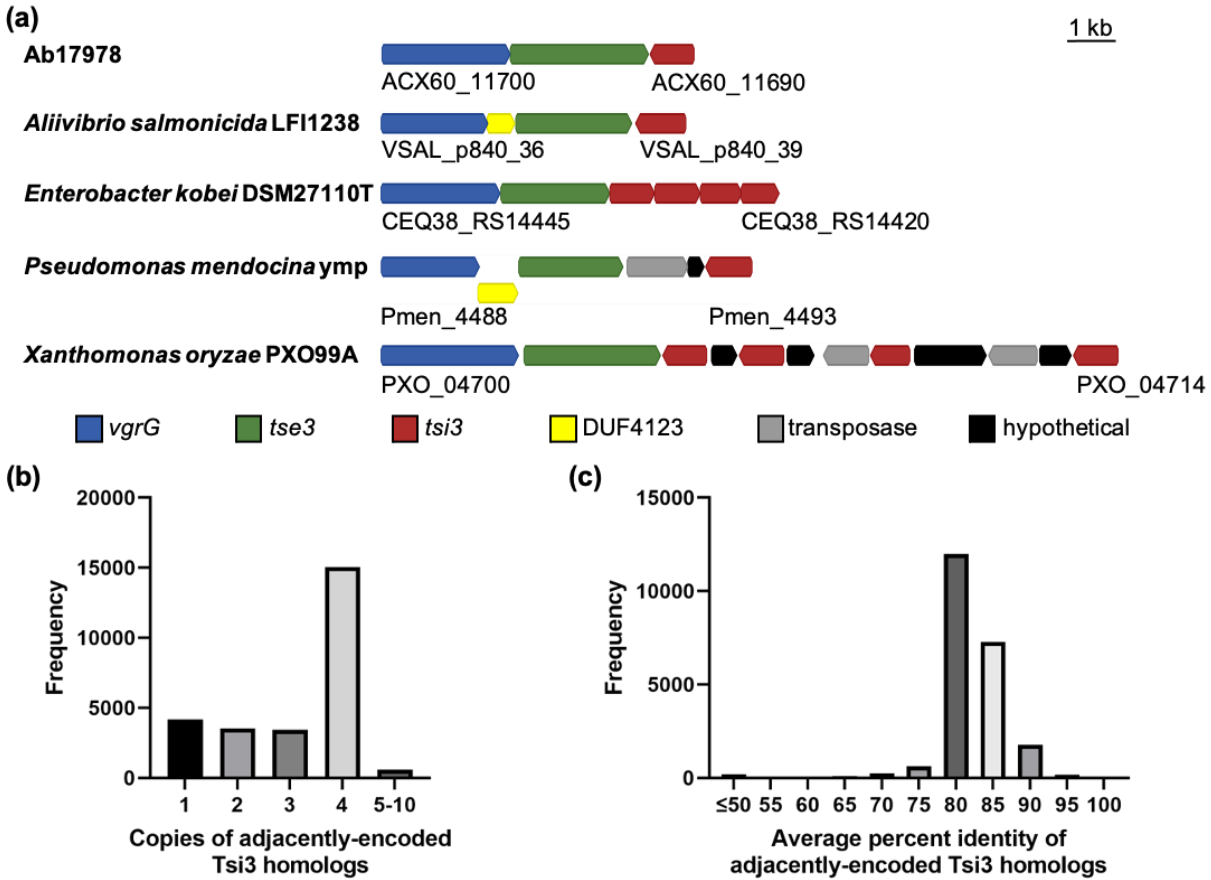
633 **Figure 1. Tsi3 confers protection from Tse3 intoxication.** (a) A schematic of the *vgrG3* gene
634 cluster of Ab17978. Locus tags for the first and final genes shown are indicated. (b) Survival of
635 the indicated prey strains following a 3.5-h incubation without a predator (None) or with WT
636 Ab17978 predator at a 1:1 (predator:prey) ratio. Data are shown as the mean ± S.D.; *n* = 3
637 biological replicates in technical duplicate. ***, *P* < 0.001; ns, not significant (determined by one-
638 way analysis of variance [ANOVA], followed by Dunnett's multiple-comparison test). (c) Far
639 Western blot probing for the interaction between Tse3 and Tsi3. Cell lysates from *E. coli*
640 expressing Tsi3-FLAG or an empty vector control were separated by SDS-PAGE and transferred
641 onto a nitrocellulose membrane in duplicate. Transferred proteins were either subjected to Far
642 Western blot analysis (left) or to Ponceau S staining (right), which serves as a loading control.

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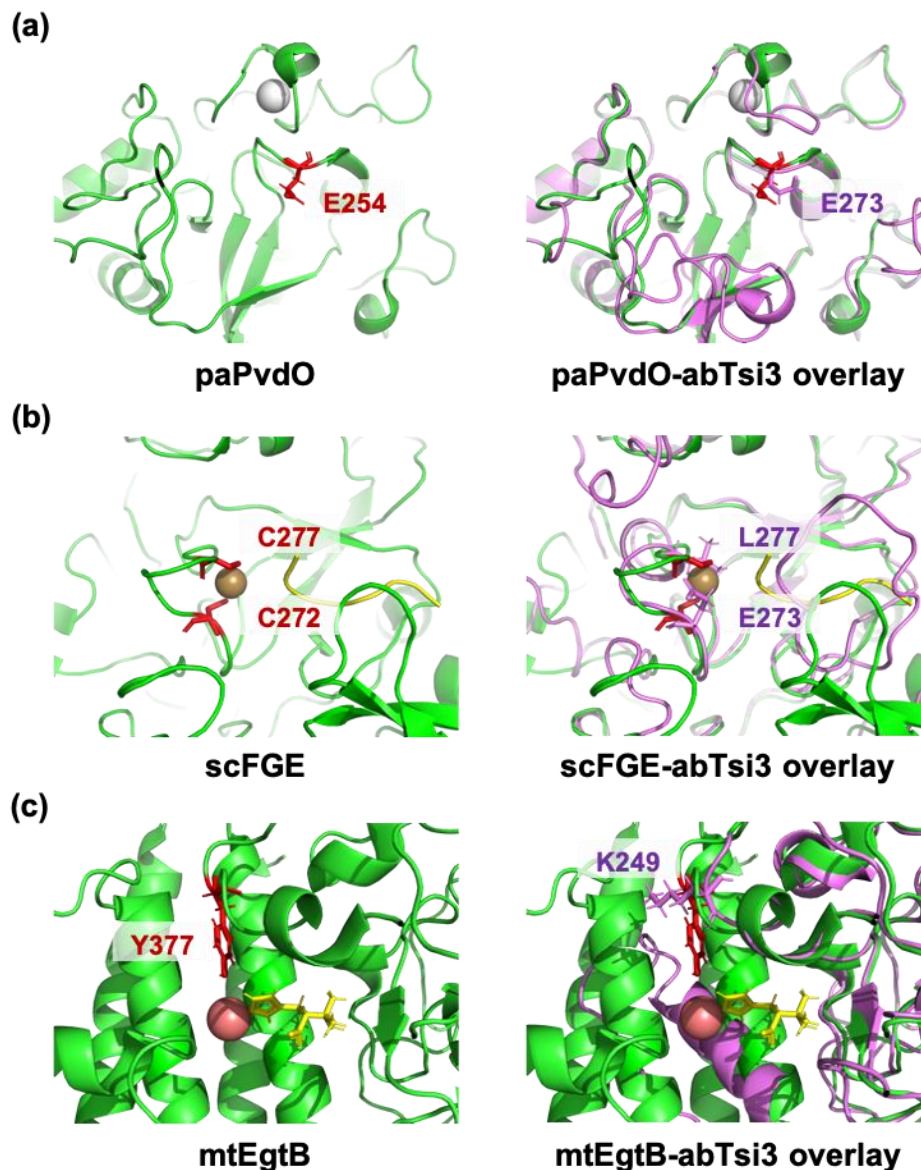
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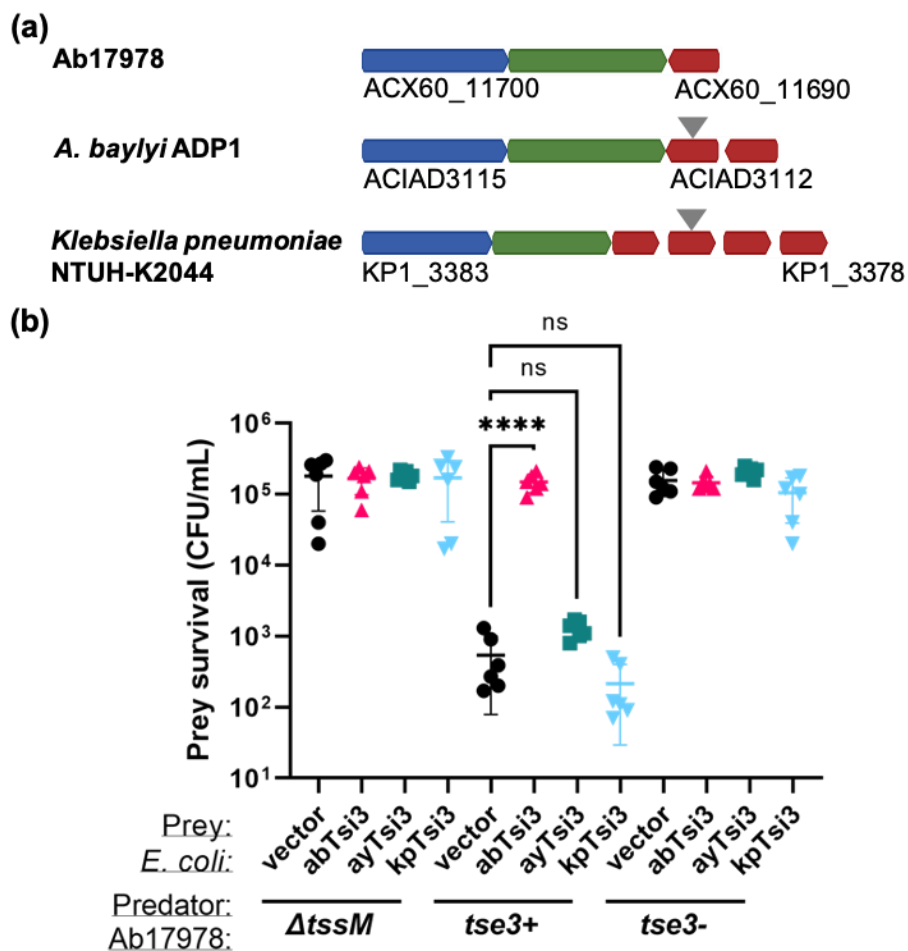


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648 **Figure 2. Tsi3 homologs are widespread.** (a) Representative *vgrG-tse3-tsi3* loci from various
649 bacteria. (b) A histogram showing the spread of Tsi3 homologs in groups of adjacently-encoded
650 Tsi3 homologs. (c) A histogram showing the spread of average percentage identity among
651 adjacently-encoded Tsi3 homologs.
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653
654 **Figure 3. Tsi3 homologs are unlikely to be functional PvdO, FGE or EgtB enzymes.** *left*, X-
655 ray structures of (a) PvdO from *P. aeruginosa* (paPvdO, PDB: 5HHA), (b) FGE from *S. coelicolor*
656 (scFGE, PDB: 6MUJ) or (c) EgtB from *M. thermophilum* (mtEgtB, PDB: 4X8E). Known and
657 putative catalytic residues are shown in red and enzyme substrates are shown in yellow. The FGE
658 substrate was extracted from PDB: 2AIK. Metal ions are shown as spheres: calcium (white),
659 copper (brown) and manganese (pink). *right*, Overlay of known X-ray structures (green) with
660 abTsi3 (purple) modelled to (a) paPvdO, (b) scFGE or (c) mtEgtB. Tsi3 residues replacing putative
661 or known catalytic residues are indicated.

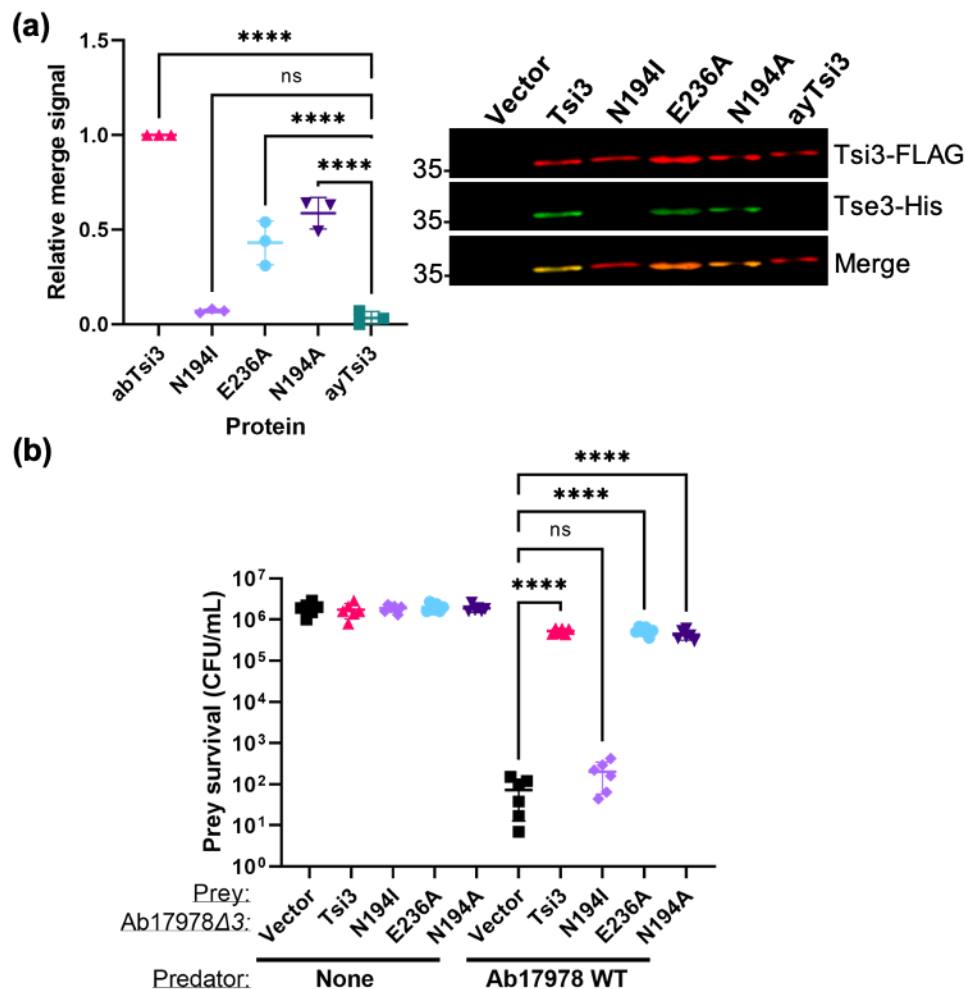
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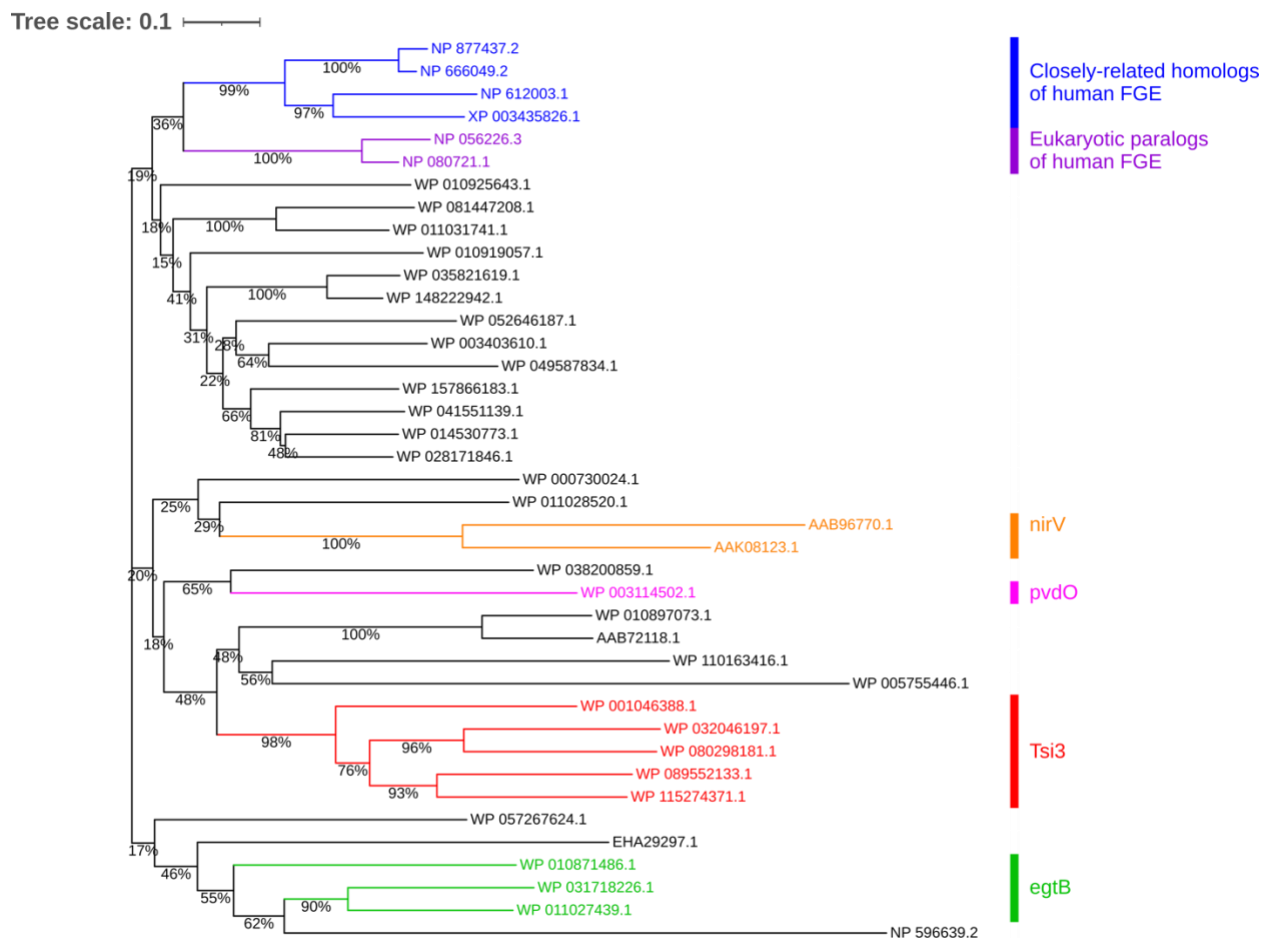
667 **Figure 4. Non-cognate Tsi3 homologs do not confer immunity to abTse3.** (a) Genetic context
 668 of Tsi3 homologs from various bacteria that were expressed in *E. coli* to determine functionality
 669 against abTse3. Locus tags for the first and final genes shown are indicated. The specific Tsi3
 670 homologs expressed are denoted by gray arrows. Color scheme is consistent with Fig. 2. (b)
 671 Survival of *E. coli* prey expressing various Tsi3 homologs following a 3.5-h incubation with the
 672 indicated Ab17978 predator strains at a 1:10 predator:prey ratio. Data are shown as the mean \pm
 673 S.D. from $n = 3$ biological replicates in technical duplicate. ****, $P < 0.0001$; ns, not significant
 674 (determined by one-way analysis of variance [ANOVA], followed by Dunnett's multiple-
 675 comparison test). Ab, Ab17978; Ay, *A. baylyi* ADP1; Kp, *K. pneumoniae* NTUH-K2044.
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679 **Figure 5. Disruption of Tse3-Tsi3 interaction prevents immunity to effector toxicity.** (a) Far
 680 Western blot assay probing for the interaction between the indicated abTsi3 variants and Tse3.
 681 Data are shown as the mean \pm S.D. from $n = 3$ independent experiments. ****, $P < 0.0001$; ns, not
 682 significant (determined by one-way analysis of variance [ANOVA], followed by Dunnett's
 683 multiple-comparison test). A representative Far Western blot is shown on the right. (b) Survival
 684 of Ab17978 Δ 3 prey expressing one of the indicated abTsi3 variants following a 3.5-h incubation
 685 with Ab17978 WT or no predator control at a 1:1 predator:prey ratio. Data are shown as the mean
 686 \pm S.D. from $n = 3$ biological replicates in technical duplicate. ****, $P < 0.0001$; ns, not significant
 687 (determined by one-way analysis of variance [ANOVA], followed by Dunnett's multiple-
 688 comparison test).
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 691 **Figure 6. Tsi3 homologs form a clade separate from previously characterized FGE domain-**
 692 **containing proteins.** A phylogenetic tree of representatives of the FGE family is shown. The
 693 evolutionary history was inferred using the Neighbor-Joining method. Bootstrap percentages (500
 694 replicates) are shown next to the branches. Description of the representatives is shown in Table
 695 S1. Clades with known function are denoted on the right.
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Table 1. Predicted structural homologs of Tsi3 with 100% confidence based on Phyre2.

Rank	Protein	Fold library ID	Sequence ID
1	Structure of PvdO from <i>Pseudomonas aeruginosa</i>	c5hhaB_	21
2	Formylglycine Generating Enzyme from <i>Streptomyces coelicolor</i>	c2q17C_	26
3	Human formylglycine generating enzyme FGE	d1z70x1	27
4	Formylglycine generating enzyme from <i>T. curvata</i> in complex with Cd(II)	c5nyyA_	26
5	Human formylglycine generating enzyme with cysteine sulfenic acid	c1y1fX_	27
6	Paralogue of the human formylglycine generating enzyme	d1y4ja1	27
7	Ergothioneine-biosynthetic sulfoxide synthase EgtB, apo form	c4x8bA_	21
8	<i>Treponema denticola</i> variable protein 1	c2y3cA_	25
9	EgtB from <i>Chloracidobacterium thermophilum</i> , a type II sulfoxide synthase in complex with N,N,N-trimethyl-histidine	c6qkjA_	21
10	Crystal Structure of CarF	c5aohA_	19
11	Major Tropism Determinant P1 Variant	d1yu0a2	9
12	Major Tropism Determinant I1 Variant	d1yu3a2	10
13	Major Tropism Determinant P1 (Mtd-P1) Variant Complexed with <i>Bordetella bronchiseptica</i> Virulence Factor Pertactin extracellular domain (Prn-E)	c2iouC_	14
14	<i>Thermus aquaticus</i> variable protein (TaqVP) from diversity-generating retroelements (DGR)	c5vf4A_	16

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