The *P. aeruginosa* Type VI Secretion System Effector Tse5 Forms Ion-Selective Membrane Pores that Disrupt the Membrane Potential of Intoxicated Cells

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Article

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The *P. aeruginosa* Type VI Secretion System effector Tse5 forms ion-selective membrane pores that disrupt the membrane potential of intoxicated cells

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Running title: Tse5 forms ion-selective pores to depolarise intoxicated bacterial cells

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24 **SUMMARY**

25 The Type VI Secretion System (T6SS) of *Pseudomonas aeruginosa* injects effector proteins into
26 neighbouring competitors and host cells, providing a fitness advantage that allows this opportunistic
27 nosocomial pathogen to persist and prevail during the onset of infections. However, despite the high
28 clinical relevance of *P. aeruginosa*, the identity and mode of action of most *P. aeruginosa* T6SS-
29 dependent effectors remain to be discovered. Here, we report the molecular mechanism of Tse5-CT,
30 which is the toxic auto-proteolytic product of the *P. aeruginosa* T6SS exported effector Tse5. Our
31 results demonstrate Tse5-CT is a pore-forming toxin that can transport ions across the membrane,
32 causing membrane depolarisation and bacterial death. The membrane potential regulates a wide range
33 of essential cellular functions, and therefore membrane depolarisation is an efficient strategy to
34 compete with other microorganisms in polymicrobial environments.

35

36 **Keywords:** Bacterial secretion; Protein export; Type VI Secretion System; *Pseudomonas*
37 *aeruginosa*, pore-forming protein, ion channel

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Bacteria usually associate forming polymicrobial structures called biofilms^{1,2}, where they frequently compete with other microorganisms for space and nutrients. In this context, many Gram-negative bacteria employ the Type VI secretion system (T6SS) to deliver toxic effectors to close competitors to either kill them or subvert their key biological functions. Thus, the T6SS provides a significant evolutionary advantage to bacteria that allows them to thrive and succeed in niche colonisation³⁻⁵. The T6SS assembles inside bacteria from 13 essential components related to the tail proteins of bacteriophage T4⁶. This molecular machine is energised by the contraction of the TssBC sheath (also known as VipAB)⁷⁻⁹, which encapsulates the Hcp tubular structure. The Hcp tube is topped by the VgrG-PAAR tip complex, which is believed to facilitate puncture of the producer and target cell membranes upon TssBC sheath contraction^{8,10}. T6SS effectors are either encapsulated within the Hcp tube¹¹ or associated with the VgrG spike complex¹². In addition, other effectors exist as extension domains on VgrG¹³⁻¹⁵, Hcp¹⁶, or PAAR proteins⁸. Importantly, bacteria that have a specific T6SS effector also encode a corresponding cognate immunity protein, which specifically binds to the effector, thereby neutralising the effector's toxicity¹⁷.

P. aeruginosa is a Gram-negative opportunistic nosocomial pathogen, and it is one of the primary causes of pneumonia in immunocompromised patients and hosts with lung diseases. Due to its high intrinsic and acquired resistance against most common antimicrobial agents, eradicating *P. aeruginosa* infections has become increasingly difficult. Consequently, the World Health Organization has ranked it second in a list of bacteria that require immediate development of novel antibiotics¹⁸. *P. aeruginosa* contains in its genome three independent T6SS clusters (H1, H2, and H3-T6SS)^{19,20}. Known effectors delivered by the H1-T6SS have an antiprokaryotic activity that target: (i) the cell wall peptidoglycan (Tse1: peptidase activity^{21,22} and Tse3: muramidase activity^{23,24}), (ii) the membrane (Tse4^{11,25}), (iii) NAD(P)⁺ (Tse6: glycohydrolase activity²⁶), (iv) the DNA (Tse7: DNase activity²⁷), and (v) protein biosynthesis (Tse8²⁸).

67

68 In the present study, we investigate Tse5 (PA2684), an H1-T6SS-dependent effector conserved
69 among *P. aeruginosa* strains isolated from patients with cystic fibrosis and bronchiectasis²⁹. Two
70 laboratories^{11,30} almost simultaneously discovered Tse5. Nonetheless, its mechanism of action
71 remains elusive. It was demonstrated that Tse5-CT is toxic when expressed in the cytoplasm of *E.*
72 *coli*³⁰ and when directed to its periplasm¹¹. Tse5 associates with VgrG4 (PA2685) for H1-T6SS-
73 dependent delivery into target cells^{11,30}. Furthermore, Tse5-producing cells protect themselves from
74 intoxication by the cognate immunity protein Tsi5 (PA2683)^{11,30}. Tsi5 contains two predicted
75 transmembrane regions. Furthermore, subcellular localisation experiments and Western blot analysis
76 have shown that it fractionates with the cytoplasmic membrane of *E. coli* cells expressing Tsi5, which
77 supports the hypothesis Tsi5 is an integral membrane protein³⁰.

78

79 Bioinformatic analysis of Tse5 predicts two conserved domains: An N-terminus domain (residues
80 Met1-Lys47) that is predicted to fold like a PAAR domain that lacks the signature Pro–Ala–Ala–Arg
81 motif; and a large central domain (Pro48-Leu1168 with homology to Rearrangement hot spot repeats
82 (PF05593) (Rhs repeats)³¹. Notably, the PAAR motif is conserved in many putative toxins, including
83 Rhs toxins, implicated in T6SS-dependent interbacterial competition^{32–37}. Rhs toxins are bacterial
84 exotoxins that belong to the polymorphic category³⁸. Moreover, Rhs toxins are large proteins of
85 usually more than 1,500 residues with variable C-terminal toxic domains, and they are ubiquitous in
86 Gram-negative and Gram-positive bacteria³³. Flanking the N-terminus of the C-terminal toxic
87 domain, there is a conserved DPXGL-(18)-DPXGL motif, which marks the limit of the Rhs core
88 domain. This conserved motif forms the active site of an aspartyl protease that releases the C-terminal
89 toxic domain^{39,40} (Supplementary Fig. 3d).

90

91 The C-terminus region of Tse5 (Tse5-CT; residues Ile1169–Gln1317) harbours the Tse5 toxicity^{11,30}.
92 However, Tse5-CT is unique to *P. aeruginosa* and has no predicted function, which challenges the
93 elucidation of its mechanism of action.

94

95 **Tse5-CT expression has a bacteriolytic effect on *Pseudomonas putida* cells**

96 To provide insight into the mode of action of Tse5-CT, we first evaluated if it can kill *P. putida*
97 EM383 cells (bacteriolytic effect) or rather cause a transient growth inhibition (bacteriostatic effect).
98 The *P. putida* strain EM383 results from eleven deletions introduced in the genome of *P. putida* strain
99 KT2440 to optimize heterologous gene expression⁴¹. We observed that inducing the expression of
100 Tse5-CT resulted in rapid bacterial growth arrest, with optical density (OD₆₀₀) dropping from values
101 of 0.3 to 0.23 10 hours after Tse5-CT induction (Fig. 1a). On the contrary, bacteria transformed with
102 the empty vector (pS238D1) reached an optical density seven times larger 10 hours post-induction
103 (OD₆₀₀ = of 1.59). Furthermore, we failed to recover bacterial growth when the inducer (m-toluic
104 acid) was removed 5 hours post-induction. This result indicates Tse5-CT has a bacteriolytic effect
105 when expressed in *P. putida* cells.

106

107 Co-expression of Tsi5 (green curves) could not reverse the Tse5-CT phenotype (red curves), resulting
108 in cultures having similar optical densities as cultures expressing Tse5-CT only (Fig. 1a). Previous
109 studies also failed to complement the Tse5-CT phenotype on *E. coli* cells, despite its capacity to
110 abrogate Tse5-based intoxication in *P. aeruginosa*¹¹. Given that Tsi5 is expected to be a
111 transmembrane protein, the inability to reverse the Tse5-CT phenotype in *P. putida* might be
112 associated with difficulties to express transmembrane proteins in a heterologous host^{11,42}

113

114 **Tse5-CT toxicity causes depolarisation of intoxicated *Pseudomonas putida* cells**

115 Tsi5 contains two predicted transmembrane regions. Furthermore, subcellular localisation
116 experiments and Western blot analysis have shown that Tsi5 fractionates with the cytoplasmic
117 membrane of *E. coli* cells expressing Tsi5³⁰, which altogether supports the hypothesis Tsi5 is an
118 integral membrane protein that inserts in the cytoplasmic membrane of *P. aeruginosa*. Moreover,
119 given that bioinformatic analysis of Tse5-CT did not predict any enzymatic activity, we hypothesised
120 that Tse5-CT might target the bacterial cytoplasmic membrane, which might affect the cell
121 permeability or the membrane potential of intoxicated cells.

122
123 The possible Tse5-CT effect on cellular permeability or membrane potential was evaluated in *P.*
124 *putida* EM383 cells. *P. putida* cells were transformed with the plasmid coding for Tse5-CT wild type
125 (pS238D1::tse5-CT) or a variant containing the pelB leader sequence (pS238D1::sptse5-CT), which
126 should direct Tse5-CT to the periplasmic space⁴³. Cells were cultured in liquid media, with the
127 expression of Tse5-CT or spTse5-CT induced with m-toluic acid. Ninety minutes after induction,
128 cells were stained with DiBAC₄(3) and SytoxTM Deep Red. DiBAC₄(3) is an anionic probe that can
129 permeate into depolarised cells, where it binds to intracellular proteins or the membrane, increasing
130 green fluorescence⁴⁴. SytoxTM Deep Red is a nucleic acid stain that readily penetrates cells with
131 compromised plasma membranes (permeabilised cells), increasing red fluorescence.

132
133 Cells expressing Tse5-CT and treated with DiBAC₄(3) and SytoxTM Deep Red were analysed by flow
134 cytometry (Fig. 1b-e). The measurements revealed that Tse5-CT expression results in *ca.* 51%
135 reduction of the healthy cell population compared to the healthy cells observed in the negative control
136 (cells transformed with an empty vector; Fig. 1b). However, the decrease in the number of healthy
137 cells is compensated by *ca.* 46% increase in depolarised cells (Fig. 1c) and *ca.* 5% increase in cells
138 depolarised and permeabilised (Fig. 1e).

139

140 Cells expressing the Tse5-CT variant containing the pelB leader sequence (spTse5-CT) and treated
141 with DiBAC₄(3) and SytoxTM Deep Red were also analysed by flow cytometry (Fig. 1b-e). In this
142 case, there was *ca.* 23% reduction of the healthy cell population compared to the healthy cells
143 observed in the negative control (Fig. 1b). Similar to what we observed in cells expressing Tse5-CT,
144 the decrease in healthy cells is mainly compensated by increased depolarised cells (*ca.* 21% increase
145 in depolarised cells (Fig. 1c), and *ca.* 2% increase in depolarised and permeabilised cells (Fig. 1e).

146

147 These results indicate that Tse5-CT and spTse5-CT change the membrane potential of intoxicated
148 cells but do not significantly affect the integrity of the cytoplasmic membrane. Furthermore, the
149 impact of Tse5-CT seems to be increased in the absence of the signal peptide.

150

151 **Tse5-CT can insert in lipid monolayers and the cytoplasmic membrane of *Escherichia coli* cells**

152 To evaluate the capacity of Tse5-CT to insert into biological membranes, we measured the ability of
153 Tse5-CT to partition in lipid monolayers reconstituted from an *E. coli* lipid extract using the
154 Langmuir-Blodgett balance⁴⁵ (Fig. 2a-b). This technique records the insertion of the peptide into the
155 monolayer as an increase in lateral pressure ($\Delta\Pi$) from an adjusted initial lateral pressure (Π_0).
156 Peptide insertion decreases as the initial lateral pressure increases (Fig. 2a) until the critical lateral
157 pressure (Π_c) is reached, at which the peptide is no longer able to insert into the monolayer. The lipid
158 packing in the outer monolayer of biological membranes approaches lateral surface pressures between
159 30 and 35 mN/m^{46,47}. Thus, a critical lateral pressure in this range upon protein addition indicates that
160 it is able to insert into biological membranes.

161

162 Therefore, these results indicate that Tse5-CT can partition into the hydrophobic core of a lipid
163 monolayer when introduced in a polar buffer, suggesting that it might also be able to insert into

164 biological membranes when is delivered by the T6SS into the periplasm/cytoplasm of intoxicated
165 cells.

166

167 Hydrophobic transmembrane (TM) regions should mediate the insertion of Tse5-CT into biological
168 membranes. Indeed, prediction of TM helices⁴⁸ suggests Tse5-CT might have a high propensity to
169 insert into biological membranes, containing several putative TM helices (TMH) and amphipathic
170 helices (AH) (Fig. 2c-d). To evaluate if Tse5-CT includes TM regions, we engineered a series of
171 Tse5-CT deletion mutants fused to a C-terminal dual reporter protein (Fig. 2d). The dual reporter
172 gene is a chimaera containing an *Escherichia coli* alkaline phosphatase (PhoA) gene and the α -
173 peptide gene fragment of the β -galactosidase (LacZ α). This PhoA-LacZ α dual reporter has been
174 extensively used to study *in vivo* membrane protein topology by the gene fusion approach^{49,50}. When
175 fused to periplasmic domains of polytopic membrane proteins, the dual reporter gene produces fusion
176 proteins with high PhoA activity, and when linked to cytoplasmic domains, it makes fusions with
177 high LacZ activity in *E. coli* strains capable of α -complementation. Thus, the growth of *E. coli* on
178 dual indicator agar plates allows for discrimination between colonies expressing cytoplasmic or
179 periplasmic fusion proteins. On such agar plates, cells with phosphatase activity can convert the X-
180 Pho (5-bromo-4-chloro-3-indolyl-phosphate) substrate into a blue-coloured, precipitated compound,
181 while cells with β -galactosidase activity transform the Red-Gal (6-chloro-3-indolyl- β -D-galactoside)
182 substrate into an insoluble red chromophore. Furthermore, both enzymatic activities can be quantified
183 in permeabilised intact cells using the colourimetric substrates ortho-nitrophenyl- β -D-galactoside
184 (ONPG) and p-nitrophenyl phosphate (pNPP) for LacZ and PhoA activities, respectively⁵¹.

185

186 Furthermore, all the Tse5-CT-PhoA-LacZ α fusion proteins contain the pectate lyase B (pelB) leader
187 sequence of *Erwinia carotovora* CE⁵². Adding the pelB signal peptide (sp) to the fusion proteins
188 directs them to the Sec translocon for translocation into the periplasm⁵³. Suppose Tse5-CT is a soluble

189 protein, the C-terminal of all engineered fusion proteins will localise in the periplasmic space. But, if
190 Tse5-CT is a transmembrane protein, it will contain one or more hydrophobic TM regions. These
191 regions would induce lateral opening of the SecYEG translocase and insert into the cytoplasmic
192 membrane^{54,55}. In this scenario, the PhoA-LacZ α fusion point could be located in a cytoplasmic,
193 periplasmic or transmembrane region of Tse5-CT, resulting in LacZ, PhoA or mixed activities,
194 respectively.

195

196 In particular, we engineered five spTse5-CT-PhoA-LacZ α fusion proteins (Fig. 2d). Each fusion
197 protein contains different fragments of Tse5-CT. The fusion protein spTse5-CT₁₁₆₉₋₁₂₂₉-PhoA-LacZ α
198 (K1229) contains the first 61 N-terminal residues, spTse5-CT₁₁₆₉₋₁₂₆₉-PhoA-LacZ α (A1269) contains
199 the first 101 residues, spTse5-CT₁₁₆₉₋₁₂₈₁-PhoA-LacZ α (A1281) contains the first 113 residues,
200 spTse5-CT₁₁₆₉₋₁₃₀₀-PhoA-LacZ α (K1300) contains the first 132 residues, and spTse5-CT₁₁₆₉₋₁₃₁₇-
201 PhoA-LacZ α (Q1317) contains the full length Tse5-CT (149 residues).

202

203 *E. coli* DH5 α cells expressing the K1229 fusion protein produce blue colonies when plated on dual
204 indicator agar plates, which shows that the C-terminus of the fusion protein localises in the periplasm
205 (Fig. 2e). This result is consistent with the quantification of PhoA-LacZ enzymatic activities in
206 permeabilised *E. coli* DH5 α cells expressing the K1229 fusion protein, which indicate the relative
207 PhoA activity (A) is *ca.* 2.78 times that of the relative LacZ activity (Table 1: Normalized Activity
208 Ratio (NAR); Fig. 2f). Furthermore, the relative PhoA activity of K1229 is the largest of all the
209 engineered fusion proteins.

210

211 Interestingly, *E. coli* DH5 α cells expressing fusion proteins A1269 or K1300 produce purple colonies
212 (Fig. 2e), consistent with NAR values of *ca.* 0.65 and 1.03, respectively. (Fig. 2f; Table 1). Previous
213 studies using this PhoA-LacZ α dual reporter strategy showed that purple colonies are obtained when

214 PhoA-LacZ α is fused within transmembrane regions (i.e., a combination of both red and blue
215 colourations as a mixture of cytoplasmic and periplasmic fusions)⁴⁹.

216

217 Finally, *E. coli* DH5 α cells expressing A1281 and the full-length spTse5-CT-PhoA-LacZ α fusion
218 protein (Q1317) produce red colonies when plated on dual indicator agar plates (Fig. 2e), consistent
219 with higher relative LacZ activities (NAR values of *ca.* 0.48 and 0.22 for A1281 and Q1317,
220 respectively; Fig. 2f; Table 1). Furthermore, the relative LacZ activity of Q1317 is the largest of all
221 the engineered fusion proteins.

222

223 In conclusion, this PhoA-LacZ α dual reporter assay provides experimental evidence indicating Tse5-
224 CT is a transmembrane protein that contains at least two transmembrane regions. Moreover, these
225 results demonstrate the capacity of Tse5-CT of inserting in the cytoplasmic membrane of *Escherichia*
226 *coli* cells.

227

228 **Tse5-CT forms ion-selective membrane pores *in vitro***

229 Given that our results support the notion that Tse5-CT is a transmembrane protein that targets the
230 cytoplasmic membrane of intoxicated cells to disrupt their membrane potential, we hypothesised that
231 Tse5-CT could be forming pores that can transport ions across the membrane. This ion transport could
232 be the molecular mechanism employed by Tse5-CT to depolarise intoxicated cells.

233

234 To probe its capacity to generate ion channel activity, Tse5-CT dissolved in dimethyl sulfoxide
235 (DMSO) was added to the solution bathing a solvent-free planar phospholipid bilayer (see Methods
236 for details). Gram-negative bacteria are known to have significantly different chemical compositions
237 in the cytoplasmic and periplasmic space^{56,57}. Therefore, to simulate these asymmetric conditions, a

238 salt concentration gradient was used in the membrane chamber, so one side flanking the membrane
239 was kept at 250 mM and the other at 50 mM, both buffered with 5 mM HEPES at pH 7.4.

240

241 Shortly after Tse5-CT protein was added and membrane reformed, spontaneous protein insertions
242 were obtained without any applied voltage, revealing ion channel activity with relatively stable
243 currents as shown in representative traces in Fig. 3a. Tse5-CT-induced currents were obtained using
244 membranes formed with a polar lipid extract from *E. coli* in a 250/50 mM (upper panel) and a
245 50/250 mM (middle panel) KCl gradient. Protein was always added at the same side of the membrane
246 (*cis*-side), meaning that gradient direction did not affect the capacity of Tse5-CT to insert into the
247 planar membrane. Control experiments with a neutral bilayer made from 1,2-dioleoyl-*sn*-glycero-3-
248 phosphoethanolamine (DOPE) were also carried out (Fig. 3a, lower panel). In this case, Tse5-CT-
249 induced currents were less frequent and more unstable than with polar membranes.

250

251 Fig. 3b shows the current-voltage (I-V) relationships arising from the representative traces shown in
252 Fig 3a. In all conditions explored, stable currents follow a purely ohmic behaviour, meaning channel
253 conductance ($G = I/V$) does not change with the applied voltage's magnitude and polarity. Calculated
254 conductances from all measured I-V curves vary from 0.6 to 6.6 nS (Supplementary Table 2). The
255 variability of conductances could arise from the insertion of multiple units of the same size or due to
256 variable pore conformations corresponding to different levels of Tse5-CT oligomerization.
257 Discrimination between these two options is out of the scope of the present study. Nonetheless, a G
258 of *ca.* 0.6 nS (the minimal conductive unit obtained, Table S1) roughly translates into pores around
259 1 nm in diameter (see Methods).

260

261 The amplitude and frequency of current oscillations in Fig. 3b provide useful information beyond the
262 conductive levels. The power spectral density (PSD) of Ts5-CT-induced currents quantifies the

current noise and provides the frequency hallmark⁵⁸ on the primary physical mechanisms responsible for pore formation. Supplementary Fig. 4a shows representative PSDs obtained from Tse5-CT-induced currents. PSDs for all experiments display characteristic 1/f type spectra similar to those found in other proteolipidic pore assemblies⁵⁹. Notably, for all conditions studied, PSDs at low frequencies (5-15 Hz band) follow a parabolic dependence with the applied voltage (Supplementary Fig. 4b). This is a characteristic feature of equilibrium conductance fluctuations^{60,61} and disregards non-equilibrium mechanisms in the pore formation by Ts5-CT (i.e. electroporation⁶²).

Experiments under a concentration gradient also allow us to assess the ionic selectivity of the measured pores. Because the mobility of anions and cations is the same in KCl, the sign of the measured current without any applied voltage (the vertical intercept of the I-V curve in Fig. 3b) provides a hint of the channel preference for anions or cations⁶³. In all studied conditions (polar and neutral membrane, 250/50 and 50/250 mM KCl gradients), the sign obtained of the measured currents without applied voltage is consistent with pores displaying cationic selectivity. However, the widely accepted magnitude to quantify selectivity is the so-called reversal potential (RP)⁶⁴, which is the voltage required to yield zero current under a transmembrane gradient (the horizontal intercept of the I-V curve). Once the measured RP is introduced into the Goldman–Hodgkin–Katz (GHK) equation, we can obtain the permeability ratio P_+/P_- ⁶⁵.

Fig. 3c shows the permeability ratios (P_+/P_-) for different series of experiments. For the 250/50 mM KCl configuration in polar membrane, the measured $P_+/P_- = 6.08 \pm 1.17$ (N=7). This permeability ratio means that the channels have multi-ionic character. Although pores have a marked preference for cations, anions are not excluded and still can permeate significantly through the pore. Reversing the direction of the concentration gradient (50/250 mM KCl) yields a comparable permeability ratio, $P_+/P_- = 4.79 \pm 0.99$ (N=8), and hence similar selectivity, to the opposite orientation. Keeping in mind

288 that protein addition always occurs in the *cis* chamber, this implies that Tse5-CT-induced pores are
289 fairly symmetrical structures regarding the charge distribution that regulates ionic selectivity, at least
290 at pH 7.

291

292 Taking into consideration that Tse5-CT net charge is negative and that the *E. coli* lipid extract also
293 contains negatively charged lipids (Phosphatidylglycerol (PG) is ~ 15% wt of the total and
294 Cardiolipin (CA) ~ 10% wt), it is fair to wonder whether Tse5-CT protein participates actively in the
295 pore structure or if it just acts in detergent-like fashion to promote channels formed exclusively by
296 lipids⁶⁶. Control measurements in neutral membrane with a 250/50 mM KCl gradient yield traces
297 with a positive current without applied voltage (Fig. 3a, lower panel), consistent with cation-selective
298 pores. Quantification through the RP (Fig. 3b) and the corresponding permeability ratio (Fig. 3c)
299 show that pores regulated exclusively by protein charges are still quite selective to cations, with $P_{+}/P_{-} \sim 1.70 \pm 0.21$ (N=4). This cationic selectivity of Tse5-CT-induced pores in neutral membranes
300 demonstrates that Tse5-CT protein forms part of the pore walls.

302

303 For the sake of completeness, it should be mentioned that although the majority of traces show “quiet”
304 pores with relatively stable currents and ohmic I-V relationships, there is a minor fraction of
305 recordings showing strongly voltage-dependent currents, as shown in Fig. 3d. In these cases,
306 extremely fluctuating currents appear for $V > 0$, increasing rapidly with time, making recording I-V
307 relationships impossible. In contrast, for $V < 0$ current is almost zero. When $V = 0$, the current is
308 small but measurable and compatible with a structure selective to cations. The successive application
309 of opposite voltage polarities shows that conductive structures do not disappear under $V < 0$, but they
310 just become closed like voltage-gated pores. Interestingly, these voltage-dependent currents that are
311 anecdotic in experiments involving a concentration gradient become much more frequent in
312 experiments performed under symmetric salt concentration conditions.

313

314 **Discussion**

315 In the current study, we have provided insight into the molecular mechanism that Tse5-CT employs
316 to kill intoxicated bacterial cells. In particular, we show that this molecular mechanism involves the
317 formation of ion-selective membrane pores, which can explain the observed membrane depolarisation
318 of *P. putida* cells expressing the toxin.

319

320 In all conditions explored (Fig. 3a), the Tse5-CT-induced currents in lipid membranes follow a purely
321 ohmic behaviour (Fig. 3b). Such voltage-independent conductance has been reported in proteolipidic
322 channels like the protein E of SARS-CoV-1^{67,68} or the classical swine fever virus p7⁵⁹, in total contrast
323 with other channels that show strong voltage-dependent conductance, such as the antibiotic peptide
324 alamethicin⁶⁹, the antimicrobial peptide Syr-E⁷⁰ or melittin peptide from bee venom⁷¹. Furthermore,
325 the minimal conductive unit obtained ($G \sim 0.6$ nS; Supplementary Table 1) is similar to that of well-
326 known protein channels, like the mitochondrial VDAC^{72,73}, which forms 14-Å pores in the
327 mitochondrial outer membrane.

328

329 Importantly, the measured permeability ratios (P_+/P_-) indicate that Tse5-CT-induced channels have
330 a multi-ionic character (Fig. 3c). Thus, although pores have a marked preference for cations, anions
331 can permeate significantly through the pore. Furthermore, Tse5-CT-induced pores are fairly
332 symmetrical structures, as indicated by similar permeability ratios obtained for 50/250 and
333 250/50 mM KCl concentration gradients. Nonetheless, this symmetry might not translate *in vivo*
334 under conditions of acidic stress, where protons unequally titrate each cytoplasmic membrane side.
335 In this scenario, the charge distribution could become asymmetric, and hence the selectivity could
336 depend on the direction of the concentration gradient⁷⁴.

337

338 In addition, we have observed that Tse5-CT can induce voltage-dependent currents that are anecdotic
339 in experiments involving a concentration gradient but become much more frequent in experiments
340 performed under symmetric salt concentration (Fig. 3d). Based on this result, we could speculate that
341 two different mechanisms of membrane permeabilization could be operating simultaneously. One
342 membrane permeabilization mechanism forming relatively quiet ohmic pores, which *in vivo* would
343 lead to cell depolarisation. A second membrane permeabilization mechanism that only functions in
344 one voltage polarity and quickly leads to irreproducible membrane disruption, which *in vivo* would
345 result in cell permeabilization. This channel-pore duality might explain why the expression of Tse5-
346 CT in *P. putida* results in a 46% increase in depolarised cells (Fig. 1c) and a 5% increase in
347 depolarised and permeabilised cells (Fig. 1e). Such behaviour has already been reported in
348 proteolipidic systems and is referred to as *channel-pore dualism*⁷⁵.

349
350 Overall, our electrophysiology experiments suggest that Tse5-CT inserts into *E. coli* polar membranes
351 yielding proteolipidic pores of unknown architecture (either in the form of barrel-stave, toroidal or
352 arch pores⁷⁶), but in any case with Tse5-CT located in the pore walls. Furthermore, Tse5-CT-induced
353 pores have an equilibrium nature (are formed spontaneously) and display marked cationic preference
354 although maintaining their multi-ionic character. This multi-ionic character means that changes in
355 cell homeostasis or cell polarity via Tse5-CT action probably involve the simultaneous transport of
356 several ionic species.

357
358 We have observed that the bacteriolytic effect of Tse5-CT on *P. putida* cells is characterised by a
359 rapid and permanent bacterial growth arrest (Fig. 1a). This bacteriolytic effect can be correlated with
360 our flow cytometry data, which show that Tse5-CT mainly causes membrane depolarisation (Fig. 1b-
361 e). These experiments were performed in parallel with *P. putida* cells expressing wild type Tse5-CT
362 and a variant containing the pelB leader sequence (spTse5-CT), which should direct Tse5-CT to the

363 periplasmic space⁴³. The results indicate that both proteins (Tse5-CT and spTse5-CT) can depolarise
364 intoxicated cells. Still, the effect is more significant in the absence of the pelB leader sequence (*ca.*
365 46% vs 21% increase in depolarised cells expressing Tse5-CT or spTse5-CT, respectively). This
366 result is consistent with our electrophysiology experiments that indicate Tse5-CT can spontaneously
367 induce pore formation in lipid membranes regardless of the direction of the KCl gradient. Previous
368 results also indicate Tse5-CT is toxic when expressed in the cytoplasm of *E. coli*³⁰ and when directed
369 to its periplasm¹¹.

370

371 Furthermore, using the Langmuir-Blodgett balance, we have demonstrated the capacity of Tse5-CT
372 to partition into the hydrophobic core of a lipid monolayer when introduced from a polar buffer (Fig.
373 2a-b). This is a significant result, suggesting that Tse5-CT might contain transmembrane (TM)
374 regions inserted into biological membranes. To evaluate the possibility Tse5-CT includes TM
375 regions, we engineered a series of Tse5-CT deletion mutants containing the N-terminal pelB leader
376 sequence (sp) and the C-terminal PhoA-LacZ α dual reporter protein^{49,50}. Evaluation of the enzymatic
377 activity of the dual reporter identified the PhoA-LacZ α fusion points located in periplasmic (K1229),
378 cytoplasmic (A1281 and Q1317), and transmembrane (A1269 and K1300) domains of Tse5-CT (Fig.
379 2e-f; Table 1). This result indicates that Tse5-CT is a transmembrane protein that contains at least
380 two TM regions. Pore-forming toxins (PFTs) are classified into two large groups, α -PFTs and β -
381 PFTs, depending on whether their membrane-spanning domain assembles from α -helices or β -
382 barrels⁷⁷. Whilst structural information will be required to understand the number and arrangement
383 of the transmembrane regions that form the Tse5-CT membrane pore, transmembrane helix
384 predictions suggest a substantial α -helical content with a predicted propensity for membrane insertion
385 (Fig. 2c). Therefore, suggesting Tse5-CT might be an α -PFT.

386

387 The membrane potential regulates a wide range of bacterial physiology and behaviours, including pH
388 homeostasis, membrane transport, motility, antibiotic resistance, cell division, electrical
389 communication, and environmental sensing⁷⁸. Therefore, membrane depolarisation is an excellent
390 strategy to outcompete other bacteria. Although Tse5-CT seems to be unique to *P. aeruginosa*,
391 previous T6SS-effectors have been shown to change the membrane potential of intoxicated cells.
392 These include the *P. aeruginosa* effector Tse4¹¹, the *Serratia marcescens* Ssp6 effector⁷⁹, and the
393 *Vibrio cholerae* VasX effector⁸⁰.

394

395 Ssp6 can cause depolarisation of targeted cells without a corresponding increase in permeability of
396 the cytoplasmic membrane and can form ion-selective pores⁷⁹. Similarly, Tse4 disrupted the
397 membrane potential without increased membrane permeability and was suggested to form cation-
398 selective pores²⁵. VasX displays some structural homology with pore-forming colicins. It was shown
399 to disrupt the membrane potential with simultaneous permeabilisation of the inner membrane,
400 suggesting that it forms large, non-selective pores, which would cause leakage of ions and other
401 cellular contents^{80,81}.

402

403 In summary, antibiotic resistance has become a serious public health concern, which according to the
404 WHO, by 2050 could be causing over 10 million deaths per year. *Pseudomonas aeruginosa* is a
405 significant contributor to this alarming forecast because it exhibits a high level of intrinsic and
406 acquired resistance against most common antibiotics and consequently is the top second priority
407 pathogen that requires urgent development of novel antibiotics¹⁸. *P. aeruginosa* produces a plethora
408 of effectors delivered by the T6SS to target essential functions in prey cells. Consequently, these
409 effectors are essential for its pathogenesis, and therefore, deciphering the molecular functions and
410 cellular targets of these toxins could provide unique insight for the next generation of antibiotics.
411 Towards this end, this study shows that Tse5-CT is a transmembrane protein that partitions into the

412 hydrophobic core of model membranes, including lipid monolayers and bilayers. Furthermore, when
413 it inserts in lipid bilayers, our electrophysiology experiments suggest that Tse5-CT assembles
414 proteolipidic pores that display a marked cationic preference although maintaining its multi-ionic
415 character. These findings correlate well with our *in vivo* results, which indicate Tse5-CT causes
416 membrane depolarisation and bacterial death. Taken together, these results suggest Tse5-CT toxicity
417 is a result of the ion-selective pores that it assembles in the cytoplasmic membrane of intoxicated
418 cells. Our work provides a significant contribution towards understanding how *P. aeruginosa* utilise
419 the H1-T6SS to colonise and prevail in different niches such as the respiratory tract of
420 immunocompromised patients and people with lung diseases. Moreover, elucidating the mechanism
421 of action of Tse5-CT and its cellular target pave the way for the future design of novel antibiotics
422 targeting the bacterial membrane.

423

424 **Methods**

425 **Growth inhibition of *P. putida* after Tse5-CT expression**

426 A detailed list of all strains and plasmids used in this study can be found in Supplementary Tables 1.
427 Growth curves were performed in triplicate. Overnight cultures of transformed *P. putida* EM383 cells
428 carrying pS238D1 empty plasmid, encoding Tse5-CT (pS238D1::*tse5-CT*), or both Tse5-CT and Tsi5
429 (pSEVA424::*tsi5*) were grown in 10 mL Luria-Bertani (LB) broth and adjusted to OD₆₀₀ = 0.1. Initial
430 cultures were allowed to grow for one hour and a half (OD₆₀₀ = 0.2-0.3) before toxin/immunity protein
431 expression induction. Expression of Tse5-CT toxin and Tsi5 was induced with 1mM m-Toluic acid
432 (TA) and 1mM isopropyl 1-thio-β-D-galactopyranoside (IPTG), respectively. Optical density at
433 600 nm (OD₆₀₀) was measured every hour. After 5 hours, half of the cultures were washed with 1 min
434 centrifugation at 9000 xg, and the pellet was resuspended in fresh LB broth with appropriate
435 antibiotics. All cultures were grown at 30°C with agitation (300 rpm) in LB media supplemented with

kanamycin (50 µg/mL) (also streptomycin at the same concentration in case of bacteria carrying the plasmid pSEVA424:*tsi5* for Tsi5 expression).

Flow cytometry studies of *P. putida* cells expressing Tse5-CT

See Supplementary Table 1 for a description of strains and plasmids used in this study. Electrocompetent *Pseudomonas putida* strain EM383 cells were transformed by electroporation with empty plasmid (pS238D1), plasmids coding Tse5-CT (pS238D1::*tse5-CT*), or plasmid coding spTse5-CT (pS238D1::*sptse5-CT*). Flasks containing 10 mL of LB media supplemented with kanamycin (50 µg/mL) were inoculated with transformed bacteria, and cell cultures were incubated overnight at 30 °C with agitation (180 rpm). The following day, cultures were diluted to OD₆₀₀ = 0.05 with fresh LB media and led them to grow in the same conditions until they reached exponential phase (OD₆₀₀ value of *ca.* 0.5). Tse5-CT and spTse5-CT expressions were induced by adding of 1 mM m-toluic acid (TA) final concentration. Ninety minutes after induction, cells were pelleted and suspended in a 1x PBS solution pH 7.4 to a final OD₆₀₀ value of 0.5. Following resuspension, cells were stained with DiBAC₄(3) (10 µM) and Sytox™ Deep Red (4 µM) and incubated for 30 minutes under constant shaking in dark conditions. Then, cells were directly analysed in a CytoFLEX cytometer equipped with 488 nm and 638 nm lasers (Beckman Coulter). Channels used were FITC for DiBAC₄(3) (Ex_λ = 490 nm, Em_λ = 516 nm) and APC for Sytox™ Deep Red (Ex_λ = 660 nm, Em_λ = 682 nm). Data Analysis was performed using CytExpert (Supplementary Fig. 1).

To define cell populations, we performed two positive controls, one negative control and a double control (Supplementary Fig. 2). The negative control consists of *P. putida* cells transformed with the empty plasmid pS238D1 and incubated with fluorescent dyes. This assay allows delimiting the healthy population as well as the size and complexity of the cells. For the permeabilisation control, cells were permeabilized by heat shock at 85 °C for 5 minutes, followed by another 5 minutes

incubation at 4°C and then stained with Sytox™ Deep Red. The depolarisation control was obtained by treating cells for 30 minutes with the antibiotic polymyxin B sulphate (100 µg/mL), followed by 30 minutes incubation with DiBAC₄(3). Finally, a double control was performed by treating cells with heat shock and polymyxin B sulphate and labelling them with DiBAC₄(3) and Sytox™ Deep Red fluorophores.

466

Identification of transmembrane regions in Tse5-CT using PhoA-LacZ dual reporter agar plates

See Supplementary Table 1 for a description of strains and plasmids used in this study. Chemocompetent *E. coli* DH5α cells were transformed by heat shock with a parental plasmid coding for the dual reporter PhoA-LacZ (pKTop), or Tse5-CT-PhoA-LacZ fusion proteins, followed by bacterial growth in kanamycin (50 µg/mL) selective LB-agar plates. Single colonies were grown overnight in LB media complemented with kanamycin (50 µg/mL). 20 µl of overnight cultures were spotted onto dual reporter LB-agar plates supplemented with kanamycin (50 µg/mL), Red-Gal (80 µg/mL), X-Pho (100µg/mL), IPTG (1 mM) and glucose (0.2%). Plates were incubated for 24 h at 37 °C in dark conditions.

477

Identification of transmembrane regions in Tse5-CT by measuring PhoA-LacZ enzymatic activity

Single colonies of *E. coli* DH5α cells transformed with pKTop plasmids were selected from freshly prepared LB-agar plates supplemented with kanamycin (50 µg/mL) and glucose (0.2%). Cells were grown overnight in LB media supplemented with kanamycin (50 µg/mL) and glucose (0.2%) at 37°C. Overnight cultures were diluted to an OD₆₀₀ value of 0.1 in fresh LB media, supplemented with kanamycin (50 µg/mL). When cultures reached an OD₆₀₀ = 0.6, the expression of fusion proteins was

485 induced by adding 1 mM IPTG and cell cultures were incubated for 90 minutes, maintaining the same
486 agitation and temperature conditions before induction.

487

488 In order to measure the β -galactosidase (LacZ) activity, 1.2 mL of each bacterial culture was
489 centrifuged for 5 minutes at 4500 xg at room temperature (RT). After removing the supernatant, the
490 cell pellet was resuspended in 1.2 mL of M63 medium (100 mM KH_2PO_4 , 15 mM $(\text{NH}_4)_2\text{SO}_4$, 1.7
491 mM FeSO_4 , 1 mM MgSO_4), and 200 μL were transferred to a 96-well microtiter plate to measure the
492 optical density at 595 nm (OD_{595}). In order to permeabilize the cell membranes, the remaining
493 volumes of cell cultures were treated with 100 μL chloroform and 100 μL 0.05% SDS and left for 5
494 minutes at 37 °C and 5 minutes on ice. Once the chloroform settles, 50 μL of the cells-containing
495 upper phase was transferred to the 96-well microtiter plate. Then, 100 μL of the reaction mixture
496 0.4% ONPG in PM2 medium (70 mM Na_2HPO_4 , 30 mM NaH_2PO_4 , 1 mM MgSO_4 , 0.2 mM MnSO_4)
497 was added to well-containing bacteria, and incubate at RT for 15 minutes. The reaction was stopped
498 with 50 μL of 1 M Na_2CO_3 , followed by reading the absorbance of the reaction product at 405 nm
499 (OD_{405}).

500

501 In order to measure the alkaline Phosphatase (PhoA) activity, 1.2 mL of bacterial cultures were
502 centrifuged for 5 minutes at 4500 xg at RT. After removing the supernatant, cell pellets were
503 resuspended in 1.2 mL of cold wash buffer (10 mM Tris·HCl pH 8.0, 10 mM MgSO_4) to remove
504 phosphate ions from the medium. The wash buffer was removed by 5 minutes RT centrifugation at
505 4500 xg and resuspension of the cell pellet in 1.2 mL of cold PM1 medium (1 M Tris·HCl, pH 8.0,
506 0.1 mM ZnCl_2 , 1 mM iodoacetamide). From resuspended cells in PM1 medium, 200 μL were
507 transferred to a 96-well microtiter plate to measure the optical density (OD_{595}). Membrane
508 permeabilization was performed with the remaining cell cultures as previously described. Following
509 permeabilization, 50 μL of the cells-containing upper phase was transferred to the 96-well microtiter

plate. Then, 100 μ L of the reaction mixture 0.4% *p*-NPP in 1 M Tris·HCl, pH 8.0, was added to well-
containing bacteria and incubated at RT for 60 minutes. The reaction was stopped by adding 50 μ L
of 2 M NaOH, followed by reading the absorbance of the reaction product at 405 nm (OD_{405}).

513

The relative LacZ or PhoA enzymatic activities (A) were calculated using Equation 1, which
considers the optical density (OD_{595}) of the sample and the absorbance of the reaction products
(OD_{405})

$$A_{LacZ \text{ or } PhoA} = 1000 \cdot \left(\frac{OD_{405} \text{ sample} - OD_{405} \text{ control well}}{OD_{595} \text{ sample} - OD_{595} \text{ control well}} \right) / t \text{ (min) of incubation}$$

Equation 1. Relative enzymatic activity of β -galactosidase (LacZ) or alkaline phosphatase (PhoA)

519

After obtaining the relative enzymatic activity for each Tse5-CT-PhoA-LacZ α fusion protein, the
normalized activity ratio (NAR) is calculated (Equation 2). Each measured activity is normalized by
the highest relative enzymatic activity (A) of the series.

$$NAR = \frac{(A_{PhoA} / \text{Highest } A_{PhoA})}{(A_{LacZ} / \text{Highest } A_{LacZ})}$$

Equation 2. Normalized activity ratio.

525

526 Expression and purification of Tse5 to study insertion in lipid monolayers and bilayers

The tse5-CT toxin was obtained as a self-cleavage product of the full-length protein Tse5.
The *Pseudomonas aeruginosa* tse5 gene (PA2684) was synthesised by GenScript (GenScript, NJ,
USA). The construct contains a 5' extension encoding for a 9xHis tag and a tobacco etch virus
protease cleavage site
(ATGGGCAGCAGCCATCATCATCATCATCATCATCACAGCAGCGGCGAAAACCTGT
ATTTTCAGGGCGGATCC). The construct was cloned into a pET29a(+) vector between the NdeI
and HindIII restriction sites.

534

535 For the expression of Tse5 protein, *Escherichia coli* Lemo21(DE3) cells were transformed with the
536 pET29a::*his-tag-tse5* plasmid and grown in LB agar (Luria Broth) medium supplemented with
537 50 µg/mL kanamycin, 34 µg/mL chloramphenicol and 2 mM rhamnose at 37 °C⁸². For protein
538 overexpression, rhamnose was removed from the LB medium. When cells reached OD₆₀₀ value
539 of *ca.* 0.7, Tse5 expression was induced by adding to the culture isopropyl β-D-1-
540 thiogalactopyranoside (IPTG) at the final concentration of 1 mM, and the temperature was dropped
541 to 18 °C. After *ca.* 18 h, the cells were harvested and frozen for later use.

542

543 Cell pellet obtained from 4 L culture was resuspended in 60 mL of 50 mM Tris-HCl pH 8.0, 500 mM
544 NaCl, 20 mM imidazole and 4 µL of benzonase endonuclease (lysis solution). Cells were then
545 disrupted by sonication, and the suspension was centrifuged for 40 min at 43000 xg. The supernatant
546 was filtered with a 0.2 µm syringe filter and subjected to immobilised metal affinity chromatography
547 using a HisTrap HP column of 5 mL (GE Healthcare) on a fast protein liquid chromatography system
548 (ÄKTA FPLC; GE Healthcare) equilibrated with 25 mL of 50 mM Tris-HCl pH 8, 500 mM NaCl
549 and 20 mM imidazole (solution A). The column was washed with solution A at 0.5 mL/min until no
550 change in absorbance at 280 nm was detected. Elution was performed with a linear gradient between
551 0 and 50% of 50 mM Tris-HCl pH 8, 500 mM NaCl and 500 mM imidazole (solution B) in 40 mL
552 and 2 mL/min. Fractions containing Tse5 protein were pooled, and protein concentration was
553 estimated by measuring absorbance at 280 nm. Tse5 protein was injected into a HiLoad Superdex
554 200 26/600 pg, previously equilibrated with 20 mM Tris-HCl pH 8 and 150 mM NaCl. Tse5 eluted
555 as a single monodispersed peak, but SDS-PAGE gel revealed three protein fragments (Supplementary
556 Fig. 3c). Interestingly, the band of *ca.* 15 kDa correspond to Tse5-CT, which results from the
557 cleavage of the full-length protein (Supplementary Fig. 3). The protein was then concentrated using
558 Amicon centrifugal filter units of 30 kDa molecular mass cut-off (Millipore) to a final concentration

559 of *ca.* 15 mg mL⁻¹ (*ca.* yield: 6 mg/L). The concentrated protein sample was diluted in solution A
560 containing 8 M urea to a final concentration of 6M urea to denature Tse5 and separate the three
561 fragments. To remove the 9xHis-tag-containing N-terminal fragment, the denatured protein solution
562 was added to a nickel resin (GE Healthcare) previously washed and equilibrated with buffer A with
563 8 M urea, and left for 5 minutes at 4°C under agitation. Following centrifugation of the nickel resin
564 at 15,000 xg, the unbound fraction containing the Tse5-CT and the central Rhs fragments were
565 recovered. Tse5-CT was separated from the Rhs fragment by differential precipitation with
566 ammonium sulphate ⁸³. First, the Rhs fragment was precipitated at 0.9 M ammonium sulphate,
567 followed by 15 min centrifugation at 15,000 xg. The supernatant containing Tse5-CT was
568 subsequently precipitated with 3 M ammonium sulphate. Precipitated Tse5-CT was washed with
569 MiliQ water, flash-frozen in liquid nitrogen, and lyophilized. Once lyophilized, samples were stored
570 at -20°C, ready to use. The purity of the protein was verified by SDS-PAGE and MS analysis
571 (Supplementary Fig. 3c and 3d).

572

573 **Study of Tse5-CT partitioning in lipid monolayers**

574 The capacity of Tse5-CT to penetrate into lipid monolayers was assessed by measuring its maximum
575 insertion pressure (MIP) using the Langmuir Blodgett balance technique with a DeltaPi-4 Kibron
576 tensiometer (Helsinki, Finland). Each experiment was performed in a fixed-area circular trough
577 (Kibron µTrough S system, Helsinki, Finland) of 2 cm in diameter where 1.25 ml of the aqueous
578 phase was added (5 mM Hepes pH 7.4, 150 mM NaCl). The temperature of the Langmuir balance
579 was controlled thermostatically by a water bath at 25°C (JULABO F12). The monolayer was formed
580 by spreading over the aqueous surface *E. coli* total lipid extract (Avanti Polar lipids) dissolved in
581 chloroform at 1 mg/ml with a Hamilton microsyringe until the desired initial monolayer surface
582 pressure was reached (Π_0). Experiments at different initial surface pressure (Π_0) values were
583 recorded by changing the amount of lipid applied to the air-water interface (Π_0 value ranging from

584 15 to 30 mN/m). Then Tse5-CT dissolved in DMSO was injected into the aqueous subphase to
585 facilitate incorporation into lipid monolayers (final concentration of 0.4 μ M) while controls were
586 carried out injecting DMSO alone. Changes in surface pressure were monitored over time and were
587 plotted as a function of Π_0 . These data were fitted to a linear regression model, and the maximum
588 insertion pressure was determined by extrapolation (y value when x=0).

589

590 **Study of Tse5-CT pore-forming activity in planar lipid bilayers**

591 Planar lipid membranes were formed by using a solvent-free modified Montal-Mueller technique ⁸⁴.
592 Lipid was prepared from chloroform solutions of either a natural polar extract from *E. coli* or pure
593 dioleoyl-phosphatidylethanolamine (DOPE). All lipids were purchased from Avanti Polar Lipids
594 (Alabaster, AL). *E. coli* polar lipid extract headgroup composition is 67% phosphatidylethanolamine,
595 23.2% phosphatidylglycerol, and 9.8% cardiolipin (Avanti Polar Lipids). Acyl chains are the mixture
596 naturally present in *E. coli* ⁸⁵. All lipids were dissolved in pentane at 5 mg/ml concentration after
597 chloroform evaporation. Two monolayers were made by adding 10-30 μ L of the lipid solution at each
598 compartment of a Teflon chamber (so-called *cis* and *trans*), each filled with 1.8 ml salt solutions. The
599 two compartments were partitioned by a 15 μ m thick Teflon film with a *ca.* 100 μ m diameter orifice
600 where the bilayer formed. The orifice was pre-treated with a 3% solution of hexadecane in pentane.
601 After pentane evaporation, the level of solutions in each compartment was raised above the orifice so
602 the planar bilayer could form by apposition of the two monolayers. Capacitance measurements
603 monitored correct bilayer formation. After bilayer formation, Tse5-CT dissolved in DMSO was added
604 to the *cis* compartment.

605

606 To carry out the electrical measurements, an electric potential was applied using in-house prepared
607 Ag/AgCl electrodes in 2 M KCl, 1.5% agarose bridges assembled within standard 250 μ l pipette tips.
608 Potential is defined as positive when it is higher at the side of the protein addition (the *cis* side) while

609 the *trans* side is set to ground. An Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) in
610 the voltage-clamp mode was used for measuring the current and applying potential. Data were filtered
611 by an integrated low-pass 8-pole Bessel filter at 10 kHz, saved with a sampling frequency of 50 kHz
612 with a Digidata 1440A (Molecular Devices, Sunnyvale, CA), and analysed using pClamp 10 software
613 (Molecular Devices, Sunnyvale, CA). The membrane chamber and the head stage were isolated from
614 external noise sources with a double metal screen (Amuneal Manufacturing Corp., Philadelphia, PA).
615 The
616 described set-up can resolve currents of the order of picoamperes with a time resolution below the
617 millisecond.

618
619 Current measurements were performed with a concentration gradient of either 250 mM KCl *cis* / 50
620 mM KCl *trans* (250/50 mM) or 50 mM KCl *cis* / 250 mM KCl *trans* (50/250 mM), or using 150 mM
621 KCl symmetrical solutions. All solutions were buffered with 5 mM HEPES at pH 7.4. The pH was
622 adjusted by adding HCl or KOH and controlled during the experiments with a GLP22 pH meter
623 (Crison). Steady current at each applied potential was calculated from a single Gaussian fitting of
624 histograms of current values. Conductance ($G = I/V$) was obtained from the slope of the calculated
625 current-voltage (I-V) curves.

626
627 Selectivity measurements were performed in experiments with a concentration gradient of
628 250/50 mM or 50/250 mM. Once the protein was inserted, a net ionic current appeared due to the
629 existence of one or several selective pores under a salt concentration gradient. Selectivity was
630 quantified by measuring the reversal potential (RP), which is the applied voltage needed to cancel the
631 current. If the channel is neutral, RP equals zero, while it becomes non-zero when the channel is
632 selective to anions or cations. When the concentration gradient is 250/50 mM, a negative RP
633 corresponds to cation-selective channels; with the opposite gradient (50/250 mM), a positive RP

634 indicates cationic selectivity. RP was obtained from either the linear regression of the measured IV
635 curves or by manually cancelling the observed current. All RP values were corrected by the liquid
636 junction potential from Henderson's equation to eliminate the contribution of the electrode's agarose
637 bridges ⁶³.

638

639 To carry out the current fluctuation analysis, the power spectral density (PSD) of the current
640 fluctuations was obtained directly from the measured current traces with the pClamp 10 software
641 (Molecular Devices, LLC.). The PSD generates a frequency-domain representation of the time-
642 domain data, revealing the power levels of the different frequency components present in the signal
643 and allowing the rationalization of physical mechanisms that are difficult to identify directly from
644 current measurements⁵⁸. PSD was measured by calculating the Fast Fourier Transform from the
645 digitized signal with a spectral resolution of 0.76 Hz. For each signal, the available spectral segments
646 were averaged with 50% overlap. To evaluate the increase of the PSD amplitude with the measured
647 current, PSDs were averaged in the 5-15 Hz. The increase of the PSD amplitude as the square of the
648 average current is a signature of conductance fluctuations^{86,87}.

649

650 **Statistical analyses**

651 Statistical analyses were performed using GraphPad Prism v.9 and are detailed in the figure legends.

652

653 **Data availability**

654 The authors declare that the data supporting the findings of this study are available within the paper
655 and its supplementary information files.

656

657

658 **References**

- 659 1. Sibley, C. D. *et al.* A polymicrobial perspective of pulmonary infections exposes an enigmatic
660 pathogen in cystic fibrosis patients. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 15070–15075 (2008).
- 661 2. Peters, B. M., Jabra-Rizk, M. A., O'May, G. A., William Costerton, J. & Shirtliff, M. E.
662 Polymicrobial interactions: Impact on pathogenesis and human disease. *Clinical Microbiology*
663 *Reviews* **25**, 193–213 (2012).
- 664 3. Bingle, L. E., Bailey, C. M. & Pallen, M. J. Type VI secretion: a beginner's guide. *Curr. Opin.*
665 *Microbiol.* **11**, 3–8 (2008).
- 666 4. Pukatzki, S., McAuley, S. & Miyata, S. The type VI secretion system: translocation of effectors
667 and effector-domains. *Curr. Opin. Microbiol.* (2009).
- 668 5. Cianfanelli, F. R., Monlezun, L. & Coulthurst, S. J. Aim, Load, Fire: The Type VI Secretion
669 System, a Bacterial Nanoweapon. *Trends Microbiol.* **24**, 51–62 (2016).
- 670 6. Filloux, A. Microbiology: A weapon for bacterial warfare. *Nature* **500**, 284–285 (2013).
- 671 7. Nazarov, S. *et al.* Cryo-EM reconstruction of Type VI secretion system baseplate and sheath
672 distal end. *EMBO J.* **37**, e97103 (2018).
- 673 8. Shneider, M. M. *et al.* PAAR-repeat proteins sharpen and diversify the type VI secretion
674 system spike. *Nature* **500**, 350–353 (2013).
- 675 9. Wang, J. *et al.* Cryo-EM structure of the extended type VI secretion system sheath–tube
676 complex. *Nat. Microbiol.* **2**, 1507–1512 (2017).
- 677 10. Kudryashev, M. *et al.* Structure of the Type VI secretion system contractile sheath. *Cell* **160**,
678 952–962 (2015).
- 679 11. Whitney, J. C. *et al.* Genetically distinct pathways guide effector export through the type VI
680 secretion system. *Mol. Microbiol.* **92**, 529–42 (2014).
- 681 12. Hachani, A. *et al.* Type VI Secretion System in *Pseudomonas aeruginosa*. *J. Biol. Chem.* **286**,
682 12317–12327 (2011).

- 683 13. Ma, A. T., McAuley, S., Pukatzki, S. & Mekalanos, J. J. Translocation of a *Vibrio cholerae*
684 Type VI Secretion Effector Requires Bacterial Endocytosis by Host Cells. *Cell Host Microbe*
685 **5**, 234–243 (2009).
- 686 14. Suarez, G. *et al.* A Type VI Secretion System Effector Protein, VgrG1, from *Aeromonas*
687 *hydrophila* That Induces Host Cell Toxicity by ADP Ribosylation of Actin. *J. Bacteriol.* **192**,
688 155–168 (2010).
- 689 15. Pukatzki, S., Ma, A. T., Revel, A. T., Sturtevant, D. & Mekalanos, J. J. Type VI secretion
690 system translocates a phage tail spike-like protein into target cells where it cross-links actin.
691 *Proc. Natl. Acad. Sci.* **104**, 15508–15513 (2007).
- 692 16. Ma, J. *et al.* The Hcp proteins fused with diverse extended-toxin domains represent a novel
693 pattern of antibacterial effectors in type VI secretion systems. *Virulence* **8**, 1189–1202 (2017).
- 694 17. Russell, A. B. *et al.* A Widespread Bacterial Type VI Secretion Effector Superfamily Identified
695 Using a Heuristic Approach. *Cell Host Microbe* **11**, 538–549 (2012).
- 696 18. World Health Organization. *Prioritization of pathogens to guide discovery, research and*
697 *development of new antibiotics for drug resistant bacterial infections, including tuberculosis.*
698 *WHO reference number: WHO/EMP/IAU/2017.12* (2017).
- 699 19. Filloux, A., Hachani, A. & Bleves, S. The bacterial type VI secretion machine: yet another
700 player for protein transport across membranes. *Microbiology* **154**, 1570–1583 (2008).
- 701 20. Boyer, F., Fichant, G., Berthod, J., Vandenbrouck, Y. & Attree, I. Dissecting the bacterial type
702 VI secretion system by a genome wide in silico analysis: what can be learned from available
703 microbial genomic resources? *BMC Genomics* **10**, 104 (2009).
- 704 21. Hood, R. D. *et al.* A Type VI Secretion System of *Pseudomonas aeruginosa* Targets a Toxin
705 to Bacteria. *Cell Host Microbe* **7**, 25–37 (2010).
- 706 22. Russell, A. B. *et al.* Type VI secretion delivers bacteriolytic effectors to target cells. *Nature*
707 **475**, 343–7 (2011).

- 708 23. Li, L. *et al.* Structural Insights on the Bacteriolytic and Self-protection Mechanism of
709 Muramidase Effector Tse3 in *Pseudomonas aeruginosa*. *J. Biol. Chem.* **288**, 30607–30613
710 (2013).
- 711 24. Lu, D. *et al.* Structural insights into the T6SS effector protein Tse3 and the Tse3-Tsi3 complex
712 from *pseudomonas aeruginosa* reveal a calcium-dependent membrane-binding mechanism.
713 *Mol. Microbiol.* **92**, 1092–1112 (2014).
- 714 25. LaCourse, K. D. *et al.* Conditional toxicity and synergy drive diversity among antibacterial
715 effectors. *Nat. Microbiol.* **3**, 440–446 (2018).
- 716 26. Whitney, J. C. *et al.* An interbacterial NAD(P)(+) glycohydrolase toxin requires elongation
717 factor Tu for delivery to target cells. *Cell* **163**, 607–19 (2015).
- 718 27. Pissaridou, P. *et al.* The *Pseudomonas aeruginosa* T6SS-VgrG1b spike is topped by a PAAR
719 protein eliciting DNA damage to bacterial competitors. *Proc. Natl. Acad. Sci.* **115**, 12519–
720 12524 (2018).
- 721 28. Nolan, L. M. *et al.* Identification of Tse8 as a Type VI secretion system toxin from
722 *Pseudomonas aeruginosa* that targets the bacterial transamidosome to inhibit protein synthesis
723 in prey cells. *Nat. Microbiol.* **6**, 1199–1210 (2021).
- 724 29. Díaz Ríos, C. Caracterización del resistoma y viruloma de aislados de *Pseudomonas*
725 *aeruginosa* de pacientes con fibrosis quística y bronquiectasias. *Internacional, Tesis Doctoral*
726 (2020).
- 727 30. Hachani, A., Allsopp, L. P., Oduko, Y. & Filloux, A. The VgrG proteins are ‘à la carte’
728 delivery systems for bacterial type VI effectors. *J. Biol. Chem.* **289**, 17872–17884 (2014).
- 729 31. Kelley, L. A., Mezulis, S., Yates, C. M., Wass, M. N. & Sternberg, M. J. The Phyre2 web
730 portal for protein modeling, prediction and analysis. *Nat. Protoc.* **10**, 845–858 (2015).
- 731 32. Poole, S. J. *et al.* Identification of Functional Toxin/Immunity Genes Linked to Contact-
732 Dependent Growth Inhibition (CDI) and Rearrangement Hotspot (Rhs) Systems. *PLOS Genet.*

733 7, e1002217 (2011).

734 33. Zhang, D., de Souza, R. F., Anantharaman, V., Iyer, L. M. & Aravind, L. Polymorphic toxin
735 systems: Comprehensive characterization of trafficking modes, processing, mechanisms of
736 action, immunity and ecology using comparative genomics. *Biol. Direct* **7**, 18 (2012).

737 34. Koskiniemi, S. *et al.* Rhs proteins from diverse bacteria mediate intercellular competition.
738 *Proc. Natl. Acad. Sci. U. S. A.* **110**, 7032–7037 (2013).

739 35. Wenren, L. M., Sullivan, N. L., Cardarelli, L., Septer, A. N. & Gibbs, K. A. Two independent
740 pathways for self-recognition in *Proteus mirabilis* are linked by type VI-dependent export.
741 *MBio* **4**, (2013).

742 36. Jiang, N. *et al.* *Vibrio parahaemolyticus* RhsP represents a widespread group of pro-effectors
743 for type VI secretion systems. *Nat. Commun.* **9**, 3899 (2018).

744 37. Ma, J. *et al.* PAAR-Rhs proteins harbor various C-terminal toxins to diversify the antibacterial
745 pathways of type VI secretion systems. *Environ. Microbiol.* **19**, 345–360 (2017).

746 38. ZC, R., DA, L. & CS, H. Polymorphic Toxins and Their Immunity Proteins: Diversity,
747 Evolution, and Mechanisms of Delivery. *Annu. Rev. Microbiol.* **74**, 497–520 (2020).

748 39. Busby, J. N., Panjikar, S., Landsberg, M. J., Hurst, M. R. H. & Lott, J. S. The BC component
749 of ABC toxins is an RHS-repeat-containing protein encapsulation device. *Nature* **501**, 547–
750 550 (2013).

751 40. SL, D. *et al.* The β -encapsulation cage of rearrangement hotspot (Rhs) effectors is required for
752 type VI secretion. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 33540–33548 (2020).

753 41. Martínez-García, E., Nikel, P. I., Aparicio, T. & de Lorenzo, V. *Pseudomonas* 2.0: Genetic
754 upgrading of *P. putida* KT2440 as an enhanced host for heterologous gene expression. *Microb.*
755 *Cell Fact.* **13**, 159 (2014).

756 42. Wagner, S., Bader, M. L., Drew, D. & Gier, J.-W. de. Rationalizing membrane protein
757 overexpression. *Trends Biotechnol.* **24**, 364–371 (2006).

- 758 43. Sockolosky, J. T. & Szoka, F. C. Periplasmic production via the pET expression system of
759 soluble, bioactive human growth hormone. *Protein Expr. Purif.* **87**, 129–135 (2013).
- 760 44. te Winkel, J. D., Gray, D. A., Seistrup, K. H., Hamoen, L. W. & Strahl, H. Analysis of
761 Antimicrobial-Triggered Membrane Depolarization Using Voltage Sensitive Dyes. *Front. Cell*
762 *Dev. Biol.* **0**, 29 (2016).
- 763 45. Maget-Dana, R. The monolayer technique: A potent tool for studying the interfacial properties
764 of antimicrobial and membrane-lytic peptides and their interactions with lipid membranes.
765 *Biochimica et Biophysica Acta - Biomembranes* **1462**, 109–140 (1999).
- 766 46. Demel, R. A., Geurts van Kessel, W. S. M., Zwaal, R. F. A., Roelofsen, B. & van Deenen, L.
767 L. M. Relation between various phospholipase actions on human red cell membranes and the
768 interfacial phospholipid pressure in monolayers. *BBA - Biomembr.* **406**, 97–107 (1975).
- 769 47. Calvez, P., Bussieres, S., Eric Demers & Salesse, C. Parameters modulating the maximum
770 insertion pressure of proteins and peptides in lipid monolayers. *Biochimie* **91**, 718–733 (2009).
- 771 48. Feng, S.-H., Zhang, W.-X., Yang, J., Yang, Y. & Shen, H.-B. Topology Prediction
772 Improvement of α -helical Transmembrane Proteins Through Helix-tail Modeling and
773 Multiscale Deep Learning Fusion. *J. Mol. Biol.* **432**, 1279–1296 (2020).
- 774 49. Alexeyev, M. F. & Winkler, H. H. Membrane topology of the Rickettsia prowazekii ATP/ADP
775 translocase revealed by novel dual pho-lac reporters. *J. Mol. Biol.* **285**, 1503–1513 (1999).
- 776 50. Karimova, G. & Ladant, D. Defining Membrane Protein Topology Using pho-lac Reporter
777 Fusions. in *Bacterial Protein Secretion Systems* (eds. Journet, L. & Cascales, E.) **1615**, 129–
778 142 (Humana Press, 2017).
- 779 51. Manoil, C. Chapter 3 Analysis of Membrane Protein Topology Using Alkaline Phosphatase
780 and β -Galactosidase Gene Fusions. *Methods Cell Biol.* **34**, 61–75 (1991).
- 781 52. Lei, S. P., Lin, H. ., Wang, S. ., Callaway, J. & Wilcox, G. Characterization of the Erwinia
782 carotovora pelB gene and its product pectate lyase. *J. Bacteriol.* **169**, 4379–4383 (1987).

- 783 53. Steiner, D., Forrer, P., Stumpp, M. T. & Plückthun, A. Signal sequences directing
784 cotranslational translocation expand the range of proteins amenable to phage display. *Nat.*
785 *Biotechnol. Vol. 24*, (2006).
- 786 54. Hartl, F. U., Lecker, S., Schiebel, E., Hendrick, J. P. & Wickner, W. The binding cascade of
787 SecB to SecA to SecY E mediates preprotein targeting to the E. coli plasma membrane. *Cell*
788 **63**, 269–279 (1990).
- 789 55. Hoffschulte, H. K., Drees, B. & Müller, M. Identification of a soluble SecA/SecB complex by
790 means of a subfractionated cell-free export system. *J. Biol. Chem.* **269**, 12833–12839 (1994).
- 791 56. Foster, J. W. Escherichia coli acid resistance: Tales of an amateur acidophile. *Nature Reviews*
792 *Microbiology* **2**, 898–907 (2004).
- 793 57. Miller, S. I. & Salama, N. R. The gram-negative bacterial periplasm: Size matters. *PLoS*
794 *Biology* **16**, 1–7 (2018).
- 795 58. DeFelice, L. J. *Introduction to Membrane Noise. Introduction to Membrane Noise* (Springer
796 US, 1981). doi:10.1007/978-1-4613-3135-3
- 797 59. Largo, E., Queralt-Martín, M., Carravilla, P., Nieva, J. L. & Alcaraz, A. Single-molecule
798 conformational dynamics of viroporin ion channels regulated by lipid-protein interactions.
799 *Bioelectrochemistry* **137**, 107641 (2021).
- 800 60. Bezrukov, S. M. & Winterhalter, M. Examining noise sources at the single-molecule level: 1/f
801 noise of an open maltoporin channel. *Phys. Rev. Lett.* **85**, 202–205 (2000).
- 802 61. Hoogerheide, D. P., Garaj, S. & Golovchenko, J. A. Probing surface charge fluctuations with
803 solid-state nanopores. *Phys. Rev. Lett.* **102**, 256804 (2009).
- 804 62. De, S. & Basu, R. Confirmation of membrane electroporation from flicker noise. *Phys. Rev. B*
805 *- Condens. Matter Mater. Phys.* **61**, 6689–6691 (2000).
- 806 63. Alcaraz, A. *et al.* Diffusion, exclusion, and specific binding in a large channel: A study of
807 OmpF selectivity inversion. *Biophys. J.* **96**, 56–66 (2009).

- 808 64. Ovchinnikov, Y. A. Ion Channels of Excitable Membranes. in *Science and Scientists Third*
809 **Edit**, 235–241 (Sinauer Associates Inc, 1981).
- 810 65. Hodgkin, A. L. & Katz, B. The effect of sodium ions on the electrical activity of the giant axon
811 of the squid. *J. Physiol.* **108**, 37–77 (1949).
- 812 66. Gazit, E., Boman, A., Boman, H. G. & Shai, Y. Interaction of the Mammalian Antibacterial
813 Peptide Cecropin PI with Phospholipid Vesicles. *Biochemistry* **34**, 11479–11488 (1995).
- 814 67. Verdiá-Báguena, C. *et al.* Coronavirus E protein forms ion channels with functionally and
815 structurally-involved membrane lipids. *Virology* **432**, 485–494 (2012).
- 816 68. Queralt-Martín, M., López, M. L., Aguilera-Arzo, M., Aguilera, V. M. & Alcaraz, A. Scaling
817 Behavior of Ionic Transport in Membrane Nanochannels. *Nano Lett.* **18**, 6604–6610 (2018).
- 818 69. Woolley, G. A. Channel-forming activity of alamethicin: Effects of covalent tethering.
819 *Chemistry and Biodiversity* **4**, 1323–1337 (2007).
- 820 70. Malev, V. V *et al.* Syringomycin E channel: A lipidic pore stabilized by lipopeptide? *Biophys.*
821 *J.* **82**, 1985–1994 (2002).
- 822 71. Pawlak, M., Stankowski, S. & Schwarz, G. Melittin induced voltage-dependent conductance
823 in DOPC lipid bilayers. *BBA - Biomembr.* **1062**, 94–102 (1991).
- 824 72. Ujwal, R. *et al.* The crystal structure of mouse VDAC1 at 2.3 Å resolution reveals mechanistic
825 insights into metabolite gating. *Proc. Natl. Acad. Sci. U. S. A.* **105**, 17742–17747 (2008).
- 826 73. Queralt-Martín, M. *et al.* Assessing the role of residue E73 and lipid headgroup charge in
827 VDAC1 voltage gating. *Biochim. Biophys. Acta - Bioenerg.* **1860**, 22–29 (2019).
- 828 74. López, M. L., Queralt-Martín, M. & Alcaraz, A. Stochastic pumping of ions based on colored
829 noise in bacterial channels under acidic stress. *Nanoscale* **8**, 13422–13428 (2016).
- 830 75. Mehnert, T. *et al.* Biophysical characterization of Vpu from HIV-1 suggests a channel-pore
831 dualism. *Proteins Struct. Funct. Genet.* **70**, 1488–1497 (2008).
- 832 76. Gilbert, R. J. C., Serra, M. D., Froelich, C. J., Wallace, M. I. & Anderluh, G. Membrane pore

- 833 formation at protein-lipid interfaces. *Trends in Biochemical Sciences* **39**, 510–516 (2014).
- 834 77. Peraro, M. D. & Van Der Goot, F. G. Pore-forming toxins: Ancient, but never really out of
835 fashion. *Nature Reviews Microbiology* **14**, 77–92 (2016).
- 836 78. Benarroch, J. M. & Asally, M. The Microbiologist’s Guide to Membrane Potential Dynamics.
837 *Trends Microbiol.* **28**, 304–314 (2020).
- 838 79. Mariano, G. *et al.* A family of Type VI secretion system effector proteins that form ion-
839 selective pores. *Nat. Commun.* **10**, 5484 (2019).
- 840 80. Miyata, S. T., Kitaoka, M., Brooks, T. M., McAuley, S. B. & Pukatzki, S. *Vibrio cholerae*
841 Requires the Type VI Secretion System Virulence Factor VasX To Kill *Dictyostelium*
842 *discoideum*. *Infect. Immun.* **79**, 2941–2949 (2011).
- 843 81. Miyata, S. T., Unterweger, D., Rudko, S. P. & Pukatzki, S. Dual Expression Profile of Type
844 VI Secretion System Immunity Genes Protects Pandemic *Vibrio cholerae*. *PLoS Pathog.*
845 (2013). doi:10.1371/journal.ppat.1003752
- 846 82. Schlegel, S. *et al.* Optimizing membrane protein overexpression in the *Escherichia coli* strain
847 Lemo21(DE3). *J. Mol. Biol.* **423**, 648–659 (2012).
- 848 83. Wingfield, P. T. Protein precipitation using ammonium sulfate. *Curr. Protoc. Protein Sci.*
849 **2016**, A.3F.1–A.3F.9 (2016).
- 850 84. Montal, M. & Mueller, P. Formation of bimolecular membranes from lipid monolayers and a
851 study of their electrical properties. *Proc. Natl. Acad. Sci. U. S. A.* **69**, 3561–3566 (1972).
- 852 85. Morein, S., Andersson, A. S., Rilfors, L. & Lindblom, G. Wild-type *Escherichia coli* cells
853 regulate the membrane lipid composition in a ‘window’ between gel and non-lamellar
854 structures. *J. Biol. Chem.* **271**, 6801–6809 (1996).
- 855 86. Tasserit, C., Koutsoubas, A., Lairez, D., Zalczer, G. & Clochard, M. C. Pink noise of ionic
856 conductance through single artificial nanopores revisited. *Phys. Rev. Lett.* **105**, 260602 (2010).
- 857 87. Rigo, E. *et al.* Measurements of the size and correlations between ions using an electrolytic

point contact. *Nat. Commun.* **10**, 1–13 (2019).

Supplementary Information

Supplementary information document includes Supplementary Tables 1 and 2, and Supplementary Fig. 1-4.

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Author contributions

A.G.M., J.A., M.Q.-M., E.L., and I.M. designed and performed the experiments presented and contributed to writing the manuscript. E.L., M. Q.-M. and A.A. contributed to project management, assisted with the experimental plan and contributed to writing the manuscript. D.A.-J. contributed to project management, designed the overall experimental plan for the manuscript, and contributed to writing the manuscript.

883 **Tables**

884 **Table 1.** Quantification of PhoA-LacZ enzymatic activities in permeabilised *E. coli* DH5α cells
885 expressing Tse5-CT-PhoA-LacZ fusion proteins

Point of PhoA-LacZ fusion	K1228	A1269	A1281	K1300	Q1317
PhoA ^a activities (A) (min ⁻¹)	15(1)	8.8(2)	6.1(3)	8.58(4)	3.3(6)
LacZ ^a activities (A) (min ⁻¹)	36(6)	90(7)	85(10)	55(11)	100(2)
PhoA ^b activities (%) (a.u.)	100(7)	58(1)	40(2)	56.5(2)	21(4)
LacZ ^b activities (%) (a.u.)	36(6)	89(7)	85(9)	54(11)	100(2)
NAR ^d	2.78	0.65	0.48	1.03	0.22
Localization	Periplasmic	TM	Cytosolic	TM	Cytosolic
^a the relative LacZ or PhoA enzymatic activities (A) were calculated using Equation 1. ^b the relative LacZ or PhoA enzymatic activities (A) were normalized to the maximum enzymatic activity of each enzyme. ^c the normalised activity ratios (NAR) were calculated using Equation 2. See Methods for details The standard deviations to the last shown digit are represented in parentheses.					

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889 **Fig. 1 | The bacteriolytic effect of Tse5-CT is associated with membrane depolarization. a.**

890 Monitoring growth (OD₆₀₀) in LB medium of *P. putida* cells harbouring pS238D1 empty vector (EV)

891 as control and plasmids directing the expression of Tse5-CT or Tse5-CT and Tsi5 together.

892 Expression of Tse5-CT and Tsi5 was induced at the time indicated (see the arrow) with 1mM m-

893 Toluic acid (TA) and 1mM isopropyl 1-thio-β-D-galactopyranoside (IPTG), respectively. Co-

894 expression of Tse5-CT and Tsi5 was induced by adding to the media both TA and IPTG. To stop the

895 induction, the cells (“recovered”) were washed and resuspended in fresh LB without an inductor (see

896 the arrow). Expression of Tse5-CT caused inhibition of bacterial growth, and cells did not recover

897 the capacity to duplicate after been washed. Tsi5 does not confer Tse5 protection to cells. **b.** Flow

898 cytometry results showing healthy cell populations. Healthy cells are cells that are not marked with

899 any fluorophore. **c.** Flow cytometry results showing depolarized cell population. Depolarized cells

900 stain with DiBAC₄(3). **d.** Flow cytometry results showing permeabilized cell population.

901 Permeabilized cells are stained with Sytox™ Deep Red. **e.** Flow cytometry results showing

902 permeabilized and depolarized cell populations. Permeabilized and depolarized cells are labelled with

903 Sytox™ Deep Red and DiBAC₄(3).

904

Fig. 2 | Tse5-CT contain transmembrane regions that allow it to insert in lipid monolayers and the cytoplasmic membrane of *E. coli* cells. **a.** Representative Langmuir-Blodgett balance experiments showing the lateral pressure increase on lipid monolayers after addition of Tse5-CT at time 0. **b.** The plot of lateral pressure increases as a function of initial lateral pressure for every single experiment (N=11). A Maximal insertion pressure (MIP) of 34,99 has been determined by extrapolating the plot of $\Delta\Pi = \Pi_c - \Pi_0$ as a function of Π_0 where the curve reaches the x -axis. The dotted line indicates the threshold value of lateral pressure consistent with unstressed biological membranes. The equation obtained from the linear regression analysis is $y = -0.3258x + 11,401$ with an R-squared of 0.96. **c.** Tse5-CT propensity to contain transmembrane (blue) and amphipathic (green) helices as predicted by MemBrain 3.1. **d.** Constructs containing the dual reporter PhoA-LacZ α at different C-terminal fusion points of spTse5-CT: K1229, A1269, A1281, K1300 and Q1317 (full-length Tse5-CT). **e.** *E. Coli* DH5 α cells transformed with spTse5-CT-PhoA-LacZ α fusion protein growing on dual reporter agar plates. **f.** Measures of the PhoA-LacZ enzymatic activities for each spTse5-CT-PhoA-LacZ α fusion protein.

921

922 **Fig. 3 | Tse5-CT forms stable pores with ohmic behaviour and preference for cations, and some**

923 **pores with noisy currents and strong voltage dependence. a.** Representative Tse5-CT-induced

924 stable current traces obtained in a 250/50 mM (upper panel) or 50/250 mM (middle panel) KCl

925 gradient using a polar lipid extract from *E. coli* to form the membrane. The lower panel shows a

926 representative trace in 250/50 mM KCl gradient when a neutral DOPE membrane was used. The

927 applied voltages are shown at the bottom in light grey. **b.** I/V curves corresponding to the traces

928 shown in (a). Linear regressions (solid lines) allow calculation of the conductance (1.76 nS (black),

929 0.99 nS (red) y 0.95 nS (blue)) and reversal potential (RP, indicated by circles and arrows). A negative

930 (positive) RP corresponds to a cation (anion) selectivity. **c.** Permeability ratios, P_{K^+}/P_{Cl^-} , calculated

931 from corresponding reversal potentials using the GHK equation⁶⁵. Solid circles correspond to the
932 individual data points. Data are means of 7 (black), 8 (red), and 4 (blue) independent experiments. **d.**
933 Representative Tse5-CT-induced noisy current trace obtained in a 250/50 mM KCl gradient using the
934 *E. coli* polar lipid extract to form the membrane. The applied voltage is shown at the bottom in light
935 grey. Current records in (**a**) and (**d**) were digitally filtered with 500 Hz using a low-pass 8-pole Bessel
936 filter for better visualisation.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [Tse5CTSNatComm20211013vf.pdf](#)