1 **Bacterial competition systems share a domain required for inner membrane**

2 **transport of the nuclease bacteriocin pyocin G.**

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11 analyzed data. I.A., C.S. and C.K. designed research. I.A., C.S. and CK. wrote the

12 paper. C.K. conceived the project.

13 **Competing Interest Statement:** The authors have declared that no competing

14 interests exist.

15 **Keywords:** pyocin, bacterial competition, antibiotic, *P. aeruginosa*, cell envelope,

16 protein import.

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Abstract

Bacteria exploit a variety of attack strategies to gain dominance within ecological niches. Prominent amongst these are contact-dependent inhibition (CDI), type VI secretion (T6SS) and bacteriocins. The cytotoxic endpoint of these systems is often the delivery of a nuclease to the cytosol. How such nucleases translocate across the cytoplasmic membrane of Gram-negative bacteria is unknown. Here, we identify a small, conserved, 15-kDa domain, which we refer to as the inner membrane translocation (IMT) domain that is common to T6SS and bacteriocins and linked to nuclease effector domains. Through fluorescence microscopy assays using intact and spheroplasted cells, we demonstrate that the IMT domain of the *Pseudomonas aeruginosa* specific bacteriocin pyocin G (PyoG) is required for import of the toxin nuclease domain to the cytoplasm. We also show that translocation of PyoG into the cytosol is dependent on inner membrane proteins FtsH, a AAA+ATPase/protease, and TonB1, the latter more typically associated with transport of bacteriocins across the outer membrane. Our study reveals that the IMT domain directs the cytotoxic nuclease of PyoG to cross the cytoplasmic membrane and, more broadly, has been adapted for the transport of other toxic nucleases delivered into Gram-negative bacteria by both contact dependent- and contact-independent means.

Importance

Nuclease bacteriocins are potential antimicrobials for the treatment of antibiotic resistant bacterial infections. While the mechanism of outer membrane

43 translocation is beginning to be understood, the mechanism of inner membrane

44 transport is not known. This study uses PyoG as a model nuclease bacteriocin and

45 defines a conserved domain which is essential for inner membrane translocation

46 and which is widespread in other bacterial competition systems. Additionally, the

47 presented data links two membrane proteins, FtsH and TonB1, with inner

48 membrane translocation of PyoG. These findings point to the general importance

49 of this domain to the cellular uptake mechanisms of nucleases delivered by

50 otherwise diverse and distinct bacterial competition systems. The work is also of

51 importance for the design of new protein antibiotics.

52

53 **Introduction**

54   Bacteria deploy various contact dependent and independent competition

55 systems to compete for space and resources (1). These systems deliver toxic

56 effectors to the cell envelope or the cytoplasm of bacterial competitors. While the

57 mechanism of effector delivery varies between different competition systems,

58 effector structures and killing mechanisms can be conserved (2). Often,

59 competition system effectors are nucleases that get transported across the cell

60 envelope to degrade cytoplasmic nucleic acids. These folded proteins cross the

61 multi-layered cell envelope via poorly understood translocation mechanisms.

62   Bacteriocins of Gram-negative bacteria are protein antibiotics deployed as

63 weapons for contact-independent bacterial competition. Bacteriocins have a

64 potential to be developed into antibiotics, for the treatment of infections resistant

3

65 to conventional antimicrobials (3). Bacteriocins can kill bacterial cells via different

66 mechanisms: by forming pores in the inner membrane; by inhibiting peptidoglycan

67 biosynthesis through lipid II degradation; by DNA or RNA degradation. Bacteriocin

68 producing strains are immune to their own bacteriocins due to the production of

69 immunity proteins that inhibit the bacteriocin's killing activity. Sensitive strains do

70 not produce the immunity protein or produce an immunity protein specific for a

71 different bacteriocin, but nevertheless have the appropriate translocation

72 machinery through the cell envelope. In the case of nuclease bacteriocins, which

73 penetrate the cytoplasm to degrade nucleic acids, this translocation machinery

74 spans both the outer and the inner membranes (4).

75 Pyocins are bacteriocins that target and kill *Pseudomonas aeruginosa*.

76 Pyocin G (PyoG) is a nuclease pyocin active against *P. aeruginosa* clinical isolates

77 (5). It is composed of an unstructured N-terminus followed by a receptor binding

78 domain (5), a conserved middle domain which is essential for its killing activity (6),

79 and a cytotoxic nuclease domain (7). The receptor binding domain is required for

80 outer membrane translocation of PyoG (5). The role of the conserved central

81 domain is unknown, but the fact that it is absent from bacteriocins with periplasmic

82 targets and present in bacteriocins that cleave nucleic acids (Pfam domain

83 PF06958.7) suggests that it may have a role in inner membrane transport (8).

84 Here, we show that this domain is required for PyoG translocation to the cytosol.

85 PyoG enters the periplasm by binding and translocating through Hur in the

86 outer membrane (5). Hur is a 22-stranded β-barrel TonB dependent transporter

87   (TBDT) that ordinarily transports hemin into the cell following engagement with

88   TonB1 in the inner membrane, in conjunction with the stator complex ExbB-ExbD

89   and the proton motive force (PMF). TonB1 activates hemin transport following

90   association with Hur's periplasmically-located TonB box epitope. PyoG parasitizes

91   hemin uptake and in so doing delivers its own TonB box to the periplasm which

92   engages with TonB1, allowing the toxin to be pulled into periplasm.

93   TonB-dependent outer membrane translocation of pyocins is well-

94   understood (5, 9, 10). By contrast, how these toxins translocate from the periplasm

95   to the cytoplasm is unknown. In the case of nuclease colicins, which are *E. coli*-

96   specific bacteriocins, inner membrane transport requires both the ATPase and

97   protease functions of FtsH (11). FtsH is a hexameric AAA+ ATPase/protease in

98   the inner membrane, where it is involved in protein quality control (12). FtsH

99   proteolytically processes nuclease colicins as they pass into the cytoplasm (13).

100   More recently, FtsH has also been linked to the killing activity of PyoG (5)

101   demonstrating a broader involvement in bacteriocin uptake.

102   In the present work, we address the poorly understood area of bacteriocin

103   transport across the inner membrane. Using a newly developed import assay,

104   whereby the uptake of fluorescently labelled PyoG into *P. aeruginosa* spheroplasts

105   is monitored, we map the requirements for pyocin transport to the cytoplasm.

106   Using this assay, we demonstrate that a small domain found in all nuclease

107   bacteriocins as well as other bacterial competition systems is absolutely required

108   for transport.

Results and Discussion

Inner membrane translocation of nucleases in bacterial competition systems is associated with a ubiquitous domain. Using bioinformatics, we have previously identified a highly conserved bacteriocin domain that is absent from pore forming bacteriocins but associated exclusively with competition systems that transport nucleases to the cytoplasm (8). We term this domain the Inner Membrane Translocation (IMT) domain. The IMT domain is annotated in the PFAM database as the pyocin-S domain (PF06958.7). The main structural features of the domain are two anti-parallel β-sheets that give the domain an L-shape (Figure 1 - A). Using a more extensive informatics search we found the IMT domain across several orders of Gammaproteobacteria (Figure 1 - B). Surprisingly, apart from being conserved amongst nuclease bacteriocins, this domain is also found in Type 6 Secretion System (T6SS) effectors (Figure 1 - C). These toxins, like nuclease bacteriocins, translocate across the inner membrane to kill competitors. Unlike bacteriocins however, which are diffusible toxins, T6SS effectors are contact dependent and delivered by a contractile needle that punctures the outer membrane. We found several characteristic PFAM domains of these toxin systems co-occur with the IMT domain (Figure 1 - D) – the HNH nuclease domain found in DNase bacteriocins and T6SS effectors, and the rRNase and tRNase bacteriocin domains. We conclude that the IMT domain is found in competition system effectors that express their cytotoxic activity in the cytoplasm of target cells regardless of the means by which the toxin initially penetrates the outer defenses of the Gram-negative bacterium.

Inner membrane translocation of PyoG requires the IMT domain. Having established that the IMT domain is associated with inner membrane translocation of toxin nucleases in bacteria, we sought to test its requirements for import of PyoG into *P. aeruginosa*. PyoG is a 640 amino acid toxin comprised of an N-terminal receptor binding domain that engages Hur and enables transport to the periplasm and a C-terminal nuclease domain that elicits cell death (5). Between them is the IMT domain which comprises residues 256-485 (Figure 1 – E). We developed a fluorescence microscopy assay to dissect the involvement of the IMT domain in transport. Pyocins can be readily conjugated with fluorescent dyes and used for labelling live *P. aeruginosa* cells that express components of the pyocin translocation machinery in microscopy experiments. Trypsin protection is then used to distinguish imported from surface bound molecules (9, 10). We generated different PyoG constructs, each containing a unique cysteine at the C-terminus of the construct that was conjugated to AlexaFluor (AF) 488 and assessed the ability of each construct to be imported in intact cells and spheroplasts (Figure 2 – A). Spheroplasts were generated by lysozyme/EDTA treatment that permeabilises the outer membrane and peptidoglycan layer (14, 15, 16). After labelling with fluorescent pyocin, cells were exposed to trypsin to remove any surface exposed pyocin that was not translocated across the cell envelope. In intact cells, the outer membrane is not permeable to trypsin (9, 10). Therefore, in intact cells translocation across the outer membrane is sufficient to protect a pyocin construct from degradation with trypsin. In spheroplasts, the periplasm is exposed to trypsin (14), and the pyocin construct must translocate across the inner membrane to be

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155   protected from degradation by trypsin. Residual fluorescence after trypsin

156   treatment is therefore an indication of outer membrane, and potentially inner

157   membrane transport, in intact cells. In the case of spheroplasts, it is an indication

158   of inner membrane transport. To test if nontranslocated pyocin gets degraded by

159   trypsin under the conditions of our experiments, a pyocin S2-GFP chimera was

160   used (Supplementary Figure 1 - A). This construct is comprised of the pyocin S2

161   N-terminal domain (residues 1-209) translationally fused to GFP. GFP acts as a

162   plug, blocking the import of the pyocin (10). Trypsin treatment removed the PyoS2-

163   GFP fluorescent signal entirely in both intact cells and spheroplasts

164   (Supplementary Figure 1 – A, B). A further control was undertaken to ascertain if

165   the outer membrane translocation step can be bypassed using spheroplasts. *Δhur*,

166   a PAO1 transposon mutant that lacks the PyoG receptor (5), was exposed to

167   fluorescent PyoG. Intact cells of this mutant were not labelled with the pyocin, since

168   Hur is essential for binding of PyoG to the surface of *P. aeruginosa* cells (5).

169   Generation of spheroplasts was sufficient to allow *Δhur* labelling with fluorescent

170   PyoG. Additionally, PyoG was protected from degradation by trypsin in *Δhur*

171   spheroplasts (Supplementary Figure 1 – D, E). Therefore, the Hur-dependent outer

172   membrane translocation step can be bypassed under these tested experimental

173   conditions, confirming that *P. aeruginosa* spheroplasts could be used to study

174   inner membrane translocation.

175   The cellular localisation of the following PyoG constructs were tested: full

176   length PyoG, PyoG$^{1-485}$ which lacks the cytotoxic domain, and PyoG$^{1-255}$ which

177   lacks the cytotoxic and IMT domains (Figure 2 – A). Constructs were expressed

178   and purified as previously described (5). Their fold and stability were tested by

179   circular dichroism (Supplementary Figure 1 – A) and differential scanning

180   calorimetry (Supplementary Figure 1 - B); all appeared folded at room temperature.

181   All three constructs contained the receptor (Hur-) binding domain of PyoG

182   (residues 1-255) and were protected from degradation by trypsin in intact cells

183   (Figure 1 – B, C), which indicated that these constructs translocated across the

184   outer membrane. This is in accordance with previously published work, where a

185   pyocin receptor binding domain was sufficient to protect the pyocin from

186   degradation by extracellular trypsin added to live *P. aeruginosa* cells (9, 10).

187   However, only full length PyoG and PyoG$^{1\text{-}485}$ were trypsin protected in

188   spheroplasts (Figure 1 – B, C), indicating that these two constructs were capable

189   of translocating across the inner membrane. Trypsin protection of PyoG$^{1\text{-}485}$ also

190   implied that the cytotoxic domain of PyoG was not essential for inner membrane

191   translocation, which has previously been suggested to be a requirement for colicin

192   transport (11). PyoG$^{1\text{-}255}$, which lacks the IMT domain, was not trypsin protected

193   in *P. aeruginosa* spheroplasts. We therefore conclude that the IMT domain is

194   required for PyoG to cross the inner membrane.

195   **Inner membrane translocation of PyoG requires FtsH and TonB1.** PyoG killing

196   activity has previously been linked to Hur, the outer membrane receptor, TonB1,

197   the inner membrane protein that provides energy for outer membrane

198   translocation, and FtsH in the inner membrane (5). Since FtsH is not essential in

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199   *P. aeruginosa*, we used PAO1 as a model organism to test if FtsH is required for

200   outer and/or inner membrane translocation of PyoG. PAO1 wt and *ΔftsH* cells were

201   exposed to fluorescent PyoG$^{1-485}$ and to trypsin, as described in Figure 2. PyoG$^{1-485}$

202   was trypsin protected in *ΔftsH* intact cells (Figure 3 – A, B), demonstrating that

203   outer membrane translocation of PyoG does not require FtsH. On the other hand,

204   PyoG$^{1-485}$ was not trypsin protected when added to *ΔftsH* spheroplasts (Figure 3 –

205   A, B), indicating that FtsH was required for inner membrane translocation of PyoG.

206   Additionally, when the *ftsH* deletion was complemented from a plasmid, PyoG

207   killing activity (Supplementary Figure 3 - A), and inner membrane translocation

208   (Figure 3 – A, B) were restored. Complementation with FtsH H416Y, a mutant of

209   FtsH that does not have protease activity (17), did not restore PyoG killing activity

210   (Supplementary Figure 3 - A), or inner membrane translocation (Figure 3 – A, B).

211   Therefore, proteolytically active FtsH is required for PyoG to cross the inner

212   membrane.

213   We also tested if TonB1, a protein previously linked to pyocin outer

214   membrane translocation (9, 10), is required for PyoG import into spheroplasts. This

215   protein was required for inner membrane translocation of PyoG, since PyoG$^{1-485}$

216   was not trypsin protected in *ΔtonB1* spheroplasts (Figure 3 – C, D).

217   Complementation of *ΔtonB1* with TonB1 expressed from a plasmid restored PyoG

218   killing activity (Supplementary Figure 3 - B) and inner membrane import (Figure 3

219   – C, D). We also identified a region of PyoG that is involved in binding to TonB1

220   (Supplementary Figure 4) and demonstrated that this region is required for both

221 outer and inner membrane import (Figure 3 – E, F). Pyocins S5 (9) and S2 (10)

222 bind TonB1 via a TonB box located in the unstructured N-terminus. Therefore, we

223 deleted the first 30 residues in the unstructured N-terminus of PyoG to test if this

224 deletion affects TonB1 binding. The deletion, which renders PyoG inactive against

225 *P. aeruginosa* (Supplementary Figure 4 – C), disrupted binding of PyoG to TonB1

226 (Supplementary Figure 4 – A), but it did not affect binding to the receptor Hur

227 (Supplementary Figure 4 – B), confirming that the TonB box of PyoG is in the

228 unstructured N-terminus. Unlike PyoG$^{1-485}$, PyoG$^{31-485}$ did not translocate into

229 intact cells or spheroplasts (Figure 3 – E, F), demonstrating that the TonB box is

230 required for both the outer and the inner membrane translocation steps.

231 **Model of nuclease bacteriocin transport across the inner membrane.** The

232 present study shows that the IMT domain, TonB1, and the ATPase/protease FtsH,

233 are all essential for the inner membrane translocation step of PyoG. Taken

234 together with previous studies that demonstrate FtsH dependent processing of

235 nuclease colicins during import (18, 13), we propose a model of PyoG inner

236 membrane translocation (Figure 4). The PyoG receptor binding domain (residues

237 1-255) binds to Hur, that interacts with TonB1 via its plug domain. The TonB box

238 in the bacteriocin N-terminus also associates with TonB1, likely pulling through the

239 TBDT to enter the periplasm in its entirety (Figure 5 - A; 10). PyoG associates with

240 TonB1 via its TonB box but whether this interaction also exploits the PMF-

241 dependence of TonB1, or other interaction partners is currently unknown. One

242 possible role of the TonB1 interaction may be to localise PyoG close to the inner

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243   membrane from where the IMT domain either interacts directly with the membrane

244   or even with FtsH itself for transfer across the membrane and proteolytic

245   processing (18, 13). However, we were unable to demonstrate a direct interaction

246   between FtsH and PyoG by pull-down assay (data not shown). It is also uncertain

247   whether the entire IMT domain region of PyoG gets imported into the cytoplasm.

248   Previous studies on colicins suggest that colicin D, E3 (18), E2, and E7 (13)

249   undergo proteolytic processing during import. A cleavage site positioned within the

250   IMT domain was defined for these colicins. Other studies on colicins have

251   suggested that direct interaction of the transported nuclease with the cytoplasmic

252   membrane is a requirement for transport to the cytoplasm (19, 20). However,

253   PyoG constructs lacking the nuclease are still able to translocate (Figure 2). The

254   IMT domain was sufficient to initiate inner membrane translocation, and potentially

255   this domain also inserts into the inner membrane. Additionally, it is possible that

256   the IMT domain initiates inner membrane transport through a mechanism involving

257   other inner membrane proteins. The IMT domain of the nuclease colicin, ColD, has

258   previously been shown to interact with inner membrane proteins essential for the

259   ColD killing activity. The IMT domain of colicin D, which shares 26 % sequence

260   identity with that of PyoG, has been shown to bind TonB (21, 22) as well as the

261   signal peptidase, LepB in the inner membrane. Therefore, it is possible that the

262   PyoG IMT domain also has additional interaction partners in the inner membrane.

263   We showed that some T6SS effectors, such as the *E. coli* nuclease Usp

264   effector (23), share structural similarities with nuclease bacteriocins. Both groups

265 of toxins can have an HNH motif in the nuclease domain, and the conserved IMT

266 domain upstream of the nuclease domain (Figure 1). Therefore, nuclease

267 bacteriocins and T6SS effectors may share similarities in their inner membrane

268 translocation mechanism. T6SS effectors are loaded to the T6SS needle or needle

269 tail proteins, which penetrate the outer membrane, reach the *cis* side of the

270 peptidoglycan layer (24), and deliver the effector to the bacterial periplasm (25).

271 While the T6SS effectors are directly delivered to the *cis* side of the peptidoglycan

272 layer, bacteriocins may require the pulling force of TonB1 to reach this cell

273 envelope compartment, in a mechanism similar to the one exerted by TolA on TolB

274 and Pal (26). How T6SS effectors further translocate the inner membrane is

275 unknown. It is possible that, like in the case of PyoG, the IMT domain of T6SS

276 effectors is required for inner membrane translocation (Figure 4 – C). Potentially,

277 this translocation also requires FtsH, but this has yet to be tested experimentally.

278 The conserved IMT domain has previously been linked to bacteriocin killing

279 activity (6), but until now its role in bacteriocin import has been unknown. In this

280 study, we demonstrate that the IMT domain is specifically required for inner

281 membrane translocation of nuclease bacteriocins. We also found this domain in

282 T6SS nuclease effectors, which offers a link between nuclease effector import in

283 contact dependent and independent competition systems. While these systems

284 use different mechanisms to deliver cytoplasmic effectors into the bacterial

285 periplasm, the transport of the effector across the inner membrane may be

286 conserved.

Materials and Methods

Sequence analysis of IMT domain containing proteins. IMT domain (PF06958.7) containing proteins were identified in uniref100 using HMMer. Domains were identified using Pfam 27 with e-value $< 1 \times 10^{-10}$. Taxonomy of species with IMT domain was determined using NCBI Commontree and visualised using iTOL (27).

Bacterial strains, media and growth conditions. All strains (Table 1) were cultured in LB (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract, pH 7.2) or M9 media (8.6 mM NaCl, 18.7 mM NH4CL, 42.3 mM Na2HPO4, 22.0 mM KH2PO4, 0.4% w/v glucose, 2 mM MgSO4, 0.1 mM CaCl2) at 37 °C with shaking (140 rpm). *P. aeruginosa ΔftsH* was grown on salt free LB media. *ΔtonB1* mutant of *P. aeruginosa*, and the parent strain PAO6609, were grown in LB media supplemented with 100 μM FeCl₃. *P. aeruginosa Δhur* mutant was grown in the presence of 10 μg/mL tetracycline. 100 μg/mL of carbenicillin and 25 μg/mL of triclosan was used for selecting plasmid transformants of *P. aeruginosa*.

Plasmids. pET21a(+) was used as a backbone for the expression of ColD and PyoG constructs in *E. coli* BL21(DE3). pMMB190 was used for complementation and gene expression in *P. aeruginosa*. All plasmids are listed in Table 2. Full length PyoG, with the addition of a G4S linker and a cysteine in the C-terminus in an operon with the ImG immunity protein, was synthesized by Genewiz, and cloned into pET21a(+). pNGH262 (5) was used as the backbone for the construction of plasmids carrying PyoG constructs for the fluorescent labelling of *P. aeruginosa*.

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309 pG1-485-Cys was constructed by PCR, amplifying the first 485 residues of PyoG

310 and by adding a cysteine at the C-terminus of the construct by PCR mutagenesis

311 (5). pG1-255-Cys was constructed by introducing a cysteine and an XhoI site after

312 residue A255 in pNGH262 and cutting out the region between the two XhoI sites.

313 Genes used for *P. aeruginosa* complementation (*ftsH*, *ftsH* H416Y and *tonB1*)

314 were synthetised by Genewiz and restriction cloned into pMMB190 using the

315 EcoRI and HindIII sites. Plasmid DNA was isolated from NEB5α cultures grown at

316 37 °C in LB with the appropriate antibiotic, using Monarch Plasmid Miniprep kit

317 (NEB). Sequencing was through the company Genewiz.

318 **Conjugation of *P. aeruginosa*.** Chemically competent *E. coli* S17-1 competent

319 cells were prepared by $CaCl_2$ treatment. 50 mL of over-night culture, grown at 37

320 °C in LB, was pelleted on 5000 x g for 10 min. Cells were resuspended in 50 mL

321 ice cold 0.1 M $CaCl_2$ and kept on ice for 30 min. Cells were then pelleted again

322 and resuspended in 1 mL 0.1 M $CaCl_2$ and incubated on ice for 5 more min before

323 transformation. Competent cells were transformed by heat shock. 50 µL of

324 competent cells were mixed with 5 µL of DNA. Cells were incubated on ice for 30

325 min, shocked for 30 s on 42 °C, and then incubated on ice for another 10 min. Cells

326 were then plated on LB agar with antibiotics. *P. aeruginosa* PAO1 was transformed

327 via conjugation with *E. coli* S17-1 carrying a plasmid of interest. PAO1 was grown

328 overnight at 42 °C, and S17-1 at 37 °C, with shaking, in LB media. 2 mL of each

329 culture was pelleted on 5000 x g, 10 min. Each strain was then resuspended in

330 100 µL of LB media and the two strains were mixed together. The entire mix was

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331   then spotted on top of an antibiotic free LB agar plate. The plate was incubated on

332   37 °C for 4 h. The lawn was then scraped from the plate and resuspended in 1X

333   phosphate buffer saline (PBS) pH 7. The suspension was serially diluted in PBS.

334   100 µL of each dilution was plated on LB agar with 25 µg/mL triclosan, for selecting

335   against S17-1, and 100 µg/mL carbenicillin, for selecting against untransformed

336   PAO1.

337   **Expression and purification of bacteriocin constructs.** PyoG was expressed

338   in complex with the His-tagged immunity protein, ImG. Two copies of ImG were

339   used: one in an operon with PyoG (pNGH262), and one on a separate plasmid

340   (pNGH263), as described previously (5). PyoG$^{1-485}$ and PyoG$^{1-255}$ were expressed

341   with an N-terminal His-tag. *E. coli* BL21(DE3) was used for heterologous pyocin

342   expression, as described previously (5).

343   **Circular dichroism (CD) spectroscopy.** Proteins were dialysed into 10 mM

344   potassium phosphate pH 8, 20 mM NaF, and were diluted to 0.1 mg/ml. CD spectra

345   were obtained using a Jasco J-815 Spectropolarimeter over a wavelength range

346   of 260-190 nm, a digital integration time of 1 second and a 1 nm bandwidth. CD

347   data in millidegrees were converted to mean residue ellipticity by dividing by molar

348   concentration and number of peptide bonds.

349   **Differential scanning calorimetry (DSC).** The melting temperature of proteins,

350   as an indication of their integrity, was determined by DSC performed on Malvern

351   VP Capillary DSC by Dr. David Staunton, Molecular Biophysics Suite, Department

352   of Biochemistry, University of Oxford. Pyocin constructs were tested at 20 µM in

353   10 mM potassium phosphate pH 8, 20 mM NaF.

354   **Conjugation of maleimide fluorophores to proteins.** Pyocins were fluorescently

355   labelled using Alexa Fluor 488 C5 maleimide (AF488) fluorophore that was linked

356   to proteins via an engineered C-terminal cysteine, as previously described (5).

357   **Labelling of live *P. aeruginosa* cells with fluorescent pyocins.** Fluorescent

358   PyoG constructs conjugated to AF488 were used to label *P. aeruginosa*. Bacteria

359   were grown overnight in M9 medium at 37 °C with shaking. 1 mL of this overnight

360   culture was pelleted and resuspended in 10 mL M9 medium and grown until an

361   OD600 of 0.5. All pelleting steps were performed at 7000 x g for 3 min. 1 mL of

362   cells was washed in PBS pH 7, and labelled with 2 µM pyocin for 30 min at room

363   temperature. The unbound pyocin was removed by three washes in PBS. For the

364   trypsin protection assay, after labelling, cells were exposed to 0.5 mg/mL trypsin

365   for 1 h at 30 °C, in PBS with 35 µg/mL chloramphenicol. After a final washing step,

366   bacteria were resuspended in 30 µL of PBS. 3 µL of cells were then loaded onto

367   agarose pads, prepared using Geneframes (Thermo Scientific). 80 µL of 1% (w/v)

368   agarose in PBS was pipetted into the Geneframe (17×28 mm). The surface was

369   flattened with a cover slip and excess agar removed. Once the agar solidified the

370   cover slip was removed, the bacterial suspension added and a new coverslip

371   attached to the adhesive side of the Geneframe.

372   **Labelling of *P. aeruginosa* spheroplasts with fluorescent pyocins.** Bacteria

373   were grown overnight in M9 medium at 37 °C with shaking. 1 mL of this overnight

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374 culture was pelleted and resuspended in 10 mL M9 medium and grown until an

375 OD600 of 0.5. All pelleting steps were performed at 3000 x g for 10 min. 1 mL of

376 cells was pelleted and resuspended in PBS pH 7, with the addition of 0.5 M

377 sucrose, 20 mM EDTA and 1.5 mg/mL lysozyme, and incubated for 45 min on

378 room temperature. Cells were then washed into PBS, 0.5 M sucrose, and mixed

379 with 2 µM fluorescent pyocin. After 30 min incubation on room temperature,

380 unbound pyocin was removed by three washes in PBS, 0.5 M sucrose. For the

381 trypsin protection assay, after labelling, cells were exposed to 0.5 mg/mL trypsin

382 for 1 h at 30 °C, in PBS, 0.5 sucrose. After a final washing step, bacteria were

383 resuspended in 30 µL of PBS, 0.5 M sucrose and loaded onto agar pads as

384 described above. Agar was supplemented with 0.5 sucrose to prevent the bursting

385 of the spheroplasts.

386 **Image collection and data analysis.** All images were collected on an Oxford

387 Nanoimager S microscope. In case of PyoG constructs, images were collected at

388 100 ms exposure and 20 % 488 nm laser power. For every image, 20 frames were

389 collected and merged using the command "Zproject" in Image J. Average

390 fluorescence was measured for a total of 50 cells per condition per repeat, and

391 was corrected by subtracting the average background fluorescence. All

392 experiments were conducted in 3 biological repeats. Fluorescence intensity of

393 experimental groups (groups exposed to fluorescent pyocin) was compared to the

394 unlabelled control by the Kruskal-Wallis test, using Dunn's test as the post hoc

395 procedure (confidence level 0.001). The analysis was performed by GraphPad

396   Prism version 6.04 for Windows, GraphPad Software, La Jolla California USA,

397   www.graphpad.com.

398   **Plate killing assays.** *P. aeruginosa* was grown in LB at 37 °C to an OD600 of 0.6.

399   Bacterial lawns were prepared by addition of 250 µl of culture to 5 ml of molten soft

400   LB-agar (0.75% (w/v) agar in LB), and were poured over LB-agar plates. Once set

401   and dry, 3 µl of 3-fold serially diluted pyocins, ranging from 10 µM to ~57 pM, was

402   spotted on top of the lawn. Lawns were grown overnight at 37 °C and cytotoxicity

403   was determined by observation of clearance zones.

404

405   **Acknowledgments**

413

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542 **Tables and Figures**

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544 **Table 1.** List of strains used in this study.

Species	Strain	Characteristics	Source
Escherichia coli	BL21(DE3)	Expression of His-tagged bacteriocins and TonB1	New England Biolabs
	NEB5α	Plasmid propagation	New England Biolabs
	S17-1	Conjugation with *P. aeruginosa*	American Type Culture Collection
Pseudomonas aeruginosa	PAO1	wild type	Washington Library (29)
	PW3356	PA1302 (*hur*)	
	PAO1 *ΔftsH*	*ftsH* deletion mutant	(30)
	pFtsH	*ΔftsH* mutant of PAO1 complemented with pREN144	This study.
	pFtsH H416Y	*ΔftsH* mutant of PAO1 complemented with pREN145	
	PAO6609	*met-9011 amiE200 strA pvd-9*	(31)

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	PAO6609 ΔtonB1	*tonB1* transposon mutant	
	pTonB1	*ΔtonB1* mutant of PAO6609 complemented with *ptonB1*	This study.

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548 **Table 2.** List of plasmids used in this study.

Plasmid	Insert	Vector	Description	Source
pET21a(+)			pBR322 origin, Hist tag, Amp^r	NEB
pMMB190			Broad-host-range cloning vector, Amp^r, pMMB66EH, tac promoter, LacZα	(32)
pNGH262	PyoG, ImG-His$_6$	pET21a(+)	His$_6$-Im-PyoG cloned at *NdeI* and *HindIII* sites	(5)
pNGH263	ImG-His$_6$	pACYCDuet1	PyoG immunity protein cloned at *NdeI* and *XhoI*	(5)
pG-G4S-Cys	PyoG-G4SC, ImG-His$_6$	pET21a(+)	PyoG with a G4S linker and cysteine in the C-terminus	This study

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pG1-485-Cys	PyoG$^{1-485}$Cys-His$_6$	pET21a(+)	PyoG$^{1-485}$-Cys-His$_6$ cloned at *NdeI* and *HindIII* sites	This study
pG1-255-Cys	PyoG$^{1-255}$Cys-His$_6$	pET21a(+)	PyoG$^{1-255}$-Cys-His$_6$ cloned at *NdeI* and *HindIII* sites	This study
pREN144	His6-TEV-FtsH	pMMB190	*ftsH* from PAO1 with an N-terminal His6 and TEV site	This study
pREN145	His6-TEV-FtsH, point mutation H416Y	pMMB190	*ftsH* H416Y from PAO1 with an N-terminal His6 and TEV site	This study
pTonB1	*tonB1*	pMMB190	*tonB1* from PAO1 genome	This study

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Figure 1. The IMT domain is a conserved structural domain found in multiple orders within Gammaproteobacteria and associated with multiple toxin systems. **A** - Structure of the conserved beta-sheets of the IMT domain from colicin D (blue -
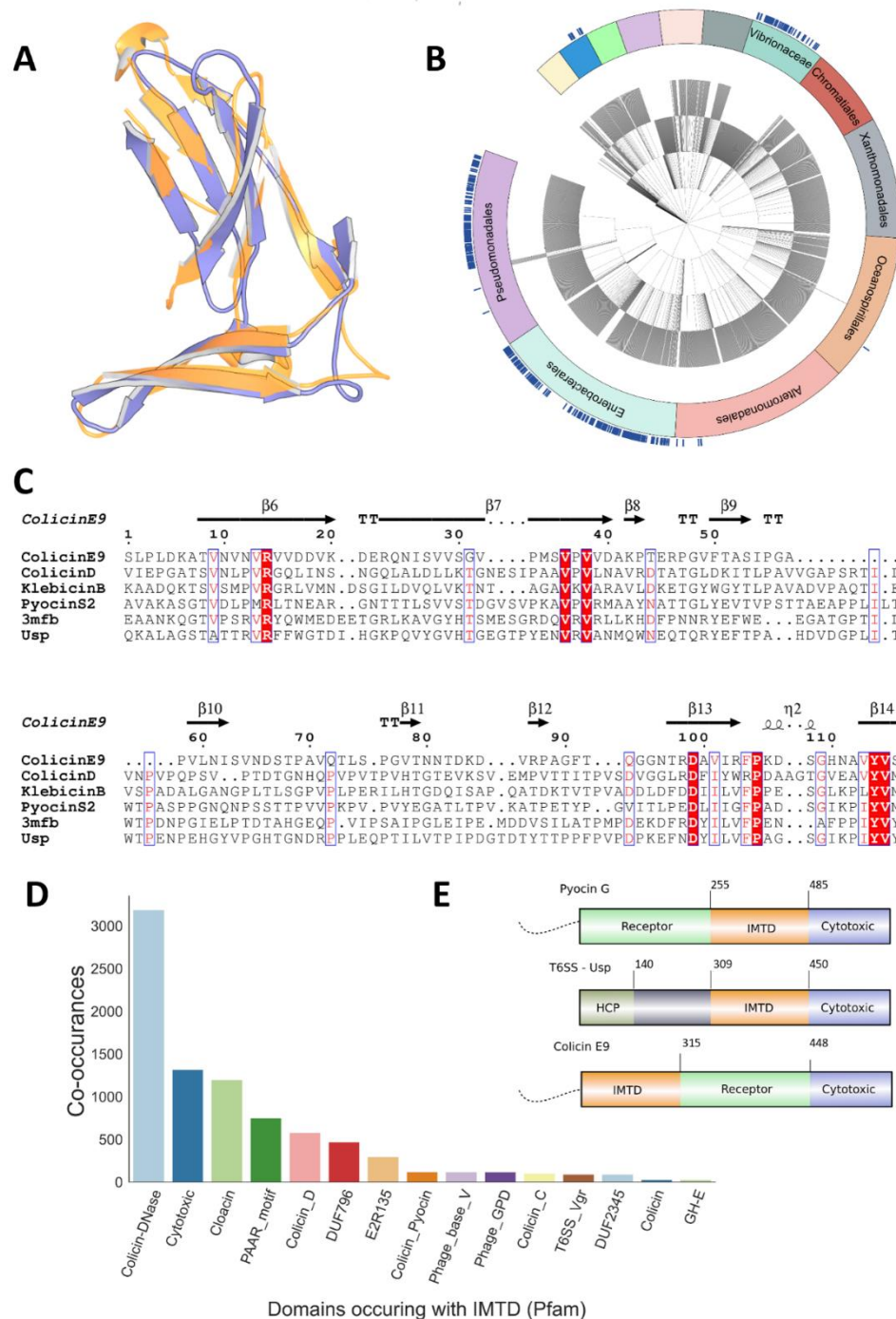
553 pdb: 5ZNM) and a T6SS effector (gold - pdb: 3MFB). **B** - Taxonomy of

554 Gammaproteobateria within uniref100. Blue ticks indicate species which contain

555 at least 1 IMT domain containing protein. IMT domain proteins are prevalent in

556 Gammaproteobacteria though restricted to certain orders (Unlabelled taxa are:

557 Cellvibrionales – Grey, Thiotrichales- Peach, Pasteurellaceae - Purple,

558 Legionellales – Green, Aeromonadales – Blue, Methylococcales – Yellow). **C** -

559 Alignment of the IMT domain domain from bacterial toxins including colicins and

560 other nuclease bacteriocins and T6SS effectors (3MFB and Usp – Uropathic

561 Specific Protein). **D** - Pfam domains which co-occur in proteins with IMT domain.

562 IMT domain is found in proteins which contain numerous toxin effectors or

563 structural domains. (Colicin-DNase – common HNH nuclease found in DNase

564 bacteriocins and T6SS effectors, Cytotoxic – rRNase toxin found in bacteriocins

565 and T6SS effectors, Colicin_D – tRNase found in bacteriocins and T6SS effectors,

566 DUF796 (T6SS_HCP) – structural motif found in T6SS effectors, E2R135 –

567 receptor domain for certain colicins, Colicin_Pyocin – a domain found in the

568 immunity proteins for many DNase bacteriocins. Phage_base_V/Phage_GPD –

569 phage/T6SS structural proteins, Colicin_C – tRNase found in bacteriocins and

570 T6SS effectors, T6SS_Vgr – structural domain of the T6SS, DUF2345 – domain

571 of unknown function associated with the T6SS, Colicin – pore forming domain

572 found in many colicins, all proteins identified with this domain had similarity to

573 Colicin B, GH-E – HNH family of nucleases). **E** – The domain organisation of PyoG

574 (5). For comparison, the position of the IMT domain is shown for a T6SS nuclease

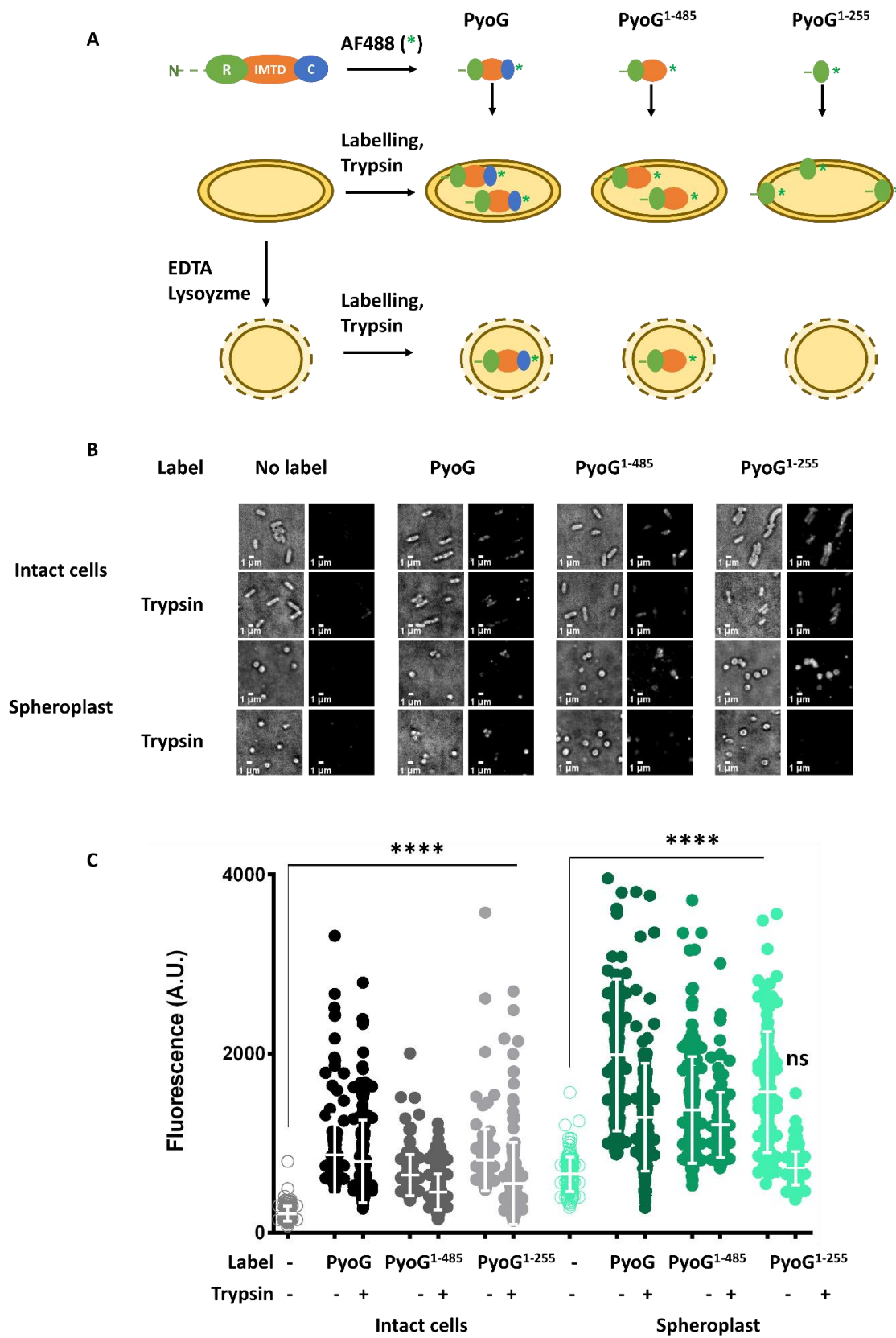575 effector (Usp) and a nuclease colicin (ColE9).

Figure 2. Import and localisation of fluorescent PyoG constructs. **A** – a fluorescence microscopy experiment set-up, used for localisation of PyoG constructs in *P. aeruginosa* cells. All constructs are conjugated to AF488 (represented with a green *) via a C-terminal cysteine. Intact cells, or spheroplasts generated by lysozyme/EDTA treatment, are exposed to 2 µM fluorescent PyoG. Unbound and untranslocated pyocin constructs are removed by trypsin treatment. Residual fluorescence in intact cells is an indication of outer membrane, and possibly inner membrane translocation. Since the spheroplast outer membrane and periplasm are disrupted by EDTA and lysozyme, the periplasm is exposed to trypsin (14, 16). Therefore, residual fluorescence in spheroplasts is an indication of inner membrane translocation. **B** - Representative micrographs before and after trypsin treatment are shown. All snapshots were adjusted to the same contrast value. Tested constructs are – full length PyoG, PyoG$^{1-485}$ lacking the cytotoxic domain, and PyoG$^{1-255}$ lacking the cytotoxic domain and the IMT domain. All constructs are trypsin protected in intact cells, indicating outer membrane translocation. In spheroplasts, full length PyoG and PyoG$^{1-485}$ are trypsin protected, but PyoG$^{1-255}$ is not. Therefore, the IMT domain is required for inner membrane translocation of PyoG. **C** - Average fluorescence intensities were measured for 150 cells per condition. Mean of three biological replicates with standard deviations are shown. Fluorescence intensities for labelled and trypsin treated groups in each condition are compared to the unlabelled control. **** represents a P value below 0.0001 in the Kruskal-Wallis Test, and ns represents no significant difference or lack of fluorescent labelling.
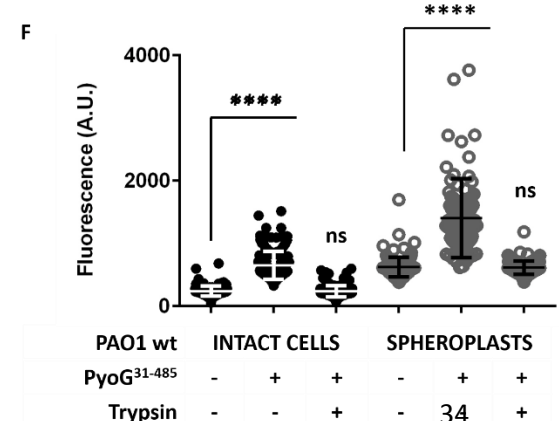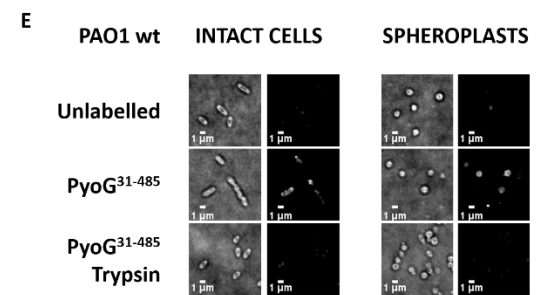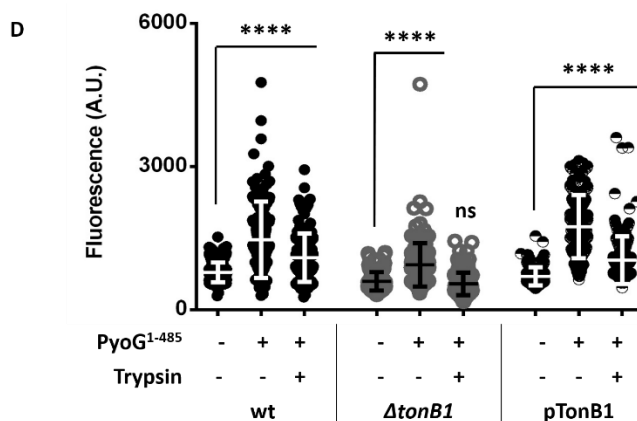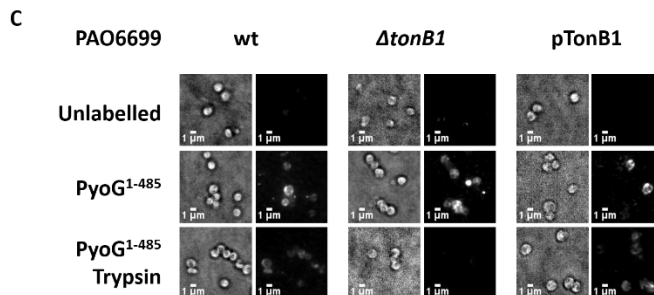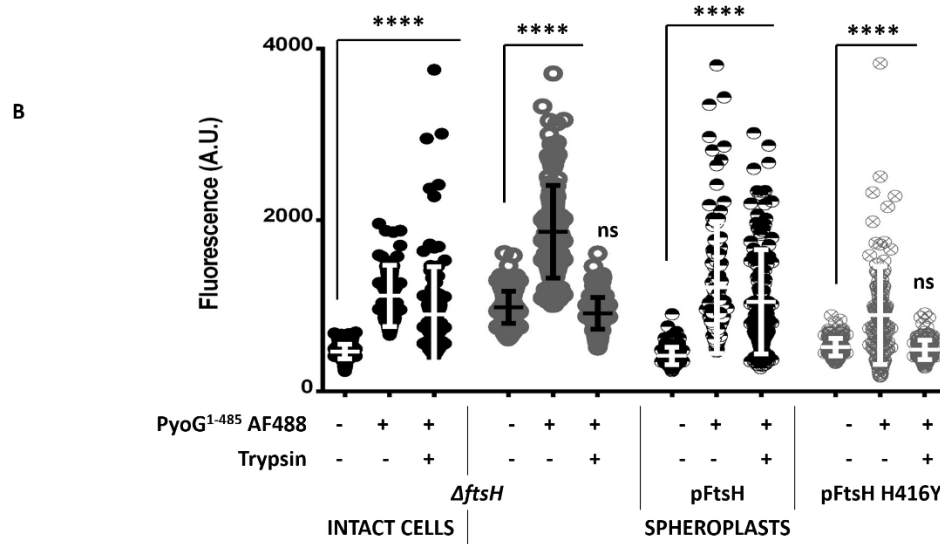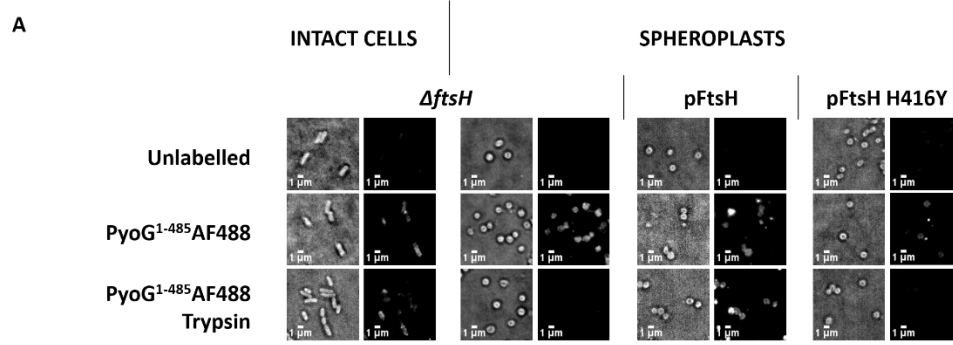
601 **Figure 3**. Inner membrane translocation of PyoG requires FtsH and binding to

602 TonB1. PyoG$^{1-485}$ is conjugated to AF488 via a C-terminal cysteine and added to

603 *P. aeruginosa* spheroplasts at 2 µM. Nontranslocated pyocin is removed by trypsin

604 treatment. **A, B** - FtsH is required for inner membrane translocation of PyoG. PyoG

605 is trypsin protected in intact *ΔftsH* cells, but not in *ΔftsH* spheroplasts. This

606 phenotype is complemented with FtsH expressed from a plasmid (pFtsH). No

607 PyoG translocation is measured if the protease activity of FtsH has been disrupted

608 with a point mutation (FtsH H416Y). Therefore, proteolytically active FtsH is

609 specifically required for the inner membrane translocation of PyoG. **C, D** - Labelling

610 of *ΔtonB1* spheroplasts with fluorescent PyoG. No residual labelling is measured

611 after trypsin treatment, which indicates that TonB1 is required for inner membrane

612 translocation of PyoG. PyoG import into spheroplasts is restored if TonB1 is

613 expressed from a plasmid (pTonB1). **E, F** – The TonB binding box of PyoG, located

614 in the unstructured N-terminus, is required for PyoG import. PyoG$^{31-485}$ lacks the

615 first 30 residues of PyoG and is conjugated to AF488 via a cysteine in the C-

616 terminus. This deletion eradicates the killing activity of PyoG and its binding to the

617 periplasmic domain of TonB1 (Supplementary Figure 4). This deletion also disrupts

618 both the outer and the inner membrane import of PyoG, since the PyoG$^{31-485}$ label

619 is not trypsin protected in both intact cells and *P. aeruginosa* spheroplasts.

620 Representative micrographs before and after trypsin treatment are shown (**A, C,**

621 **E, G**). All snapshots were adjusted to the same intensity scale. Average

622 fluorescence intensities were measured for 150 cells per condition (**B, D, F, H**).

623 Mean of three biological replicates with standard deviations are shown.

624   Fluorescence intensities for labelled and trypsin treated groups in each condition

625   are compared to the unlabeled control. **** represents a P value below 0.0001 in

626   the Kruskal-Wallis Test, and ns represents no significant difference or lack of
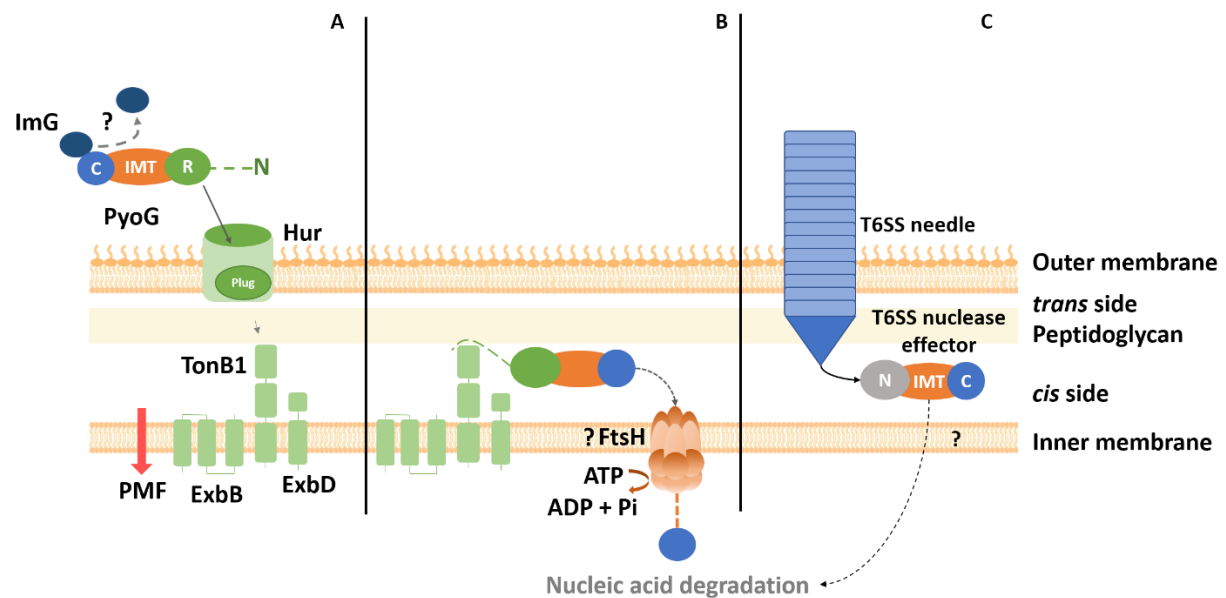
627   fluorescent labelling.

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Figure 4. Probable import mechanism of nuclease pyocin and T6SS effector. **A** – PyoG uses Hur, a TBDT, as its outer membrane receptor and translocator (5). The pyocin is composed of an unstructured N terminus (dashed line), a receptor binding domain (R), a conserved inner membrane translocation domain (IMT domain), and a cytotoxic nuclease domain (C). Like in the case of nuclease colicins (28), the immunity protein (Im) probably disassociates from the C domain during translocation. The plug domain of Hur interacts with the periplasmic domain of TonB1 (5). The binding of TonB1 and the pyocin R domain to the TBDT induces a conformational change that dislocates the plug of the TBDT, allowing the passage of the pyocin through the TBDT (10). The TonB box, located in the N-terminus of PyoG, is essential for this translocation step, probably because TonB1 pulls PyoG through Hur and into the periplasm (9, 10). **B** – Translocation of PyoG from the periplasm into the cytoplasm also requires TonB1 binding. The exact role of TonB1 in inner membrane transport remains unknown. Potentially, TonB1 positions the

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644 pyocin on the surface of the inner membrane so it can interact with other proteins

645 involved in inner membrane transport. This translocation step requires the IMT

646 domain, a conserved domain present in all nuclease bacteriocins. Inner membrane

647 transport of PyoG requires FtsH, an inner membrane ATPase/protease previously

648 associated with the killing activity of PyoG (5) and nuclease colicins (10). FtsH

649 must be proteolytically active for PyoG to translocate into the cytoplasm. Inner

650 membrane transport of PyoG could be coupled with FtsH-dependent proteolytic

651 processing that releases the C domain into the bacterial cytoplasm, as previously

652 demonstrated for nuclease colicins (14, 18). **C** – Like nuclease bacteriocins,

653 nuclease T6SS effectors contain the IMT domain (C – cytotoxic domain, IMT –

654 inner membrane translocation domain, N – other domains and motifs in the N-

655 terminus). The needle and needle tail proteins can deliver these effectors to the

656 *cis* side of the peptidoglycan layer (25). How the nuclease effectors cross the inner

657 membrane and reach the cytoplasm is unknown. It is possible that, like in the case

658 of nuclease PyoG, this translocation step depends on the IMT domain.

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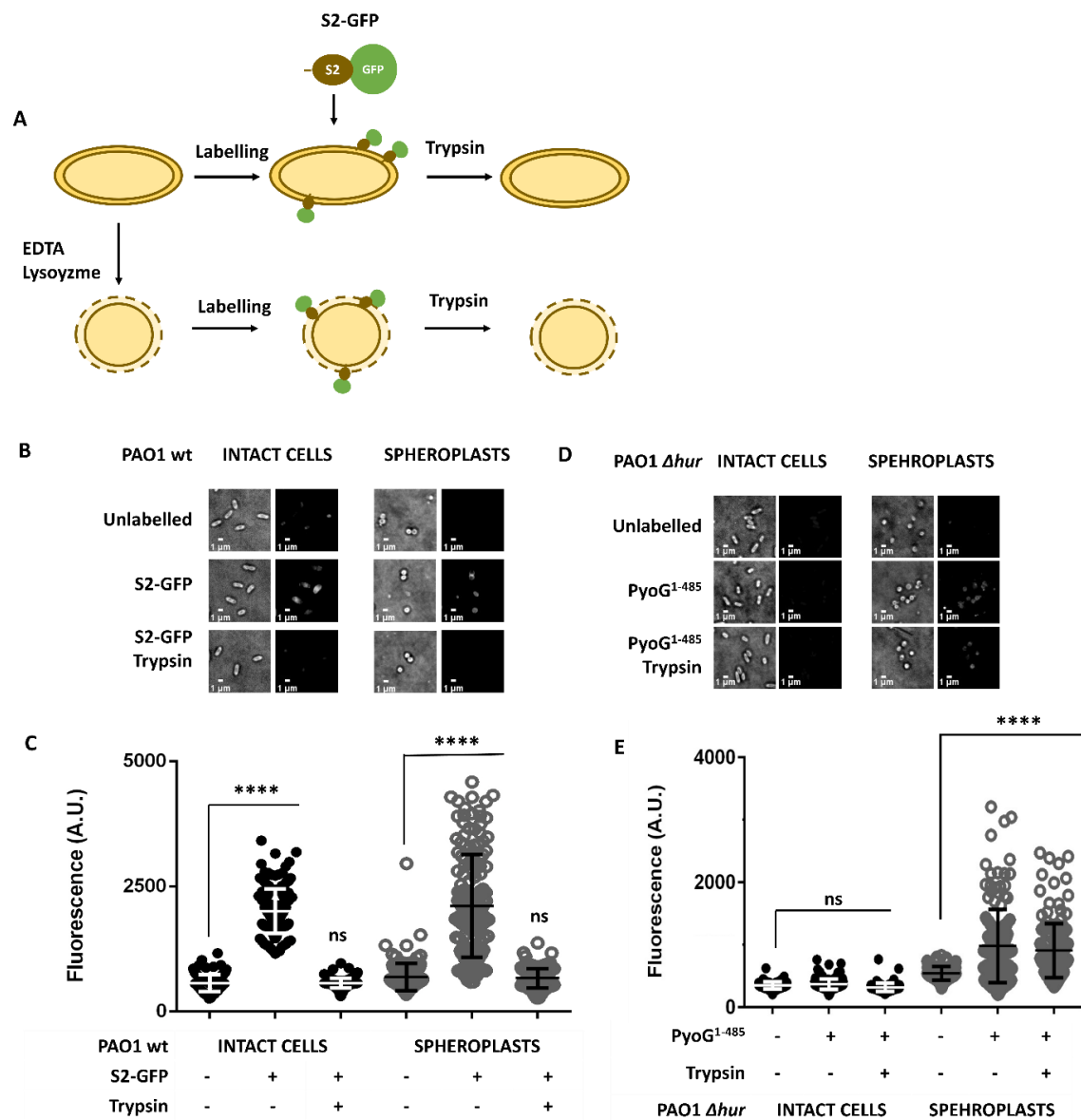
Supplementary Figure 1. S2-GFP is not trypsin protected in *P. aeruginosa* PAO1 intact cells and spheroplasts. **A** – S2-GFP is comprised of the S2 N-terminal domain (S2$^{1-209}$), translationally fused to GFP. GFP acts as a plug that blocks translocation of S2 across the cell envelope (10). This construct is used to test if surface exposed pyocin gets degraded by trypsin in the fluorescence microscopy

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667   experiment described in Figure 1. **B, C** - Intact cells, or spheroplasts generated

668   with lysozyme/EDTA treatment, are exposed to 2 µM S2-GFP. The S2-GFP

669   fluorescent signal is removed by trypsin, in both intact cells and spheroplasts. This

670   indicates that untranslocated pyocin is removed by trypsin under tested

671   experimental conditions. **D, E** - Hur, the outer membrane receptor of PyoG, is not

672   required for PyoG import into spheroplasts. Therefore, the outer membrane

673   translocation step is bypassed in spheroplasts, which enables specific detection of

674   inner membrane transport. Intact cells of PAO1 *Δhur* are not labelled with the

675   pyocin, since Hur is required for binding of PyoG to the surface of *P. aeruginosa*

676   cells (5). Generation of spheroplast with lysozyme/EDTA treatment is sufficient to

677   allow *Δhur* labelling with fluorescent PyoG. **B, D** - Representative micrographs

678   before and after trypsin treatment are shown. All snapshots were adjusted to the

679   same intensity scale. **C, E** - Average fluorescence intensities were measured for

680   150 cells per condition. Mean of three biological replicates with standard deviations

681   are shown. Fluorescence intensities for labelled and trypsin treated groups in each

682   condition are compared to the unlabelled control. **** represents a P value below

683   0.0001 in the Kruskal-Wallis Test, and ns represents no significant difference or
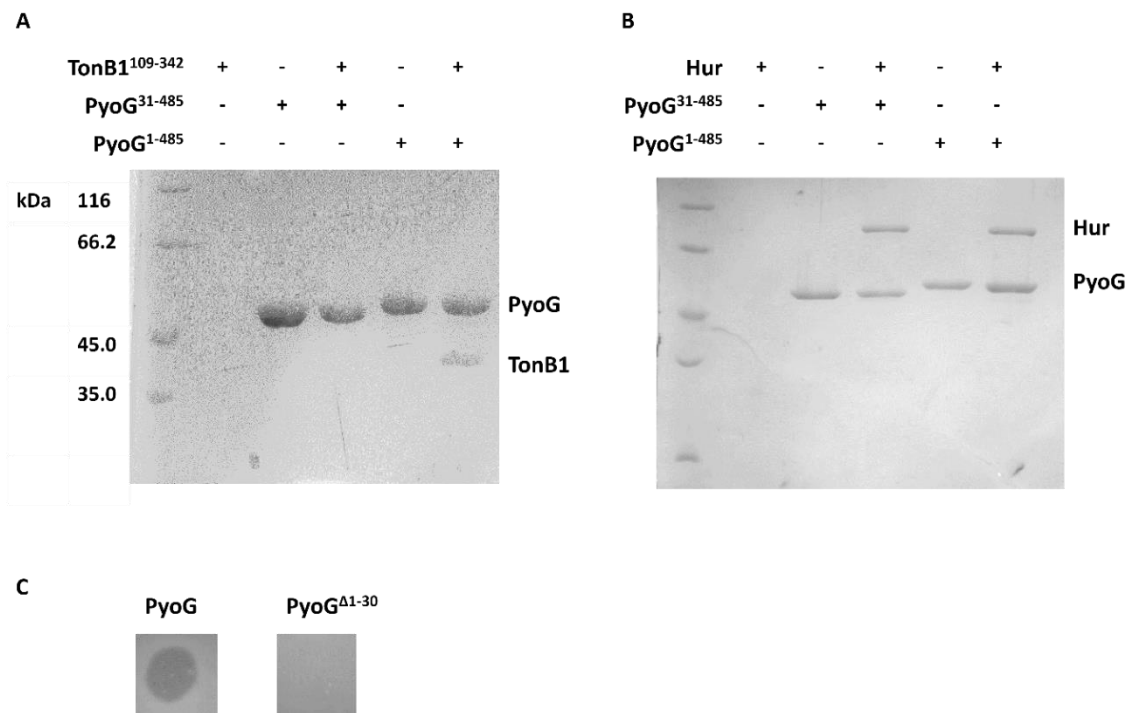
684   lack of fluorescent labelling.

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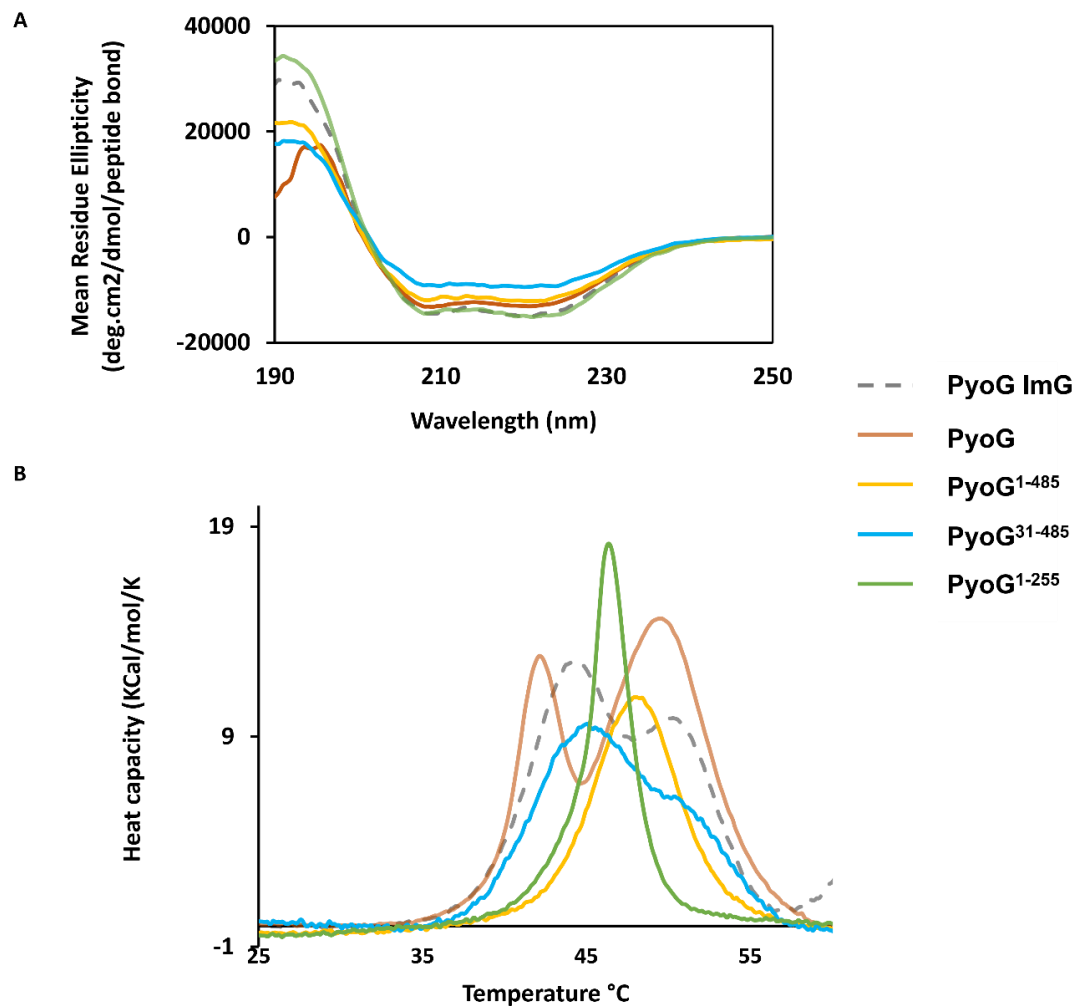
Supplementary Figure 2. Quality control of PyoG construct used for fluorescent labelling of *P. aeruginosa*. **A** - CD spectra of 0.1 mg/ml PyoG-ImG (dashed line), PyoG (orange), PyoG$^{1-485}$ (yellow), PyoG$^{31-485}$ (blue), and PyoG$^{1-255}$ (green) at room-temperature in 20 mM NaF and 10 mM KPO$_4$ buffer (pH 7). PyoG has a G4S linker and a cysteine at the C terminus. All the other constructs have a cysteine directly added to the C-terminus. An average of 9 measurements are shown. **B** - DSC curve of 20 µM PyoG constructs in 50 mM Tris-HCl pH 7, 150 mM NaCl, in the 25-60 °C temperature range. The melting temperature of PyoG is 42.14 ± 0

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695   and 49.56 ± 0.02 °C, PyoG$^{1\text{-}485}$ is 48.04 ± 0.14 °C, and PyoG$^{1\text{-}255}$ is 46.22 ± 0.02

696   °C. All constructs have a similar melting curve to the wt PyoG-ImG, indicating that

697   they are folded at room temperature.

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A

| wt | *ΔftsH* | pFtsH | pFtsH H416Y |

B

| wt | *ΔtonB1* | pTonB1 |

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700
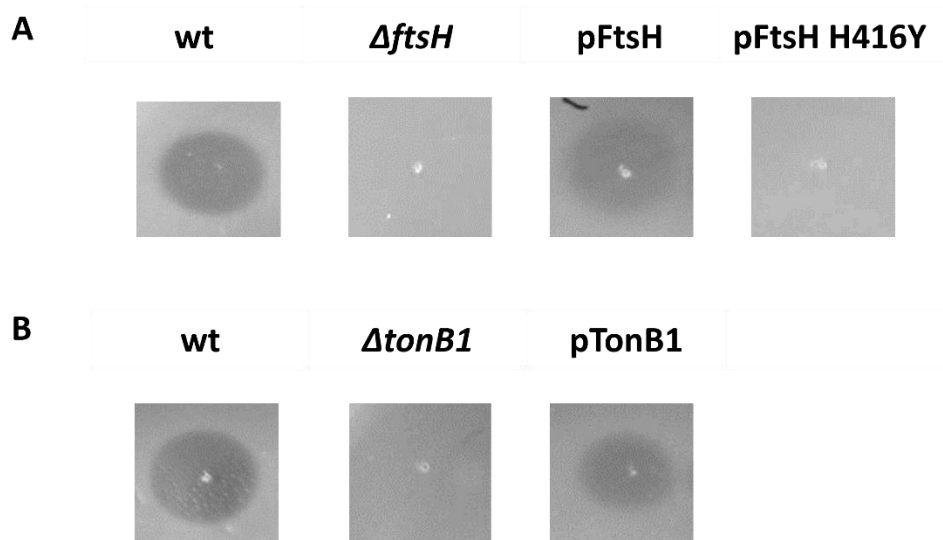
701 **Supplementary Figure 3**. Plate killing assay of *P. aeruginosa* complemented

702 strains. **A** - PAO1 *ΔftsH* complemented with FtsH expressed from a plasmid

703 (pFtsH), or a protease inactivated version of FtsH (pFtsH H416Y). Only

704 complementation with wt FtsH restored PyoG sensitivity. **B** - *P. aeruginosa*

705 PAO6699 *ΔtonB1* was complemented with TonB1 expressed from a plasmid

706 (pTonB1). Pyocin sensitivity is restored in this strain.

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Supplementary Figure 4. Deletion of the first 30 residues of PyoG disrupts TonB1 binding and the pyocin killing activity. **A, B** - Pulldowns with the PyoG$^{31-485}$ construct. This construct was used for fluorescent labelling of *P. aeruginosa* (**Figure 3 - G, H**). It has a cysteine and a His$_6$-tag in the C-terminus and was used as bait protein in pull-down assays. Periplasmic TonB1 (**A**) and Hur (**B**) were purified as described previously (5). Both proteins had no purification tag and were used as prey proteins. All proteins were mixed to a final concentration of 10 µM in binding buffer (50 mM Tris-HCl pH 7.8, 250 mM NaCl; 1% β-OG was added if Hur was used) and bound to nickel beads on room temperature. Unbound protein was washed in the same buffer and bound proteins were eluted in the presence of 250 mM imidazole. Eluate was analysed on 12% SDS-PAGE gels. Proteins that were added to beads are indicated above each lane. Positions of proteins are labelled

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721   on the right side of each gel. PyoG$^{31\text{-}485}$ does not bind TonB1 in the pull-down

722   assay (**A**), but it binds to Hur (**B**). This indicates that the first 30 residues of PyoG

723   contain the TonB1 binding box, and that this region is not essential for receptor

724   binding. **C** – The killing activity of PyoG which lacks the first 30 residues (PyoG$^{\Delta 1\text{-}}$

725   ^{30}). Unlike PyoG$^{31\text{-}485}$, PyoG$^{\Delta 1\text{-}30}$ contains the cytotoxic domain, and its activity can

726   be tested in a plate killing assay. 3 µL drops of 10 µM wt and Δ1-30 PyoG were

727   spotted on PAO1 lawns. The deletion of the first 30 residues of PyoG disrupted its

728   killing activity against PAO1, which indicates that this region of the pyocin is

729   essential for its import into bacterial cells.

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