Disruption of the *Pseudomonas aeruginosa* Tat system perturbs PQS-dependent quorum sensing and biofilm maturation through loss of the Rieske cytochrome *bc₁* sub-unit

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Abstract

Extracellular DNA (eDNA) is a major constituent of the extracellular matrix of *Pseudomonas aeruginosa* biofilms and its release is regulated via the pseudomonas quinolone signal (PQS) dependent quorum sensing (QS). By screening a *P. aeruginosa* transposon library to identify factors required for DNA release, mutants with insertions in the twin-arginine translocation (Tat) pathway were identified as exhibiting reduced eDNA release, and defective biofilm architecture with enhanced susceptibility to tobramycin. *P. aeruginosa tat* mutants showed substantial reductions in pyocyanin, rhamnolipid and membrane vesicle (MV) production consistent with perturbation of 2-heptyl-3-hydroxy-4-quinolone (PQS) dependent QS as demonstrated by changes in *pqsA* expression and 2-alkyl-4-quinolone (AQ) production. Provision of exogenous PQS to the *tat* mutants did not return *pqsA*, *rhlA* or *phzA1* expression or pyocyanin production to wild type levels. However, transformation of the *tat* mutants with the AQ-independent *pqs* effector *pqsE* restored *phzA1* expression and pyocyanin production. Since mutation or inhibition of Tat prevented PQS-driven auto-induction, we sought to identify the Tat secretion substrate responsible. A *pqsA::lux* fusion was introduced into each of 34 validated *P. aeruginosa* Tat substrate deletion mutants. Analysis of each mutant for reduced bioluminescence revealed that the signalling defect was associated with the Rieske iron-sulfur subunit of the cytochrome bc_1 complex. In common with the parent strain, a Rieske mutant exhibited defective PQS signalling, AQ production, *rhlA* expression and eDNA release that could be restored by genetic complementation. Thus, lack of the Rieske sub-unit export is clearly responsible for the Tat-mediated perturbation of PQS-dependent QS, the loss of virulence factor production, biofilm eDNA and the tobramycin tolerance of *P. aeruginosa* biofilms.

Author Summary

Pseudomonas aeruginosa is a highly adaptable human pathogen responsible for causing chronic biofilm-associated infections. Biofilms are highly refractory to host defences and antibiotics and thus difficult to eradicate. The biofilm extracellular matrix incorporates extracellular DNA (eDNA). This stabilizes biofilm architecture and helps confer tolerance to antibiotics. Since mechanisms that control eDNA release are not well understood, we screened a *P. aeruginosa* mutant bank for strains with defects in eDNA release and discovered a role for the twin-arginine translocation (Tat) pathway that exports folded proteins across the cytoplasmic membrane. Perturbation of the Tat pathway resulted in defective biofilms susceptible to antibiotic treatment as a consequence of perturbed pseudomonas quinolone (PQS) signalling. This resulted in the failure to produce or release biofilm components including eDNA, phenazines and rhamnolipids as well as microvesicles. Furthermore, we discovered that perturbation of PQS signalling was a consequence of the inability of *tat* mutants to translocate the Rieske subunit of the cytochrome bc_1 complex involved in electron transfer and energy transduction. Given the importance of PQS signalling and the Tat system to virulence and biofilm maturation in *P. aeruginosa*, our findings underline the potential of the Tat system as a drug target for novel antimicrobial agents.

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that causes a wide range of human infections including lung, urinary tract and wound, bacteremia and infections associated with medical devices [1]. It is notorious for its tolerance to antimicrobial agents, a property that is largely a consequence of its ability to form biofilm communities [1,2]. Bacterial exoproducts including cell surface appendages, extracellular polymeric substances, biosurfactants and secondary metabolites all contribute to *P. aeruginosa* biofilm formation and maturation [3-7].

Apart from exopolysaccharides such as Psl, Pel and alginate, the extracellular polymeric matrix of *P. aeruginosa* biofilms incorporates proteins, rhamnolipids, membrane vesicles (MVs) and extracellular DNA (eDNA) [5,8-10]. Rhamnolipid biosurfactants are required during the initial stages of micro-colony formation and are involved in the migration-dependent formation of the caps of mushroom-shaped micro-colonies formed in flow-cell grown biofilms [10]. They also aid the maintenance of channels between multicellular structures within biofilms and contribute to biofilm dispersal [5]. With respect to the biofilm micro-colonies that characteristically form in flow-chambers fed with glucose minimal medium, eDNA is present at high concentrations in the outer layers of microcolonies in young biofilms. However, in mature biofilms, eDNA is primarily located in the stalks at the borders between micro-colony caps and stalks [8].

The release of eDNA occurs via the lysis of a sub-population of bacterial cells [10-14]. It is involved in attachment, aggregation and stabilization of biofilm microcolonies. eDNA can act as a nutrient source, chelate metal cations and confer tolerance to antibiotics such as the polymyxins and aminoglycosides [10,12,13]. eDNA also binds other biopolymers (exopolysaccharides and proteins) stabilizing biofilm architecture and conferring protection against adverse chemical and physical

109 challenges [12,13]. By intercalating with eDNA, secondary metabolites such as
110 phenazines enhance biofilm integrity [12,15]. Pyocyanin for example can contribute
111 to DNA release through the formation of reactive oxygen species such as hydrogen
112 peroxide that damage cell membranes [12]. Although the mechanism(s) responsible
113 for eDNA release has not been fully elucidated both eDNA and MVs can be generated
114 via explosive cell lysis mediated via a cryptic prophage endolysin encoded within the
115 R- and F-pyocin gene clusters [14].
116
117 In *P. aeruginosa*, rhamnolipids and pyocyanin production, eDNA and MV release, and
118 hence biofilm development, are all controlled by quorum sensing (QS) [1,8,16].
119 Consequently, *P. aeruginosa* mutants with defects in this cell-to-cell communication
120 system form aberrant, flat undifferentiated biofilms [10]. In *P. aeruginosa*, the QS
121 regulatory network consists of a hierarchical cascade incorporating the overlapping
122 *las*, *rhl* and *pqs* pathways that employ *N*-acylhomoserine lactones (AHLs) and 2-
123 alkyl-4-quinolones (AQs) as signal molecules [1,16,17]. All three QS systems
124 contain auto-induction loops whereby activation of a dedicated transcriptional
125 regulator by the cognate QS signal molecule induces expression of the target
126 synthase such that QS signal molecule production can be rapidly amplified to
127 promote co-ordination of gene expression at the population level.
128
129 *P. aeruginosa* produces a diverse family of AQs and AQ *N*-oxides [18,19] of which
130 2-heptyl-3-hydroxy-4-quinolone (the *Pseudomonas* Quinolone Signal, PQS) and its
131 immediate precursor 2-heptyl-4-hydroxyquinoline (HHQ) are most closely
132 associated with PQS signalling [17]. Most of the genes required for AQ biosynthesis
133 (*pqsABCDE*) and response (*pqsR;* also called *mvfR*) are located at the same genetic
134 locus although *pqsH* and *pqsL* are distally located [17]. PqsA catalyses the formation
135 of anthraniloyl-CoA that is condensed with malonyl-CoA by PqsD to form 2-
136 aminobenzoylacetyl-CoA (2-ABA-CoA) [20,21]. The latter is converted to 2-
137 aminobenzoylacetate (2-ABA) via the thioesterase functionality of PqsE [22]. The

138 PqsBC heterodimer condenses 2-ABA with octanoyl-coA to generate HHQ [23,24].
139 PQS is formed through the oxidation of HHQ by PqsH [25] while formation of the AQ
140 *N*-oxides such as 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) requires the
141 alternative mono-oxygenase PqsL [18]. The PqsE protein has dual functions; while
142 it is not essential for AQ biosynthesis, it is required for the AQ-independent
143 production of several factors that contribute to biofilm maturation including
144 pyocyanin, rhamnolipids and lectin A [26]. While activation of RhlR-dependent
145 genes depends on PqsE [27], the AQ-independent, thioesterase-independent
146 mechanism by which PqsE acts has not yet been elucidated [17,22].
147
148 The *pqs* system is subject to positive autoinduction, since the LysR-type
149 transcriptional regulator PqsR (MvfR), binds to the promoter region of *pqsABCDE*
150 (P*pqsA*) triggering transcription once activated by HHQ or PQS [28-30] (**Fig. 1**).
151 Therefore, by analogy with other QS systems, HHQ and PQS can both act as
152 autoinducers by generating a positive feedback loop that accelerates their
153 biosynthesis. However, in contrast to HHQ which only regulates the *pqsABCDE*
154 operon [17], PQS is a ferric iron chelator [31] that not only drives AQ biosynthesis
155 via PqsR but also the expression of genes involved in the iron-starvation response
156 and virulence factor production *via* PqsR-dependent and PqsR-independent
157 pathways [17]. In addition, PQS can act as a cell-sensitizing pro-oxidant [32] and
158 is required for MV production via a direct physical interaction with lipopolysaccharide
159 (LPS) within the outer membrane [33]. The packaging of PQS within MVs also
160 provides a means for trafficking this hydrophobic QS signal within
161 a *P. aeruginosa* population [34].
162
163 With respect to biofilm development, the *pqs* system is of particular interest because
164 *pqs* biosynthetic mutants fail to produce eDNA, rhamnolipids, pyocyanin and MVs,
165 and form thin defective biofilms containing little eDNA [8, 27,33,35]. The mechanism
166 involved in PQS-mediated DNA-release in biofilms is not understood but has been

167 suggested to be linked to phage induction causing cell lysis [8,36-40]. Although
168 explosive cell lysis releases eDNA in biofilms and generates MVs through
169 vesicularization of shattered membrane fragments, *pqsA* mutants are not defective
170 for explosive lysis [14] and therefore this phenomenon is unlikely to account for PQS-
171 dependent eDNA release.
172
173 In the present study we sought to identify additional factors involved in eDNA release
174 by screening a transposon (Tn) mutant library for eDNA-release defective mutants.
175 Apart from *pqs* biosynthetic mutants, we obtained Tn insertion mutants within the
176 twin-arginine translocation (Tat) pathway that exhibited reduced levels of eDNA
177 release, fail to produce rhamnolipids or pyocyanin and form defective, eDNA- poor,
178 antibiotic susceptible biofilms. Since mutation or deletion of *tat* resulted in altered
179 AQ production, reduced pyocyanin, rhamnolipid and MVs, and as the *tat* mutants
180 were refractory to exogenously supplied PQS, the aberrant biofilm phenotype
181 observed could be accounted for by perturbation of PQS autoinduction. By screening
182 a library of *P. aeruginosa* Tat substrate mutants, we identified the Rieske sub-unit of
183 the cytochrome bc_1 complex as being required for *pqs*-dependent QS and hence
184 eDNA release and biofilm maturation.
185

186 ## Results

187

188 ### *Transposon mutagenesis screen for P. aeruginosa mutants exhibiting*
189 ### *reduced DNA release*

190
191 To identify *P. aeruginosa* genes that contribute to eDNA release, a mariner
192 transposon (Tn) mutant library was generated in strain PAO1. Approximately 10,000
193 mutants grown in microtitre plates were assayed for reduced eDNA release using
194 propidium iodide (PI) to quantify eDNA because it is unable to penetrate live bacteria
195 and because its fluorescence is enhanced 30-fold on binding DNA [41]. From the

196 initial screen, 84 Tn insertion mutants were selected and re-screened to eliminate
197 strains with double Tn insertions and to confirm their eDNA phenotype. For 34 of
198 the remaining mutants, the regions flanking each Tn insertion were sequenced and
199 the corresponding genes identified. For many of the eDNA-release deficient mutants,
200 the Tn insertions were located within genes required for AQ biosynthesis (*pqsC* and
201 *pqsH)* or regulation (*pqsR)* (**Fig. 2**) These data confirm our previous work that first
202 uncovered a role for PQS signalling in eDNA release [8]. Apart from the *pqs*
203 mutants, two mutants were obtained with insertions in the *tatA* and *tatB* genes
204 respectively (**Fig. 2**). These code for components of the twin-arginine translocation
205 (Tat) system that mediates the export of folded proteins and was originally named
206 with respect to the presence of an Arg-Arg motif in the signal sequence of Tat-
207 exported products also called Tat substrates [42,43]. In *P. aeruginosa* diverse
208 proteins involved in phosphate and iron metabolism, virulence and energy
209 transduction are exported to the periplasm, or secreted via the Tat export system
210 and *tat* mutants exhibit pleiotropic phenotypes [44,45]. **Fig. 2** shows that genetic
211 complementation of the *P. aeruginosa tatA* mutant with a plasmid-borne copy
212 restored eDNA release.
213
214 ***The Tat pathway contributes to biofilm development in P. aeruginosa***
215
216 Since eDNA makes an important contribution to biofilm development and
217 architecture [10,12,13], biofilm formation by the *P. aeruginosa tatA* mutant in flow-
218 chambers was investigated. After 4-days growth, the *P. aeruginosa* wild-type and
219 complemented *tatA*/pTatA mutant formed biofilms with mushroom-shaped
220 structures whereas the *tatA* mutant formed thin, flat biofilms (**Fig. 3A**). Ethidium
221 bromide staining of the latter revealed that they contained much less eDNA than the
222 structured biofilms formed by the wild-type and complemented *tatA* mutant strain
223 (**Fig. 3A**). Consistent with this biofilm phenotype, exposure of the biofilms formed
224 by each of the three strains to tobramycin showed that the *tatA* mutant biofilm was

225 more sensitive to tobramycin than either the wild-type or *tatA*/pTatA mutant biofilm
226 (**Fig. 3B**).
227
228
229 ***P. aeruginosa tatA mutants are defective in the production of rhamnolipids,***
230 ***pyocyanin and MVs***
231
232 Since rhamnolipids, pyocyanin and MVs are all important components of
233 *P. aeruginosa* biofilms, their production was quantified in the *tatA* Tn insertion
234 mutant and in a Δ*tatABC* deletion mutant. **Fig. 4 (A and B)** shows that both *tat*
235 mutants produce substantially less pyocyanin and rhamnolipid than the parent or
236 *tatA* complemented strain. Furthermore, MV levels (**Fig. 4C**) were reduced by ~50%
237 in the *tatA* mutant compared with the wild type and could be restored by genetic
238 complementation.
239
240 ***Inactivation of the tat pathway by mutation or small molecule-mediated***
241 ***inhibition perturbs PQS signalling***
242
243 The reductions in eDNA release, rhamnolipids, pyocyanin and MVs noted in the *tat*
244 mutant as well as its biofilm phenotype are similar to those observed for
245 *P. aeruginosa* strains with mutations in *pqs* genes such as *pqsA*, the first gene in
246 the AQ biosynthetic pathway (see **Figs. 2** and **Fig 4** and [8, 40]). These data
247 suggested that the *tat* mutant biofilm phenotype was likely, at least in part, to be
248 due to a defect in PQS signalling.
249
250 To investigate the impact of the *tatA* mutation on the expression of *pqsA,* a
251 CTX::*pqsA′-lux* fusion was introduced into the chromosomal CTX site of both the
252 wild type and *tatA* mutant. **Fig. 5A** shows that *pqsA* expression in the *tatA* mutant
253 is reduced ~4 fold compared with the wild type strain and restored by genetic
254 complementation of the mutant. In agreement with these data, the Tat inhibitor,

255 Bayer 11-7082, identified by Vasil *et al* [46] reduced *pqsA* expression in the wild
256 type PAO1 strain by ~4 fold consistent with the reduction noted for the CTX::*pqsA-*
257 *lux* fusion in the *tatA* mutant (**Fig. 5B**). Bayer-11 7082 had no effect on growth or
258 light output in *P. aeruginosa* expressing the *lux* genes from a derepressed *lac*
259 promoter (**S1 Fig.**). In addition**,** the concentrations of PQS in whole culture extracts
260 of *P. aeruginosa* after growth in LB medium as determined by LC-MS/MS was
261 respectively ~56% lower in the *tatA* mutant compared with the wild type and
262 complemented *tat* mutant (**Fig. 5C**). Since *pqsA* expression and hence AQ
263 production is also PqsR/MvfR-dependent, we compared the expression of *pqsR* in
264 the Δ*tatABC* with the parent strain and found no difference (**S2 Fig.**).
265
266 Since mutation of *tatA* results in reduced *pqsA* expression, it is possible that the
267 auto-induction of PQS signalling via PqsR/MvfR is compromised. To uncouple the
268 autoinduction of AQ production, the *pqsABCD* genes were introduced into the
269 *P. aeruginosa* Δ*pqsA* and Δ*pqsA*Δ*tatA* mutants respectively on a plasmid
270 (pBBRMCS5::*pqsABCD*) that constitutively expresses the *pqsABCD* genes [47]. **S3**
271 **Fig.** shows that PQS, HHQ and HQNO are present in the culture medium of the
272 Δ*pqsA* mutant transformed with pBBRMCS5::*pqsABCD*. However neither the cell
273 free supernatant nor whole cells of the *tatA* Δ*pqsA* double mutant transformed with
274 pBBRMCS5::*pqsABCD* contained or accumulated intracellular AQs (**S4 Fig.**)
275 suggesting that in the absence of autoinduction in the *tat* mutant background. *P.*
276 *aeruginosa* is unable to synthesize AQs.
277
278 ***Exogenous PQS does not restore PQS signalling in a P. aeruginosa* tatA**
279 ***mutant***
280
281 QS systems are characteristically autoinducible such that exogenous provision of the
282 cognate signal molecule induces expression of the signal synthase and hence
283 downstream target genes [48]. When the *tatA* mutant was provided with exogenous

284 PQS, eDNA release did not increase (**Fig. 2**). To investigate this finding further,
285 either PQS or HHQ was exogenously supplied to the wild type, *tatA* mutant or the
286 complemented *tatA* mutant strains carrying chromosomal *pqsA′-lux* fusions. The
287 data presented in **Fig. 6A** show that the response of the *tatA* mutant to PQS or HHQ
288 respectively at 5 or 20 μM with respect to *pqsA* expression was at least 2-fold lower
289 than the controls. Since both wild type and *tatA* mutant still produce AQs
290 endogenously, the experiments were repeated in the *P. aeruginosa* Δ*pqsA* and *tatA*
291 Δ*pqsA* mutants since no AQs are produced in these genetic backgrounds. **Fig. 6B**
292 shows that the response to both PQS and HHQ is substantially reduced (e.g. ~4 fold
293 at 5 μM PQS) in the absence of *tatA* in the *P. aeruginosa tatA* Δ*pqsA* double mutant.
294
295 To determine the consequences of perturbed PQS signalling on the expression of the
296 rhamnolipid (*rhlA*) and pyocyanin biosynthetic genes (*P. aeruginosa* has two, almost
297 identical redundant 7 gene phenazine biosynthetic operons termed *phzA1-G1* and
298 *phzA2-G2;* [49]), the corresponding miniCTX-*lux* promoter fusions for *rhlA* and
299 *phzA1* respectively were constructed and introduced onto the chromosomes of the
300 wild type, Δ*pqsA* and *tatA* Δ*pqsA* mutants respectively. **Fig. 7A** shows the
301 expression profiles of *rhlA′-lux* as a function of time. Both the wild type and Δ*pqsA*
302 mutant show an ~2 fold increase in *rhlA* expression when supplied with exogenous
303 PQS (20 μM) and share similar profiles over the growth curve. In contrast, the *rhlA′-*
304 *lux* fusion in the *tatA* mutant does not show the same expression profile or response
305 to exogenous PQS as the wild type and Δ*pqsA* mutant strains. The *rhlA′-lux*
306 expression profile in the *tatA* mutant supplied with exogenous PQS is however
307 clearly restored when the mutation is complemented by pTatA (**Fig. 7A**).
308
309 AQ-dependent QS is required for *phzA1* expression [27,49]. Exogenous PQS
310 increased *phzA1′-lux* expression by ~4 fold in both wild type and Δ*pqsA* mutant

311 backgrounds (**Fig. 7B**). However, the *tatA* Δ*pqsA* double mutant responded
312 comparatively poorly to PQS (**Fig. 7B**).
313
314
315 ***PqsE restores pyocyanin in the tat mutants***
316
317

318 Although PqsE is not essential for AQ biosynthesis, it is required for the production
319 of pyocyanin, rhamnolipids and biofilm maturation and its function is independent
320 of PQS, HHQ or PqsR [27,50]. Consequently, the expression of certain PQS
321 signalling-dependent exoproducts such as pyocyanin can be restored in a *pqsA*
322 negative (and hence AQ-negative) mutant by expressing *pqsE*. To determine
323 whether expression of *pqsE* alone could restore *phzA1* expression and pyocyanin
324 production in the *tat* mutants, *pqsE* was introduced on pUCP18. **Fig. 7B** shows that
325 *phzA1* expression can be restored in the *tatA* Δ*pqsA* mutant in the absence of AQs
326 while **Fig. 8** confirms that plasmid-borne *pqsE* restores pyocyanin production.
327

328 **The reduction in rhamnolipid production in the *tat* mutants does not**
329 **account for the perturbation of *PQS signalling***
330

331 In *P. aeruginosa* biofilms, rhamnolipids provide protective shielding against
332 neutrophils [51,52] and contribute to the effectiveness of PQS signalling by
333 enhancing the solubility and bioactivity of PQS [53]. In **Fig. 4B**, we show that
334 rhamnolipid production is substantially reduced in the *P. aeruginosa tat* mutant
335 backgrounds. To determine whether the perturbation of PQS signalling in the *tat*
336 mutants is a consequence of reduced rhamnolipid production, we investigated the
337 impact of exogenous rhamnolipids on *pqsA* expression. **S5 Fig.** shows that the
338 addition of purified rhamnolipids (10 or 50 μg/ml) to the *tatA* Δ*pqsA* mutant with or
339 without PQS (40 μM) had little effect on *pqsA* expression.
340

341 **Identification of the Tat substrate responsible for perturbation of *PQS***
342 ***signalling***
343 Recently Gimenez et al [45] experimentally validated the Tat-mediated export of 34
344 *P. aeruginosa* gene products predicted to have Tat signal peptides. To determine
345 which of the exported Tat substrates was responsible for perturbation of PQS
346 signalling, allelic replacement mutants were constructed in *P. aeruginosa* strain
347 PA14 for each substrate. Before introducing the *pqsA′-lux* fusion onto the
348 chromosomal CTX site of each Tat substrate mutant, we first confirmed that PQS
349 signalling in a PA14 Δ*tatABC* mutant was perturbed in a similar manner to that
350 observed for PAO1, the genetic background used so far in the study (**Fig. 9A**).
351 Determination of the maximum expression of *pqsA′-lux* for each of the 34 Tat
352 substrate mutants (**Fig. 9A**) revealed that although a number of mutants exhibited
353 reduced light output, the greatest reduction was observed for deletion of
354 PA14_57570 (equivalent to PA4431 in PAO1, here designated *petA* following the
355 nomenclature of orthologues described in other species). This gene codes an iron-
356 sulfur cluster protein, the Rieske subunit of the cytochrome bc_1 complex.
357
358 To confirm that the Δ*petA* mutant in common with the *P. aeruginosa tat* mutants
359 exhibited similar defects as a consequence of perturbed PQS signalling, we
360 compared their AQ production (**Fig. 9B**), *rhlA* expression, and eDNA release in
361 biofilms (**S6 Fig.** and **S7 Fig.**). Comparison of PQS, HHQ and HQNO production in
362 PA14 with the Δ*petA* mutant and the strain with a *cis* complementation of the *petA*
363 mutation at the CTX site confirmed the loss and increase in AQ production (**Fig. 9B**).
364 In *P. aeruginosa* PA14, the *rhlA* expression profiles for the parent and Δ*tatABC*
365 deletion mutant in the absence or presence of exogenously supplied PQS were
366 similar to those of strain PAO1 (compare **S6 Fig.** with **Fig. 7A**). Confocal
367 microscopy images of biofilm formation by *P. aeruginosa* PA14 under static growth
368 conditions shows that in common with the Δ*tatABC* mutant, *petA* mutant biofilms
369 lack the eDNA content observed in the wild type and complemented mutant (**S7**

370 **Fig.**) demonstrating that PetA is the Tat substrate required for PQS-dependent eDNA
371 release.
372
373
374 ## Discussion
375

376 Extracellular DNA is one of the most abundant components of the extracellular
377 matrix of bacterial biofilms [10-14]. However, the detailed mechanism(s) by which
378 eDNA is released is not fully understood but likely to involve multiple pathways.
379 *P. aeruginosa pqsL* and *pqsA* mutants release more or less eDNA respectively,
380 consistent with the levels of PQS they produce [8]. Furthermore, the biofilms
381 formed by *pqsA* mutants are thin and flat and contain little eDNA consistent with
382 *pqs* signalling playing an important regulatory role in biofilm maturation [8]. To gain
383 further insights into eDNA release, a *P. aeruginosa* transposon mutant library was
384 screened and two groups of mutants exhibiting reduced eDNA release were
385 identified. The first of these contained Tn insertions in genes involved in *pqs*
386 signalling including *pqsC*, *pqsH* and *pqsR*. Mutations in *pqsC* or *pqsR* in common
387 with *pqsA* both abrogate AQ biosynthesis while *pqsH* mutants are unable to produce
388 3-hydroxy-AQs such as PQS but maintain production of AQs and AQ N-oxides such
389 as HHQ and HQNO respectively [17, 54]. These data suggest that eDNA release is
390 likely to require PQS/HHQ rather than the effector protein PqsE. This is because
391 although *pqsE* mutants in common with *pqsA* mutants form poor biofilms, *pqsE pqsA*
392 double mutants could not be complemented by *pqsE* alone to restore biofilm
393 development [26]. This contrasts with pyocyanin and lectin A for example, as
394 production of both can be restored by PqsE in the absence of AQ biosynthesis [26].
395

396 The transposon insertions in the second group of *P. aeruginosa* eDNA release
397 mutants obtained were located in *tatA* and *tatB*, the first two genes in the *tatABC*
398 operon that codes for the twin-arginine translocase secretion pathway [42,43]. The
399 Tat secretion system was originally discovered in the thylakoid membranes of plant

400 chloroplasts and subsequently identified in bacteria and archaea [42]. Tat exports
401 folded proteins out of the cytoplasm and across the cytoplasmic membrane in an
402 ATP-independent manner [42]. In bacteria such as *E. coli* and *P. aeruginosa* proteins
403 exported by the Tat system are most frequently terminally localized in the
404 periplasm, but some (e.g. phospholipase C) can be transported across the outer
405 membrane by the Xcp type II secretion system, thereby becoming extracellular. Tat
406 signal peptides harbor a conserved S/TRRxFLK consensus motif, where the twin
407 arginine is invariant and in the vast majority of cases essential for export [45].
408 Using prediction algorithms for Tat substrates, 44 putative *P. aeruginosa* PA14 Tat
409 signal peptides have been identified, and of these, 34 confirmed experimentally
410 using a novel amidase-based Tat reporter [45]. These include phospholipases and
411 proteins involved in pyoverdine-mediated iron-uptake, respiration, osmotic stress
412 defence, motility, and biofilm formation [44, 45]. However, none of these are known
413 to be involved in AQ biosynthesis, transport or *pqs* signal transduction.
414
415 In our flow-chamber grown biofilms, clear differences between the *P. aeruginosa*
416 PAO1 wild type and *tatA* mutant biofilms were apparent. While the wild type formed
417 characteristic mushroom-shaped structures under these conditions, the *tatA* mutant
418 formed flat, thin biofilms. In addition, eDNA was observed primarily in the stalks of
419 the mushroom-shaped structures in the wild-type biofilms, whereas the *tatA* mutant
420 biofilms contained no stalks and little extracellular DNA. Moreover, the biofilms
421 formed by the *tatA* mutant were more sensitive to tobramycin treatment than wild-
422 type biofilms, presumably in part due to the inability of the *tatA* mutant to release
423 DNA and form differentiated multicellular structures. Indeed, DNA binds positively
424 charged antibiotics and can act as a 'shield' against aminoglycosides such as
425 tobramycin [53]. In *P. aeruginosa* biofilms, exogenously provided DNA integrates
426 into *P. aeruginosa* biofilms increasing their tolerance toward aminoglycosides [55].
427 The formation of flat, eDNA deficient, tobramycin-sensitive biofilms and the
428 reduction in rhamnolipid, pyocyanin and MV production by the *P. aeruginosa tatA*

429 mutant are consistent with the *pqsA* mutant biofilm phenotype [8] and imply the
430 existence of a link between Tat export and *pqs* signalling. Since transcriptomic
431 studies of PQS signalling in *P. aeruginosa* have not provided any evidence that the
432 Tat export system is QS controlled [17,26], we considered it likely that mutation of
433 the *tat* genes resulted in perturbed PQS signalling. This observation could also
434 account, at least in part, for the reduced virulence of *P. aeruginosa tat* mutants in a
435 rat pulmonary infection model [44].
436
437 To explore this hypothesis, the expression of *pqsA* and the concentrations of PQS
438 and HHQ produced were compared in *P. aeruginosa* PAO1 wild type, *tatA* and
439 complemented *tatA* mutant strains. Similar results were also obtained for
440 *P. aeruginosa* strain PA14. Disruption of the *tat* operon had a pronounced effect on
441 the expression of *pqsA,* with a nearly 3–fold reduction in *pqsA* expression. Moreover,
442 addition of the Tat inhibitor, BAY 11-7082 [46] also resulted in a comparable
443 reduction in *pqsA* expression. LC-MS/MS quantification of PQS levels in early
444 stationary phase after 8 h growth, showed a ~50% reduction, thus correlating with
445 the *pqsA′-lux* promoter fusion assays. Although PQS and HHQ both serve as PqsR
446 ligands for driving the positive feedback loop for *pqsABCDE* expression that is central
447 to AQ signalling [28-30], exogenous provision of neither PQS nor HHQ could fully
448 restore *pqsA* expression in the *tatA* mutant. Since this mutant still produces AQs,
449 albeit at a reduced level, a *pqsA* mutation was introduced into the *tatA* mutant
450 background to examine the response to exogenous AQs in an AQ-negative genetic
451 background (*tatA* Δ*pqsA*). The data obtained confirmed that the *P. aeruginosa tatA*
452 Δ*pqsA* mutant responded only weakly to exogenously added PQS or HHQ, albeit to
453 a much lesser extent than the isogenic Δ*pqsA* mutant with an intact *tat* operon.
454 These data suggest that disruption of *tat* impairs the ability of *P. aeruginosa* to fully
455 induce AQ-dependent QS. The consequences of *tat*-dependent perturbation of *pqs*
456 signalling are clearly evident in the modified expression profile of the *rhlA′-lux* fusion
457 in the *tatA* mutant with or without exogenously supplied PQS. Similar results were

458 also obtained for *phzA1* expression in that the reduction in expression observed in
459 a *tatA* Δ*pqsA* mutant background was not restored to wild type levels by exogenous
460 PQS.

461 Since pyocyanin can be produced in the absence of PQS by ectopic expression of
462 *pqsE* [26], it was possible that the *tat* phenotype is either a consequence of the
463 inability of PQS/HHQ to activate the *pqsABCDE* operon via PqsR to generate
464 sufficient PqsE protein or because the activity of PqsE depends on a functional Tat
465 system. To investigate the impact of PqsE on pyocyanin production in the PAO1 *tatA*
466 mutants, a plasmid borne copy of *pqsE* was introduced into the *tat* mutants.
467 Pyocyanin production was fully restored suggesting that the *tat* mutant phenotype
468 is not due to the inability of PqsE to function but rather a failure of the *pqs* auto-
469 induction circuitry to produce sufficient PQS/HHQ to efficiently activate *pqsABCDE*
470 transcription and hence *pqsE* expression.

471 AQs such as PQS are extremely hydrophobic and virtually insoluble in aqueous
472 environments. Hence PQS signalling can be enhanced by increasing the solubility
473 and delivery of PQS. Calfee *et al* [53] showed that rhamnolipids increase the
474 solubility and bioactivity of PQS in *P. aeruginosa* with respect to *lasB* expression.
475 Since rhamnolipid production is substantially reduced in the *P. aeruginosa tat*
476 mutants, we added back purified rhamnolipids to the *tatA* Δ*pqsA* mutant. However,
477 no effect on *pqsA* expression was observed indicating that the PQS signalling defect
478 in the *tat* mutants is not simply due to the loss of rhamnolipid production and an
479 inability to solubilize PQS.
480
481 In *Escherichia coli,* inactivation of *tat* leads to a characteristic cell envelope defect
482 because of the mis-localization of two amidases involved in cell wall metabolism.
483 This results in the formation of bacterial chains, leakage of periplasmic proteins and
484 enhanced susceptibility to detergents [56]. Since PQS is a signal molecule that must
485 be transported across the cell envelope in both directions, it was possible that an

486 envelope defect in *P. aeruginosa tat* mutants impacts on the PQS signalling auto-
487 induction cascade. However, Ball *et al* [57] showed that *P. aeruginosa tat* mutants
488 do not show the same Tat-dependent envelope defects found in *E. coli.* In addition,
489 we found no evidence for intracellular accumulation of AQs indicative of a transport
490 defect.
491
492 To uncover the mechanistic link between Tat and PQS signalling, we sought to
493 determine whether mutation of *tat* resulted in the inability to export a specific Tat
494 substrate, the loss of which resulted in perturbation of PQS autoinduction and hence
495 the expression of PQS-dependent genes and the formation of defective biofilms. By
496 screening 34 validated Tat substrate mutants for reduced *pqsA* expression, we
497 identified the *petA* gene as responsible for the defect in *pqs*-dependent QS. This
498 gene codes for the Rieske protein, an iron-sulfur cluster protein sub-unit component
499 of the cytochrome bc_1 complex involved in electron transfer and respiration under
500 aerobic conditions and also essential for enabling *P. aeruginosa* to grow under
501 anaerobic conditions in the presence of nitrite [58]. Self-poisoning of cytochrome
502 bc_1 occurs via HQNO which is produced via the same biosynthetic pathway as PQS
503 (**Fig. 1**). HQNO binds to the quinone reduction (Qi) site of the respiratory
504 cytochrome bc_1 complex [59]. This results in the generation of reactive oxygen
505 species that cause *P. aeruginosa* cell death and autolysis favouring biofilm formation
506 and antibiotic tolerance. Rieske subunit and cytochrome b_1 mutants do not undergo
507 autolysis and are insensitive to exogenous HQNO [59]. Similarly, *P. aeruginosa pqsL*
508 mutants that are unable to produce HQNO also fail to undergo autolysis [59]. Our
509 data also show that in common with the *tat* mutants, the *petA* mutant produces
510 reduced levels of HQNO (as well as PQS and HHQ). This suggests that lack of eDNA
511 in the *tat* mutant biofilms is because the cells do not undergo limited autolysis as
512 they lack the self-poisoning mechanism that depends on both an intact cytochrome
513 bc_1 and sufficient HQNO. Thus, lack of the Rieske sub-unit export is clearly
514 responsible for the Tat-mediated perturbation of PQS-dependent QS, the loss of

515 virulence factor production, biofilm eDNA and the tobramycin tolerance of
516 *P. aeruginosa* biofilms. Given the importance of PQS signalling and the Tat system
517 to virulence and biofilm maturation in *P. aeruginosa*, our findings underline the
518 potential of the Tat system as a drug target for novel antimicrobial agents.
519

520 ## Materials and Methods

521 **Bacterial strains and growth conditions**

522 The *P. aeruginosa* and *E. coli* strains used are listed in **S1 Table** and were grown in
523 LB or ABTG [8] at 37°C unless otherwise stated. *P. aeruginosa* biofilms were
524 cultivated at 30°C in flow-chambers irrigated with FAB medium [60] supplemented
525 with 0.3 mM glucose or in M9 medium with succinate (for static biofilms). Selective
526 media were supplemented with ampicillin (Ap; 100 µg ml^{-1}), gentamicin (Gm; 60 µg
527 ml^{-1}), or streptomycin (Sm; 100 µg ml^{-1}). PQS, HHQ and 2-heptyl-4-
528 hydroxyquinoline N-oxide (HQNO) were synthesized and characterized in house as
529 described before [19,30] and dissolved in methanol before being added to growth
530 media at the appropriate concentration.

531 **Mutant construction, screening and validation**

532 The *P. aeruginosa* PAO1 Tn mutant library was constructed using the Mariner
533 transposon vector pBT20 as previously described [61]. Transconjugants carrying Tn
534 insertions were picked from the selective plates and inoculated into microtiter plates
535 containing ABTG medium [62] supplemented with propidium iodide (PI) and the
536 level of red fluorescence quantified. Mutants producing reduced levels of eDNA were
537 selected and the sequences flanking the Tn insertion identified by arbitrary PCR
538 essentially as described by Friedman and Kolter [63] but using the specific primers
539 TnM1 and TnM2 (**S2 Table**). DNA Sequencing was performed by Macrogen, Seoul,
540 Korea with primer TnMseq (**S2 Table**). An in-frame *P. aeruginosa tatABC* deletion
541 mutant was generated by allelic exchange using the oligonucleotide primers Tat3D-

542 UF, Tat3D-UR, Tat3D-DF and Tat3D-DR (**S2 Table**) to introduce the up- and down-
543 stream regions of the *tatABC* locus into the suicide vector pME3087 to generate
544 pME3087::*tatABC* (**S1 Table**). The latter was introduced into *P. aeruginosa* via
545 conjugation with *E. coli* S17-1 λpir followed by enrichment for tetracycline-sensitive
546 cells as described by Ye *et al* [64]. The Δ*tatABC* deletion in *P. aeruginosa* PAO1 was
547 confirmed by PCR and sequence analysis. To generate the PA14 Δ*petA* mutant,
548 500 bp upstream and downstream of the gene were amplified using respectively
549 primers S.PA4431upFor/S.PA4431upRev and S.PA4431downFor/S.PA4431downRev
550 listed in **S2 Table**. The PCR product was cloned in pKNG101 suicide vector by one-
551 step sequence and ligation-independent cloning (SLIC) [65] which was then
552 sequenced. The resulting plasmid, pKNGΔ*petA*, maintained in the *E. coli* CC118λpir
553 strain, was then mobilized in *P. aeruginosa* strains. The mutant, in which the double
554 recombination events occurred, was confirmed by PCR analysis. A similar strategy
555 was used to construct allelic replacement mutants for each of 34 validated Tat
556 substrates [45]. These strains are summarized in **S3 Table** and their validation will
557 be described in detail elsewhere. For the generation of the *petA* cis-complemented
558 strain, PA14Δ*petA::(CTX1*::*petA)*, the *petA* genes along with a 500 bp fragment
559 corresponding to the putative promoter region for the *petA* gene were PCR amplified
560 using S-4431CTXFor/S-4431CTXRev and cloned by SLIC into the mini-CTX1 vector
561 yielding pminiCTX1-*petA*. Transfer of this plasmid in *P. aeruginosa* Δ*petA* strain was
562 carried out by triparental mating using the conjugative properties of the helper
563 plasmid pRK2013. The recombinant clones containing the mini-CTX inserted at the
564 *attB* locus on the *P. aeruginosa* genome were selected on tetracycline-containing
565 PIA generating PA14Δ*petA::(attB::petA)*.
566
567 **Construction of a *tatA* complementation plasmid**

568 The *tatA* gene was amplified by PCR using primers F*tatA* and R*tatA* (**S2 Table**),
569 introduced into pUCP22 (**S1 Table**) and electroporated into the *P. aeruginosa tatA*

570 and *tatA* Δ*pqsA* mutants. Transformants were selected on LB plates containing 200
571 μg ml^{-1} carbenicillin

572 **Bioluminescent reporter gene fusion assays**

573 To investigate the impact of the *tat* mutation and Tat inhibitor Bayer 11-7082 [46]
574 on PQS signalling, transcriptional fusions between the promoter regions of *pqsA*,
575 *pqsR*, *rhlA*, *phzA1 and phzA2* and the *luxCDABE* operon were constructed using the
576 miniCTX-*lux* plasmid as previously described [17]. In addition, a constitutively
577 bioluminescent reporter using a miniCTX::*tac-luxCDABE* promoter fusion was
578 constructed as a control for Bayer 11-7082. Bioluminescence as a function of
579 bacterial growth was quantified in 96 well plates using a combined luminometer-
580 spectrometer.

581 **Cultivation of biofilms**

582 Biofilms were grown in flow-chambers with individual channel dimensions of 1 x 4 x
583 40 mm as described previously [66]. One hour after inoculation, with bacteria, the
584 growth medium flow (0.2 mm/s corresponding to laminar flow with a Reynolds
585 number of 0.02) was started. When required, eDNA in biofilms was stained with 1
586 μM ethidium bromide prior to microscopy. Tobramycin (10 μM) was added to the
587 biofilm medium after 4 days of cultivation. After 24 h of tobramycin treatment,
588 propidium iodide (10 μM) was added to the flow cells to visualize the dead cells via
589 confocal laser scanning microscopy. *P. aeruginosa* PA14 and *petA* mutant biofilms
590 were grown under static conditions over 48 h at 37 °C on glass slides (Ibidi)
591 incorporating 300 μl chambers. After 48 h incubation, spent medium was removed
592 and the biofilm eDNA stained with YoYo-1 (40 μM).
593

594 **Microscopy and image processing of flow cell biofilms**

595 All images of flow-chamber-grown and static biofilms were captured with a confocal
596 laser scanning microscope (CLSM) equipped with detectors and filter sets for

597 monitoring green fluorescent protein, Syto9, propidium iodide, and ethidium
598 bromide. Images were obtained using a 63x/1.4 objective or a 40x/1.3 objective.
599 Simulated 3-D images and sections were generated using the IMARIS software
600 package (Bitplane AG, Zürich, Switzerland).
601
602 **AQ, pyocyanin, rhamnolipid and MV analysis**

603 The AQs (PQS, HHQ and HQNO) were quantified by LC-MS/MS after extracting cell
604 free supernatants or whole bacterial cells in triplicate with acidified ethyl acetate or
605 methanol respectively as described by Ortori *et al* [19]. Pyocyanin was extracted
606 with chloroform and quantified spectrophotometrically [26]. Rhamnolipids were
607 quantified indirectly using the orcinol method [35]. For PQS solubilization
608 experiments, rhamnolipids were purified as described by Muller *et al* [67]. MVs were
609 harvested by ultracentrifugation, the pellets resuspended in 10mM HEPES buffer and
610 the lipid content quantified using FM4-64 essentially as described previously [68].
611 MV production was normalized by dividing the lipid fluorescence units by CFU values
612 determined by dilution plating. Assays were performed in triplicate at least twice.

613 **Statistical Analysis**.

614 Significance for differences between wild type and isogenic mutants was determined
615 by two-tailed *t*-tests where ****$p < 0.001$, ***$p < 0.001$, **$p < 0.01$, and *$p <$
616 0.05 and n.s., not significant.
617
618

619 **Supporting Information**
620
621 **S1 Fig.** The Tat inhibitor Bayer 11-7082 has no effect on light output at 20 or 40 μM
622 from *P. aeruginosa* CTX:*ptac'-luxCDABE* chromosomal reporter fusion. The co-
623 solvent DMSO, had no effect at 0.8% on the *lux* reporter fusion either. Data are
624 presented as maximal light output as a function of growth (RLU/OD_{600}). Experiments
625 were repeated in triplicate at least twice.
626
627 **S2 Fig**. Deletion of the *P. aeruginosa tatABC* genes does not influence *pqsR*
628 expression. The data show that there are no differences in the expression of a
629 chromosomal CTX::*pqsR'-luxCDABE* fusion in the *P. aeruginosa* wild type
630 compared with the Δ*tatABC* mutant Data are presented as maximal light output as
631 a function of growth (RLU/OD_{600}). Experiments were repeated in triplicate at least
632 twice.
633
634 **S3 Fig.** AQ biosynthesis is not restored in a *tatA* Δ*pqsA* double mutant by plasmid-
635 borne *pqsABCD* in the absence of autoinduction. Semi-quantitative analysis by LC-
636 MS/MS of PQS, HHQ and HQNO production by *P. aeruginosa pqsA* and *tatA* Δ*pqsA*
637 mutants respectively without or with the *pqsABCD* biosynthetic genes provided *in*
638 *trans* via pBBRMCS::*pqsABCD.* Experiments were repeated in triplicate at least
639 twice.
640
641 **S4 Fig.** HHQ and PQS do not accumulate intracellularly a *tatA* Δ*pqsA* double
642 mutant harboring the plasmid-borne *pqsABCD* genes in the absence of
643 autoinduction. Semi-quantitative analysis by LC-MS/MS of PQS (**A**) and HHQ (**B**)
644 extracted from whole cells of *P. aeruginosa* wild type and the *tatA* Δ*pqsA* mutant
645 without or with the *pqsABCD* biosynthetic genes provided via pBBRMCS::*pqsABCD.*
646 Cells were harvested at 8 h and 16 h respectively. Experiments were repeated in
647 triplicate
648

649 **S5 Fig.** Exogenous rhamnolipids do not enhance PQS-dependent expression of
650 *pqsA* in a *tatA* Δ*pqsA* mutant. PQS (40 μM) was added with or without purified
651 rhamnolipids (50 μg/ml) to a *pqsA* mutant or a *tatA* Δ*pqsA* mutant carrying
652 chromosomal *pqsA′-lux* fusions. Maximal light output as a function of growth
653 (RLU/OD_{600}) is presented. Experiments were repeated in triplicate at least twice.
654
655 **S6 Fig** Rhamnolipid biosynthesis gene *rhlA* shows altered expression profiles in *P.*
656 *aeruginosa* PA14 Δ*tatABC* and Δ*petA* mutants compared with wild type and fail to
657 respond to exogenous PQS. Bioluminescence from a chromosomal *rhlA′-lux* fusion
658 as a function of growth (RLU/OD) over time when introduced into (**A**) the PA14 wild
659 type, (**B**) Δ*tatABC* and (**C**) Δ*petA* mutants in the absence or presence of exogenous
660 PQS (20 μM).
661
662 **S7 Fig** Deletion of *petA* in *P. aeruginosa* PA14 results in a reduction in the eDNA
663 content of biofilms. Biofilms of wild type PA14, the Δ*tatABC* and Δ*petA* mutants
664 and the genetically complemented PA14 mutant (Δ*petA*::(CTX::*petA*)) were grown
665 cultured statically and stained for eDNA with YOYO-1. (**A**) CLSM images and (**B**)
666 eDNA quantification. Experiments were repeated in triplicate at least twice. ***$p < 0.001$, **$p < 0.01$.
667
668
669 **S1 Table** Strains and plasmids used in this study
670 **S2 Table** Oligonucleotide primers used in this study
671 **S3 Table** *P. aeruginosa* PA14 Tat substrate mutants used in this study

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1034 **Figure Legends**

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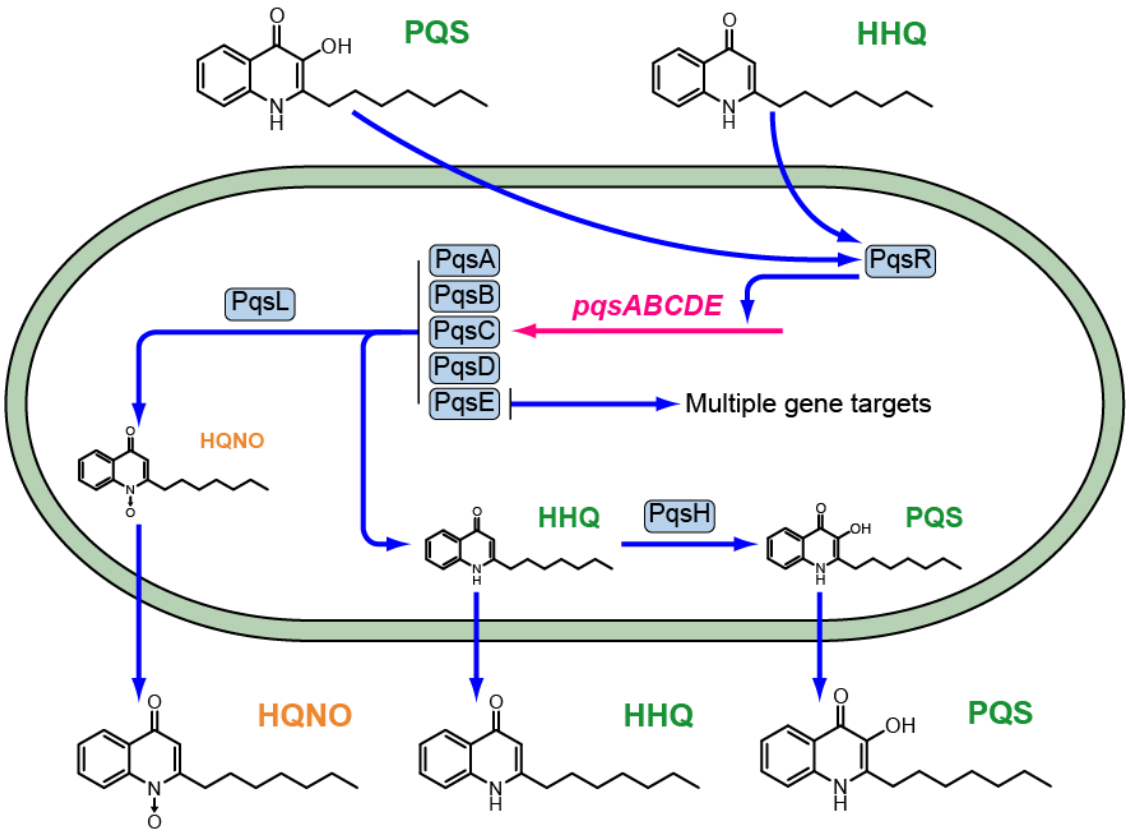

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1040 **Fig 1.** The PQS signalling pathway in *P. aeruginosa*. The PqsABCDE proteins
1041 synthesize HHQ, which is converted to PQS by PqsH and also HQNO in conjunction
1042 with PqsL. Both HHQ and PQS are released by the cells into the extracellular
1043 environment and are taken back up by neighboring cells. Autoinduction occurs when
1044 either HHQ or PQS binds to PqsR and amplifies expression of the *pqsABCDE* operon.
1045 The *pqsE* gene product has dual functions contributing to AQ biosynthesis as a
1046 thioesterase and also via an AQ-independent, thioesterase-independent mechanism
1047 to e.g. pyocyanin, rhamnolipid, and lectin production and to biofilm maturation. The
1048 conversion of HHQ to PQS confers additional functionalities since PQS unlike HHQ
1049 induces microvesicle formation and is a potent iron chelator.

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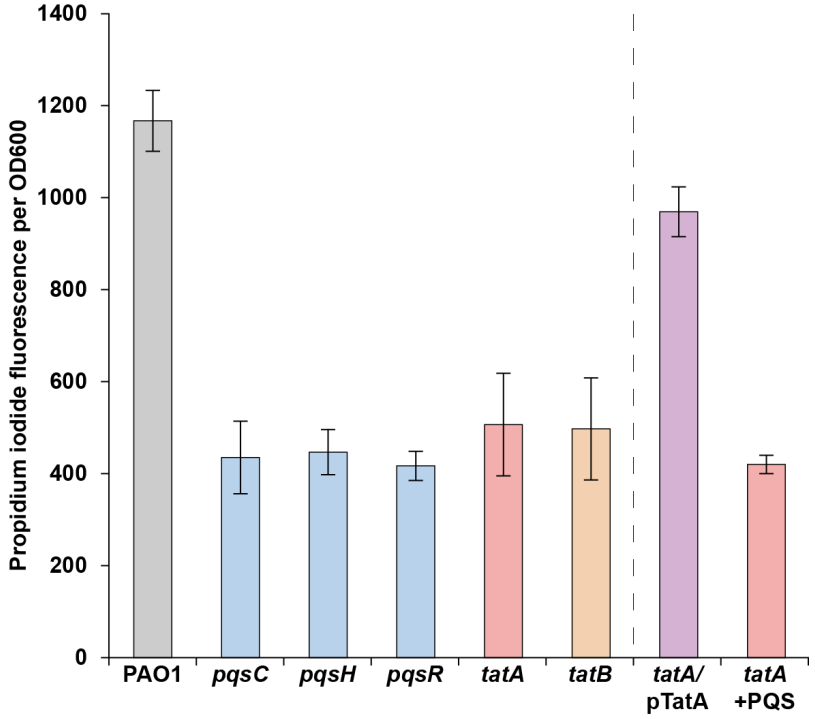

1065 **Fig 2.** Transposon mutant screen for *P. aeruginosa* strains defective for eDNA
1066 release. *P. aeruginosa* wild-type and mutant strains were grown for 24 h in 96 well
1067 microtiter plates containing ABTG medium, after which the relative levels of eDNA
1068 in the cultures were determined using a PI binding assay. The means and standard
1069 deviations of eight replicates are shown.

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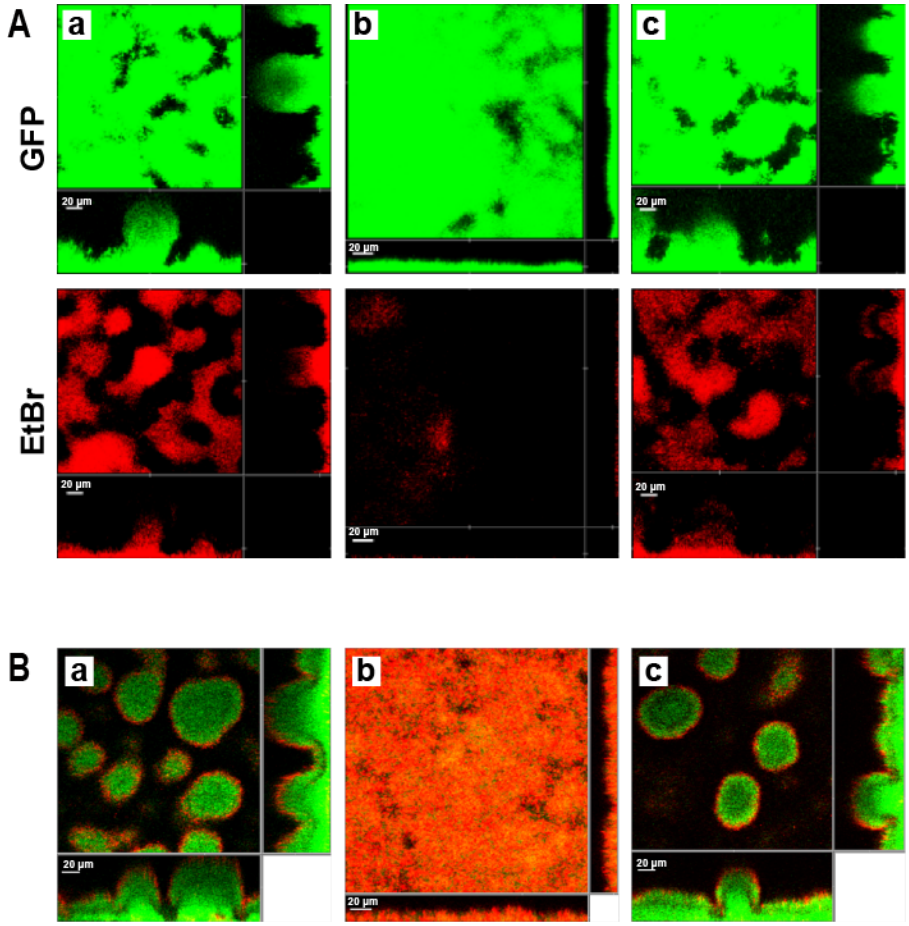

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1081 **Fig 3.** *P. aeruginosa tat* mutants form defective biofilms with increased susceptibility
1082 to tobramycin. CLSM images showing four-day-old biofilms formed in flow chambers
1083 of the *gfp*-tagged *P. aeruginosa* wild-type (a), *tatA* mutant (b) and genetically
1084 complemented *tatA* mutant (c). In **(A)** biofilms were stained for total biomass with
1085 Syto9 (green) and for eDNA with ethidium bromide (red). In **(B)** biofilms were
1086 treated with tobramycin and the medium was supplemented with propidium iodide
1087 prior to CLSM such that dead cells appear red while live cells appear green. Each
1088 panel shows one horizontal optical section two flanking vertical optical sections.
1089 Bars, 20 µm.

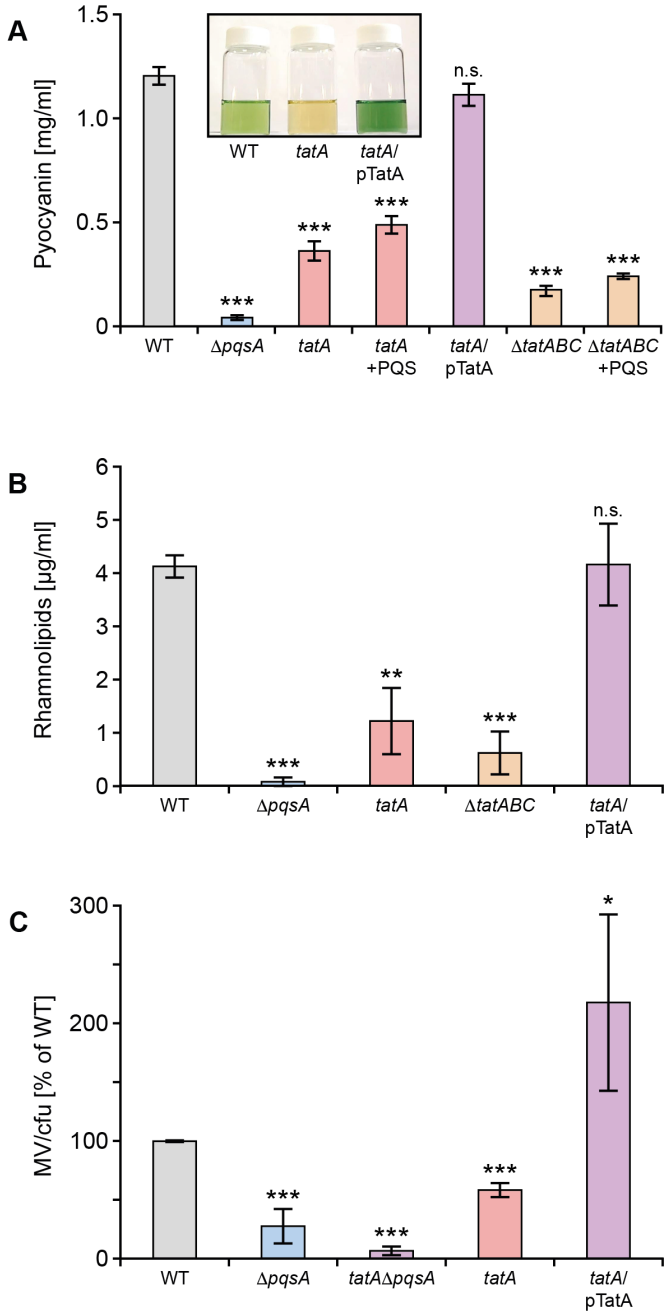

Fig 4. Production of pyocyanin (**A**), rhamnolipids (**B**) and MVs (**C**) are reduced in *P. aeruginosa tat* mutants. (**A**) Pyocyanin levels are shown in the *P. aeruginosa* wild type, Δ*pqsA*, *tatA*, and *tatABC* deletion mutants and the *tatA* mutant complemented with plasmid-borne *tatA*. The impact of exogenous PQS (40 µM) on the *tatA* and Δ*tatABC* mutants is also shown. Insert panel shows the absence of green pigment in the *tatA* mutant compared with the wild type and complemented *tatA* mutant. (**B**) Rhamnolipid production in the *tatA* and Δ*tatABC* mutants compared with the wild type, Δ*pqsA* mutant and *tatA* complemented with plasmid-borne *tatA.* (**C**) Comparison of MV production in the *tatA* mutant, complemented *tatA* mutant and in a double *tatA* Δ*pqsA* mutant compared with the wild type strain. Experiments were repeated in triplicate at least twice. ***$p < 0.001$, **$p < 0.01$; n.s. not significant.

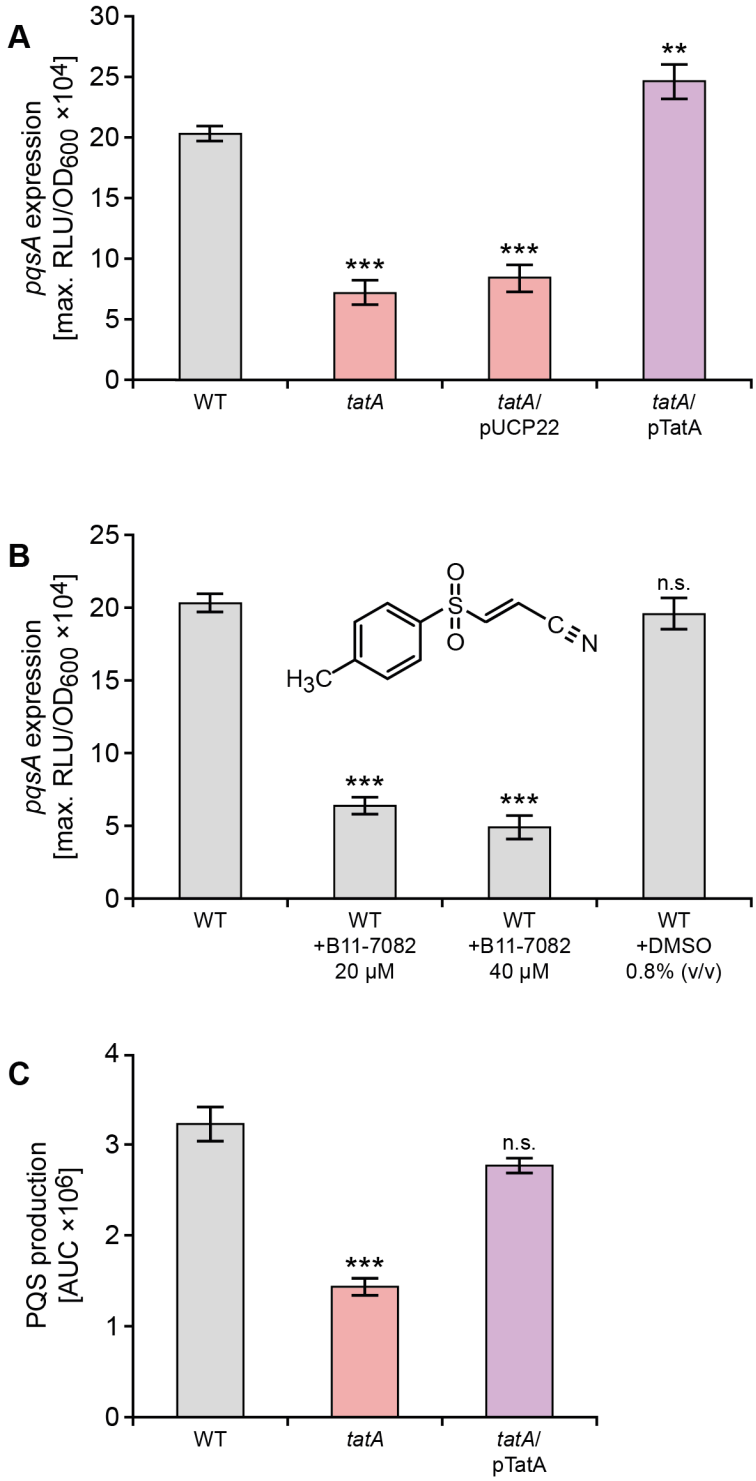

Fig 5. Mutation of *tatA* or exposure to the Tat inhibitor Bayer 11-7082 inhibits *pqsA* expression and AQ production. (**A**) Mutation of *tatA* or (**B**) treatment with Bayer 11-7082 supplied at either 20 µM or 40 µM reduces the maximal expression of a *P. aeruginosa* PAO1 chromosomal *pqsA′-lux* promoter fusion without affecting growth. (**C**) LC-MS/MS analysis of PQS production by *P. aeruginosa* PAO1 wild type compared with the *tatA* mutant and complemented *tatA* mutant. Experiments were repeated in triplicate at least twice. ***$p < 0.001$; n.s. not significant.

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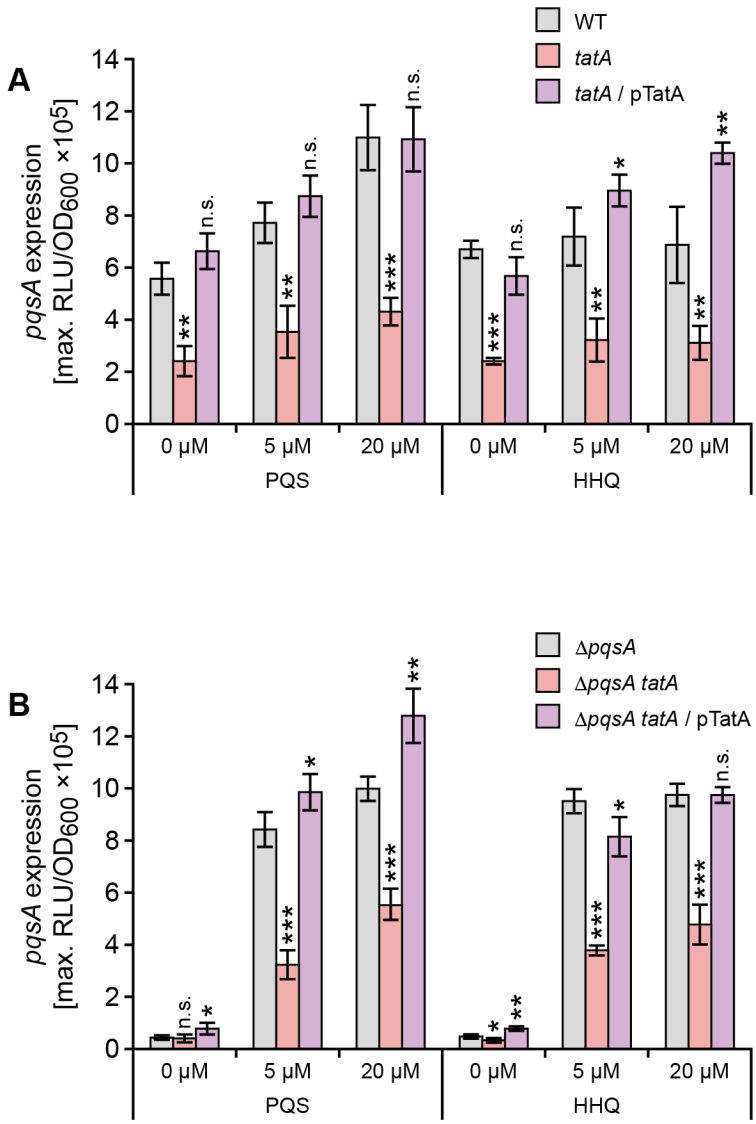

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1122 **Fig 6.** Exogenous AQs do not restore *pqsA* expression in *P. aeruginosa tatA* (A) or
1123 *tatA* Δ*pqsA* (B) mutants. Exogenous PQS or HHQ was added at 5 μM or 20 μM to
1124 (**A**) wild type, the *tatA* mutant and complemented *tatA* mutant or (**B**) Δ*pqsA*, Δ*pqsA*
1125 *tatA* or *tatA* Δ*pqsA* mutant complemented with *tatA.* Maximal light output from the
1126 chromosomal *pqsA′-lux* fusion was recorded as a function of growth (RLU/OD_{600}).
1127 Experiments were repeated in triplicate at least twice. ***$p < 0.001$, **$p < 0.01$,
1128 and *$p < 0.05$; n.s. not significant.

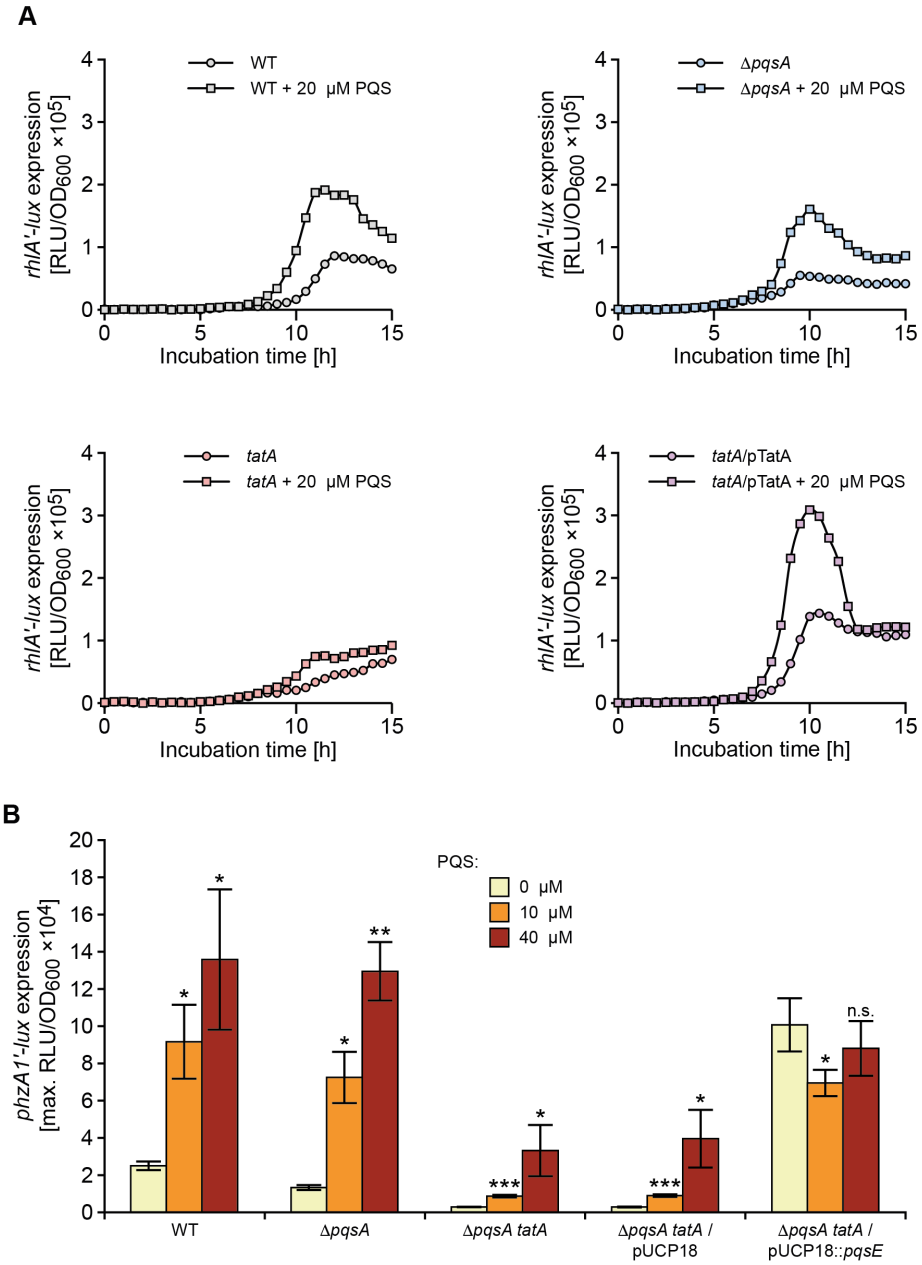

Fig 7. Rhamnolipid (*rhlA*) and pyocyanin biosynthetic (*phzA1*) genes show altered expression profiles in *P. aeruginosa tat* mutants and fail to respond to exogenous PQS. (**A**) Light output from a chromosomal *rhlA'-lux* fusion as a function of growth (RLU/OD) over time when introduced into the wild type, *pqsA* and *tatA* mutants or complemented *tatA* mutant in the absence or presence of exogenous PQS (20 μM). (**B**) Maximal light output from a chromosomal *phzA1'-lux* fusion as a function of growth (RLU/OD_{600}) when introduced into the wild type, *pqsA*, or *pqsA tatA* mutants in the absence or presence of exogenous PQS (10 or 40 μM) or plasmid-borne *pqsE* or the pUCP18 vector control. Experiments were repeated in triplicate at least twice. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$; n.s. not significant.

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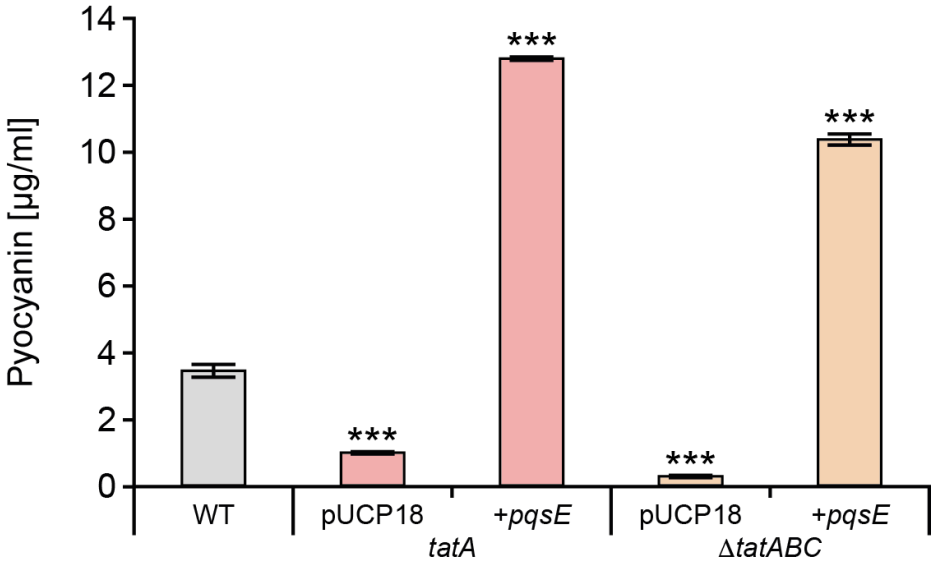

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1153 **Fig 8.** PqsE restores pyocyanin production in *P. aeruginosa tat* mutants. Production
1154 of pyocyanin by the wild type compared with the *tatA* and Δ*tatABC* mutants
1155 transformed with plasmid-borne *pqsE* or the empty vector. Experiments were
1156 repeated in triplicate at least twice. ***$p < 0.001$,
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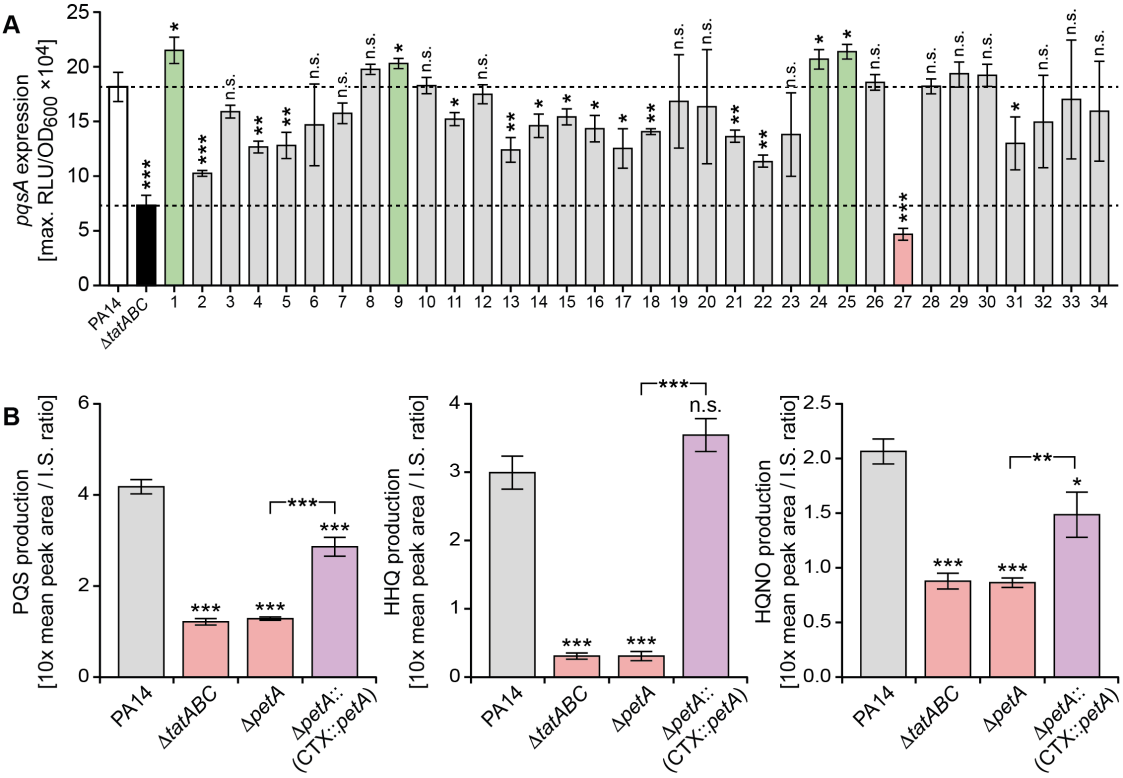

Fig 9. Tat substrate screen for *P. aeruginosa* PA14 mutants with defects in *pqs* signalling uncovers a role for the cytochrome bc_1 Rieske sub-unit. (**A**) Comparison of peak *pqsA* expression in each of 34 Tat substrate mutants (**Table S3**) transformed with a CTX::*pqsA′-lux* fusion compared with the PA14 wild type (white bar) and the Δ*tatABC* mutant (black bar). The bars represent mutants where *pqsA* expression is higher (green bar) or lower (grey bar) or the same (grey bar) as the wild type. PA14 mutant 27 (pink bar) has the lowest *pqsA* expression and carries a deletion in PA14-57570 (*petA*), the Rieske subunit of cytochrome bc_1. (**B**) Production of PQS, HHQ and HQNO after 16 h growth by *P. aeruginosa* PA14, the Δ*tatABC* and Δ*petA* mutants and the genetically complemented PA14 mutant (Δ*petA*::(CTX::*petA*)). ***p < 0.001, **p < 0.01, and *p < 0.05; n.s. not significant.