Disruption of the *Pseudomonas aeruginosa* Tat system perturbs PQS-dependent quorum sensing and biofilm maturation through loss of the Rieske cytochrome bc_1 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

PqsBC heterodimer condenses 2-ABA with octanoyl-coA to generate HHQ [23,24]. PQS is formed through the oxidation of HHQ by PqsH [25] while formation of the AQ *N*-oxides such as 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) requires the alternative mono-oxygenase PqsL [18]. The PqsE protein has dual functions; while it is not essential for AQ biosynthesis, it is required for the AQ-independent production of several factors that contribute to biofilm maturation including pyocyanin, rhamnolipids and lectin A [26]. While activation of RhlR-dependent genes depends on PqsE [27], the AQ-independent, thioesterase-independent mechanism by which PqsE acts has not yet been elucidated [17,22].

The *pqs* system is subject to positive autoinduction, since the LysR-type transcriptional regulator PqsR (MvfR), binds to the promoter region of *pqsABCDE* (P*pqsA*) triggering transcription once activated by HHQ or PQS [28-30] (**Fig. 1**). Therefore, by analogy with other QS systems, HHQ and PQS can both act as autoinducers by generating a positive feedback loop that accelerates their biosynthesis. However, in contrast to HHQ which only regulates the *pqsABCDE* operon [17], PQS is a ferric iron chelator [31] that not only drives AQ biosynthesis via PqsR but also the expression of genes involved in the iron-starvation response and virulence factor production *via* PqsR-dependent and PqsR-independent pathways [17]. In addition, PQS can act as a cell-sensitizing pro-oxidant [32] and is required for MV production via a direct physical interaction with lipopolysaccharide (LPS) within the outer membrane [33]. The packaging of PQS within MVs also provides a means for trafficking this hydrophobic QS signal within a *P. aeruginosa* population [34].

With respect to biofilm development, the *pqs* system is of particular interest because *pqs* biosynthetic mutants fail to produce eDNA, rhamnolipids, pyocyanin and MVs, and form thin defective biofilms containing little eDNA [8, 27,33,35]. The mechanism involved in PQS-mediated DNA-release in biofilms is not understood but has been

suggested to be linked to phage induction causing cell lysis [8,36-40]. Although explosive cell lysis releases eDNA in biofilms and generates MVs through vesicularization of shattered membrane fragments, *pqsA* mutants are not defective for explosive lysis [14] and therefore this phenomenon is unlikely to account for PQS-dependent eDNA release.

In the present study we sought to identify additional factors involved in eDNA release by screening a transposon (Tn) mutant library for eDNA-release defective mutants. Apart from *pqs* biosynthetic mutants, we obtained Tn insertion mutants within the twin-arginine translocation (Tat) pathway that exhibited reduced levels of eDNA release, fail to produce rhamnolipids or pyocyanin and form defective, eDNA- poor, antibiotic susceptible biofilms. Since mutation or deletion of *tat* resulted in altered AQ production, reduced pyocyanin, rhamnolipid and MVs, and as the *tat* mutants were refractory to exogenously supplied PQS, the aberrant biofilm phenotype observed could be accounted for by perturbation of PQS autoinduction. By screening a library of *P. aeruginosa* Tat substrate mutants, we identified the Rieske sub-unit of the cytochrome bc_1 complex as being required for *pqs*-dependent QS and hence eDNA release and biofilm maturation.

Results

Transposon mutagenesis screen for P. aeruginosa mutants exhibiting reduced DNA release

To identify *P. aeruginosa* genes that contribute to eDNA release, a mariner transposon (Tn) mutant library was generated in strain PAO1. Approximately 10,000 mutants grown in microtitre plates were assayed for reduced eDNA release using propidium iodide (PI) to quantify eDNA because it is unable to penetrate live bacteria 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

more sensitive to tobramycin than either the wild-type or *tatA*/pTatA mutant biofilm (**Fig. 3B**).

P. aeruginosa tatA mutants are defective in the production of rhamnolipids, pyocyanin and MVs

Since rhamnolipids, pyocyanin and MVs are all important components of *P. aeruginosa* biofilms, their production was quantified in the *tatA* Tn insertion mutant and in a Δ*tatABC* deletion mutant. **Fig. 4 (A and B)** shows that both *tat* mutants produce substantially less pyocyanin and rhamnolipid than the parent or *tatA* complemented strain. Furthermore, MV levels (**Fig. 4C**) were reduced by ~50% in the *tatA* mutant compared with the wild type and could be restored by genetic complementation.

Inactivation of the tat pathway by mutation or small molecule-mediated inhibition perturbs PQS signalling

The reductions in eDNA release, rhamnolipids, pyocyanin and MVs noted in the *tat* mutant as well as its biofilm phenotype are similar to those observed for *P. aeruginosa* strains with mutations in *pqs* genes such as *pqsA*, the first gene in the AQ biosynthetic pathway (see **Figs. 2** and **Fig 4** and [8, 40]). These data suggested that the *tat* mutant biofilm phenotype was likely, at least in part, to be due to a defect in PQS signalling.

To investigate the impact of the *tatA* mutation on the expression of *pqsA,* a CTX::*pqsA'-lux* fusion was introduced into the chromosomal CTX site of both the wild type and *tatA* mutant. **Fig. 5A** shows that *pqsA* expression in the *tatA* mutant is reduced ~4 fold compared with the wild type strain and restored by genetic complementation of the mutant. In agreement with these data, the Tat inhibitor,

Bayer 11-7082, identified by Vasil *et al* [46] reduced *pqsA* expression in the wild type PAO1 strain by ~4 fold consistent with the reduction noted for the CTX::*pqsA-lux* fusion in the *tatA* mutant (**Fig. 5B**). Bayer-11 7082 had no effect on growth or light output in *P. aeruginosa* expressing the *lux* genes from a derepressed *lac* promoter (**S1 Fig.**). In addition**,** the concentrations of PQS in whole culture extracts of *P. aeruginosa* after growth in LB medium as determined by LC-MS/MS was respectively ~56% lower in the *tatA* mutant compared with the wild type and complemented *tat* mutant (**Fig. 5C**). Since *pqsA* expression and hence AQ production is also PqsR/MvfR-dependent, we compared the expression of *pqsR* in the Δ*tatABC* with the parent strain and found no difference (**S2 Fig.**).

Since mutation of *tatA* results in reduced *pqsA* expression, it is possible that the auto-induction of PQS signalling via PqsR/MvfR is compromised. To uncouple the autoinduction of AQ production, the *pqsABCD* genes were introduced into the *P. aeruginosa* Δ*pqsA* and Δ*pqsA*Δ*tatA* mutants respectively on a plasmid (pBBRMCS5::*pqsABCD*) that constitutively expresses the *pqsABCD* genes [47]. **S3 Fig.** shows that PQS, HHQ and HQNO are present in the culture medium of the Δ*pqsA* mutant transformed with pBBRMCS5::*pqsABCD*. However neither the cell free supernatant nor whole cells of the *tatA* Δ*pqsA* double mutant transformed with pBBRMCS5::*pqsABCD* contained or accumulated intracellular AQs (**S4 Fig.**) suggesting that in the absence of autoinduction in the *tat* mutant background. *P. aeruginosa* is unable to synthesize AQs.

Exogenous PQS does not restore PQS signalling in a P. aeruginosa* tatA *mutant

QS systems are characteristically autoinducible such that exogenous provision of the cognate signal molecule induces expression of the signal synthase and hence downstream target genes [48]. When the *tatA* mutant was provided with exogenous

PQS, eDNA release did not increase (**Fig. 2**). To investigate this finding further, either PQS or HHQ was exogenously supplied to the wild type, *tatA* mutant or the complemented *tatA* mutant strains carrying chromosomal *pqsA′-lux* fusions. The data presented in **Fig. 6A** show that the response of the *tatA* mutant to PQS or HHQ respectively at 5 or 20 µM with respect to *pqsA* expression was at least 2-fold lower than the controls. Since both wild type and *tatA* mutant still produce AQs endogenously, the experiments were repeated in the *P. aeruginosa* Δ*pqsA* and *tatA* Δ*pqsA* mutants since no AQs are produced in these genetic backgrounds. **Fig. 6B** shows that the response to both PQS and HHQ is substantially reduced (e.g. ~4 fold at 5 µM PQS) in the absence of *tatA* in the *P. aeruginosa tatA* Δ*pqsA* double mutant.

To determine the consequences of perturbed PQS signalling on the expression of the rhamnolipid (*rhlA*) and pyocyanin biosynthetic genes (*P. aeruginosa* has two, almost identical redundant 7 gene phenazine biosynthetic operons termed *phzA1-G1* and *phzA2-G2;* [49]), the corresponding miniCTX-*lux* promoter fusions for *rhlA* and *phzA1* respectively were constructed and introduced onto the chromosomes of the wild type, Δ*pqsA* and *tatA* Δ*pqsA* mutants respectively. **Fig. 7A** shows the expression profiles of *rhlA′-lux* as a function of time. Both the wild type and Δ*pqsA* mutant show an ~2 fold increase in *rhlA* expression when supplied with exogenous PQS (20 µM) and share similar profiles over the growth curve. In contrast, the *rhlA′-lux* fusion in the *tatA* mutant does not show the same expression profile or response to exogenous PQS as the wild type and Δ*pqsA* mutant strains. The *rhlA′-lux* expression profile in the *tatA* mutant supplied with exogenous PQS is however clearly restored when the mutation is complemented by pTatA (**Fig. 7A**).

AQ-dependent QS is required for *phzA1* expression [27,49]. Exogenous PQS increased *phzA1′-lux* expression by ~4 fold in both wild type and Δ*pqsA* mutant

backgrounds (**Fig. 7B**). However, the *tatA* Δ*pqsA* double mutant responded comparatively poorly to PQS (**Fig. 7B**).

PqsE restores pyocyanin in the tat mutants

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

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

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

Identification of the Tat substrate responsible for perturbation of *PQS signalling*

Recently Gimenez et al [45] experimentally validated the Tat-mediated export of 34 *P. aeruginosa* gene products predicted to have Tat signal peptides. To determine which of the exported Tat substrates was responsible for perturbation of PQS signalling, allelic replacement mutants were constructed in *P. aeruginosa* strain PA14 for each substrate. Before introducing the *pqsA′-lux* fusion onto the chromosomal CTX site of each Tat substrate mutant, we first confirmed that PQS signalling in a PA14 Δ*tatABC* mutant was perturbed in a similar manner to that observed for PAO1, the genetic background used so far in the study (**Fig. 9A**). Determination of the maximum expression of *pqsA′-lux* for each of the 34 Tat substrate mutants (**Fig. 9A**) revealed that although a number of mutants exhibited reduced light output, the greatest reduction was observed for deletion of PA14_57570 (equivalent to PA4431 in PAO1, here designated *petA* following the nomenclature of orthologues described in other species). This gene codes an iron-sulfur cluster protein, the Rieske subunit of the cytochrome bc_1 complex.

To confirm that the Δ*petA* mutant in common with the *P. aeruginosa tat* mutants exhibited similar defects as a consequence of perturbed PQS signalling, we compared their AQ production (**Fig. 9B**), *rhlA* expression, and eDNA release in biofilms (**S6 Fig.** and **S7 Fig.**). Comparison of PQS, HHQ and HQNO production in PA14 with the Δ*petA* mutant and the strain with a *cis* complementation of the *petA* mutation at the CTX site confirmed the loss and increase in AQ production (**Fig. 9B**). In *P. aeruginosa* PA14, the *rhlA* expression profiles for the parent and Δ*tatABC* deletion mutant in the absence or presence of exogenously supplied PQS were similar to those of strain PAO1 (compare **S6 Fig.** with **Fig. 7A**). Confocal microscopy images of biofilm formation by *P. aeruginosa* PA14 under static growth conditions shows that in common with the Δ*tatABC* mutant, *petA* mutant biofilms lack the eDNA content observed in the wild type and complemented mutant (**S7**

Fig.) demonstrating that PetA is the Tat substrate required for PQS-dependent eDNA release.

Discussion

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

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

chloroplasts and subsequently identified in bacteria and archaea [42]. Tat exports folded proteins out of the cytoplasm and across the cytoplasmic membrane in an ATP-independent manner [42]. In bacteria such as *E. coli* and *P. aeruginosa* proteins exported by the Tat system are most frequently terminally localized in the periplasm, but some (e.g. phospholipase C) can be transported across the outer membrane by the Xcp type II secretion system, thereby becoming extracellular. Tat signal peptides harbor a conserved S/TRRxFLK consensus motif, where the twin arginine is invariant and in the vast majority of cases essential for export [45]. Using prediction algorithms for Tat substrates, 44 putative *P. aeruginosa* PA14 Tat signal peptides have been identified, and of these, 34 confirmed experimentally using a novel amidase-based Tat reporter [45]. These include phospholipases and proteins involved in pyoverdine-mediated iron-uptake, respiration, osmotic stress defence, motility, and biofilm formation [44, 45]. However, none of these are known to be involved in AQ biosynthesis, transport or *pqs* signal transduction.

In our flow-chamber grown biofilms, clear differences between the *P. aeruginosa* PAO1 wild type and *tatA* mutant biofilms were apparent. While the wild type formed characteristic mushroom-shaped structures under these conditions, the *tatA* mutant formed flat, thin biofilms. In addition, eDNA was observed primarily in the stalks of the mushroom-shaped structures in the wild-type biofilms, whereas the *tatA* mutant biofilms contained no stalks and little extracellular DNA. Moreover, the biofilms formed by the *tatA* mutant were more sensitive to tobramycin treatment than wild-type biofilms, presumably in part due to the inability of the *tatA* mutant to release DNA and form differentiated multicellular structures. Indeed, DNA binds positively charged antibiotics and can act as a 'shield' against aminoglycosides such as tobramycin [53]. In *P. aeruginosa* biofilms, exogenously provided DNA integrates into *P. aeruginosa* biofilms increasing their tolerance toward aminoglycosides [55]. The formation of flat, eDNA deficient, tobramycin-sensitive biofilms and the reduction in rhamnolipid, pyocyanin and MV production by the *P. aeruginosa tatA*

mutant are consistent with the *pqsA* mutant biofilm phenotype [8] and imply the existence of a link between Tat export and *pqs* signalling. Since transcriptomic studies of PQS signalling in *P. aeruginosa* have not provided any evidence that the Tat export system is QS controlled [17,26], we considered it likely that mutation of the *tat* genes resulted in perturbed PQS signalling. This observation could also account, at least in part, for the reduced virulence of *P. aeruginosa tat* mutants in a rat pulmonary infection model [44].

To explore this hypothesis, the expression of *pqsA* and the concentrations of PQS and HHQ produced were compared in *P. aeruginosa* PAO1 wild type, *tatA* and complemented *tatA* mutant strains. Similar results were also obtained for *P. aeruginosa* strain PA14. Disruption of the *tat* operon had a pronounced effect on the expression of *pqsA,* with a nearly 3–fold reduction in *pqsA* expression. Moreover, addition of the Tat inhibitor, BAY 11-7082 [46] also resulted in a comparable reduction in *pqsA* expression. LC-MS/MS quantification of PQS levels in early stationary phase after 8 h growth, showed a ~50% reduction, thus correlating with the *pqsA′-lux* promoter fusion assays. Although PQS and HHQ both serve as PqsR ligands for driving the positive feedback loop for *pqsABCDE* expression that is central to AQ signalling [28-30], exogenous provision of neither PQS nor HHQ could fully restore *pqsA* expression in the *tatA* mutant. Since this mutant still produces AQs, albeit at a reduced level, a *pqsA* mutation was introduced into the *tatA* mutant background to examine the response to exogenous AQs in an AQ-negative genetic background (*tatA* Δ*pqsA*). The data obtained confirmed that the *P. aeruginosa tatA* Δ*pqsA* mutant responded only weakly to exogenously added PQS or HHQ, albeit to a much lesser extent than the isogenic Δ*pqsA* mutant with an intact *tat* operon. These data suggest that disruption of *tat* impairs the ability of *P. aeruginosa* to fully induce AQ-dependent QS. The consequences of *tat*-dependent perturbation of *pqs* signalling are clearly evident in the modified expression profile of the *rhlA′-lux* fusion 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

envelope defect in *P. aeruginosa tat* mutants impacts on the PQS signalling auto-induction cascade. However, Ball *et al* [57] showed that *P. aeruginosa tat* mutants do not show the same Tat-dependent envelope defects found in *E. coli.* In addition, we found no evidence for intracellular accumulation of AQs indicative of a transport defect.

To uncover the mechanistic link between Tat and PQS signalling, we sought to determine whether mutation of *tat* resulted in the inability to export a specific Tat substrate, the loss of which resulted in perturbation of PQS autoinduction and hence the expression of PQS-dependent genes and the formation of defective biofilms. By screening 34 validated Tat substrate mutants for reduced *pqsA* expression, we identified the *petA* gene as responsible for the defect in *pqs*-dependent QS. This gene codes for the Rieske protein, an iron-sulfur cluster protein sub-unit component of the cytochrome bc_1 complex involved in electron transfer and respiration under aerobic conditions and also essential for enabling *P. aeruginosa* to grow under anaerobic conditions in the presence of nitrite [58]. Self-poisoning of cytochrome bc_1 occurs via HQNO which is produced via the same biosynthetic pathway as PQS (**Fig. 1**). HQNO binds to the quinone reduction (Qi) site of the respiratory cytochrome bc_1 complex [59]. This results in the generation of reactive oxygen species that cause *P. aeruginosa* cell death and autolysis favouring biofilm formation and antibiotic tolerance. Rieske subunit and cytochrome b_1 mutants do not undergo autolysis and are insensitive to exogenous HQNO [59]. Similarly, *P. aeruginosa pqsL* mutants that are unable to produce HQNO also fail to undergo autolysis [59]. Our data also show that in common with the *tat* mutants, the *petA* mutant produces reduced levels of HQNO (as well as PQS and HHQ). This suggests that lack of eDNA in the *tat* mutant biofilms is because the cells do not undergo limited autolysis as they lack the self-poisoning mechanism that depends on both an intact cytochrome bc_1 and sufficient HQNO. Thus, lack of the Rieske sub-unit export is clearly 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*

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

Bioluminescent reporter gene fusion assays

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

Cultivation of biofilms

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

Microscopy and image processing of flow cell biofilms

All images of flow-chamber-grown and static biofilms were captured with a confocal 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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Figure Legends

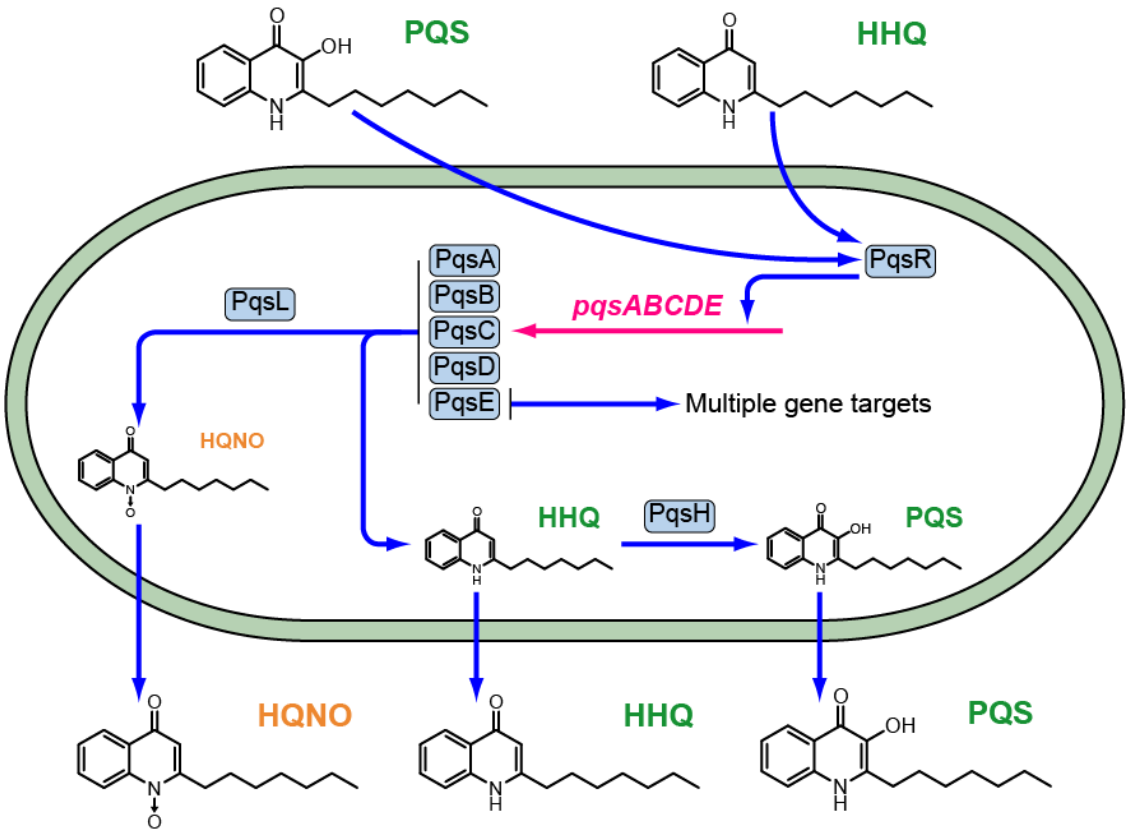

Fig 1. The PQS signalling pathway in *P. aeruginosa*. The PqsABCDE proteins synthesize HHQ, which is converted to PQS by PqsH and also HQNO in conjunction with PqsL. Both HHQ and PQS are released by the cells into the extracellular environment and are taken back up by neighboring cells. Autoinduction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

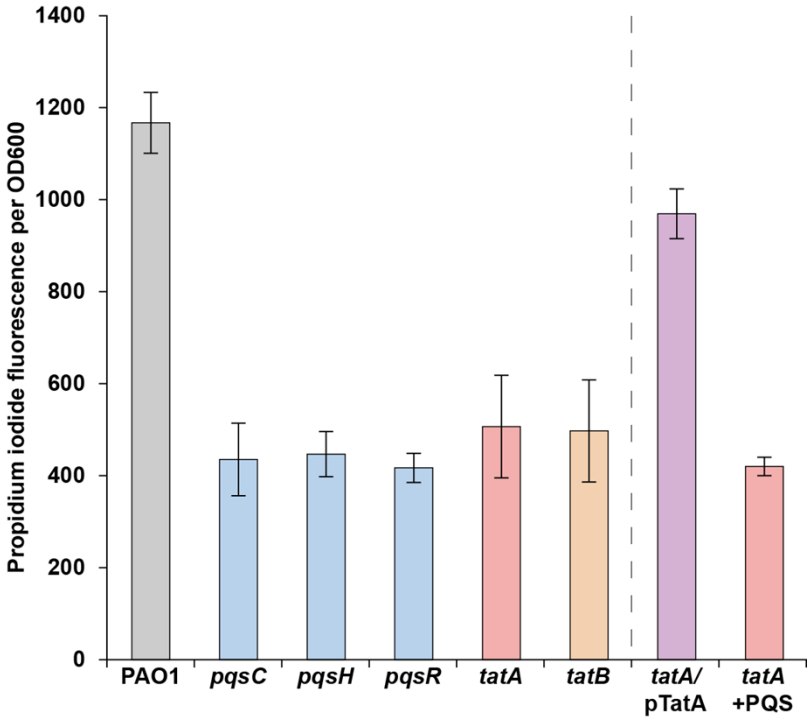

Fig 2. Transposon mutant screen for *P. aeruginosa* strains defective for eDNA release. *P. aeruginosa* wild-type and mutant strains were grown for 24 h in 96 well microtiter plates containing ABTG medium, after which the relative levels of eDNA in the cultures were determined using a PI binding assay.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

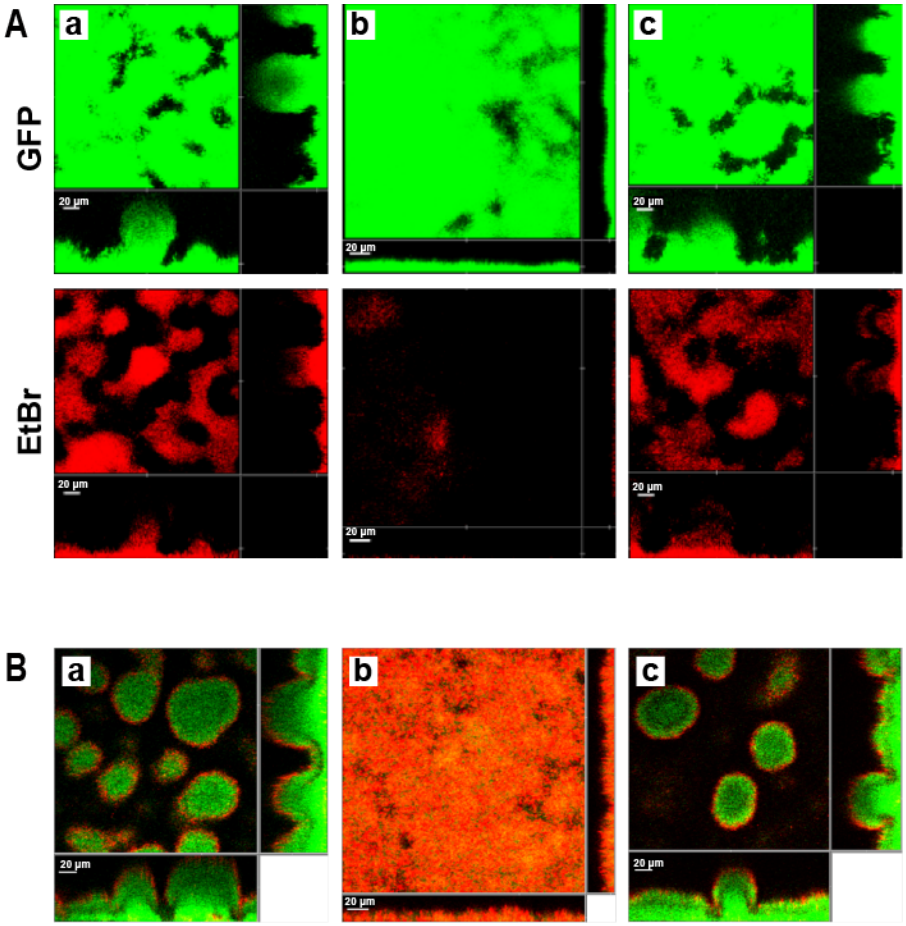

Fig 3. *P. aeruginosa tat* mutants form defective biofilms with increased susceptibility to tobramycin. CLSM images showing four-day-old biofilms formed in flow chambers of the *gfp*-tagged *P. aeruginosa* wild-type (a), *tatA* mutant (b) and genetically complemented *tatA* mutant (c). In **(A)** biofilms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

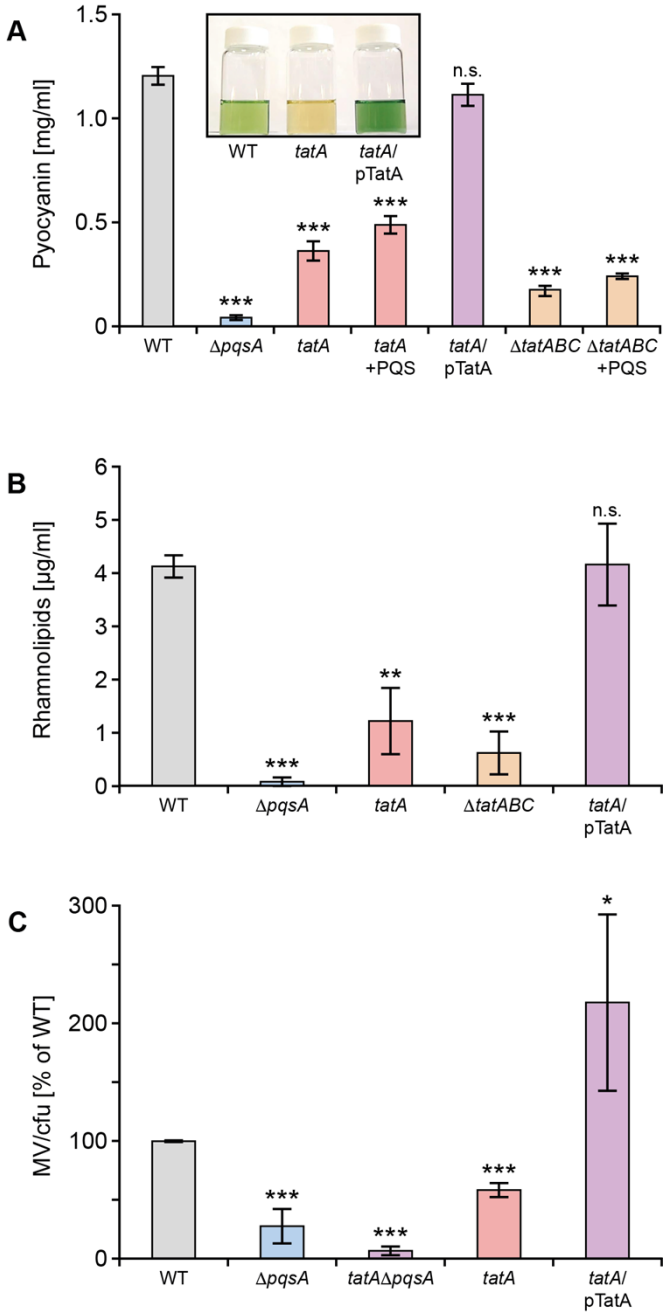

Fig 4. Production of pyocyanin (**A**), rhamnolipids (**B**) and MVs (**C**) are reduced in *P. aeruginosa tat* mutants. (**A**) Pyocyanin levels are shown in the *P. aeruginosa* wild type, Δ*pqsA*, *tatA*, and *tatABC* deletion mutants and the *tatA* mutant complemented with plasmid-borne *tatA*. The 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.

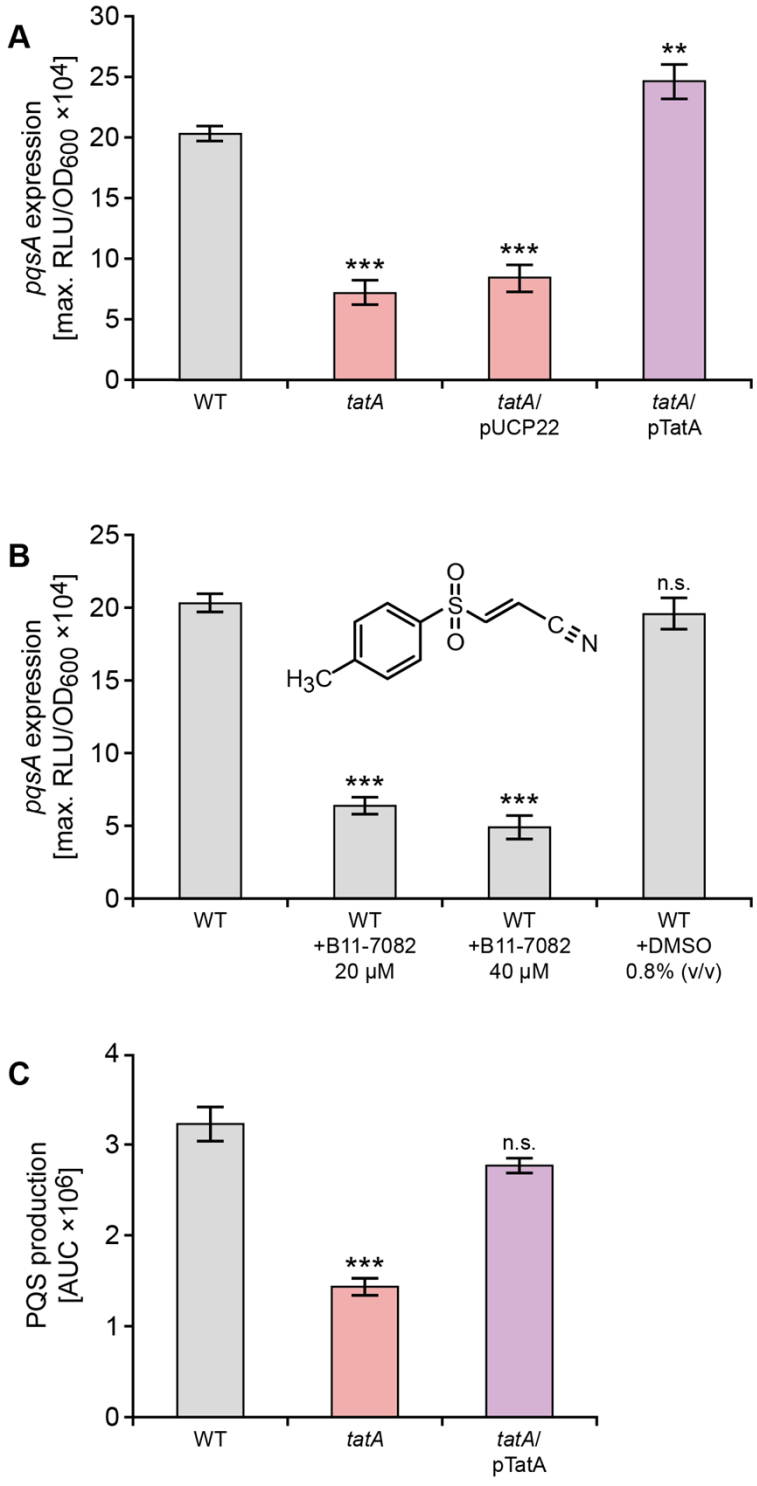

Fig 5. Mutation of *tatA* or exposure to the Tat inhibitor Bayer 11-7082 inhibits *pqsA* expression and AQ production. (**A**) Mutation of *tatA* or (**B**) treatment with Bayer 11-7082 supplied at either 20 µM or 40 µM reduces the maximal expression of a *P. aeruginosa* PAO1 chromosomal *pqsA'-lux* 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.

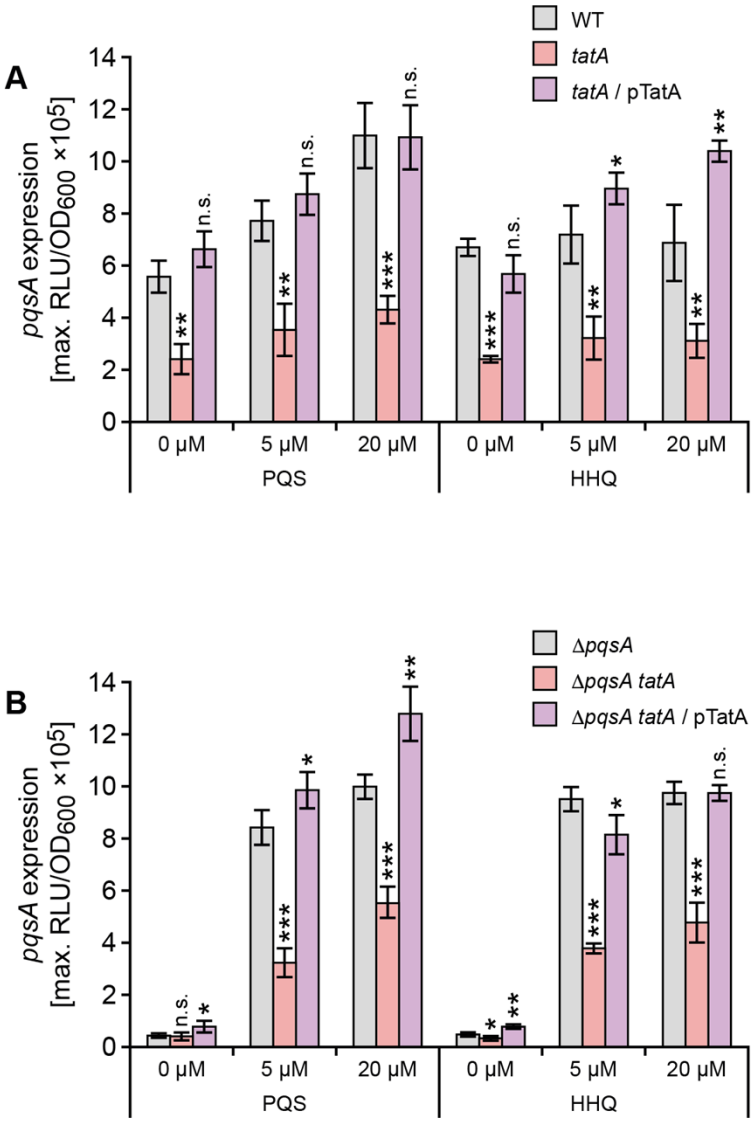

Fig 6. Exogenous AQs do not restore *pqsA* expression in *P. aeruginosa tatA* (A) or *tatA* Δ*pqsA* (B) mutants. Exogenous PQS or HHQ was added at 5 μM or 20 μM to (**A**) wild type, the *tatA* mutant and complemented *tatA* mutant or (**B**) Δ*pqsA*, Δ*pqsA tatA* or *tatA* Δ*pqsA* mutant complemented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

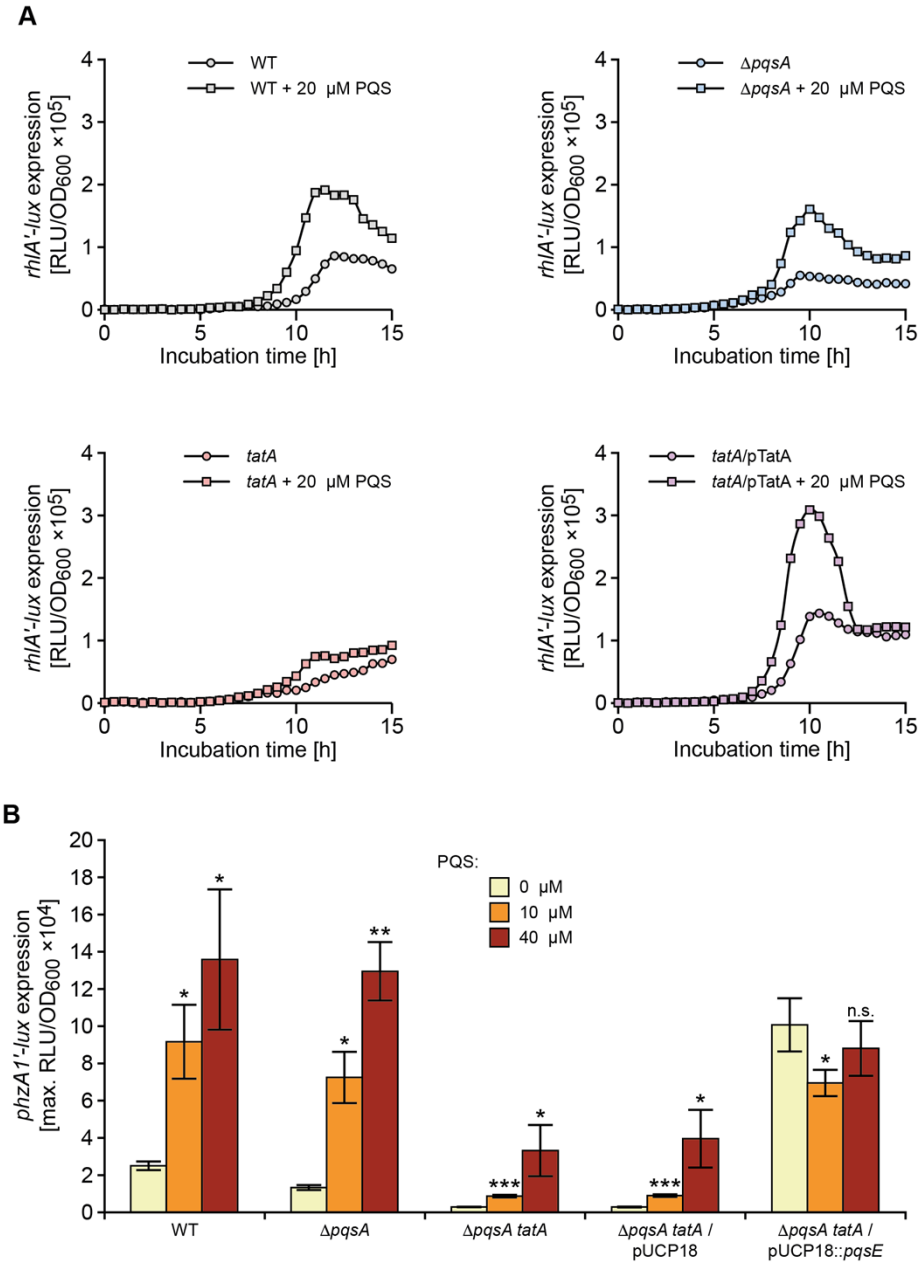

Fig 7. Rhamnolipid (*rhlA*) and pyocyanin biosynthetic (*phzA1*) genes show altered expression profiles in *P. aeruginosa tat* mutants and fail to respond to exogenous PQS. (**A**) Light output from a chromosomal *rhlA'-lux* fusion as a function of growth (RLU/OD) over time when introduced into the wild 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.

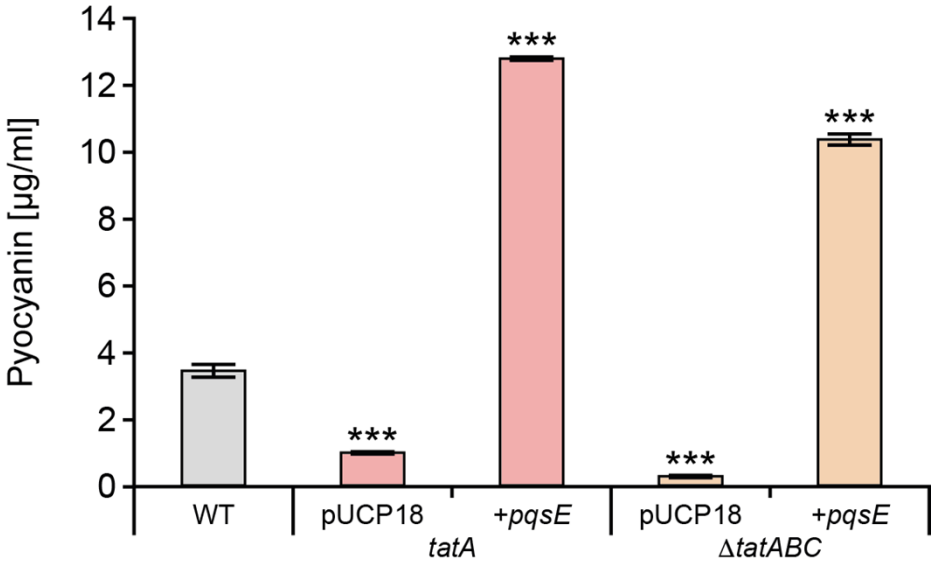

Fig 8. PqsE restores pyocyanin production in *P. aeruginosa tat* mutants. Production of pyocyanin by the wild type compared with the *tatA* and Δ*tatABC* mutants transformed with plasmid-borne *pqsE* or the empty vector. Experiments were repeated in triplicate at least twice. ***p < 0.001,

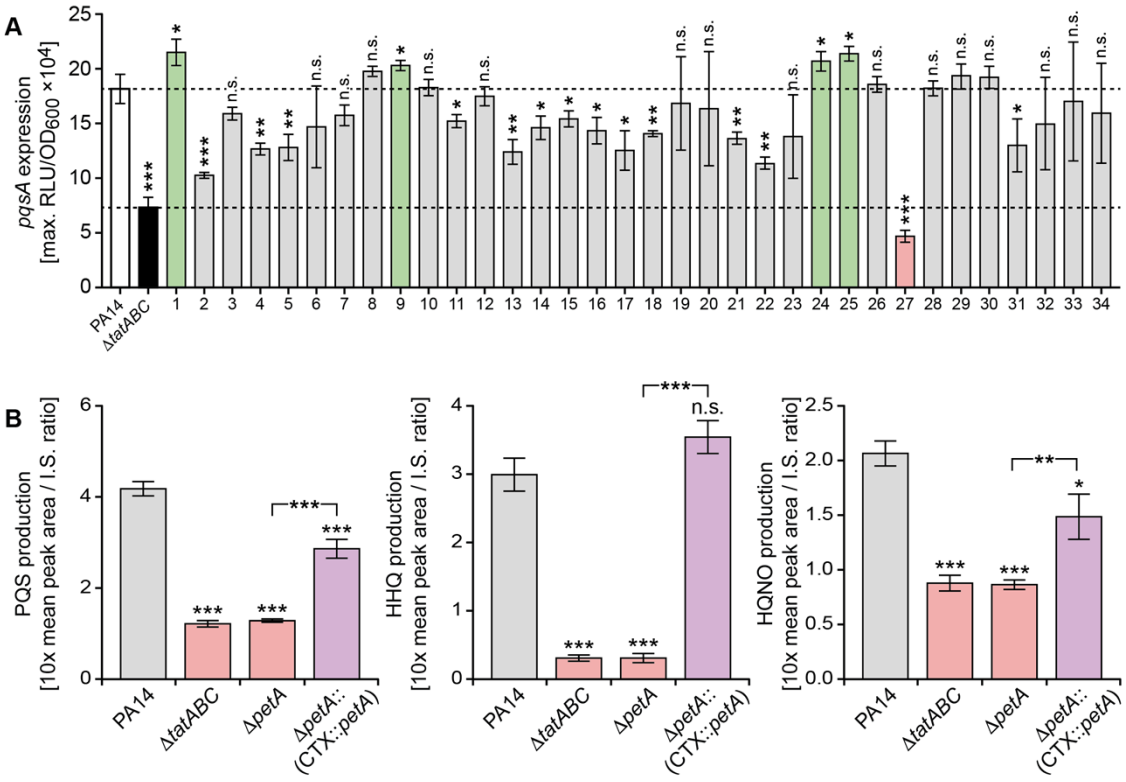

Fig 9. Tat substrate screen for *P. aeruginosa* PA14 mutants with defects in *pqs* signalling uncovers a role for the cytochrome bc_1 Rieske sub-unit. (**A**) Comparison of peak *pqsA* expression in each of 34 Tat substrate mutants (**Table S3**) transformed with a CTX::*pqsA'-lux* fusion compared with 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.