1 **Disruption of the *Pseudomonas aeruginosa* Tat system perturbs** 2 **PQS-dependent quorum sensing and biofilm maturation through** 3 **loss of the Rieske cytochrome *bc*₁ sub-unit**

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28 **Running Head:** Tat, quorum sensing and biofilms in *P. aeruginosa*

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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_L* 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 *bc₁* 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

challenges [12,13]. By intercalating with eDNA, secondary metabolites such as phenazines enhance biofilm integrity [12,15]. Pyocyanin for example can contribute to DNA release through the formation of reactive oxygen species such as hydrogen peroxide that damage cell membranes [12]. Although the mechanism(s) responsible for eDNA release has not been fully elucidated both eDNA and MVs can be generated via explosive cell lysis mediated via a cryptic prophage endolysin encoded within the R- and F-pyocin gene clusters [14].

In *P. aeruginosa*, rhamnolipids and pyocyanin production, eDNA and MV release, and hence biofilm development, are all controlled by quorum sensing (QS) [1,8,16]. Consequently, *P. aeruginosa* mutants with defects in this cell-to-cell communication system form aberrant, flat undifferentiated biofilms [10]. In *P. aeruginosa*, the QS regulatory network consists of a hierarchical cascade incorporating the overlapping *las*, *rhl* and *pqs* pathways that employ *N*-acylhomoserine lactones (AHLs) and 2-alkyl-4-quinolones (AQs) as signal molecules [1,16,17]. All three QS systems contain auto-induction loops whereby activation of a dedicated transcriptional regulator by the cognate QS signal molecule induces expression of the target synthase such that QS signal molecule production can be rapidly amplified to promote co-ordination of gene expression at the population level.

P. aeruginosa produces a diverse family of AQs and AQ *N*-oxides [18,19] of which 2-heptyl-3-hydroxy-4-quinolone (the *Pseudomonas* Quinolone Signal, PQS) and its immediate precursor 2-heptyl-4-hydroxyquinoline (HHQ) are most closely associated with PQS signalling [17]. Most of the genes required for AQ biosynthesis (*pqsABCDE*) and response (*pqsR*; also called *mvfR*) are located at the same genetic locus although *pqsH* and *pqsL* are distally located [17]. PqsA catalyses the formation of anthraniloyl-CoA that is condensed with malonyl-CoA by PqsD to form 2-aminobenzoylacetyl-CoA (2-ABA-CoA) [20,21]. The latter is converted to 2-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 RhIR-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* (*PpqsA*) 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 *bc₁* 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

initial screen, 84 Tn insertion mutants were selected and re-screened to eliminate strains with double Tn insertions and to confirm their eDNA phenotype. For 34 of the remaining mutants, the regions flanking each Tn insertion were sequenced and the corresponding genes identified. For many of the eDNA-release deficient mutants, the Tn insertions were located within genes required for AQ biosynthesis (*pqsC* and *pqsH*) or regulation (*pqsR*) (**Fig. 2**) These data confirm our previous work that first uncovered a role for PQS signalling in eDNA release [8]. Apart from the *pqs* mutants, two mutants were obtained with insertions in the *tatA* and *tatB* genes respectively (**Fig. 2**). These code for components of the twin-arginine translocation (Tat) system that mediates the export of folded proteins and was originally named with respect to the presence of an Arg-Arg motif in the signal sequence of Tat-exported products also called Tat substrates [42,43]. In *P. aeruginosa* diverse proteins involved in phosphate and iron metabolism, virulence and energy transduction are exported to the periplasm, or secreted via the Tat export system and *tat* mutants exhibit pleiotropic phenotypes [44,45]. **Fig. 2** shows that genetic complementation of the *P. aeruginosa* *tatA* mutant with a plasmid-borne copy restored eDNA release.

The Tat pathway contributes to biofilm development in P. aeruginosa

Since eDNA makes an important contribution to biofilm development and architecture [10,12,13], biofilm formation by the *P. aeruginosa* *tatA* mutant in flow-chambers was investigated. After 4-days growth, the *P. aeruginosa* wild-type and complemented *tatA*/pTatA mutant formed biofilms with mushroom-shaped structures whereas the *tatA* mutant formed thin, flat biofilms (**Fig. 3A**). Ethidium bromide staining of the latter revealed that they contained much less eDNA than the structured biofilms formed by the wild-type and complemented *tatA* mutant strain (**Fig. 3A**). Consistent with this biofilm phenotype, exposure of the biofilms formed 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 μ M 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* $\Delta pqsA$ and *tatA* $\Delta pqsA$ 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 μ M PQS) in the absence of *tatA* in the *P. aeruginosa* *tatA* $\Delta pqsA$ double mutant.

To determine the consequences of perturbed PQS signalling on the expression of the rhamnolipid (*rhIA*) 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 *rhIA* and *phzA1* respectively were constructed and introduced onto the chromosomes of the wild type, $\Delta pqsA$ and *tatA* $\Delta pqsA$ mutants respectively. **Fig. 7A** shows the expression profiles of *rhIA'-lux* as a function of time. Both the wild type and $\Delta pqsA$ mutant show an ~ 2 fold increase in *rhIA* expression when supplied with exogenous PQS (20 μ M) and share similar profiles over the growth curve. In contrast, the *rhIA'-lux* fusion in the *tatA* mutant does not show the same expression profile or response to exogenous PQS as the wild type and $\Delta pqsA$ mutant strains. The *rhIA'-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 $\Delta pqsA$ mutant

backgrounds (**Fig. 7B**). However, the *tatA ΔpqsA* 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 ΔpqsA* 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 ΔpqsA* 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 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 $\Delta tatABC$ 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 *bc₁* complex.

To confirm that the $\Delta petA$ mutant in common with the *P. aeruginosa* *tat* mutants 358 exhibited similar defects as a consequence of perturbed PQS signalling, we 359 compared their AQ production (**Fig. 9B**), *rhIA* expression, and eDNA release in 360 biofilms (**S6 Fig.** and **S7 Fig.**). Comparison of PQS, HHQ and HQNO production in 361 PA14 with the $\Delta petA$ mutant and the strain with a *cis* complementation of the *petA* 362 mutation at the CTX site confirmed the loss and increase in AQ production (**Fig. 9B**). 363 In *P. aeruginosa* PA14, the *rhIA* expression profiles for the parent and $\Delta tatABC$ 364 deletion mutant in the absence or presence of exogenously supplied PQS were 365 similar to those of strain PAO1 (compare **S6 Fig.** with **Fig. 7A**). Confocal 366 microscopy images of biofilm formation by *P. aeruginosa* PA14 under static growth 367 conditions shows that in common with the $\Delta tatABC$ mutant, *petA* mutant biofilms 368 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 ΔpqsA*). The data obtained confirmed that the *P. aeruginosa* *tatA ΔpqsA* mutant responded only weakly to exogenously added PQS or HHQ, albeit to a much lesser extent than the isogenic *ΔpqsA* 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 *rhIA'-lux* fusion in the *tatA* mutant with or without exogenously supplied PQS. Similar results were

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

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

AQs such as PQS are extremely hydrophobic and virtually insoluble in aqueous environments. Hence PQS signalling can be enhanced by increasing the solubility and delivery of PQS. Calfee *et al* [53] showed that rhamnolipids increase the solubility and bioactivity of PQS in *P. aeruginosa* with respect to *lasB* expression. Since rhamnolipid production is substantially reduced in the *P. aeruginosa tat* mutants, we added back purified rhamnolipids to the *tatA ΔpqsA* mutant. However, no effect on *pqsA* expression was observed indicating that the PQS signalling defect in the *tat* mutants is not simply due to the loss of rhamnolipid production and an inability to solubilize PQS.

In *Escherichia coli*, inactivation of *tat* leads to a characteristic cell envelope defect because of the mis-localization of two amidases involved in cell wall metabolism. This results in the formation of bacterial chains, leakage of periplasmic proteins and enhanced susceptibility to detergents [56]. Since PQS is a signal molecule that must 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 *bc₁* 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 *bc₁* 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 *bc₁* 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 *b₁* 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 *bc₁* 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

virulence factor production, biofilm eDNA and the tobramycin tolerance of *P. aeruginosa* biofilms. 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.

Materials and Methods

Bacterial strains and growth conditions

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

Mutant construction, screening and validation

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

UF, Tat3D-UR, Tat3D-DF and Tat3D-DR (**S2 Table**) to introduce the up- and down-stream regions of the *tatABC* locus into the suicide vector pME3087 to generate pME3087::*tatABC* (**S1 Table**). The latter was introduced into *P. aeruginosa* via conjugation with *E. coli* S17-1 λ pir followed by enrichment for tetracycline-sensitive cells as described by Ye *et al* [64]. The Δ *tatABC* deletion in *P. aeruginosa* PAO1 was confirmed by PCR and sequence analysis. To generate the PA14 Δ *petA* mutant, 500 bp upstream and downstream of the gene were amplified using respectively primers S.PA4431upFor/S.PA4431upRev and S.PA4431downFor/S.PA4431downRev listed in **S2 Table**. The PCR product was cloned in pKNG101 suicide vector by one-step sequence and ligation-independent cloning (SLIC) [65] which was then sequenced. The resulting plasmid, pKNG Δ *petA*, maintained in the *E. coli* CC118 λ pir strain, was then mobilized in *P. aeruginosa* strains. The mutant, in which the double recombination events occurred, was confirmed by PCR analysis. A similar strategy was used to construct allelic replacement mutants for each of 34 validated Tat substrates [45]. These strains are summarized in **S3 Table** and their validation will be described in detail elsewhere. For the generation of the *petA* cis-complemented strain, PA14 Δ *petA*::(*CTX1*::*petA*), the *petA* genes along with a 500 bp fragment corresponding to the putative promoter region for the *petA* gene were PCR amplified using S-4431CTXFor/S-4431CTXRev and cloned by SLIC into the mini-CTX1 vector yielding pminiCTX1-*petA*. Transfer of this plasmid in *P. aeruginosa* Δ *petA* strain was carried out by triparental mating using the conjugative properties of the helper plasmid pRK2013. The recombinant clones containing the mini-CTX inserted at the *attB* locus on the *P. aeruginosa* genome were selected on tetracycline-containing PIA generating PA14 Δ *petA*::(*attB*::*petA*).

Construction of a *tatA* complementation plasmid

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

and *tatA* $\Delta pqsA$ mutants. Transformants were selected on LB plates containing 200 $\mu\text{g ml}^{-1}$ 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*, *rhIA*, *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

monitoring green fluorescent protein, Syto9, propidium iodide, and ethidium bromide. Images were obtained using a 63x/1.4 objective or a 40x/1.3 objective. Simulated 3-D images and sections were generated using the IMARIS software package (Bitplane AG, Zürich, Switzerland).

AQ, pyocyanin, rhamnolipid and MV analysis

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

Statistical Analysis.

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

Supporting Information

S1 Fig. The Tat inhibitor Bayer 11-7082 has no effect on light output at 20 or 40 μ M from *P. aeruginosa* CTX:*ptac'-luxCDABE* chromosomal reporter fusion. The co-solvent DMSO, had no effect at 0.8% on the *lux* reporter fusion either. Data are presented as maximal light output as a function of growth (RLU/OD₆₀₀). Experiments were repeated in triplicate at least twice.

S2 Fig. Deletion of the *P. aeruginosa* *tatABC* genes does not influence *pqsR* expression. The data show that there are no differences in the expression of a chromosomal CTX::*pqsR'-luxCDABE* fusion in the *P. aeruginosa* wild type compared with the Δ *tatABC* mutant. Data are presented as maximal light output as a function of growth (RLU/OD₆₀₀). Experiments were repeated in triplicate at least twice.

S3 Fig. AQ biosynthesis is not restored in a *tatA* Δ *pqsA* double mutant by plasmid-borne *pqsABCD* in the absence of autoinduction. Semi-quantitative analysis by LC-MS/MS of PQS, HHQ and HQNO production by *P. aeruginosa* *pqsA* and *tatA* Δ *pqsA* mutants respectively without or with the *pqsABCD* biosynthetic genes provided *in trans* via pBBRMCS::*pqsABCD*. Experiments were repeated in triplicate at least twice.

S4 Fig. HHQ and PQS do not accumulate intracellularly in a *tatA* Δ *pqsA* double mutant harboring the plasmid-borne *pqsABCD* genes in the absence of autoinduction. Semi-quantitative analysis by LC-MS/MS of PQS (**A**) and HHQ (**B**) extracted from whole cells of *P. aeruginosa* wild type and the *tatA* Δ *pqsA* mutant without or with the *pqsABCD* biosynthetic genes provided via pBBRMCS::*pqsABCD*. Cells were harvested at 8 h and 16 h respectively. Experiments were repeated in triplicate.

S5 Fig. Exogenous rhamnolipids do not enhance PQS-dependent expression of *pqsA* in a *tatA ΔpqsA* mutant. PQS (40 μM) was added with or without purified rhamnolipids (50 μg/ml) to a *pqsA* mutant or a *tatA ΔpqsA* mutant carrying chromosomal *pqsA'-lux* fusions. Maximal light output as a function of growth (RLU/OD₆₀₀) is presented. Experiments were repeated in triplicate at least twice.

S6 Fig Rhamnolipid biosynthesis gene *rhIA* shows altered expression profiles in *P. aeruginosa* PA14 *ΔtatABC* and *ΔpetA* mutants compared with wild type and fail to respond to exogenous PQS. Bioluminescence from a chromosomal *rhIA'-lux* fusion as a function of growth (RLU/OD) over time when introduced into (A) the PA14 wild type, (B) *ΔtatABC* and (C) *ΔpetA* mutants in the absence or presence of exogenous PQS (20 μM).

S7 Fig Deletion of *petA* in *P. aeruginosa* PA14 results in a reduction in the eDNA content of biofilms. Biofilms of wild type PA14, the *ΔtatABC* and *ΔpetA* mutants and the genetically complemented PA14 mutant (*ΔpetA::*(CTX::*petA*)) were grown cultured statically and stained for eDNA with YOYO-1. (A) CLSM images and (B) eDNA quantification. Experiments were repeated in triplicate at least twice. ***p < 0.001, **p < 0.01.

S1 Table Strains and plasmids used in this study

S2 Table Oligonucleotide primers used in this study

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, $\Delta pqsa$, *tatA*, and *tatABC* deletion mutants and the *tatA* mutant complemented with plasmid-borne *tatA*. The impact of exogenous PQS (40 μ M) on the *tatA* and $\Delta tatABC$ 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 $\Delta tatABC$ mutants compared with the wild type, $\Delta pqsa$ 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* $\Delta pqsa$ 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/OD₆₀₀). 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 (*rhIA*) 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 *rhIA*'-*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 μ M). **(B)** Maximal light output from a chromosomal *phzA1*'-*lux* fusion as a function of growth (RLU/OD₆₀₀) when introduced into the wild type, *pqsA*, or *pqsA* *tatA* mutants in the absence or presence of exogenous PQS (10 or 40 μ M) 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 $\Delta tatABC$ 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 *bc₁* 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 *bc₁*. **(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.