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The *Pseudomonas aeruginosa* 4-Quinolone Signal Molecules HHQ and PQS Play Multifunctional Roles in Quorum Sensing and Iron Entrapment

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SUMMARY

Pseudomonas aeruginosa produces 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS), a quorum-sensing (QS) signal that regulates numerous virulence genes including those involved in iron scavenging. Biophysical analysis revealed that 2-alkyl-3-hydroxy-4-quinolones form complexes with iron(III) at physiological pH. The overall stability constant of 2-methyl-3-hydroxy-4-quinolone iron(III) complex was log β_3 = 36.2 with a pFe^{3+} value of 16.6 at pH 7.4. PQS was found to operate via at least three distinct signaling pathways, and its precursor, 2-heptyl-4-quinolone (HHQ), which does not form an iron complex, was discovered to function as an autoinducer molecule per se. When PQS was supplied to a *P. aeruginosa* mutant unable to make pyoverdine or pyochelin, PQS associated with the cell envelope and inhibited bacterial growth, a finding that reveals a secondary function for PQS in iron entrapment to facilitate siderophore-mediated iron delivery.

INTRODUCTION

Although unicellular, bacteria are highly interactive and the term "quorum sensing" (QS) is used to describe the cell-to-cell communication mechanisms that control bacterial population-dependent gene expression. QS improves bacterial access to nutrients and specific environmental niches, promotes collective defense, and facilitates survival through differentiation into morphological forms better able to combat environmental threats [1, 2]. It depends on the synthesis of small diffusible molecules that are often referred to as "autoinducers" as they induce their own synthesis. As the bacterial population density increases, so does QS signal molecule synthesis, and consequently, the concentration in the external environment rises. Once a critical threshold concentration has been reached, a target sensor kinase or response regulator is activated (or repressed) so controlling the expression of QS-dependent target genes [2].

QS signal molecules are chemically diverse and are involved in controlling the expression of genes involved in secondary metabolite production, plasmid transfer, bioluminescence, motility, biofilm maturation, and virulence in diverse bacterial genera [2]. Some species of bacteria possess multiple interdependent QS systems. The opportunistic human pathogen *Pseudomonas aeruginosa* possesses two *N*-acylhomoserine lactone (AHL)-dependent QS systems consisting of two pairs of LuxRI homologs, LasRI and RhlRI, respectively. LasR and RhlR are both LuxR-type transcriptional activators that are activated by *N*-acylhomoserine lactones (AHLs) synthesized via LasI (*N*-(3-oxododecanoyl)homoserine lactone) and RhlI (*N*-butanoylhomoserine lactone), respectively [3–5]. Transcriptomic studies [6–8] have revealed that the *las*- and *rhl*-regulated genes and operons constitute over 6% of the genome and are scattered throughout the chromosome, supporting the view that the *P. aeruginosa* QS circuitry constitutes a global regulatory system [9].

The *P. aeruginosa las* and *rhl* regulatory circuitry is linked to a second QS signaling system, which employs 2-heptyl-3-hydroxy-4(1*H*)-quinolone, the pseudomonas quinolone signal (PQS; for structure, see Figure 1) [10, 11]. In common with the AHLs, PQS regulates the production of virulence determinants including elastase, rhamnolipids, the galactophilic lectin, LecA, and pyocyanin (a blue-green phenazine pigment) and influences biofilm development [10, 12]. In contrast to the AHLs, when supplied exogenously, PQS overcomes the cell-population density-dependent production of *P. aeruginosa* exoproducts [12].

PQS is synthesized via a "head-to-head" condensation of anthranilate and β-keto dodecanoate [13] and requires the products of the *pqsA*, *pqsB*, *pqsC*, and *pqsD* genes, which also generate over 50 other 2-alkyl-4-quinolones (AHQs) including 2-heptyl-4(1*H*)-quinolone (HHQ) (Figure 1) [14, 15]. Many of these AHQs are, however, produced at low levels, and consequently their biological

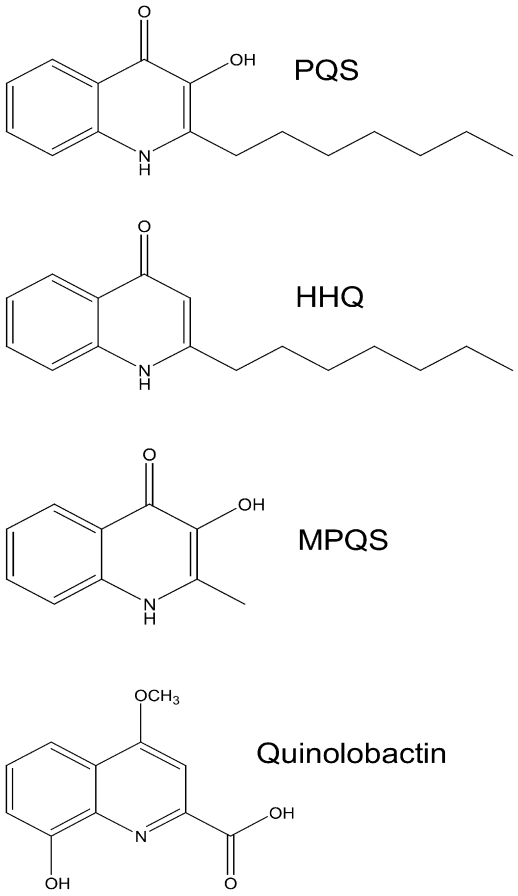

Figure 1. Structures of 2-Heptyl-3-Hydroxy-4-Quinolone, PQS; 2-Heptyl-4-Quinolone, HHQ; 2-Methyl-3-Hydroxy-4-Quinolone, MPQS; and Quinolobactin

significance is not yet clear [16]. The function of the last gene in the *pqs* operon (*pqsE*) is not known, but while *pqsE* mutants produce parental levels of AHQs, they do not exhibit any PQS-associated phenotypes [12, 14, 15], consequently PqsE is considered to facilitate the response to PQS [12, 14].

The immediate precursor of PQS is HHQ, and its conversion to PQS depends on the action of PqsH, a putative mono-oxygenase [14, 15] that is LasR regulated so linking the AHL and AHQ regulatory systems. Expression of the *pqsABCDE* operon and hence AHQ production is controlled by the LysR-type regulator PqsR(MvfR), which binds directly to the *pqsA* promoter [17, 18]. In DNA mobility shift assays, PqsR binding is enhanced in the presence of PQS, implying that PQS acts as a PqsR coinducer. The *pqsR* gene is itself positively regulated by *lasR* and negatively regulated by *rhlR*, establishing a further link between AHL-dependent quorum sensing and AHQ biosynthesis [17, 18] and hence AHQ signaling.

Since HHQ is released by *P. aeruginosa* cells into the extracellular milieu, it has been suggested to function as a "messenger" molecule that is subsequently converted to PQS by the cells that take it up [15]. This hypothesis is based on experiments in which pyocyanin synthesis was restored by mixing cultures of strains with mutations in LasR and PqsR. However, LasR and PqsR are global regulators, and strains carrying mutations in these genes are highly pleiotropic, and therefore this hypothesis requires confirmation by using defined *pqsA* and *pqsH* mutants that do not produce PQS and HHQ (*pqsA* mutant) or PQS (*pqsH* mutant).

Recently, Diggle et al. [19] discovered that pathogenic bacteria other than *P. aeruginosa* synthesize AHQs. *Burkholderia pseudomallei*, for example, produces AHQs and employs HHQ as a QS signal molecule but does not produce PQS because it lacks a *pqsH* homolog [19]. This finding suggested to us that HHQ might also function as a QS signal molecule in *P. aeruginosa* and indicated that PQS might also possess additional functionalities. By comparing the transcriptomes of the wild-type *P. aeruginosa* PAO1 strain and a *pqsA* mutant, we noted that numerous genes involved in siderophore-mediated iron scavenging were upregulated (S.P.D., V.J.W., P.C., M.C., and P.W., unpublished data). These included 15 genes within the pyoverdine locus (clustered in the region from PA2385 to PA2427) [20, 21] and include *pvdQ* (PA2365), which is both essential for pyoverdine synthesis and is an AHL-degrading enzyme [20, 22]. PA2426 (*pvdS*), which codes for an extracytoplasmic function sigma factor required for the expression of the pyoverdine biosynthesis genes, was also induced by PQS. In addition, the genes PA4221 to PA4229 corresponding to the pyochelin siderophore locus [23, 24] were upregulated. During the preparation of this manuscript, similar results were also reported by Bredenbruch et al. [25] who compared the transcriptomes of a wild-type *P. aeruginosa* PAO1 strain grown in the presence or absence of PQS. Using electrospray mass spectrometry, they also noted that PQS bound iron(III).

Here, we used spectrophotometric and potentiometric titrations to establish the affinity for iron(III) of 2-alkyl-3-hydroxy-4-quinolones such as PQS and to dissect the signaling pathways by which PQS and the PQS iron complex control the expression of iron-regulated and iron-independent genes. These experiments also revealed an unexpected function for HHQ as an autoinducer molecule and uncovered a dual function for PQS as a ferric iron chelator, which does not function as a siderophore but instead appears to trap iron at the cell surface in order to facilitate siderophore-mediated iron uptake.

RESULTS

Iron-Chelating Properties of 2-Alkyl-3-Hydroxy-4-Quinolones

The induction of many *P. aeruginosa* iron-regulated genes, including siderophore-dependent iron-scavenging systems by PQS, prompted us to evaluate the iron-chelating properties of 2-alkyl-4-quinolones. When assayed by using the universal CAS siderophore assay for iron chelators [26], it is clear that PQS but not HHQ is capable of disrupting the chrome azurol S iron complex by chelating iron(III)

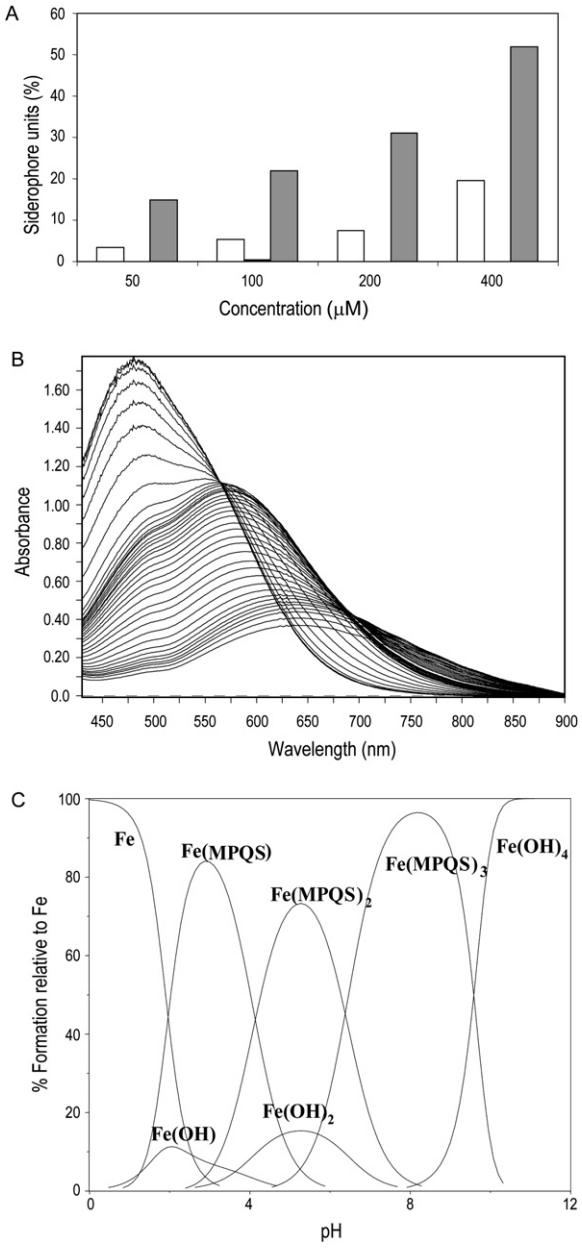

Figure 2. Iron Chelation Properties of AHQ Molecules

(A) CAS assay showing that PQS and MPQS but not HHQ chelate iron. PQS, white bars; HHQ, black bars; and MPQS, gray bars.

(B) Visible spectra of iron-MPQS complex; from pH 1.181– 9.205 [MPQS] = 246 μM, [Fe^{3+}] = 48.9 μM in 50% (v/v) DMSO/aqueous solution I = 0.1(KCl) at 25°C.

(C) Speciation plot of iron-MPQS complexes, [Fe^{3+}] = 10^{-8}M, [MPQS] = 10^{-6}M.

(Figure 2A). To determine the affinity of PQS for iron, we first attempted to obtain pKa values for the PQS-iron complex in aqueous methanol mixtures. However, because of the low solubility of the natural product under these conditions, we used the methyl analog, 2-methyl-3-hydroxy-4-quinolone (MPQS) (Figure 1), which has similar iron-chelating activity in the CAS assay (Figure 2A). For MPQS, the pKa values were 2.3 and 10.1, indicating that the neutral 4-quinolone rather than the 4-hydroxy-quinoline is the predominant species over the pH range 4–6 and therefore under physiological conditions. The three affinity constants of MPQS for iron(III) were determined by spectrophotometric titrations (Figure 2B) as log K_1 = 14.6, log K_2 = 12.0, and log K_2 = 9.6. The overall stability constant of the MPQS iron complex was found to be log β_3 = 36.2, resulting in a pFe^{3+} value [27] of 16.6 at pH 7.4. Consequently 2-alkyl-3-hydroxy-4-quinolones are ferric iron chelators. This contrasts with HHQ, the PQS precursor [15], which lacks the 3-hydroxyl moiety and does not chelate iron (Figure 2A). Furthermore, speciation plots (Figure 2C) indicate that in the pH range 6–8, a mixture of the 2:1 and 3:1 MPQS:Fe^{3+} complexes are favored. At pH 7, even at very low iron and equimolar concentrations (10^{-8} M), the speciation is such that there are approximately equimolar amounts of the 2:1 and 3:1 complexes present. Taken together, these data demonstrate that PQS and MPQS but not HHQ chelate iron(III) under biological conditions.

PQS Regulates *pvd* and *pch* Expression and Siderophore Production

To begin dissection of the specific contributions of PQS, the PQS iron complex, and HHQ to signaling in *P. aeruginosa*, we constructed four different *lux* gene reporter fusions. These were to the PQS-regulated genes *pvdE* and *pvdS* that are required for the biosynthesis and regulation of pyoverdine production, respectively, and to *pqsA* (the first gene in the *pqsABCDE* biosynthesis operon) and *lecA* (encodes the galactophilic lectin LecA, which is QS dependent and is involved in biofilm formation [28, 29]). These were introduced in single copy onto the chromosome of a *P. aeruginosa pqsA* mutant that cannot synthesize AHQs. Figure 3A shows that in iron-sufficient LB medium, all four fusions were strongly induced by PQS. In addition, in an independent quantitative real-time polymerase chain reaction experiment using the same growth conditions, two additional iron-transport genes involved in pyoverdine and pyochelin production (*pvdA* and *pchE*, respectively) were upregulated 6-fold and 17-fold, respectively, on addition of 20 μM PQS to the *pqsA* mutant (data not shown). These data demonstrate that PQS induces the expression of genes involved both in the regulation (*pvdS*) and biosynthesis of pyoverdine (e.g., *pvdA* and *pvdE*) and pyochelin (*pchE*), respectively.

In the *P. aeruginosa pqsA* mutant, a decrease in production of the fluorescent yellow pigmentation characteristic of the parent was noted. This could be accounted for by a reduction in pyoverdine production. To investigate this phenotype further, we assayed the levels of pyoverdine in the *P. aeruginosa* parent and *pqsA* mutant both in the absence and presence of PQS in LB medium. Figure 3B shows that pyoverdine levels were reduced by 2-fold in LB medium in the *pqsA* mutant when compared with the wild-type but were substantially increased in both following the provision of exogenous PQS (20 μM).

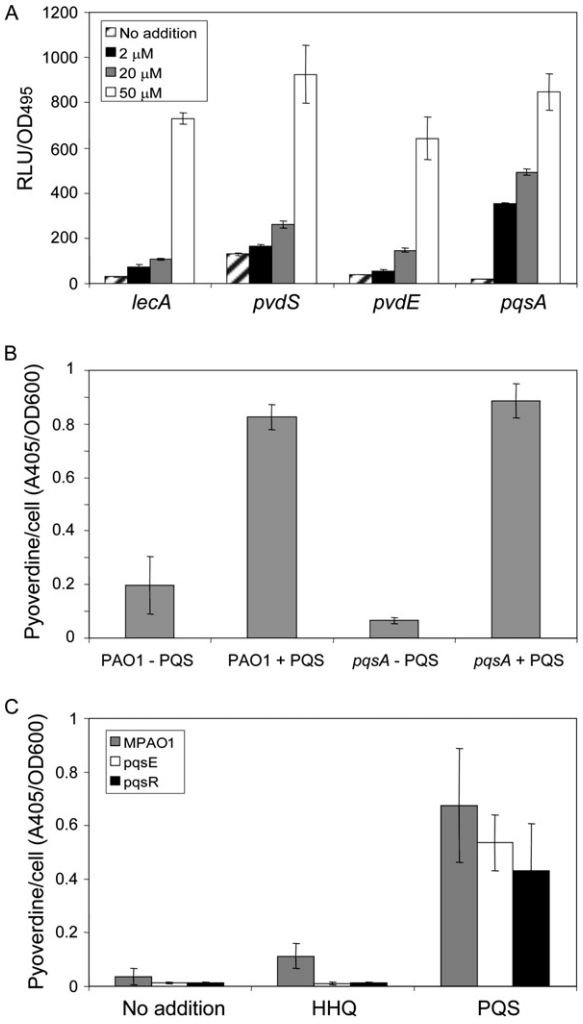

Figure 3. PQS Regulates *pvd* and *pch* Expression and Siderophore Production

(A) PQS-mediated induction of *lecA*, *pvdS*, *pvdE*, and *pqsA* in the *P. aeruginosa pqsA* mutant by PQS supplied at 2, 20, and 50 μM in LB broth. The promoter for each target gene was fused to Mini-CTX*lux* and introduced onto the chromosome of the *pqsA* mutant.

(B) Production of the *P. aeruginosa* siderophore pyoverdine is induced by PQS (20 μM) in both wild-type and *pqsA* mutant in LB broth.

(C) Production of pyoverdine is induced by PQS (50 μM) in both *pqsE* and *pqsR* mutants. Error bars represent two standard errors of the mean value of three independent measurements.

PQS Signals via Three Distinct Pathways

The expression of PQS-dependent genes such as *lecA* requires both *pqsR* (which positively regulates *pqsABCDE* and hence PQS biosynthesis) and *pqsE* (for the response to PQS), while *pqsA* expression, and hence PQS biosynthesis, depends on *pqsR* but not *pqsE* [12, 14]. To determine whether pyoverdine production was dependent on the presence of PqsE and/or PqsR, we grew *pqsE* (MPAO1 *pqsE*) and *pqsR* (MPAO1 *pqsR*) mutants in the presence/absence of 50 μM PQS or HHQ. Figure 3C shows that in the MPAO1 parent strain and both the *pqsE* and *pqsR* mutant, addition of PQS strongly induced pyoverdine production. Addition of HHQ (which does not chelate iron) did not affect pyoverdine production in either mutant. The increase in pyoverdine seen in MPAO1 can probably be attributed to increased HHQ conversion into PQS in this strain. Taken together, these data suggests that PQS signals via three different pathways, via *pqsR*, *pqsE*, and via iron chelation.

Does the Signaling Functionality of PQS Depend on Iron Chelation?

To evaluate the importance of iron chelation for the PQS-dependent regulation of *pqsA* and *lecA*, we examined their induction in response to MPQS, which chelates iron but lacks the heptyl side chain, and to HHQ, which lacks the 3-hydroxy moiety and does not chelate iron. Figures 4A and 4B show that in iron-sufficient LB medium, MPQS (which we have shown is a neutral molecule at physiological pH and therefore should be capable of accessing the cytoplasm) only weakly induces expression of both genes, highlighting the importance of the alkyl side chain in signaling and suggesting that activation of these genes does not depend on the iron-chelating properties of PQS alone. To explore further the role of iron chelation in PQS signaling, we examined the expression of *pqsA* in the PAO1 *pqsA* mutant grown in iron-deficient CAA medium since *pqsA* expression was not influenced by the growth medium iron content (data not shown). When CAA was supplemented with PQS, a 3:1 PQS iron chelate or HHQ but not MPQS (Figure 4C) or ferric chloride (data not shown), *pqsA* was strongly induced. This suggests that for certain genes such as *pqsA*, induction by either PQS or HHQ is due to direct signaling rather than removal of iron from the medium. In contrast to MPQS, HHQ was highly effective at inducing the expression of *pqsA* (Figure 4A) and also induced *lecA* although to a lesser extent (Figure 4B), implying that HHQ functions as a signal molecule per se rather than simply as a PQS precursor, at least for certain genes.

HHQ Is a Signal Molecule in *P. aeruginosa*

In Figure 4, we show that HHQ strongly induces *pqsA* expression at low concentrations (2 μM). HHQ also induced *lecA* expression, but a concentration of 50 μM was not as effective as 50 μM PQS. These data suggest that HHQ may function as a signal molecule in its own right possibly in a *pqsE*-independent but *pqsR*-dependent manner. However, in our experiments, it was possible that HHQ is being converted into PQS since the *pqsA* mutant contains an intact *pqsH* gene. To test this possibility using *lecA* as a target gene and pyocyanin as a phenotype, we mixed both *pqsA* and *pqsH* mutants together.

Figure 5A (top panel) shows that when viewed under a bioluminescent camera, *lecA::lux* (top row) is not expressed in a *pqsA* mutant but is weakly expressed in a *pqsH* mutant, which produces HHQ but not PQS. When the two mutants are mixed, *lecA* is fully induced. This experiment indicates that HHQ released by the *P. aeruginosa pqsH* mutant is converted into PQS by the *pqsA*

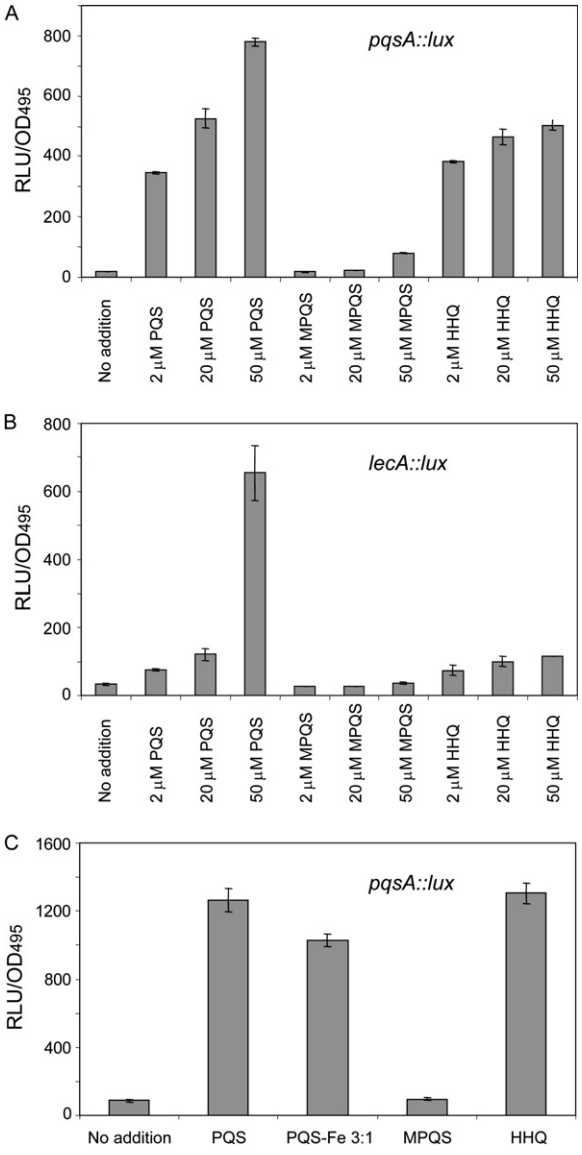

Figure 4. The Induction of *pqsA* or *lecA* Expression by PQS in LB Is Not Due to the Iron-Chelating Properties of PQS

(A and B) *P. aeruginosa pqsA* mutant containing Mini-CTX*lux* fusion to *pqsA* (A) or PAO1 *lecA::lux*Δ*pqsA* (B) were supplied with or without PQS, MPQS, or HHQ (at 2, 20, or 50 μM).

(C) PQS signaling does not depend on the PQS-iron complex. The maximum expression of mini-CTX *pqsA::lux* in a *P. aeruginosa pqsA* mutant was determined after growth in iron-deficient CAA medium supplemented without or with PQS, a 3:1 PQS:Fe^{3+} complex, MPQS, or HHQ supplied at 1.5 μM. Error bars represent two standard errors of the mean value of three independent measurements.

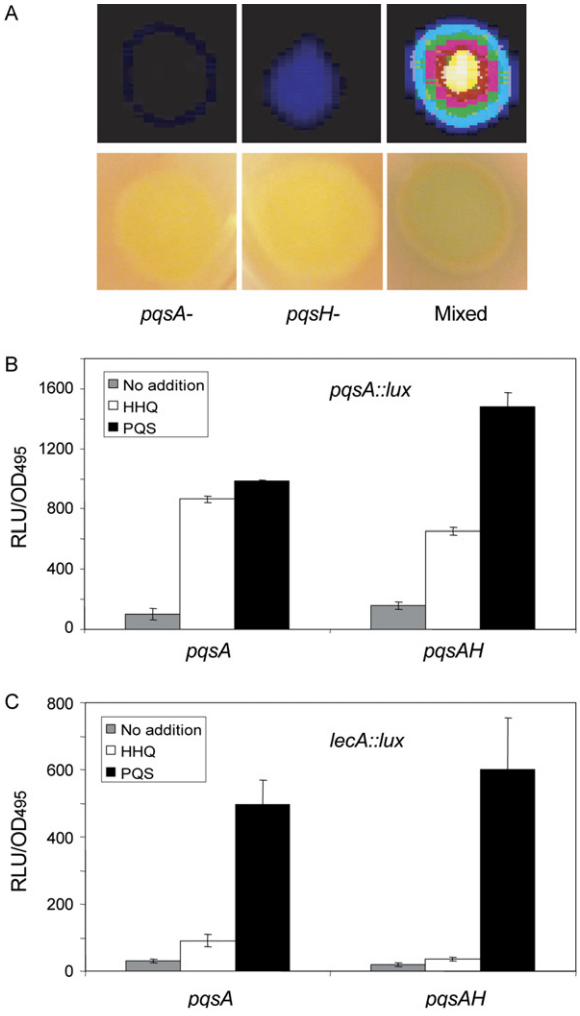

Figure 5. HHQ Functions as a Cell-to-Cell Signaling Molecule and an Autoinducer

(A) Coculture experiments with *pqsA* and *pqsH* mutants carrying *lecA::lux* fusion showing restoration of *lecA* expression (top row) and pyocyanin (bottom row).

(B) Effect of HHQ (50 μM) and PQS (50 μM) on *pqsA* expression in *pqsA* and *pqsAH* mutants.

(C) Effect of HHQ (50 μM) and PQS (50 μM) on *lecA* expression in *pqsA* and *pqsAH* mutants. Error bars represent two standard errors of the mean value of three independent measurements.

mutant due to the action of the PqsH mono-oxygenase. This finding was confirmed by both TLC and HPLC analysis (data not shown). Consequently, the maximal expression of *lecA* requires PQS rather than HHQ. Furthermore, we also noted that the blue-green pigment pyocyanin (absent in both *pqsA* and *pqsH* mutants) was restored upon mixing on the two mutants (Figure 5A, bottom panel). Again, this suggests that the production of pyocyanin relies mainly upon the presence of PQS and not HHQ.

To determine whether HHQ could function as a signal per se, we constructed a *P. aeruginosa pqsAH* double mutation in PAO1 *lecA::lux* and in the parent PAO1 strain. In the PAO1 *pqsAH* double mutant we introduced the mini-CTX *pqsA::lux* fusion. These mutants are unable to make any AHQs and do not make PQS when provided with exogenous HHQ (data not shown). Figure 5B reveals that HHQ in common with PQS significantly induces *pqsA* expression in a *pqsA* mutant. In a *pqsAH* double mutant, HHQ also causes a large induction of *pqsA* expression.

This indicates that HHQ is capable of inducing *pqsA* (and therefore its own biosynthesis) in the absence of PQS. Remarkably, the *pqsA* gene will respond to <1 μM HHQ in the absence of PQS (data not shown). HHQ also induces *lecA* expression but is much less active than PQS at a concentration of 50 μM (Figure 5C). In a *pqsAH* double mutant, addition of HHQ results in a 2-fold induction of *lecA*, but it is clear that *lecA* primarily responds to PQS. Furthermore, in a *pqsE* mutant, HHQ is unable to activate *lecA* expression, indicating that in common with PQS, HHQ requires a functional PqsE to drive expression of *lecA* (data not shown).

When taken together these data demonstrate that: (1) HHQ can function as a signal molecule without being converted to PQS, (2) HHQ is an autoinducer molecule since it drives it own synthesis via induction of *pqsA*, and (3) HHQ also acts as a messenger molecule since the conversion of HHQ to PQS is essential for maximal induction of genes such as *lecA* and those responsible for pyocyanin production.

PQS Does Not Function as a Siderophore But as an Iron Trap

Since PQS and other 2-alkyl-3-hydroxy-4-quinolones chelate iron, it is possible that they could contribute to iron acquisition by functioning as siderophores to deliver iron to the cell. To address this possibility, the *P. aeruginosa* wild-type and a *pvdD pchEF* double mutant (unable to produce either pyoverdine or pyochelin) were grown in CAA medium in the absence or presence of PQS. Figure 6A shows that PQS (20 μM) completely inhibited the growth of the double siderophore-negative mutant in an iron-deficient medium in a manner comparable to the inhibition caused by the nonutilizable iron(III) chelator EDDHA [30]. Interestingly, addition of PQS to single pyoverdine or pyochelin mutants did not significantly inhibit growth (data not shown). The PQS-dependent growth inhibition in the double mutant could, however, be completely overcome by supplementing the medium with the PQS-Fe 3:1 (20 μM) iron complex. This gave the same result as adding 10 μM $FeCl_3$ to the growth medium (Figure 6B). Previously, Lepine et al. [31] reported that 63% of PQS was associated with the *P. aeruginosa* strain PA14 bacterial cell. We have also obtained similar results in that a substantial proportion of PQS (approximately 50%–60%) was found to be associated with the PAO1 cell envelope (data not shown). When taken together, these data are consistent with the hypothesis that PQS traps the available iron from the growth medium within the cell envelope but is unable to deliver iron into the bacterial cell in the absence of a functional siderophore iron transport system.

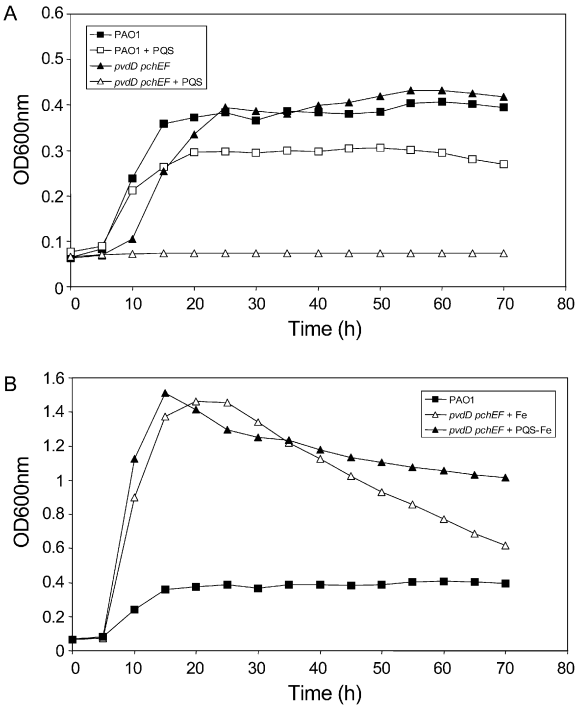

Figure 6. PQS Does Not Act as a Siderophore

(A) Growth of PAO1 and the siderophore-negative PAO1 *pvdD pchEF* double mutant in iron-deficient CAA medium in the presence or absence of 20 μM PQS.

(B) Restoration of growth of PAO1 *pvdD pchEF* mutant with 20 μM PQS-Fe^{3+}.

DISCUSSION

Among the many different AHQs produced by *P. aeruginosa*, two of the major compounds are PQS and its precursor, HHQ, although similar concentrations of 2-nonyl-4-quinolone (HNQ), 2-nonenyl-4-quinolone, and 2-heptyl-4-quinolone-*N*-oxide (HQNO) have been reported to be present in culture supernatants [16]. Consequently, the physiological functions of the AHQs have attracted significant interest especially given the role of PQS in quorum sensing. However, transcriptomic studies have revealed that PQS induces the transcription of many *P. aeruginosa* iron-regulated genes including those involved in siderophore-mediated iron uptake. This occurs when exogenous PQS is added to a wild-type *P. aeruginosa* strain [25]. Accordingly, we also noted that a subset of iron-regulated genes was upregulated in the transcriptome of the wild-type PAO1 strain when compared with the *pqsA* mutant (S.P.D., V.J.W., P.C., M.C., and P.W., unpublished data). These data suggest that *P. aeruginosa* must be sensing a lack of readily available iron despite being grown in an iron-sufficient growth medium (LB). Possible explanations are that the production of PQS is responsible for this iron shortage either because of its signaling function and/or a capacity to complex the metal such that *P. aeruginosa* self-induces a state of iron deprivation sufficient to induce siderophore production.

Given that PQS induced the expression of iron-regulated genes in iron-sufficient LB medium, we sought to determine the affinity of PQS for iron(III). The CAS siderophore assay clearly showed that PQS but not HHQ chelated iron and subsequent biophysical analysis revealed that 2-alkyl-3-hydroxy-4-quinolones are indeed iron(III) chelators forming both 2:1 and 3:1 complexes with ferric iron

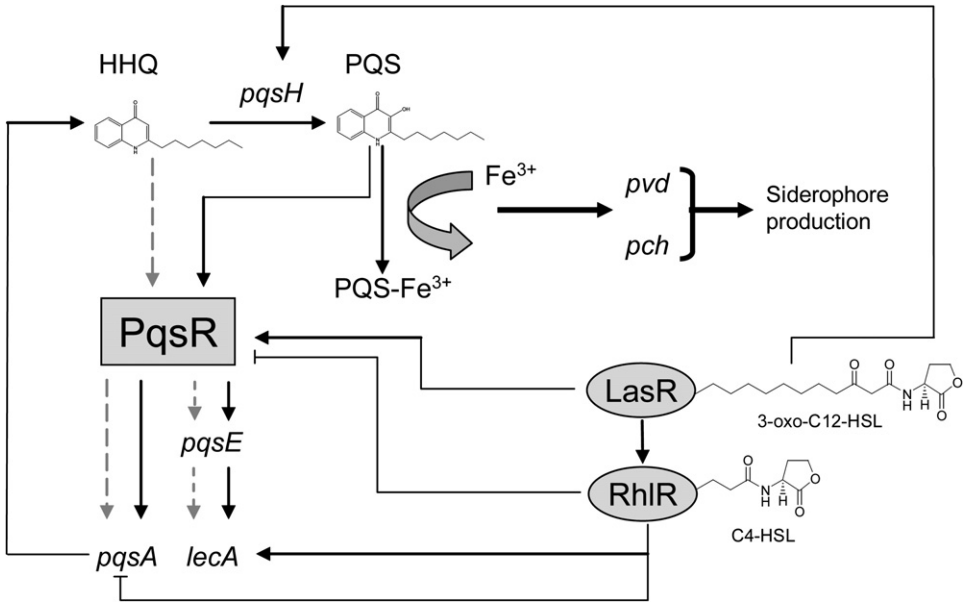

Figure 7. A Model for AHQ- and AHL-Dependent Quorum Sensing in *P. aeruginosa*

HHQ, the immediate precursor of PQS, drives the expression of *pqsA* via PqsR, and hence HHQ functions as an autoinducer, driving its own biosynthesis. HHQ is converted to PQS by the action of PqsH. PQS in common with HHQ induces the expression of *pqsA* in a PqsR-dependent manner. However, genes such as *lecA* and those required for pyocyanin biosynthesis also require PqsE. Furthermore, PQS released from the cell is capable of binding iron, forming a PQS-Fe^{3+} complex. The removal of iron from the extracellular environment by PQS induces iron acquisition genes in both a PqsR- and PqsE-independent manner and is not related to cell-to-cell signaling in *P. aeruginosa.* AHQ- and AHL-dependent quorum sensing are intimately linked since LasR/3-oxo-C12-HSL is required for full expression of *pqsH*, while *pqs*R is positively regulated by LasR/3-oxo-C12-HSL but repressed by RhlR/C4-HSL. → denotes positive regulation; —| denotes repression.

at physiological pH values. The 2-alkyl-3-hydroxy-4-quinolones such as PQS structurally resemble the 3-hydroxypyridin-4-ones, selective iron chelators, which form similar ferric iron complexes at pH 7.4 and possess similar pFe^{3+} values (~19–21) [27]. This means that they are capable of scavenging iron effectively at low ligand concentrations. The 2-alkyl-3-hydroxy-4-quinolones such as MPQS (pFe^{3+} 16.6) and PQS are, however, much weaker iron chelators than pyoverdine ($pFe^{3+} \geq 25$) [32] but show similar affinities to siderophores such as aminochelin (pFe^{3+} 17.6) [33] and in particular to quinolobactin (pFe^{3+} 16.2) (Figure 1). The latter molecule is structurally related to PQS and is able to function as a siderophore for *Pseudomonas fluorescens* ATCC 17400 [34].

To determine how PQS induces siderophore production, we examined the induction of pyoverdine synthesis in the parent, *pqsA*, *pqsE*, and *pqsR* mutants. Pyoverdine production was induced by PQS but not HHQ in each strain, indicating that PQS operates via at least three different signaling pathways. These are (1) the *pqsR* pathway in which PQS induces the expression of genes such as *pqsA* but does not require *pqsE*, (2) the *pqsR*/*pqsE* pathway in which PQS induces the expression of genes such as *lecA* and those required for pyocyanin, and (3) the iron-deprivation pathway in which the iron-chelating activity of PQS induces siderophore production (Figure 7). This pathway is likely to involve the iron-dependent repressor, Fur, which is derepressed when iron becomes limiting [35].

While the PQS precursor HHQ did not induce siderophore biosynthesis, it induced *pqsA* expression, a finding that establishes that HHQ is not just a precursor of PQS but can be classified as an autoinducer molecule since it induces its own synthesis (Figure 7). These data suggest that in common with PQS, HHQ is likely to act as PqsR coinducer. Further work will be required to determine whether HHQ interacts directly with PqsR and whether it is capable of regulating the expression of genes unrelated to AHQ biosynthesis. During the revision of this manuscript, Xiao et al. [38] reported that HHQ in common with PQS binds to the PqsR (MvfR) ligand-binding domain [36].

In an iron-deficient growth medium, there was little difference between the level of *pqsA* induced by PQS, the 3:1 PQS-iron(III) complex, and HHQ (which lacks the 3-hydroxy moiety and does not chelate iron), while MPQS (which chelates iron but lacks the C7 acyl side chain) had little effect. These data indicate that the PQS-iron complex is not required for the regulation of PQS-dependent genes. We therefore sought an alternative function for the iron-chelating properties of PQS, the most likely explanation being that PQS functions as a siderophore. However, when PQS was added to a *P. aeruginosa pvdD pchE* double mutant, which is unable to produce any siderophores (but not single pyoverdine or pyochelin mutants), bacterial growth was completely inhibited. This growth inhibition could however be overcome if the medium was supplied with sufficient iron to saturate the

PQS. These data suggest that PQS is unlikely to function as a siderophore in *P. aeruginosa*.

Since the C7 alkyl chain makes PQS a very hydrophobic molecule, the solubility and signaling properties of which have been suggested to depend either on solubilisation by rhamnolipids [37] or to localization within membrane vesicles [38]. Previously Lepine et al. [31] showed that 62.5% of PQS was associated with the bacterial cell, and so we therefore examined the distribution of PQS in the supernatant and cell envelope of our wild-type PAO1 strain. In agreement with Lepine et al. [31], 50%–60% of the molecule was found to be associated with the cell envelope (data not shown), a finding also which concurs with a previous report showing the presence of ethanol extractable HHQ and 4-hydroxy-2-nonylquinoline (i.e., 2-nonyl-4-quinolone; HNQ) in the membrane of *P. aeruginosa* grown under conditions of iron sufficiency [39]. Since Bredenbruch et al. [25] reported that exogenous PQS depletes iron from the growth medium, it is likely that PQS functions as an iron trap, mopping up the iron from growth environment and retaining it primarily in association with the *P. aeruginosa* cell surface. Here, the iron may be captured by pyoverdine and/or pyochelin and shuttled back into the cell via the respective siderophore receptor. Both the pyoverdine receptor, FpvA, and the pyochelin receptor, FptA, bind the apo forms of the cognate siderophore, which are therefore present at the cell surface and available to accept ferric ion iron from envelope-located PQS, which has a lower affinity for iron than both pyoverdine and pyochelin [40–42]. In this way, the bacterial cell, which undertook the energetically expensive biosynthesis of the siderophore, would benefit directly rather than losing the exported siderophore to other cells within the immediate growth environment. Such a dual iron chelator mechanism is reminiscent of iron scavenging in mycobacteria where the water-soluble exochelins operate in conjunction with the highly lipid-soluble outer cell envelope mycobactins [43]. However, further work will be required to determine the molecular interactions that occur between the PQS iron chelate and receptor associated apo siderophores within the cell envelope.

SIGNIFICANCE

2-alkyl-4-quinolones (AHQs) such as PQS and its precursor HHQ were originally discovered during the search for novel natural products with antibacterial activities. In *Pseudomonas aeruginosa*, which produces over 50 different AHQs, PQS functions as a QS-signaling molecule involved in the regulation of numerous virulence genes through cell-to-cell communication. AHQs, and in particular HHQ, are also used as QS signals by other pathogenic bacteria. Consequently, AHQ signaling is an attractive antibacterial target since its blockade could lead to the attenuation of virulence. Hence, there is a need to gain better insights into the molecular basis for AHQ signaling in important human pathogens such as *P. aeruginosa*. PQS signaling depends on the activity of the transcriptional regulator PqsR, which controls PQS biosynthesis, and PqsE, which is required for PQS action. Since PQS also induces both iron-regulated and iron-independent genes and as iron is an essential nutrient for *P. aeruginosa*, we used spectrophotometric and potentiometric titrations to establish the affinity for iron(III) of 2-alkyl-3-hydroxy-4-quinolones such as PQS and to dissect the signaling pathways by which PQS and the PQS iron complex regulate gene expression. From the data obtained, we established that PQS signals via at least three distinct pathways. These are (1) the *pqsR* pathway in which PQS induces the expression of genes such as *pqsA* but does not require *pqsE*, (2) the *pqsR*/*pqsE* pathway in which PQS induces the expression of genes such as *lecA*, and (3) the iron-deprivation pathway. These experiments also revealed an unexpected function for HHQ as an autoinducer molecule and revealed that a function for PQS as an iron(III) chelator, which appears to trap iron at the cell surface, maybe facilitating siderophore-mediated iron transport.

EXPERIMENTAL PROCEDURES

Bacteria, Growth Conditions, and Plasmids

The *P. aeruginosa* strains used in this study are shown in Table S1 available with this article online. Bacteria were routinely grown in iron-sufficient Luria-Bertani broth (LB) or on Pseudomonas Isolation Agar (PIA) (Difco). The iron content of LB was determined to be 6.1 μM by inductively coupled plasma-atomic emission spectrometry by the Department of Geology, Royal Holloway College, University of London. When iron-deficient conditions were required, CAA medium was used [30]. All strains were grown at 37°C in 25 ml of broth and 250 ml Schott Duran flasks with shaking at 200 rpm. Where required, synthetic PQS, HHQ, and MPQS were added at the concentrations indicated.

Synthesis of PQS, MPQS, and HHQ Analogs

PQS, MPQS, and HHQ were synthesized as previously described [12, 19].

CAS Iron-Chelation Assay

A 0.5 ml aliquot of PBS (pH 7.4) containing the relevant AHQ concentration was mixed with 0.5 ml of CAS assay solution prepared according to Schwyn and Nylands [26]. A reference was prepared by using PBS (pH 7.4) but without AHQs. The samples (s) and reference (r) absorbances at 630 nm were determined after 15 min incubation at room temperature. The percentage of iron-chelating activity was calculated by subtracting the sample A_{630} from that of the reference A_{630} value. Siderophore units are defined as $[A_r - A_s / A_r] \times 100$ = percent of siderophore units.

Iron Chelating and Biophysical Assays

The automatic titration system used in this study comprised of an autoburette (Metrohm Dosimat 765 liter ml syringe) and Mettler Toledo MP230 pH meter with Metrohm pH electrode (6.0133.100) and a reference electrode (6.0733.100). 0.1 M KCl electrolyte solution was used to maintain the ionic strength. The temperature of the test solutions was maintained in a thermostatic jacketed titration vessel at 25°C ± 0.1°C by using a Techne TE-8J temperature controller. The solution under investigation was stirred vigorously during the experiment. A Gilson Mini-plus#3 pump with speed capability (20 ml/min) was used to circulate the test solution through a Hellem quartz flow cuvette. For stability constant determinations, a 50 mm path length cuvette was used, and for pKa determinations, a cuvette path length of 10 mm was used. The flow cuvette was mounted on an HP 8453

UV-visible spectrophotometer. All instruments were interfaced to a computer and controlled by a Visual Basic program. Automatic titration and spectral scans adopted the following strategy: the pH of a solution was increased by 0.1 pH unit by the addition of KOH from the autoburette; when pH readings varied by <0.001 pH unit over a 3 s period, an incubation period was activated. For pKa determinations, a period of 1 min was adopted; for stability constant determinations, a period of 5 min was adopted. At the end of the equilibrium period, the spectrum of the solution was then recorded. The cycle was repeated automatically until the defined end point pH value was achieved. All the titration data were analyzed with the pHab program [44]. The species plot was calculated with the HYSS program [45]. Analytical grade reagent materials were used in the preparation of all solutions.

Pyoverdine Assay

To assay for pyoverdine, PAO1 and the *pqsA* mutant were incubated for 6 hr in LB medium (20 ml in 250 ml flasks) in the presence or absence of PQS (20 μM). MPAO1, *pqsE*, and *pqsR* mutants were grown for 18 hr in the presence or absence of PQS or HHQ (50 μM). Cells were removed by centrifugation, and the absorbance of the supernatant at 405 nm determined [46]. A_{405} value obtained was divided by the OD_{600} of the culture giving the amount of pyoverdine produced per cell. Experiments were performed in triplicate.

Construction of Mini-CTX*lux* Reporter Gene Fusions

Single copy fusions to *luxCDABE* of the *pqsA*, *pvdS*, and *pvdE* promoters were introduced onto the PAO1 *pqsA* mutant chromosome with mini-CTX*lux* [47]. A 532 bp fragment containing the *pqsA* promoter region was amplified and cloned into pBluescript KS+ digested with *Hind*III and *Bam*HI. The fragment was excised with *Hind*III and *Bam*HI and cloned into similarly digested Mini-CTX*1ux* [47] resulting in Mini-CTX *pqsA::lux.* A similar approach was taken for the construction of the Mini-CTX *pvdS::lux* reporter fusion. Mini-CTX *pvdE::lux* was constructed by subcloning the 0.55 kb *pvdE* promoter fragment from pMP190::*pvdE* [48] into Mini-CTX*lux.* All three constructs were introduced as single copy chromosomal insertions as described by Becher and Schweizer [47].

Time and Cell-Density-Dependent Measurement of Bioluminescence

Bioluminescence was determined as a function of cell density by using a combined, automated luminometer-spectrometer (the Anthos Labtech LUCYI) [49]. Overnight cultures of *P. aeruginosa* were diluted 1:1000 in fresh LB or CAA medium, and 0.2 ml cultures were grown in microtiter plates. Luminescence and turbidity were automatically determined every 30 min. Luminescence is given in relative light units (RLU) divided by OD_{495}. Where required, PQS, MPQS, or HHQ were supplied at the concentrations indicated.

Supplemental Data

Supplemental Data contain Table S1, which shows the bacterial strains and plasmids used in this study, and are available at http://www.chembiol.com/cgi/content/full/14/1/87/DC1/.

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