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Dioxygenase-Mediated Quenching of Quinolone-Dependent Quorum Sensing in *Pseudomonas aeruginosa*

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SUMMARY

2-Heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) is a quorum-sensing signal molecule used by *Pseudomonas aeruginosa*. The structural similarity between 3-hydroxy-2-methyl-4(1*H*)-quinolone, the natural substrate for the 2,4-dioxygenase, Hod, and PQS prompted us to investigate whether Hod quenched PQS signaling. Hod is capable of catalyzing the conversion of PQS to *N*-octanoylanthranilic acid and carbon monoxide. In *P. aeruginosa* PAO1 cultures, exogenously supplied Hod protein reduced expression of the PQS biosynthetic gene *pqsA*, expression of the PQS-regulated virulence determinants lectin A, pyocyanin, and rhamnolipids, and virulence in planta. However, the proteolytic cleavage of Hod by extracellular proteases, competitive inhibition by the PQS precursor 2-heptyl-4(1*H*)-quinolone, and PQS binding to rhamnolipids reduced the efficiency of Hod as a quorum-quenching agent. Nevertheless, these data indicate that enzyme-mediated PQS inactivation has potential as an antivirulence strategy against *P. aeruginosa*.

INTRODUCTION

Pseudomonas aeruginosa is an important opportunistic human pathogen found in soil and water habitats. It is one of the leading causes of nosocomial infections and the predominant respiratory pathogen in cystic fibrosis (CF) (Lyczak et al., 2002). In common with many pathogenic bacteria, the production of colonization, survival, and virulence factors in *P. aeruginosa* is coordinated in a growth- and cell density-dependent manner via cell-to-cell communication or quorum sensing (QS) (Williams et al., 2007). The hierarchical QS system of *P. aeruginosa* consists of an interdependent and overlapping regulatory network using *N*-acylhomoserine lactone (AHL) and 2-alkyl-4(1*H*)-quinolone (AQ) QS signal molecules (Venturi, 2006; Diggle et al., 2006; Williams and Cámara, 2009). With respect to the latter, *P. aeruginosa* produces over 50 different AQ congeners that differ mainly in the length of the 2-alkyl side chain (C5 to C13), which can be saturated or unsaturated, and in the presence or absence of a 3-position hydroxyl substituent (Lépine et al., 2004). AQs are known to possess antibacterial, anti-algal, iron-chelating, and immune modulatory activities. Among these, 2-heptyl-3-hydroxy-4(1*H*)-quinolone (the *P. aeruginosa* quinolone signal, PQS; Figure 1A) was identified by Pesci et al. (1999) as a QS signal molecule. PQS is now known to be involved in biofilm development and in the regulation of many virulence factors and secondary metabolites including the galactophilic lectin LecA, pyocyanin, elastase, rhamnolipids, and the MexGHI-OpmD multidrug efflux pump (Diggle et al., 2003; Allesen-Holm et al., 2006). A study of the global transcriptional profile of *P. aeruginosa* in response to PQS revealed a significant upregulation of genes involved in the oxidative stress response and high-affinity iron acquisition (Bredenbruch et al., 2006; Diggle et al., 2007). PQS also functions as an iron trap, sequestering iron from the growth environment and retaining it as the iron(III)-PQS complex in association with the cell surface of *P. aeruginosa* (Diggle et al., 2007). In addition, PQS has been suggested to balance life and death in *P. aeruginosa* populations by inducing a protective response in some cells while eliminating other, damaged cells via pro- and antioxidant activities (Häussler and Becker, 2008).

The biosynthesis of AQs occurs via the "head-to-head" condensation of anthranilate and a 3-oxo-fatty acid (Ritter and Luckner, 1971; Bredenbruch et al., 2005) and requires the *phnAB* operon or the *kynABU* genes for providing anthranilate, together with the *pqsABCD* operon and the *pqsH* gene (Gallagher et al., 2002; Farrow and Pesci, 2007). The latter gene product is a putative monooxygenase that oxidizes 2-heptyl-4(1*H*)-quinolone (HHQ; Figure 1A) to PQS. PqsA is an anthranilate CoA ligase, which primes anthranilate for entry into the PQS biosynthetic pathway (Coleman et al., 2008), whereas PqsD was identified as a condensing enzyme that may either catalyze the head-to-head condensation of anthranoyl-CoA with a 3-oxo acid or may be involved in the formation of a 3-oxo acid precursor (Zhang et al., 2008). PqsB and PqsC are highly homologous to 3-oxoacyl-(acyl-carrier-protein) synthases and while their precise contribution to AQ biosynthesis is not known, they are probably involved in fatty acid recruitment and condensation (Gallagher et al., 2002). The fifth gene in the *pqs* operon, PqsE, is not required for AQ biosynthesis but instead is an effector of the PQS response (Gallagher et al., 2002; Diggle et al., 2003; Déziel et al., 2004; Farrow et al., 2008). The major AQs found in *P. aeruginosa* cultures are the C7 and C9 congeners of PQS

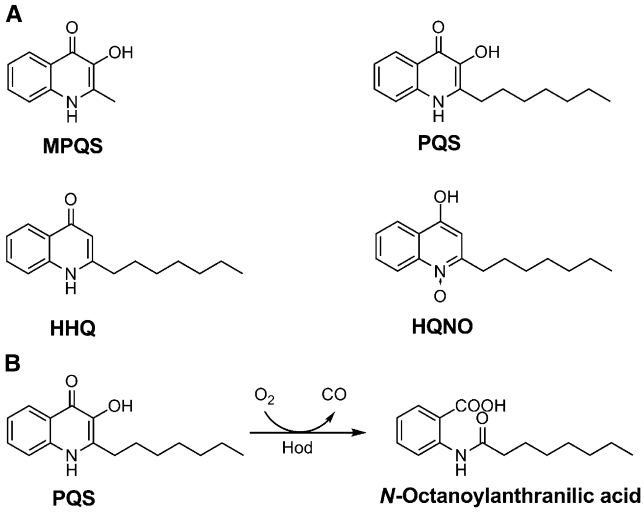

Figure 1. Structures of AQs and Mode of Dioxygenase-Catalyzed PQS Cleavage

(A) Structures of MPQS, PQS, HHQ and HQNO.

(B) 2,4-Dioxygenolytic cleavage of PQS to carbon monoxide and *N*-octanoylanthranilic acid, catalyzed by Hod from *Arthrobacter nitroguajacolicus* Rü61a. See also Figure S1.

and HHQ together with the *N*-oxides of HHQ (Figure 1A) and 2-nonyl-4(1*H*)-quinolone (HNQ).

In AQ signaling, both PQS and its precursor HHQ function as autoinducers that drive expression of the *pqsABCDE* operon through an interaction with the LysR-type transcriptional activator PqsR (MvfQ) (Déziel et al., 2005; Diggle et al., 2007; Wade et al., 2005). Consequently, when added exogenously to a *P. aeruginosa pqsA* mutant, both PQS and HHQ effectively drive the expression of a *pqsA::lux* fusion in a *pqsR*-dependent manner (Fletcher et al., 2007). However, in *P. aeruginosa*, PAO1, HHQ, in contrast to PQS, does not efficiently restore the expression of key downstream virulence genes such as *lecA* (Diggle et al., 2007). The *N*-oxides of HHQ and HNQ have little or no activity in these assays and their signaling functions, if any, have yet to be established.

PQS and other AQs are present in the sputum and bronchoalveolar lavage fluid from CF patients infected with *P. aeruginosa* (Machan et al., 1992; Collier et al., 2002). Strains isolated from infants with CF also showed increased production of PQS (Guina et al., 2003). Inactivation of AQ signaling by mutagenesis of either AQ synthesis (e.g., *pqsA*) or signal transduction genes (*pqsR* and *pqsE*) inhibits virulence gene expression and attenuates pathogenicity in experimental infection models (Cao et al., 2001; Diggle et al., 2003; Déziel et al., 2005). Furthermore, halogenated anthranilate analogs that inhibited AQ biosynthesis and signaling in laboratory cultures also restricted *P. aeruginosa* systemic dissemination and mortality in mice without perturbing bacterial viability (Lesic et al., 2007). Taken together, these observations suggest that PQS and hence AQ signaling makes an important contribution to pathogenesis and so constitutes an attractive antibacterial target. Quorum quenching and attenuation of *P. aeruginosa* virulence by enzymes that catalyze cleavage of signal molecules has been reported for AHL lactonases (Reimann et al., 2002) and AHL acylases (Lin et al., 2003; Sio et al., 2006). However, an enzyme that inactivates the PQS molecule and is capable of inhibiting AQ signaling has not been described.

3-Hydroxy-2-methyl-4(1*H*)-quinolone 2,4-dioxygenase (Hod, "1*H*-3-Hydroxy-4-oxoquinaldine 2,4-dioxygenase") of *Arthrobacter nitroguajacolicus* strain Rü61a is a cytoplasmic enzyme involved in the pathway of 2-methylquinoline (quinaldine) utilization, catalyzing the cleavage of 3-hydroxy-2-methyl-4(1*H*)-quinolone (MPQS, i.e., the C1 congener of PQS) to carbon monoxide and *N*-acetylanthranilic acid. Hod is a monomeric protein with an α/β-hydrolase fold (Fischer et al., 1999; Frerichs-Deeken et al., 2004; Steiner et al., 2007). It contains an intramolecular disulfide bond, which is important for stability and the rather unique ability of Hod to re-fold to the catalytically active native state after thermal denaturation (Beermann et al., 2007; Boehm et al., 2008). Hod, in contrast to most other known oxygenases, neither contains nor requires a metal ion or organic cofactor for catalysis (Fetzner, 2002). The *hod* gene together with the other genes coding for 2-methylquinoline conversion to anthranilate are clustered on the linear conjugative plasmid pAL1 of strain Rü61a (Overhage et al., 2005; Parschat et al., 2007). Interestingly, linear pAL1-like plasmids have been found in several other *Arthrobacter* spp. isolated from soil (Overhage et al., 2005), suggesting that the ability to degrade 2-methylquinoline and MPQS, or even other AQs, is not uncommon in soil bacteria.

The structural similarity of the natural substrate and PQS prompted us to investigate whether Hod is active against PQS and other related AQs and so capable of quenching AQ signaling in *P. aeruginosa*.

RESULTS

Products of PQS Cleavage by Hod

Hod-catalyzed conversion of PQS resulted in formation of carbon monoxide, which was detected spectrophotometrically in the form of CO hemoglobin (see Figure S1 available online), suggesting that PQS, consistent with MPQS, the physiological substrate of Hod, undergoes 2,4-dioxygenolytic ring cleavage (Figure 1B). Electrospray mass spectrometry of the extracted organic product from enzymatic turnover of PQS indicated a compound with an ion at *m/z* of 264.159 for $[M+H]^+$ and 286.141 for $[M+Na]^+$; analysis of authentic *N*-octanoylanthranilic acid showed *m/z* of 264.159 for $[M+H]^+$, consistent with the chemical composition $[C_{15}H_{22}O_3N]^+$, and *m/z* 286.142 for $[M+Na]^+$. Fragmentation of $[M+H]^+$ (*m/z* 264.159) of the product from PQS cleavage resulted in *m/z*(%) of 120.044(100) and 138.055(19) and corresponded to the fragmentation pattern of $[M+H]^+$ of the reference compound of *m/z*(%) 120.044(100) $[C_7H_6ON]^+$ and 138.055(20) $[C_7H_8O_2N]^+$ (Figure S1). The product from Hod-catalyzed PQS cleavage and authentic *N*-octanoylanthranilic acid eluted in a single peak when co-chromatographed on a reversed-phase HPLC column.

Mass spectral analyses of the extract of the enzyme assay, obtained by combined anion exchange/reversed-phase chromatography, did not indicate the additional presence of an α-oxo acid $[C_{16}H_{21}O_4N]$ that would result from 2,3-dioxygenolytic cleavage of PQS. To examine the possibility that an oxo compound might have been formed as side product, but lost

during extraction, the aqueous enzyme assays after complete conversion of the substrate were directly reacted with 2,4-dinitrophenylhydrazine. Based on calibration of the assay with pyruvic acid hydrazone (detection limit of $\geq$4.3 μM), approximately 1.9% of side product would have been detected from the Hod-catalyzed conversion of 0.23 mM PQS, if the hydrazone of an α-oxo acid formed from PQS were detected with the same sensitivity as that of pyruvic acid. However, hydrazone formation was not observed, suggesting specificity of Hod for 2,4-dioxygenolytic cleavage of PQS.

Catalytic Activity of Hod toward AQs In Vitro, in Bacterial Culture Media, and in the Presence of Iron(III) or Rhamnolipids

To determine the activity of purified Hod toward 2-alkyl-3-hydroxy-4(1*H*)-quinolones, a series of compounds with alkyl chain lengths ranging from C1 to C11 was synthesized. Notably, Hod-catalyzed dioxygenolysis of 2-ethyl-3-hydroxy-4(1*H*)-quinolone was faster than that of MPQS, but further extension of alkyl chain length impeded the reaction. Compared with the activity of Hod [in Tris/HCl buffer (pH 8)] toward MPQS of 70 U mg^{-1} (100%), relative activities of 126%, 60%, 23%, and ~0.009% were observed with 2-ethyl-, 2-propyl, 2-pentyl-, and 2-nonyl-3-hydroxy-4(1*H*)-quinolone, respectively. Conversion of the 2-undecyl-substituted congener was not detected. The catalytic activity of Hod toward PQS was 0.2 U mg^{-1}, i.e., about 0.3% of MPQS activity.

The apparent k_{cat} and K_m values of Hod for PQS, determined in Tris/HCl buffer (pH 8), were 0.16 s^{-1} and 13.4 μM, respectively. Since in air-saturated buffer $k_{cat\ app}$ and $K_{m\ app}$ of the enzyme for the physiological substrate MPQS are 38.4 s^{-1} and 2.7 μM, respectively, the catalytic efficiency $k_{cat\ app}/K_{m\ app}$ of the enzyme for PQS is about 1200-fold lower than for MPQS. UV/Vis spectral analyses indicated that HHQ, the precursor of PQS, is not converted by the enzyme. Notably, HHQ acts as competitive inhibitor of Hod-catalyzed PQS conversion [$K'_m = K_m \times (1 + [I]/K_{ic})$], with an inhibition constant K_{ic} of 24 μM. 2-Heptyl-4-hydroxyquinoline *N*-oxide (HQNO; Figure 1A) was neither a substrate nor an inhibitor of Hod.

Specific Hod activities toward the physiological substrate MPQS in bacterial culture media including sterile Luria broth (LB) (pH 7.4), casamino acids medium (pH 7; Cornelis et al., 1992), high-iron mineral salts medium (pH 7, with 25 μM $FeSO_4$; Bredenbruch et al., 2006), and QS selection medium (pH 6.7; Diggle et al., 2007) were 42 U mg^{-1}, 31 U mg^{-1}, 27 U mg^{-1}, and 28 U mg^{-1}, respectively. Hod has a broad pH optimum at pH 8.0–10.5 and shows relative activities of 92%, 84%, and 42% at pH 7.5, 7.0, and 6.5, respectively (measured in 10 mM disodium phosphate/borate buffer). Hence pH may contribute to the decreased activities observed, as compared with the activity of 70 U mg^{-1} observed in the standard biochemical assay.

MPQS and PQS were previously shown to form 2:1 and 3:1 chelate complexes with Fe(III) at physiological pH (Diggle et al., 2007). In *P. aeruginosa* cultures, the PQS dissolved in the medium and associated with the cell surface may well exist as Fe(III) complexes (Diggle et al., 2007). Since binding of Fe(III) ions to the 3-hydroxyl and 4-oxo groups of MPQS and PQS might affect substrate recognition and/or turnover by the enzyme, its activity was determined at different molar ratios of $FeCl_3$ to MPQS or PQS. In the Tris-buffered biochemical assay, >80% of catalytic activity was retained at up to 5-fold molar excess of Fe(III) over organic substrate (Figure S2), suggesting that the iron-to-PQS ratios expected to occur in *P. aeruginosa* cultures in commonly used media as well as in vivo should not significantly affect the performance of the enzyme.

When PQS (25 μM) was equilibrated in buffer with *P. aeruginosa* rhamnolipids, the apparent activity of Hod toward PQS was decreased to about 65% and 55% in the presence of 10 μg ml^{-1} and 20 μg ml^{-1} rhamnolipids, respectively. However, a further increase in rhamnolipid concentration had comparatively minor effects, since the enzyme showed about 48% of activity in a mixture of 25 μM PQS with 100 μg ml^{-1} rhamnolipids. The activity of Hod toward MPQS was not affected by the presence of rhamnolipids, excluding the possibility of denaturation of Hod by the surfactants (data not shown).

Hod-Dependent Quenching of PQS-Dependent QS in *P. aeruginosa*

To determine whether the catalytic activity of Hod was sufficient to perturb AQ signaling in *P. aeruginosa*, we first determined the impact of Hod on PQS-dependent activation of chromosomally integrated *pqsA::lux* and *lecA::lux* fusions, in a *P. aeruginosa pqsA* mutant, to circumvent any competitive inhibition due to the presence of HHQ and in the absence of endogenously produced PQS. Figure 2A shows that addition of 25 U Hod to a 0.3 ml culture results in an ~4-fold reduction in *pqsA::lux* expression when induced by 2 μM PQS. Similar experiments with the *lecA::lux* fusion (Figure 2B) and pyocyanin (Figure 2C) also show that Hod downregulates the production of both of these PQS-dependent virulence determinants in a *pqsA* mutant. When added to cultures of the wild-type *P. aeruginosa* PAO1 strain, exogenous Hod (25 U/0.3 ml) only reduced *pqsA* expression to 70% of the control (Figure 3A), whereas *lecA* expression was reduced to 37% of the control (Figure 3B). This finding is in good agreement with the levels of LecA protein (Figure 3C). While Hod had little effect on pyocyanin production (Figure 3D), at 50 U ml^{-1} it reduced rhamnolipid levels in PAO1 wild-type cultures by ~35% from 4.9 ± 0.4 to 3.2 ± 0.5 μg rhamnolipids ml^{-1} (Figure 3E).

To gain further insights into the activity of Hod on PQS in *P. aeruginosa* PAO1 wild-type cultures and to determine whether the efficacy of Hod was reduced in culture, we extracted stationary phase cultures treated with Hod and quantified PQS and HHQ levels using LC-MS. Figure 3F shows that Hod reduced PQS but not HHQ levels in PAO1 cultures in a concentration-dependent manner. However, although the enzyme retained >80% of catalytic activity after incubation in sterile LB for 2 days at 30°C or 37°C, it lost activity when incubated in cultures or spent culture supernatants of *P. aeruginosa* PAO1. The half-life of Hod activity in a batch culture was about 6 hr (Figure S3). In culture supernatant, Hod activity decreased continuously, with a similar half-life. SDS-PAGE analysis suggested that the loss of activity was due to proteolytic degradation (Figure S3). This was confirmed by incubating Hod with the *P. aeruginosa* PAO1 type II secretion (*xcp*) mutant D40ZQ that is unable to secrete a number of exoproteases (Ball et al., 2002). In this case, and in contrast to the parent PAO1 strain, Hod reduced PQS-dependent pyocyanin production by ~60% (data not shown).

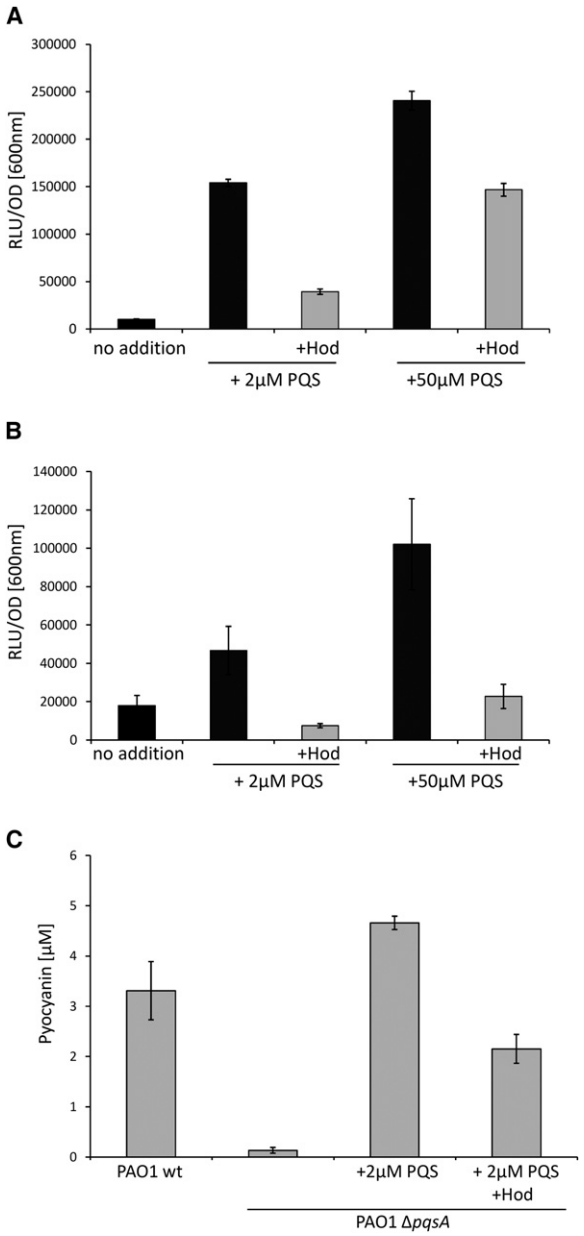

Figure 2. Hod-Mediated Quorum Quenching in an AQ-Negative *P. aeruginosa pqsA* Mutant

(A) *pqsA* expression in PAO1 *pqsA* CTX-*lux*::*pqsA* grown in the presence of 2 or 50 μM PQS and with or without Hod (25 U/0.3 ml).

(B) *lecA* expression in PAO1 *pqsA lecA*::*lux* grown in the presence of 2 or 50 μM PQS and with or without Hod (25 U/0.3 ml).

(C) Pyocyanin production in PAO1 and in PAO1 *pqsA* in the presence of 2 μM PQS and with or without Hod (25 U/0.3 ml). All experiments were carried out in triplicate at least twice. Error bars represent one standard deviation of the mean value from three independent measurements.

Hod Reduces *P. aeruginosa* Virulence in a Plant Infection Model

Although *P. aeruginosa* is not generally considered as a plant pathogen, plants have been used successfully as in vivo disease models to study pseudomonas virulence, and experimental data demonstrated conservation of virulence mechanisms between plant and animal infection models (Starkey and Rahme, 2009). PqsR (MvfR), for example, was discovered using plants as model hosts (Starkey and Rahme, 2009). Using the lettuce leaf model we demonstrated a reduced virulence of the PQS negative mutant PAO1 *pqsA* in comparison to the parent strain (data not shown). To verify if Hod can attenuate virulence in the same model we coinjected *P. aeruginosa* PAO1 with Hod, which (1) caused much less leaf rib tissue damage than the control and (2) reduced bacterial growth in the leaf tissues (Figure 4).

DISCUSSION

The multifactorial virulence of *P. aeruginosa* is tightly regulated via a sophisticated, hierarchical QS network that incorporates both AHL and AQ signal molecules and hence offers a number of different potential targets for novel antibacterials. These include the QS signal synthases (e.g., LasI, RhlI, and PqsA) and response regulator proteins (e.g., LasR, RhlR, and PqsR) as well as the QS signal molecules themselves. With respect to the latter, antibodies raised against QS signal molecule conjugates have shown efficacy in experimental animal infection models (Kaufmann et al., 2008) while enzymes such as lactonases and acylases capable of inactivating AHL-dependent QS reduce virulence gene expression in vitro and in planta (Dong et al., 2007). However, to our knowledge, no enzymes capable of quenching AQ- or PQS-dependent QS have previously been described. Here we have reported on the potential of the dioxygenase Hod to inactivate PQS, downregulate the expression of key *P. aeruginosa* PQS-dependent virulence genes, and reduce in planta growth and plant tissue damage.

When incubated with Hod, PQS undergoes 2,4-dioxygenolytic cleavage with concomitant formation of carbon monoxide, consistent with the natural substrate MPQS. Carbon monoxide is an inhibitor of the respiratory chain and also affects global transcriptional regulators of *P. aeruginosa*; however, effective inhibition requires CO to be delivered intracellularly (Davidge et al., 2009; Desmard et al., 2009).

The presence of the C7 alkyl chain substantially reduced the activity of the enzyme such that its catalytic efficiency was some 1200-fold lower than for MPQS. Interestingly, Hod was most active against the C2 (ethyl) congener of PQS with efficacy reducing as alkyl chain length was extended such that the enzyme was virtually inactive against the C9 PQS congener. Although *P. aeruginosa* produces PQS congeners with C5 to C11 alkyl chains, the C7 and to a lesser extent the C9 compounds are the most active in driving *pqsA* expression and therefore the primary 2-alkyl-3-hydroxy-4(1*H*)-quinolone QS signals (Fletcher et al., 2007). AQ biosynthesis and *pqsA* expression are not, however, exclusively dependent on PQS acting as a coinducer of the transcriptional activator protein PqsR (Wade et al., 2005; Xiao et al., 2006). This is because HHQ, the direct precursor of PQS, also drives the expression of *pqsA* in a PqsR-dependent manner and is more effective than PQS with a lower EC_{50} (0.4 μM compared with 18 μM for PQS) (Fletcher et al., 2007). Consequently, since both HHQ and PQS accumulate in *P. aeruginosa* culture supernatants, the ability of Hod to reduce AQ biosynthesis by downregulating *pqs* gene expression in culture is therefore likely to be influenced by HHQ. *P. aeruginosa* cultures also accumulate substantial concentrations of

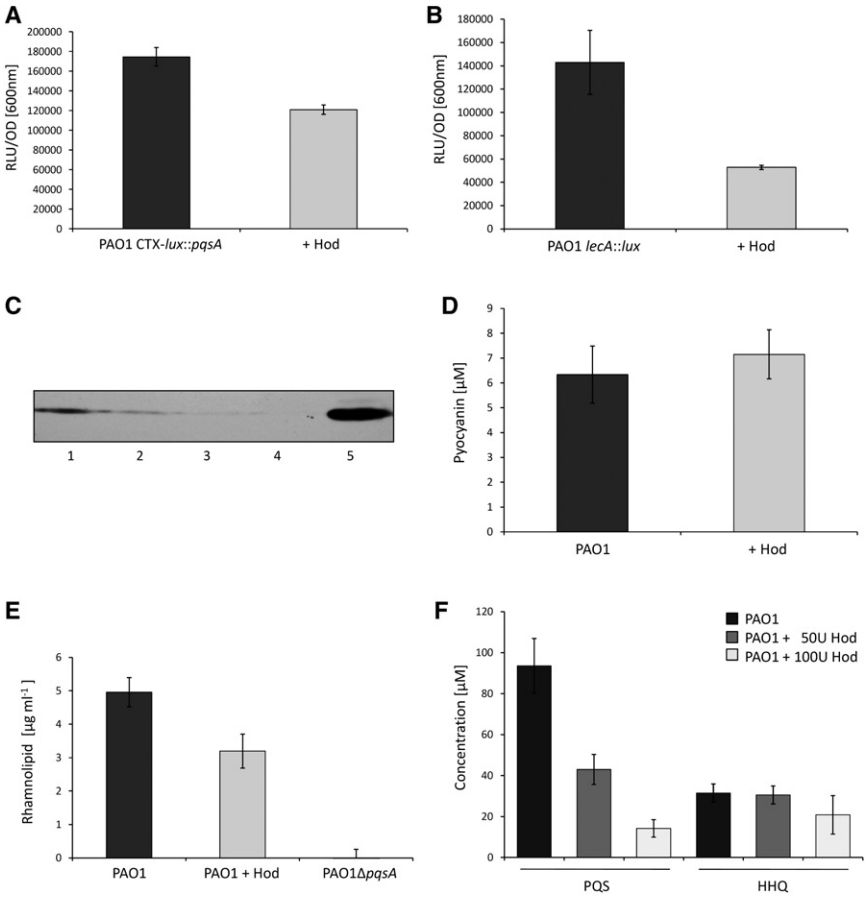

Figure 3. Hod-Mediated Quorum Quenching in *P. aeruginosa* PAO1

The impact of Hod on *pqsA* and *lecA* expression (A and B), on lectin A protein (C), on pyocyanin production (D), on rhamnolipid production (E), and on PQS and HHQ concentrations (F). For (A), (B) and (D), 25 U Hod/0.3 ml was used. The effect of Hod on rhamnolipid production (E) was analyzed with 50 U Hod/ml.

(C) Lane 1, PAO1; lane 2, PAO1 + 25 U Hod/0.3ml; lane 3, PAO1 + 50 U Hod/0.3ml; lane 4, PAO1 *pqsH* (negative control); lane 5, lectin A (purified protein control). Western blot analysis was repeated three times and each experiment was carried out in triplicate at least twice. Error bars represent one standard deviation of the mean value from three independent measurements.

the *N*-oxide HQNO. This AQ, which does not contribute to *pqsA* expression, is derived from the same substrate pool as HHQ. While HQNO proved to be neither a substrate nor an inhibitor of Hod, HHQ acted as a competitive inhibitor with a K_{ic} of 24 μM (at pH 8), implying that at least at high HHQ concentrations, Hod may not be effective at quenching PQS-dependent QS in *P. aeruginosa* cultures. In addition, other bacterial culture medium components, medium pH, and bacterial exoproducts could conceivably interfere with Hod-mediated quorum quenching. Therefore, we determined the activity of Hod toward MPQS in un-inoculated conventional rich and chemically defined laboratory bacterial culture media and found a reduction to between 38.5% and 60% of that observed at pH 8 in the standard biochemical assay. Apart from pH, the iron content of such un-inoculated growth media may also reduce the efficacy of Hod since PQS (and MPQS) forms 2:1 and 3:1 complexes with Fe(III). However, in the Tris-buffered assay even at a 5-fold molar excess of iron, over 80% of catalytic activity was retained. These data are important since they suggest that Hod should be capable of interfering with PQS signaling via all three pathways, i.e., (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 production of lectin, pyocyanin, and rhamnolipids; and (3) the iron-deprivation pathway in which the iron-chelating activity of PQS induces siderophore production (Diggle et al., 2007).

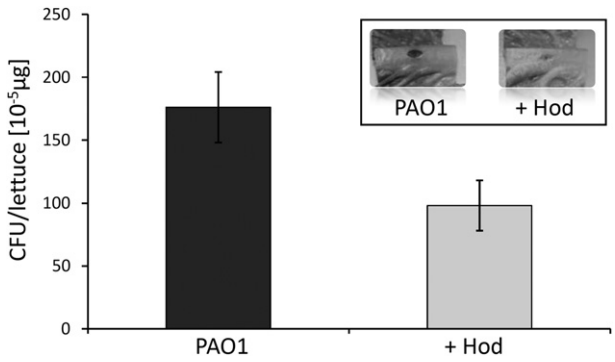

Figure 4. Hod (25 U/Lettuce Leaf) Reduces *P. aeruginosa* Growth and Tissue Damage in a Plant Leaf Infection Model

Hod was co-inoculated with PAO1 into lettuce leaf ribs and incubated for 2 days. They were monitored for soft rot damage (inset) and for bacterial viable counts (determined as cfu/μg leaf mid-rib tissue). Error bars represent the standard error of the mean value from six independent measurements.

P. aeruginosa exports biosurfactant rhamnolipids that function to facilitate swarming motility, promote the uptake of hydrophobic compounds, contribute to biofilm architecture, and induce the rapid necrotic killing of polymorphonuclear leucocytes (Jensen et al., 2007). In addition, rhamnolipids solubilize PQS (which is very poorly water soluble) and significantly enhance its bioavailability as revealed by the PQS-dependent induction of *lasB* expression in a *P. aeruginosa lasR* mutant and by the augmentation of PQS-induced apoptosis in eukaryotic cells (Calfee et al., 2005). Given the importance of rhamnolipids in PQS solubilization and PQS bioactivity, it was relevant to determine whether by sequestering PQS they reduced the efficacy of Hod. In buffer, we noted that the apparent activity of the enzyme toward PQS (25 μM) was reduced to ~55% in the presence of 20 μg ml^{-1} rhamnolipids. Further increases in

rhamnolipid concentration had only minor affects on Hod activity, which is consistent with the observations of Calfee et al. (2005) that the rhamnolipid-dependent augmentation of PQS bioactivity was diminished at high rhamnolipid concentrations.

One further group of exoproducts likely to influence Hod activity were secreted exoproteases, and we observed that in spent stationary phase *P. aeruginosa* cell-free culture supernatants, Hod was rapidly inactivated via proteolytic cleavage with a half-life of approximately 6 hr. Taken together, the above data suggested that the prevailing environmental conditions and presence of bacterial exoproducts, in particular the rhamnolipids and exoproteases, were likely to impact on the efficacy of Hod as a quorum quenching enzyme. However, both exoproteases and rhamnolipid production are regulated via QS and therefore the enzymatic inactivation of a key QS signal molecule such as PQS is likely to reduce the rate of production and accumulation of these exoproducts during bacterial growth.

To evaluate the potential efficacy of Hod as a quorum-quenching enzyme, we initially examined *pqsA::lux* and *lecA::lux* expression in a *pqsA* mutant supplied with exogenous PQS since a *pqsA* mutant does not produce HHQ or other AQs. In these experiments *pqsA* and *lecA* expression as well as pyocyanin production were substantially downregulated. In contrast, when the parent *P. aeruginosa* strain was incubated with the enzyme, *pqsA* expression was reduced by only 30% (as opposed to 63% with the *pqsA* mutant) and pyocyanin was unaffected. In contrast, *lecA* expression, lectin A protein levels, and rhamnolipids were each substantially quenched. These data can be explained by the presence of HHQ, which acts as a potent *pqsA* inducer (as well as a competitive inhibitor of Hod), and by the fact that PQS is a much more potent inducer of lectin A and rhamnolipids than HHQ. LC-MS analysis of *P. aeruginosa* wild-type cultures treated with Hod confirmed that PQS levels, unlike HHQ, were effectively reduced in a concentration-dependent manner. Because rhamnolipids are regulated via PQS (Diggle et al., 2003), the Hod-mediated reduction in PQS will affect PQS signaling directly and indirectly since lower rhamnolipid levels will in turn reduce the bioactivity of PQS and hence its activity as a QS signal. Thus, the quorum quenching of specific virulence factors observed in laboratory culture could be extended to a plant infection model where Hod reduced both virulence and bacterial growth in leaf tissues.

Despite its relatively weak PQS-inactivating activity and susceptibility to inactivation by *P. aeruginosa* exoproducts, Hod was capable of quorum quenching in vitro and in vivo. This finding is all the more interesting considering that PQS can be packaged into membrane vesicles that arise through the pinching off of the outer membrane and fusion with the envelope of other bacterial cells, serving as a mechanism for trafficking PQS within a *P. aeruginosa* population (Mashburn and Whiteley, 2005; Mashburn-Warren et al., 2008).

Recently, Hod has been crystallized (Steiner et al., 2007). Although PQS is much less susceptible to cleavage by Hod than MPQS, further insights into the three-dimensional structure of the enzyme active site may reveal opportunities for modification to accommodate the C7 side chain of PQS and improve catalytic efficiency. Similarly, it may also be possible to engineer Hod to reduce its susceptibility to *P. aeruginosa* exoproteases or to encapsulate the protein such that it is delivered in a protected environment permitting PQS substrate but not exoprotease access.

SIGNIFICANCE

Pseudomonas aeruginosa produces a variety of 2-alkyl-4(1*H*)-quinolones (AQ) that were originally discovered during the search for novel secondary metabolites with therapeutic potential. AQs have antibacterial, iron chelating, immune modulatory, and signaling properties. PQS and its precursor, HHQ, both function as QS signal molecules and are components of a sophisticated gene regulatory network coupling cell-to-cell communication to population density and the elaboration of multiple exoproduct virulence factors. Mutation of key AQ biosynthesis or signal transduction genes results in the attenuation of _P. aeruginosa_ virulence in animal and plant experimental infection models. Consequently there is considerable interest in the development of novel selective agents that prevent infection by targeting bacterial virulence rather than growth since these are less likely to select rapidly for resistance. In a search for enzymes capable of inactivating PQS-dependent QS we discovered that 3-hydroxy-2-methyl-4(1*H*)-quinolone 2,4-dioxygenase (Hod) from _Arthrobacter nitroguajacolicus_ strain Rü61a is capable of catalyzing the conversion of PQS to *N*-octanoylanthranilic acid and carbon monoxide, albeit with substantially less catalytic efficiency than its natural substrate MPQS. Despite the susceptibility of Hod to pseudomonas exoproteases and to competitive inhibition by HHQ as well as the sequestration of PQS by rhamnolipids in bacterial culture, Hod was capable of significantly reducing the expression of key _P. aeruginosa_ virulence factors and reducing growth and tissue damage in a plant leaf infection model. These data highlight the potential of quenching AQ-dependent QS and hence virulence through the enzymatic degradation of extracellular AQ signaling molecules. Besides the ability to cleave and thus inactivate PQS, Hod is also interesting as a carbon monoxide-forming enzyme. Since intracellular carbon monoxide is a potent inhibitor of the respiratory chain and also affects gene expression in _P. aeruginosa_, CO release from 3-hydroxy-4(1*H*)-alkylquinolones by intracellular Hod might significantly affect bacterial growth and metabolism.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions

The strains used in this study are listed in Table S1. The *P. aeruginosa* PAO1 *rhlR* and *pqsH* mutants were constructed by allelic exchange as described before (Fletcher et al., 2007). Conjugal transfer was performed as described by Kaniga et al. (1991). *E. coli* M15 (pREP4, pQE30-hodC69S) was grown in LB (Sambrook et al., 1989) in the presence of ampicillin (100 $\mu g\ ml^{-1}$) and kanamycin (25 $\mu g\ ml^{-1}$) at 37°C. At an optical density ($OD_{600\ nm}$) of 0.5, gene expression was induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside, and the cultivation temperature was decreased to 20°C. Cells were harvested by centrifugation at an OD_{600nm} of ~3.2. Unless otherwise stated, *P. aeruginosa* strains were grown with shaking in LB at 37°C.

Synthesis of AQs and *N*-octanoylanthranilic Acid

PQS, HHQ, and related compounds were synthesized as described before (Diggle et al., 2006). MPQS was synthesized from 3-formyl-2-methyl-4(1*H*)-quinolone

(Eiden et al., 1978) as described by Cornforth and James (1956). *N*-Octanoylanthranilic acid, mp 93-94°C, was prepared as a creamy solid in 82% yield by acylation of anthranilic acid solution in sodium hydroxide with octanoyl chloride according to Wells et al. (1952).

Purification of Recombinant Hod

Since Hod (GenBank: CAL09864) tends to form dimers due to oxidative formation of an intermolecular disulfide bridge (Frerichs-Deeken et al., 2004), we used protein with a substitution of cysteine-69 by serine in this study. Purification of the Hod protein, carrying an N-terminal hexahistidine tag besides the C69S substitution, from *E. coli* M15 (pREP4, pQE30-hodC69S) was performed as described in Beermann et al. (2007). The purity of the protein preparations was verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie staining. Concentrations of Hod were determined by absorption measurements using an extinction coefficient (ε_{280nm}) of 1.937 ml mg^{-1} cm^{-1} (Beermann et al., 2007).

Enzyme Assays and Kinetics

The catalytic activity of Hod toward MPQS was determined spectrophotometrically in 50 mM Tris/HCl (pH 8), or in 10 mM disodium phosphate/borate buffer (pH 8) as described previously (Frerichs-Deeken et al., 2004). All assays were performed in air-saturated buffer. Activity toward 2-ethyl-, 2-propyl-, 2-pentyl-, 2-heptyl-, and 2-nonyl-3-hydroxy-4(1*H*)-quinolone was determined spectrophotometrically, using molar extinction coefficients [in 50 mM Tris/HCl buffer (pH 8)] of ε_{335nm} = 9.72 × 10^3 M^{-1} cm^{-1}, ε_{337nm} = 9.55 × 10^3 M^{-1} cm^{-1}, ε_{382nm} = 5.86 × 10^3 M^{-1} cm^{-1}, ε_{377nm} = 10.89 × 10^3 M^{-1} cm^{-1}, and ε_{370nm} = 10.88 × 10^3 M^{-1} cm^{-1}, respectively. Enzyme-catalyzed conversion of 2-undecyl-3-hydroxy-4(1*H*)-quinolone was tested by recording UV/Vis-spectra of the compound, incubated in buffer with 5 μM Hod for up to 48 hr. Stock solutions of AQs (10 mM) were prepared in methanol. In the standard assays, final concentrations of MPQS and PQS were 100 μM and 45 μM, respectively. One unit of enzyme activity was defined as the amount of enzyme required to consume 1 μmol of substrate per minute at 30°C under the conditions described. To determine the apparent kinetic constants of Hod for PQS, final PQS concentrations of 3.5–45 μM were used in the assays and apparent K_m and k_{cat} values were deduced from Hanes plots. Four independent series of measurements were performed, using different preparations of Hod, and each assay within a series was done at least in triplicate. Standard deviations for all apparent K_m and k_{cat} values were below ±17% of the average value within a series of experiments (i.e., for individual protein preparations), but up to ±30% ($K_{m\ app}$) and up to ±43% ($k_{cat\ app}$) of the value among independent experiments, i.e., among different protein preparations.

Hod-catalyzed conversion of HHQ was assessed by recording UV/Vis spectra (200–400 nm) for up to 16 hr of an assay mixture that contained 5 μM of enzyme. To examine whether HHQ and HQNO act as inhibitors of Hod, its activity toward PQS (10–50 μM) was determined in the presence of 20, 35, 40, and 50 μM HHQ and 50 μM HQNO. Two independent series of experiments were performed, and each assay was performed in triplicate. The activity of Hod in different culture media was also estimated in spectrophotometric assays, after determining the corresponding molar absorption coefficients, ε_{334nm}, of MPQS.

To determine the effect of *P. aeruginosa* rhamnolipids on the activity of Hod toward PQS, 25 μM PQS was equilibrated for 20 hr (at 37°C and 550 rpm) in 50 mM Tris/HCl buffer (pH 7.4) with different concentrations of rhamnolipids (5–300 μg ml^{-1}). After addition of Hod (40 μg ml^{-1}), PQS cleavage was monitored at 377 nm. Since the presence of rhamnolipids changes the UV spectrum of PQS, indicating close interaction, the molar extinction coefficient (at 377 nm) of PQS was determined for all PQS-rhamnolipid mixtures tested.

Identification of Products Formed from PQS

Enzyme-catalyzed formation of carbon monoxide from PQS was detected using a highly specific spectrophotometric assay developed by Klendshoj et al. (1950). The organic product of Hod-catalyzed cleavage of PQS was purified by combined anion exchange/reversed-phase chromatography and analyzed by mass spectrometry. For details, see Supplemental Experimental Procedures.

To assess whether the catalytic activity of Hod possibly involves 2,3-dioxygenolytic cleavage of PQS, resulting in formation of an α-oxo acid, the product from PQS conversion was reacted with 2,4-dinitrophenylhydrazine (Friedemann and Haugen, 1943). 39 μg of PQS (0.23 mM) was incubated for 8 hr with 1 mg of Hod in assay buffer. Subsequent to addition of 2,4-dinitrophenylhydrazine (dissolved in 2 M HCl) and incubation for 5 min, the reaction mixture was adjusted to alkaline pH with NaOH, and 2,4-dinitrophenylhydrazones were detected spectrophotometrically at 440 nm. The sensitivity of the assay was determined using pyruvic acid as a reference oxo acid.

Bioluminescence Reporter Gene Assays

The impact of Hod on *pqsA::lux* and *lecA::lux* promoter fusions chromosomally integrated into *P. aeruginosa* was determined using a combined, automated luminometer-spectrometer (Genios Pro; TECAN Ltd). Overnight cultures of *P. aeruginosa* were diluted 1:1000 in fresh LB medium, and 0.2 ml cultures were grown in microtiter plates. Where required, AQs and/or Hod were added at the concentrations indicated. Luminescence and turbidity were automatically determined every 30 min. Luminescence is given in relative light units divided by OD_{600nm}. All assays were carried out in triplicate at least three times.

Exoproduct Assays

Pyocyanin was extracted with chloroform from the culture supernatants of strains grown in the presence or absence of Hod in 96 well microtiter plate format and quantified spectrophotometrically at 520 nm (Essar et al., 1990) using a NanoDrop spectrophotometer (Thermo Scientific). Lectin A (PA-1L) protein levels were determined by western blot analysis of whole cell lysates as described by Winzer et al. (2000). Rhamnolipid levels in the *P. aeruginosa* wild-type with and without Hod and in the *pqsA* mutant were quantified indirectly using the orcinol method as described in Wilhelm et al. (2007) using a *P. aeruginosa* PAO1 *rhlR* mutant as a rhamnolipid negative control.

LC-MS Quantification of PQS and HHQ

AQs were extracted from *Pseudomonas aeruginosa* PAO1 culture supernatants grown in the presence or absence of Hod in 96 well microtiter plate format. 150 μl of supernatant was shaken with 300 μl of ethyl acetate acidified with acetic acid (0.1%) and centrifuged. 150 μl of the organic phase was transferred to a fresh eppendorf tube. The extraction step was repeated three times and after drying the organic fraction (total volume 450 μl), 50 μl methanol was used to solubilize the compounds for LC-MS analysis.

Lettuce Leaf Infection Model

Ten microliter aliquots of a *P. aeruginosa* culture resuspended to OD_{600nm} 0.1 in 10 mM $MgSO_4$ with or without Hod (25 U) were injected into the midribs of fresh romaine lettuce leaves incubated for 2–5 d as described by Starkey and Rahme (2009) and monitored for the appearance of soft-rot symptoms. In addition, the numbers of bacterial cells (cfu) μg^{-1} mid-rib were determined after a defined incubation period.

SUPPLEMENTAL DATA

Supplemental Data include three figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00404-9.

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