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Functional Genetic Analysis Reveals a 2-Alkyl-4-Quinolone Signaling System in the Human Pathogen *Burkholderia pseudomallei* and Related Bacteria

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Summary

Pseudomonas aeruginosa **synthesizes diverse 2-alkyl-4(1*H*)-quinolones (AHQs), including the signaling molecule 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS), via the *pqsABCDE* locus. By examining the genome databases, homologs of the *pqs* genes were identified in other bacteria. However, apart from *P. aeruginosa*, only *Burkholderia pseudomallei* and *B. thailandensis* contained a complete *pqsA–E* operon (termed *hhqA–E*). By introducing the *B. pseudomallei hhqA and hhqE* genes into *P. aeruginosa pqsA* and *pqsE* mutants, we show that they are functionally conserved and restore virulence factor and PQS production. *B. pseudomallei*, *B. thailandensis*, *B. cenocepacia*, and *P. putida* each produced 2-heptyl-4(1*H*)-quinolone (HHQ), but not PQS. Mutation of *hhqA* in *B. pseudomallei* resulted in the loss of AHQ production, altered colony morphology, and enhanced elastase production, which was reduced to parental levels by exogenous HHQ. These data reveal a role for AHQs in bacterial cell-to-cell communication beyond that seen in *P. aeruginosa*.**

Introduction

Bacterial cells are highly interactive and capable of cell-to-cell communication through the production and detection of small, diffusible signal molecules that coordinate gene expression as a function of cell population density. This phenomenon has been termed "quorum sensing" (QS) [1]. While there is considerable chemical diversity in the nature of signal molecules employed, QS systems are conserved throughout the bacterial kingdom [2]. In Gram-negative bacteria such as the opportunistic human pathogen *Pseudomonas aeruginosa*, QS signal molecules regulate virulence, secondary metabolism, swarming motility, and biofilm development via an intricate global gene regulatory network [2–4]. This network incorporates two *N*-acylhomoserine lactone (AHL)-dependent QS systems, the *las* and *rhl* systems [5–7]. These consist of a response regulator protein (LasR or RhlR) activated upon binding the cognate AHL, either *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) or *N*-butanoyl-L-homoserine lactone (C4-HSL), which are synthesized via LasI or RhlI, respectively [6–8] (Figure 1A). *P. aeruginosa* also produces a variety of 2-alkyl-4(1*H*)-quinolones (AHQs), some of which were originally identified from their antibacterial properties [9–12] (Figure 1A). Indeed, *P. aeruginosa* has been shown to produce over 50 AHQs [12], although the biological function of many of these is not known. One of these compounds, 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS), was discovered to function as a diffusible signal molecule and was termed the pseudomonas quinolone signal (PQS; Figure 1A) [3]. Subsequently, PQS was shown to regulate *P. aeruginosa* virulence gene expression [13–15] and to function as an integral component of the QS network, since PQS production is modulated by both the *las* and *rhl* systems [3, 14, 16]. Furthermore, *P. aeruginosa* strains carrying mutations in the MexGHI-OpmD efflux pump are unable to produce PQS [15] and are severely attenuated in both mouse and plant experimental infection models. PQS has been found in the lungs of cystic fibrosis patients infected with *P. aeruginosa* [17], and it exhibits potent immune modulatory activity [18]. It is derived from anthranilate [19], and its biosynthesis requires a functional *pqsABCDE* operon [20]. The PQS biosynthetic genes *pqsA–D* [20] are responsible for the production of more than 50 AHQs, including the PQS precursor 2-heptyl-4(1*H*)-quinolone (HHQ) (Figure 1A) (R = *n*-C_7H_{15}) [14, 19]. Although the precise enzymatic contribution of each *pqs* biosynthetic gene to AHQ biosynthesis has not been determined, it is clear from isotope-labeling experiments that AHQs are synthesized in *P. aeruginosa* via a "head-to-head" condensation of anthranilate and β-keto fatty acids [21] (Figure 1B). From sequence comparisons, *pqsA* codes for a putative coenzyme A ligase, *pqsB* and *pqsC* code for two β-keto-acyl-acyl carrier protein synthases, and *pqsD* is predicted to be a transacetylase homologous to FabH1. The last gene in the *pqs* operon is *pqsE*. Since *pqsE* mutants produce parental levels of PQS and HHQ but do not exhibit any PQS-associated phenotypes [14, 19, 20], PqsE is considered to facilitate the response to PQS [14, 20].

Many QS signal molecules are derived from primary cellular metabolites such as *S*-adenosyl-L-methionine (SAM), which is required for AHL and autoinducer-2 (AI-2) synthesis, while fatty acids are required for both AHL and AHQ production [23]. AHQ biosynthesis also requires anthranilate, an intermediate in aromatic amino acid metabolism. Given that AHQ synthesis employs common substrates from primary metabolism, this suggests that other bacteria may also be capable of making

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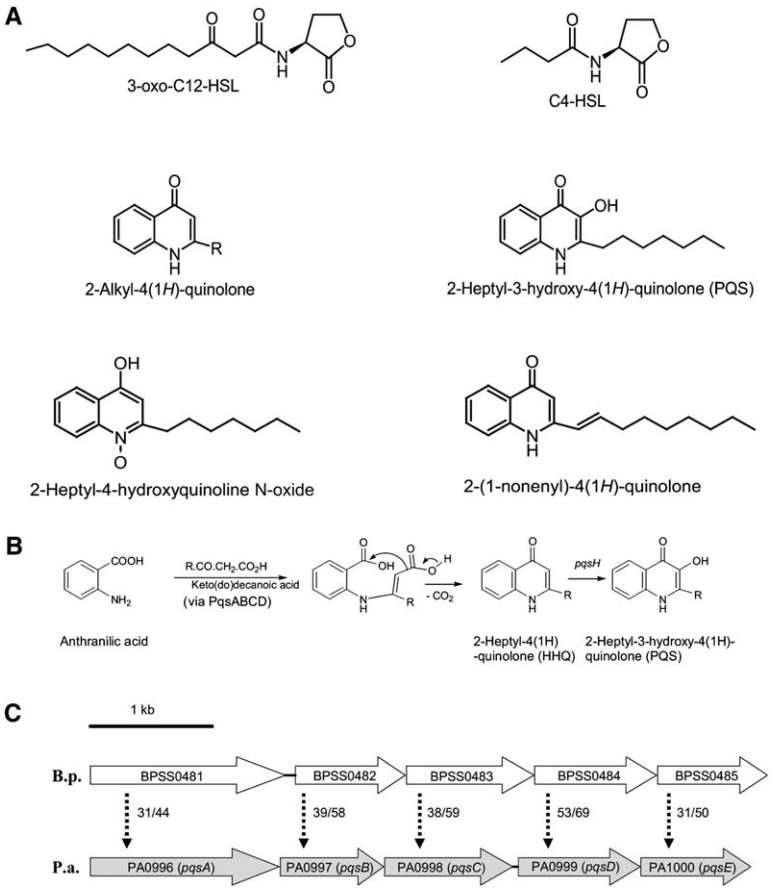

Figure 1. Quorum Sensing Signal Molecules Produced by *P. aeruginosa*, and a Putative *pqsABCDE* Operon in *B. pseudomallei*

(A) *N*-acyl homoserine lactones (AHLs) and 2-alkyl-4(1*H*)-quinolones (AHQs) known to be produced by *P. aeruginosa*.

(B) Scheme showing the biosynthesis of HHQ and PQS from anthranilate via PqsABCD.

(C) Comparison of the *B. pseudomallei* K96243 (**B.p.**) *hhqABCDE* operon and the *P. aeruginosa* PAO1 (**P.a.**) *pqsABCDE* operon. Values represent identity/similarity from a direct protein-protein comparison with the blastp program (http://www.ncbi.nlm.nih.gov/BLAST/).

AHQ signal molecules, although, to our knowledge, these have not been isolated from any other species of bacteria except *P. aeruginosa*. Previously, Lépine et al. [22] assayed culture supernatants of several species of *Pseudomonas*, including *P. fluorescens*, *P. fragi*, and *P. syringae*, for PQS and the *N*-oxide isomer of PQS, but they did not detect either quinolone.

Examination of the currently completed bacterial genomes revealed that many species of both Gram-positive and Gram-negative bacteria contain putative *pqs* gene homologs, but we focused on several species of the genera *Pseudomonas* and *Burkholderia*, which contain genes that could potentially be involved in AHQ biosynthesis. Here, we show that the human pathogens *B. pseudomallei* and *B. cenocepacia* and the nonpathogenic *B. thailandensis* and *P. putida* produce AHQs. While none of the strains examined synthesized PQS, all produced HHQ; *B. thailandensis* produced a range of AHQs; and at least one *B. pseudomallei* strain made 2-nonenyl-4(1*H*)-quinolone (NEHQ). Mutation of *hhqA* in *B. pseudomallei* resulted in the loss of AHQ synthesis, altered colony morphology, and enhanced elastase synthesis. Exogenous HHQ reduced elastase production in the *B. pseudomallei hhqA* mutant, consistent with a role for HHQ in cell-cell signaling. In addition, the *B. pseudomallei hhqA* and *hhqE* genes both complemented the corresponding *P. aeruginosa pqsA* and *pqsE* mutants, demonstrating that the genes required for AHQ synthesis and response are functionally conserved across the two genera, and that AHQ signaling is not restricted to *P. aeruginosa*.

Results

Identification of Putative AHQ Biosynthesis Genes in *Burkholderia* and *Pseudomonas*

A search of completed and uncompleted microbial genomes (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) revealed that several species belonging to the genera *Pseudomonas* and *Burkholderia* as well as the plant pathogen *Ralstonia* contain putative homologs of the *P. aeruginosa pqs* biosynthetic genes *pqsA*, *pqsC*, and *pqsD*, respectively (Table S1; see the Supplemental Data available with this article online). However, apart from *P. aeruginosa*, only strains of the primary human pathogen *B. pseudomallei* and the serologically and genetically related *B. thailandensis* were found to contain a complete, putative *pqsABCDE* operon (Figure 1C) located on chromosome 2. When compared with *P. aeruginosa*, the *B. pseudomallei* gene products exhibit from 31% to 53% identity (Figure 1C). Similarly, the *B. thailandensis pqsA–E* genes demonstrate a high level of DNA sequence identity with those from *B. pseudomallei* (*pqsA*, 92%; *pqsB*, 89%; *pqsC*, 94%; *pqsD*, 94%; *pqsE*, 91%). Interestingly, neither *B. pseudomallei* nor *B. thailandensis* possess a homolog of the *P. aeruginosa pqsH* gene. This gene facilitates the conversion of HHQ to PQS in *P. aeruginosa*, and its absence from *B. pseudomallei* would suggest that production of PQS was unlikely in this organism. Therefore, we have designated the putative *B. pseudomallei* and *B. thailandensis* AHQ biosynthesis operon *hhqABCDE*.

Transcomplementation of *P. aeruginosa pqsA* and *pqsE* Genes with the Corresponding *B. pseudomallei* Homologs

The identification of a putative *pqsABCDE* AHQ biosynthetic operon in *B. pseudomallei* (Figure 1C) suggested that these genes may be functionally equivalent to those of *P. aeruginosa* PAO1 and PA14 strains. To test this hypothesis, we separately cloned the *hhqA* (BPSS0481) and *hhqE* (BPSS0485) genes into the plasmid pUCP18 [24] and introduced the constructs into *P. aeruginosa pqsA* and *pqsE* mutants, respectively. Figure 2A shows that the loss of PQS (and HHQ) production in the *pqsA* mutant can be restored by complementation with the *hhqA* gene from *B. pseudomallei*, which also restores pyocyanin synthesis (Figure 2B). In *P. aeruginosa*, mutation of *pqsE* has little effect on PQS synthesis, as PqsE is required for the response of the organism to PQS [20]. The introduction of the *B. pseudomallei hhqE* gene did not affect PQS biosynthesis when introduced into the PAO1 *pqsE* mutant (data not shown). However, Figure 2B shows that pyocyanin production was restored in the *P. aeruginosa pqsE* mutant when transcomplemented with the *hhqE* gene. Previously, we have demonstrated that mutation of the *pqsE* gene results in the loss of production of the galactophilic lectin, LecA (PA-IL) [14]. To investigate whether this defect could also be complemented by the *hhqE* gene from *B. pseudomallei*, we introduced a *lecA::lux* reporter gene fusion into the chromosome of a PAO1 *pqsE* mutant. In this genetic background, there is an 80% reduction in the level of *lecA* expression (Figure 2C). The introduction of a plasmid-borne copy of the *B. pseudomallei hhqE* gene restores *lecA* expression to ~45% that of the parent strain, indicating that the *hhqE* gene has a similar function in both *P. aeruginosa* and *B. pseudomallei*.

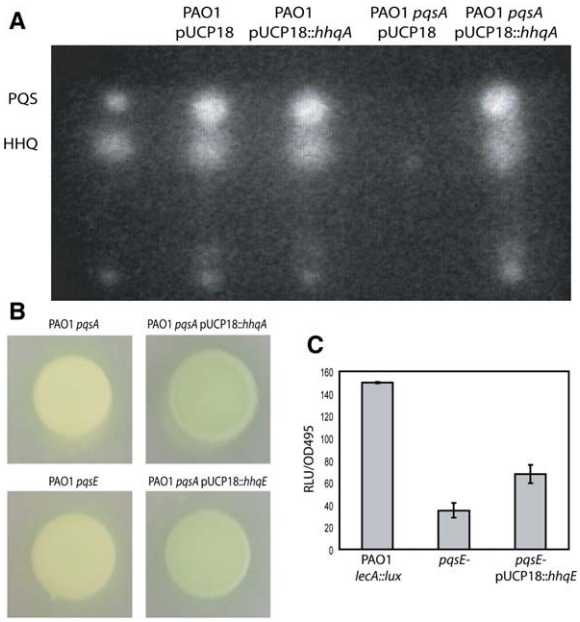

Figure 2. The *B. pseudomallei* Genes *hhqA* and *hhqE* Are Functionally Equivalent to the *P. aeruginosa* Genes Required for PQS Biosynthesis and Response

(A) TLC overlaid with an AHQ bioreporter and viewed with a Berthold Luminograph photon video camera. AHQs are visualized as bioluminescent spots. This demonstrates that the *B. pseudomallei hhqA* gene introduced on pUCP18 restores AHQ production in a *P. aeruginosa pqsA* mutant.

(B) *P. aeruginosa pqsA* and *pqsE* mutants grown on LB agar do not produce the green phenazine pigment pyocyanin. Complementation with the *B. pseudomallei hhqA* or *hhqE* genes restores pyocyanin production.

(C) Expression of a *lecA::lux* fusion in the parent strain PAO1 *lecA::lux*, the *P. aeruginosa pqsE* mutant (*pqsE*) and the *P. aeruginosa pqsE* mutant complemented with *hhqE* on pUCP18 (*pqsE+phhqE*). Error bars represent two standard errors of three independent measurements.

Burkholderia pseudomallei and *Burkholderia thailandensis* Produce AHQs

The presence of a putative PQS biosynthetic operon and the ability of the *hhqA* and *hhqE* genes to complement the corresponding *P. aeruginosa* mutants suggested that *B. pseudomallei* and *B. thailandensis* were likely to employ AHQ-dependent QS. To determine whether they produced AHQs, cell-free culture supernatants were extracted with acidified ethyl acetate and subjected to thin-layer chromatography (TLC), and the plate was overlaid with the AHQ bioreporter PAO1 *lecA::lux ΔpqsA* as described in the Experimental Procedures. This reporter responds sensitively to a range of AHQs, including both PQS and HHQ (to be described in detail elsewhere). Figure 3A shows that three *B. pseudomallei* strains and one *B. thailandensis* strain (E30) produce at least one compound capable of activating the AHQ bioreporter. However, the migration of this compound on TLC suggested that it is not PQS, but a closely related AHQ, possibly HHQ. To unequivocally identify the *B. pseudomallei* K96243 compound(s), the solvent-extracted culture supernatant was examined by LC-MS/MS. A compound with a molecular ion of *m/z* 244 [M + H] was identified with fragmentation ions of *m/z* 172 and 159 (Figure 3B), which are characteristic of HHQ (Figure 3D) rather than PQS (Figure 3C) and are produced by cleavage of the C7 alkyl chain [12]. *B. thailandensis* E30 culture supernatants contained a number of AHQs incorporating either a saturated or unsaturated alkyl chain at position 2. Also an *N*-oxide, analyzed to be 2-nonyl-4-hydroxyquinoline *N*-oxide, was identified. All of these derivatives have previously been described in *P. aeruginosa.* The results are summarized in Table 1.

HHQ Production in *B. cenocepacia* and *P. putida*

Using TLC in conjunction with the AHQ bioreporter PAO1 *pqsA lecA::lux*, a range of *Burkholderia* and *Pseudomonas* species were screened for AHQ production. The results are summarized in Table 2. Apart from *P. aeruginosa* strains PAO1 and PA14, which produce both PQS and HHQ, no other species tested produced PQS. *P. putida* KT2440 and a clinical isolate of *B. cenocepacia* (J415) both produced detectable amounts of a compound comigrating with HHQ on TLC (data not shown). Consequently, cell-free supernatants from *P. putida* and *B. cenocepacia* were subjected to LC-MS/MS, and consistent with the TLC data, HHQ was identified from the molecular ion *m/z* 244 [M + H] and daughter ions (*m/z* 172 and 159; data not shown). Neither PQS nor any other AHQs were detected in *B. cenocepacia* or *P. putida* cell-free supernatants. Furthermore, no AHQs could be detected in spent supernatants from *P. mendocina*,

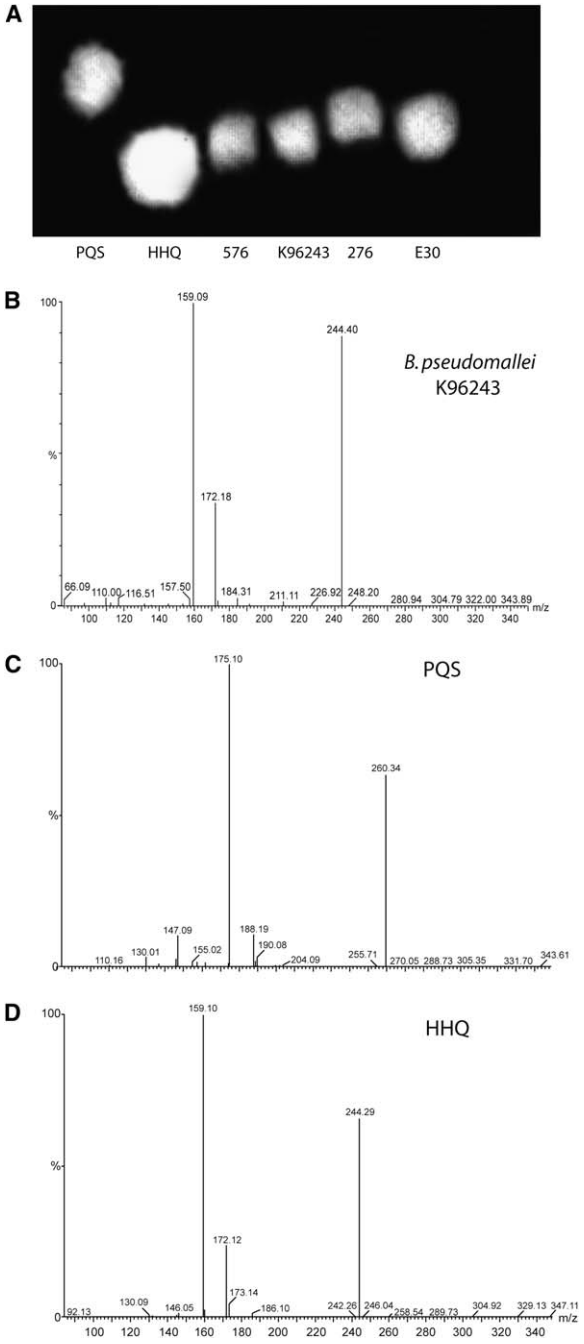

Figure 3. HHQ Is Produced by *B. pseudomallei* and *B. thailandensis*

(A) TLC analysis of solvent-extracted, cell-free supernatants from *B. pseudomallei* strains 576, K96243, and 276 and *B. thailandensis* strain E30. After chromatography, the TLC plate was overlaid with PAO1 *lecA::lux⊿pqsA* and viewed under a Berthold Luminograph. AHQs are visualized as bioluminescent spots.

(B) MS spectrum of a solvent-extracted culture supernatant prepared from *B. pseudomallei* K96243 showing the presence of HHQ.

(C) LC-MS/MS fragmentation pattern of PQS.

(D) LC-MS/MS fragmentation pattern of HHQ.

P. fragi, *P. diminuta*, *P. aureofaciens*, *P. fluoresecens*, *P. syringae*, *P. stutzeri*, *P. picketti*, *P. chlororaphis*, *B. stabiliz*, *B. vietnamiensis*, or *B. anthina*.

Table 1. AHQ Molecules Identified in *B. thailandensis* E30

Saturated AHQs	$[M + H]^+$	Major Fragment Ions
C_7 (HHQ); 2-heptyl-4(1*H*)-quinolone	244	159, 172
C_9 (NHQ); 2-nonyl-4(1*H*)-quinolone	272	159, 172
C_9 (NQNO); 2-nonyl-4 hydroxyquinoline *N*-oxide	288	159, 172, 186
C_{11} (UDHQ); 2-undecyl-4(1*H*)-quinolone	300	159, 172
Unsaturated AHQs		
$C_{7:1}$ (HEHQ); 2-(1-heptenyl)-4-(1*H*)-quinolone	242	159, 172, 184
$C_{9:1}$ (NEHQ); 2-(1-nonenyl)-4(1*H*)-quinolone	270	159, 172, 184
$C_{11:1}$ (UDEHQ); 2-(1-undecenyl)-4(1*H*)-quinolone	298	159, 172, 184

The *B. pseudomallei hhqA* Gene Is Required for AHQ Biosynthesis–Identification of NEHQ

To demonstrate that the putative *hhq* biosynthesis operon from *B. pseudomallei* was responsible for the production of AHQs, we mutated the *hhqA* gene in *B. pseudomallei* 844 by inserting a 1026 bp internal fragment of BPSS0481 on pKNOCK-Tc into the gene. Figure 4A shows an HPLC comparison of solvent-extracted supernatants prepared from the parent strain and an *hhqA* mutant recorded at 335 nm. A compound eluting at 7.75 min was identified in the parent strain and is absent from the *hhqA* mutant. This compound possesses a similar spectral profile (212, 247, 335 nm) to HHQ (data not shown) that has characteristic absorbance peaks at 213, 231, and 315 nm. However, using these HPLC conditions, HHQ elutes at 6.5 min; therefore, these data suggest that the compound eluting at 7.75 min is not HHQ, but a related AHQ. MS/MS analysis revealed that this compound has a molecular ion of *m/z* 270 [M + H] and major fragment ions at *m/z* 172, 159, and 130 (Figure 4B). These are consistent with NEHQ (Figure 1A), which has an unsaturated C9 alkyl side chain and has previously been identified in *P. aeruginosa* culture supernatants [22]. The position of the double bond in NEHQ is inferred from its MS data by the presence of a fragment ion at 172, assignable to 2-vinyl-4(1*H*)-quinolone arising from the loss of the C7H14 portion of the chain.

Disruption of AHQ Signaling in *B. pseudomallei* Results in Altered Colony Morphology and Increased Elastase Synthesis

To begin characterizing the QS signaling function of HHQ in *B. pseudomallei*, we investigated the effect of mutating *hhqA* on the synthesis of exoproteases, siderophores, elastase, and AHLs as well as the response to oxidative stress. Plate assays to detect siderophores and exoproteases revealed no obvious differences between the parent and *hhqA* mutant (data not shown). However, growth on LB plates revealed striking morphological differences between wild-type and *hhqA* mutant colonies. Wild-type *B. pseudomallei* colonies were typically pigmentless, rounded, and smooth-edged, with a mucoid phenotype, while the *hhqA* mutant presented with a wrinkled appearance with bacterial growth over the primary colony (Figure 5). Complementation with the *hhqA* gene restored colony morphology to

Table 2. PQS and AHQ Production in Clinical and Environmental Strains of *Pseudomonas* and *Burkholderia*

Strain	PQS	AHQ[a]	Source	Reference
P. aeruginosa PAO1	+	+	Wound infection	Holloway collection
P. aeruginosa PA14	+	+	Burn wound	[39]
P. mendocina ATCC25411	—	—	Environmental	ATCC (American Type Culture Collection)
P. aureofaciens 30–84	—	—	Wheat rhizosphere	W.W. Bockus
P. diminuta CIP7129	—	—	Soil	Source unknown
P. fluorescens CHAO	—	—	Soil	[40]
P. putida KT2440	—	+	Biosafety strain	[41]
P. syringae DC3000	—	—	Tomato	[42]
P. stutzeri ATCC17588	—	—	Environmental	ATCC
P. chlororaphis PCL1391	—	—	Spanish tomato rhizosphere	[43]
P. fragi ATCC4973	—	—	Environmental	ATCC
P. pickettii ATCC27511	—	—	Soil	ATCC
B. pseudomallei K96243	—	+	Clinical melioidosis isolate, Thailand	[44]
B. pseudomallei 576	—	+	Clinical melioidosis isolate, Thailand	[45]
B. pseudomallei 10276	—	+	Clinical melioidosis isolate, Bangladesh	[45]
B. pseudomallei 844	—	+	Clinical melioidosis isolate, Thailand	Srinagarind Hospital, Thailand
B. thailandensis E30	—	+	Environmental	[46, 47]
B. cenocepacia J415	—	+	CF isolate	[48]
B. stabilis	—	—	CF isolate	[48]
B. vietnamiensis G4	—	—	CF isolate	[48]
B. anthina	—	—	Environmental soil	[49]

B. thailandensis E30 produced HHQ, NHQ, NQNO, UDHQ, HEHQ, NEHQ, and UDEHQ.
[a] All AHQ-positive strains produced HHQ, except for 844, which produced NEHQ.

that of the wild-type (data not shown). The *hhqA* mutant exhibited an increase in elastase production when grown on elastin agar plates. A zone of clearing of 9.8 mm (±1 mm) was observed for the mutant, compared to 5.3 mm (±0.5 mm) for the wild-type and 5.4 mm (±0.5 mm) for the complemented mutant. The enhanced elastase synthesis observed in the *hhqA* mutant could be abolished by incorporating HHQ into the elastin agar plate assay (data not shown).

B. pseudomallei possesses an AHL-based QS system that is required for full virulence [25] and is also involved in controlling the response to oxidative stress (P.L., unpublished data). As PQS signaling has previously been shown to be integrated into the AHL-dependent QS system of *P. aeruginosa*, we wanted to determine whether mutation of the *B. pseudomallei hhqA* gene had any effect on AHL production and on the response of *B. pseudomallei* to oxidative stress. The *B. pseudomallei* 844 strain produces C8-HSL, 3-oxo-C8-HSL, 3-hydroxy-C8-HSL, C10-HSL, 3-hydroxy-C10-HSL, and 3-hydroxy-C12-HSL (P.L., unpublished data). Analysis of the *B. pseudomallei* 844 *hhqA* mutant culture supernatant revealed that this strain produced the same spectrum of AHLs produced by the parent strain (data not shown). Furthermore, both the parent and the *hhqA* mutant were similarly susceptible to the organic hydroperoxide *tert*-butyl hydroperoxide.

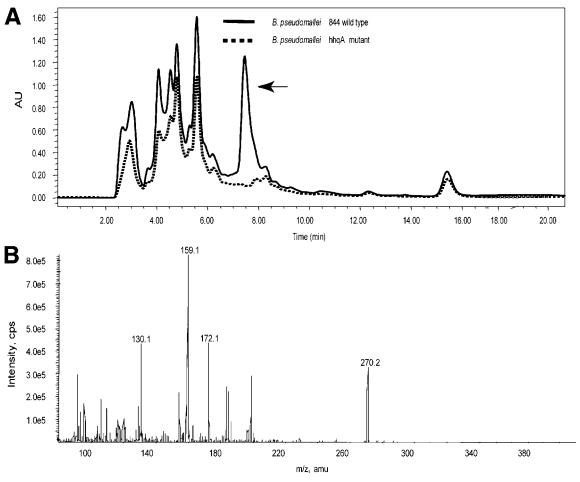

Figure 4. *B. pseudomallei* 844 Produces 2-(1-Nonenyl)-4(1*H*)-Quinolone, which Is Abolished in an *hhqA* Mutant

(A) HPLC analysis of solvent-extracted, cell-free culture supernatants from *B. pseudomallei* strain 844 and the corresponding *hhqA* mutant. The arrow marks the position of the major peak eluting at 7.75 min, which is lacking in the *hhqA* mutant.
(B) LC-MS/MS spectrum for the peak eluting at 7.75 min, which has a parent *m/z* 270 ion [M + H] and major daughter ions at *m/z* 172, 159, and 130, consistent with NEHQ.

Discussion

P. aeruginosa produces over 50 different AHQ molecules that exhibit diverse biological activities [4, 9–12, 18, 19]. Of these, PQS is a key QS signal molecule that regulates elastase, LecA lectin, rhamnolipid, and pyocyanin production [3, 14, 20] and enhances biofilm

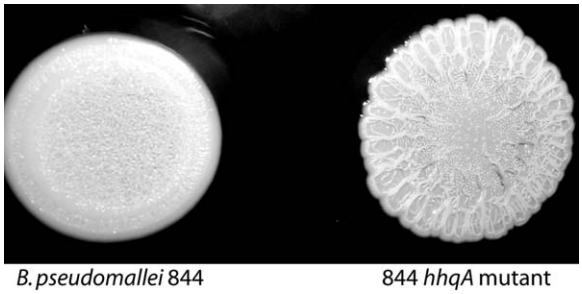

Figure 5. A *B. pseudomallei hhqA* Mutant Displays an Altered Colony Morphology

development [14]. A mutation in *pqsR* (*mvfR*) that positively regulates PQS biosynthesis renders *P. aeruginosa* PA14 avirulent in both plant and animal assays, with 320-fold less growth of *Arabidopsis* and a 65% reduction of mortality in mice [26]. In addition, mutation of the MexGHI-OpmD multidrug efflux pump, which results in the loss of PQS biosynthesis, also leads to the attenuation of virulence in both rat and plant experimental infection models [15]. PQS therefore plays a key role in regulating the virulence of *P. aeruginosa*, and, consequently, PQS biosynthesis and signaling are potential targets for novel antimicrobials. It was therefore of considerable interest to determine whether AHQ-dependent signaling is also employed by other bacteria.

Since AHQs are synthesized from common bacterial metabolites (anthranilate and β-keto fatty acids), and given that genes exhibiting homology with the *P. aeruginosa pqs* biosynthetic genes are present in related bacteria that share similar ecological niches, it is likely that AHQ signaling is employed by organisms other than *P. aeruginosa*. For bacteria such as *P. fluorescens*, *P. syringae*, and *P. fragi*, neither PQS nor HHQ, nor any other AHQs, were detected in cell-free spent culture supernatants, even though they possess genes that share some homology with *pqsA*, *pqsC*, and *pqsD.* However, these bacteria lack a complete AHQ biosynthetic operon, and the *pqs* genes are scattered throughout their respective chromosomes. Furthermore, no homolog of *pqsE*, which is required for the response to PQS in *P. aeruginosa* [14, 20, 27], could be identified in any of the genomes of sequenced strains belonging to these genera. A systematic search of completed bacterial genomes revealed that, apart from *P. aeruginosa*, only two bacterial species (*B. pseudomallei* and *B. thailandensis*) contained putative *pqsB* and *pqsE* homologs.

B. pseudomallei and the closely related *B. thailandensis* both contain a putative *pqsABCDE* operon on chromosome 2 similar to that of *P. aeruginosa*. *B. pseudomallei* is the causative agent of melioidosis, an infectious disease of major public health importance in southeast Asia and northern Australia [28], and it is even found in survivors of the recent Asian tsunami [29]. It is also regarded as a potential biothreat agent [28]. There is, therefore, considerable interest in gaining new insights into the mechanisms by which virulence and persistence are regulated in *B. pseudomallei.* Here, we have obtained evidence for the functional equivalence of the *B. pseudomallei* genes corresponding to the *P. aeruginosa pqsA* and *pqsE* genes that are involved in the control of *P. aeruginosa* virulence via AHQ-dependent QS. This was obtained by transcomplementing the *P. aeruginosa pqsA* and *pqsE* mutants with the *B. pseudomallei hhqA* and *hhqE* genes, respectively. Both PQS and pyocyanin synthesis were restored in the *pqsA* mutant, and the production of pyocyanin and lectin was restored in the *pqsE* mutant. These data indicate that both the biosynthesis of and the response to PQS can be reestablished in *P. aeruginosa* by introducing the *hhqA* and *hhqE* genes, respectively, from *B. pseudomallei*. While PqsA is required for PQS biosynthesis in *P. aeruginosa*, the function of PqsE remains unclear, as a *pqsE* mutant produces wild-type levels of both PQS [20] and HHQ [19] but does not produce pyocyanin or the galactophilic lectin, LecA [14, 20] and makes reduced amounts of elastase [14]. Furthermore, addition of exogenous PQS to a *P. aeruginosa pqsE* mutant does not restore pyocyanin, lectin, or elastase production [14], suggesting that either PqsE is required for the cellular response to PQS [20] or that PqsE is involved in generating an, as yet, unidentified signaling molecule from PQS, as the protein has structural similarities to members of the metallo-β-lactamase protein superfamily. Nevertheless, these data indicated that AHQ-dependent cell-to-cell communication was likely to be functional in *B. pseudomallei* and in other bacteria that possess homologs of the *P. aeruginosa pqs* biosynthetic genes.

A *P. aeruginosa pqsA* mutant does not make any AHQs, but when complemented with *B. pseudomallei hhqA*, it produces both HHQ and PQS. This finding raised the question as to the identity of the AHQ(s) synthesized in *B. pseudomallei* itself since this human pathogen does not possess a homolog of the putative monooxygenase PqsH, which is required for the conversion of HHQ to PQS (Figure 1B). This is also the case for other bacteria that possess *pqs* homologs but lack *pqsH*. To assay for AHQ biosynthesis in different bacterial genera, we developed a rapid method for screening culture supernatants that employs a *P. aeruginosa* bioreporter (to be described in detail elsewhere) that cannot synthesize AHQs as a consequence of the *pqsA* mutation, but responds to exogenously supplied AHQs that activate a chromosomally located *lecA::lux* promoter fusion. The bioreporter can be incorporated within agar and used as an overlay after TLC of the solvent-extracted culture supernatants. AHQs such as PQS and HHQ are readily identified as bioluminescent spots upon activation of the reporter. By using this bioreporter, we observed that none of the *Pseudomonas* or *Burkholderia* species examined produced PQS. However, *B. pseudomallei* strains 276, 576, and K96243, *B. thailandensis* strain E30, *B. cenocepacia* strain J415, and *P. putida* strain KT2440 all activated the reporter, and the active spot migrated on TLC with an R_f value close to that of the HHQ standard. The identity of the active compound as HHQ in *B. pseudomallei* K96243, *P. putida*, *B. thailandensis*, and *B. cenocepacia* was confirmed by LC-MS/MS. In addition, *B. pseudomallei* strain 844 and *B. thailandensis* produced NEHQ, a C9 analog of HHQ with an unsaturated alkyl side chain. Furthermore, we also identified HNQ, NQNO, UDHQ, HEHQ, NEHQ, and UDEHQ in *B. thailandensis.* These AHQs are all produced by *P. aeruginosa* [12]. It is interesting that both pathogenic (*B. pseudomallei*, *B. cenocepacia*, and *P. aeruginosa*) and nonpathogenic species (*B. thailandensis* and *P. putida*) can be AHQ producers. Furthermore, our data also show that AHQ synthesis does not only take place in bacteria where the *pqs*/*hhq* genes are clustered as an operon, since *P. putida* KT2440 produces HHQ, but the *hhq* homologs are scattered throughout the chromosome. This organism also lacks a readily identifiable *pqsE* homolog, suggesting that *hhqE*/*pqsE* may not always be required for AHQ signal transduction.

Both the complementation of a *P. aeruginosa pqsA* mutant with *hhqA* and the loss of AHQ synthesis upon mutation of *hhqA* unequivocally demonstrate the requirement of this gene for AHQ biosynthesis in *B. pseudomallei*. Furthermore, the loss of AHQ synthesis in *B. pseudomallei* resulted in a striking change in colony

morphology and increased elastase production, which could be reversed by providing exogenous HHQ, suggesting that AHQ signaling is involved in the control of at least two different phenotypes. The enhanced elastase production observed in the *hhqA* mutant may be due to increased levels of the *B. pseudomallei* metalloprotease, MprA [30]. Whether AHQ-dependent QS is required for the regulation of virulence in *B. pseudomallei* has yet to be established. In *P. aeruginosa*, the AHL- and AHQ-dependent signaling pathways are closely interlinked [3, 14]. *B. pseudomallei* and *B. thailandensis* both possess three AHL synthases and five AHL response regulators and produce multiple AHLs [25, 31]. In *B. pseudomallei*, mutations in the AHL-dependent QS system reduced the time to death in Syrian hamsters [25], while, in *B. thailandensis*, mutation of the QS system affected several cellular processes, including lipase production, swarming, and twitching motility [31]. It is therefore possible that AHL and AHQ signaling in *B. pseudomallei*/*B. thailandensis* are also interlinked. It is interesting to note that in *B. thailandensis*, mutation of the LasR/RhlR homologous *btaR3* gene resulted in a significant wrinkled phenotype [31] similar to that observed in the *B. pseudomallei hhqA* mutant in this study. This suggests that AHL and AHQ signaling may constitute a regulatory cascade in these bacteria. However, examination of the AHL profile of the *hhqA* mutant revealed no obvious differences when compared with that of the parent strain. Whether *B. pseudomallei* AHL mutants show either altered colony phenotypes or AHQ levels has yet to be determined. Indeed, such a finding would imply the existence of a regulatory link between AHL and AHQ signaling in *B. pseudomallei*, such as occurs in *P. aeruginosa* [14].

In *P. aeruginosa*, HHQ is released into the external milieu by the producing cells and is subsequently taken up by neighboring bacterial cells, where it is converted into PQS [19]. The conversion of HHQ to PQS is mediated by the *pqsH*-encoded putative FAD-dependent monooxygenase [19], which is under partial AHL-dependent control via LasR/3-oxo-C12-HSL [19, 20]. HHQ has been suggested to function as a messenger molecule that is converted to PQS rather than being a signal molecule per se, since its activity was reported to depend on the conversion of HHQ to PQS [19]. However, *B. pseudomallei*, for example, lacks a *pqsH* homolog, does not synthesize PQS, and employs HHQ as a signal molecule, at least in the context of colony morphology and elastase production. This raises the question as to whether HHQ can function as a signal molecule in *P. aeruginosa* and whether the ability of the latter to convert HHQ to PQS offers an ecological advantage by conferring a novel functionality. Recent work in this laboratory has revealed that, when introduced onto the chromosome of a *P. aeruginosa pqsH* mutant, a *lecA::lux* gene fusion responds to exogenously supplied HHQ (unpublished data). This suggests that HHQ is able to function in *P. aeruginosa* as a signal molecule per se since the *pqsH* mutant cannot produce PQS. It is therefore possible that the oxidation of both endogenous and exogenous HHQ to PQS by *P. aeruginosa* ensures that other bacteria occupying the same ecological niche are unable to exploit PQS as a signal molecule. Since *P. aeruginosa* can respond to both AHQs, it may be able not only to "tune in" to cell-to-cell communication between other bacterial genera, but also to interfere with such signaling. However, confirmation of this hypothesis will require extensive investigations of the response of HHQ-producing bacteria to PQS.

Significance

Cell-to-cell communication (quorum sensing) is now recognized to play a pivotal role in coordinating the physiological behavior of unicellular microorganisms with respect to secondary metabolite production, biofilm development, and virulence. Communication within and between bacterial species depends on the deployment of chemically diverse QS signal molecules. Understanding the chemical and biological bases for such signaling systems and their function and conservation within the bacterial kingdom is central to their exploitation particularly as targets for novel antibacterial agents. Using a combination of bioinformatics, bioreporters, and analytical chemistry together with bacterial genetics, we show for the first time, to our knowledge, that AHQ signaling is employed not just by *P. aeruginosa*, but also by other important human pathogens, including *B. pseudomallei*, which is responsible for the life-threatening disease melioidosis, a major public health threat in southern Asia and northern Australia. Furthermore, this organism is a concern, as it has potential uses as a bioterrorist weapon. Little is understood about the lifestyle of this dangerous organism, and, consequently, new findings, particularly those associated with virulence, are important. Our study has revealed that while the *B. pseudomallei* genes required for AHQ biosynthesis (*hhqA*) and response (*hhqE*) are functionally homologous to those of *P. aeruginosa*, the strains *B. pseudomallei*, *B. thailandensis*, *B. cenocepacia*, and *P. putida* do not produce the *P. aeruginosa* signal molecule PQS but do produce the PQS precursor, HHQ. Mutation of *hhqA in B. pseudomallei* resulted in the loss of AHQ production, altered colony morphology, and enhanced elastase production. These data demonstrate a role for AHQ signaling in *B. pseudomallei* and highlight the future challenges with respect to the nature of the AHQ regulon, its contribution to virulence gene regulation, and the mechanism through which AHQ signaling is transduced via HhqE/PqsE in both *B. pseudomallei* and *P. aeruginosa*.

Experimental Procedures

Bacterial Strains, Plasmids, and Growth Conditions

The strains and plasmids used in this study include the *P. aeruginosa* strains wild-type PAO1, a *pqsA* mutant [20], a *pqsE* mutant [20], and an AHQ bioreporter PAO1 *lecA::lux⊿pqsA* strain (to be described elsewhere). Other strains of bacteria used in this study can be found in Table 2. For complementation studies, plasmids were transformed into *P. aeruginosa* by electroporation [32]. All bacterial strains were routinely grown in Luria-Bertani (LB) at either 37°C or 30°C with shaking (200 rpm) in 25 ml broth in 250 ml Erlenmeyer flasks. Carbenicillin (300 μg/ml) and tetracycline (60 μg/ml) were added to cultures where required.

DNA Isolation and Manipulation

DNA was isolated as previously described [33]. Sequence analysis and database searches were performed with the Lasergene

(DNAstar) software package and the NCBI BLAST server (http://www.ncbi.nlm.nih.gov/).

Complementation of PAO1 *pqsA* and *pqsE* Mutants by *B. pseudomallei hhqA* and *hhqE* Genes

Using *B. pseudomallei* chromosomal DNA isolated from strain 844, *hhqA* (BPSS0481; 1697 bp, BamHI, EcoRI) and *hhqE* (BPSS0485; 912 bp, SmaI, EcoRI) were cloned into similarly digested pUCP18 and electroporated into the relevant *P. aeruginosa* strain. Mutants containing the complementing plasmids were identified by their ability to grow in LB containing carbenicillin (300 μg/ml).

Construction of a *B. pseudomallei hhqA* Insertion Mutant

An *hhqA* insertion mutant was created in *B. pseudomallei* 844 by cloning a 1026 bp internal fragment of the BPSS0481 gene into pKNOCK-Tc by using SmaI restriction sites [34]. The construct was conjugated into *B. pseudomallei*, and mutants were selected by their ability to grow in LB agar containing tetracycline (60 μg/ml). The fidelity of the mutation was confirmed by PCR. The *B. pseudomallei hhqA mutant* was complemented by introducing a plasmid-borne copy of *hhqA* cloned into pUCP18. The construct was conjugated into *B. pseudomallei* and selected on pseudomonas base agar containing 60 μg/ml tetracycline and 500 μg/ml carbenicillin.

Extraction of AHQs from *Pseudomonas* and *Burkholderia* Cultures

Aliquots of stationary phase cell-free supernatants were extracted as previously described [14]. Briefly, aliquots of 10 ml *P. aeruginosa* were extracted with 10 ml acidified ethyl acetate, vortexed vigorously, and centrifuged at 10,000 rpm for 5 min. The organic phase was transferred to a fresh tube and dried to completion under a stream of nitrogen gas. The solute was resuspended in 50 μl methanol for future analysis. Sterile supernatants of other *Pseudomonas* and *Burkholderia* strains, including *B. pseudomallei* and *B. thailandensis* cultures, were extracted in the same way, although 50 ml supernatant was extracted in 50 ml acidified ethyl acetate and resuspended in 50 μl methanol.

Synthesis of AHQs and AHLs

AHLs and AHQs were synthesized as previously described [14, 35]. Briefly, AHQs were synthesized as follows: 2-heptyl-4(1*H*)-quinolone (HHQ), m.p. 145°C–146°C, was synthesized by the acid-catalyzed cyclocondensation of ethyl 3-oxodecanoate with aniline in 50% yield. Starting from HHQ, 3-formyl-2-heptyl-4(1*H*)-quinolone, m.p. 245°C–248°C (dec.), and 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS), m.p. 195°C–197°C, were synthesized in 40% and 70% yields, respectively. PQS analogs were dissolved in methanol before being added to growth media at the indicated concentrations.

Thin-Layer Chromatography Analysis of AHQs with a Bioreporter Assay

Samples of ethyl acetate-extracted culture supernatants were spotted onto a normal phase silica 60_{F254} (Merck) TLC plate that had been previously soaked for 30 min in 5% w/v KH_2PO_4 and activated at 100°C for 1 hr. Synthetic HHQ and PQS (1 or 2 μl of a 10 mM stock concentration, respectively) were used as positive controls. Spots were dried, and the TLC plate was developed by using dichloromethane:methanol (95:5) as the mobile phase. TLC plates were visualized with a UV transilluminator and photographed. To confirm the presence of AHQs, TLC plates were overlaid with a thin film of 0.3% (w/v) LB agar seeded with the AHQ bioreporter PAO1 *lecA::lux⊿pqsA* (which responds to a range of AHQs, including PQS and HHQ, and will be described in detail elsewhere), and incubated at 37°C for 6 hr. Bioluminescence was detected with a Luminograph LB 980 photon video camera (EG and G Berthold).

HPLC and LC-MS/MS of AHQs

HPLC analysis of AHQs was conducted with a Waters 996 Photodiode Array Detector (PDA) coupled with a Waters 625 Quaternary pump, and the data were collated by using Empower software (Waters Corporation, UK). Acidified ethyl acetate extracts of cell-free culture supernatants were eluted isocratically with 80% v/v acetonitrile in water as the mobile phase and an Exsil Pure C18 MS 5 μm column (250 × 2.1 mm internal diameter; Alltech Associates, Inc.). For LC-MS/MS, analyses were performed by using reverse phase high-performance liquid chromatography (RP-HPLC) with an Exsil Pure C18 MS 5 μM column (250 × 2.1 mm) coupled with LC-MS/MS (Applied Biosystems 4000 Q-TRAP) and eluted with a 35%–70% w/v acetonitrile/water gradient. The fragmentation ions of each of the anticipated AHQs were monitored by using the positive ion electrospray mode.

Phenotypic Assays

For elastase production, 10 μl of an overnight culture was spotted onto nutrient agar containing 0.3% w/v elastin (Sigma) [36] with or without 20 μl of a 100 μM solution of HHQ. Plates were incubated at 37°C for 48 hr and stored at 4°C for 4 days, and the zones of clearing were measured. Siderophore activity was determined by spotting 10 μl aliquots of an overnight culture onto CAS plates that were incubated overnight at 37°C [37]. To evaluate bacterial resistance to oxidative stress, cultures were grown overnight in M9 low-glucose medium and adjusted to an OD_{600} of 1.0 and 10-fold serially diluted. A total of 10 μl of each culture was spotted onto LB agar containing 150 μM *tert*-butyl hydroperoxide, and the extent of growth was noted after 24 hr of incubation at 37°C [38].

Time and Cell Population 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) as previously described [14].

Supplemental Data

Supplemental Data include Table S1, which shows *pqs* gene homologs in *Pseudomonas*, *Burkholderia*, and *Ralstonia* species, and are available at http://www.chembiol.com/cgi/content/full/13/7/701/DC1/.

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Accession Numbers

The *hhqA* gene from *Burkholderia pseudomallei* K29643 has been deposited in GenBank under ID code 823560.