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Biotic inactivation of the *Pseudomonas aeruginosa* quinolone signal molecule

Eliza Ye-Chen Soh,[1,2] Siri R. Chhabra,[1] Nigel Halliday,[1] Stephan Heeb,[1] Christine Müller,[3] Franziska S. Birmes,[3] Susanne Fetzner,[3] Miguel Cámara,[1] Kok-Gan Chan[2] and Paul Williams[1]*

[1]*Centre for Biomolecular Sciences, School of Life Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK.*

[2]*Division of Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur 50603, Malaysia.*

[3]*Institute of Molecular Microbiology and Biotechnology, University of Muenster, D-48149 Muenster, Germany.*

Summary

In *Pseudomonas aeruginosa*, quorum sensing (QS) regulates the production of secondary metabolites, many of which are antimicrobials that impact on polymicrobial community composition. Consequently, quenching QS modulates the environmental impact of *P. aeruginosa*. To identify bacteria capable of inactivating the QS signal molecule 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS), a minimal medium containing PQS as the sole carbon source was used to enrich a Malaysian rainforest soil sample. This yielded an *Achromobacter xylosoxidans* strain (Q19) that inactivated PQS, yielding a new fluorescent compound (I-PQS) confirmed as PQS-derived using deuterated PQS. The I-PQS structure was elucidated using mass spectrometry and nuclear magnetic resonance spectroscopy as 2-heptyl-2-hydroxy-1,2-dihydroquinoline-3,4-dione (HHQD). *Achromobacter xylosoxidans* Q19 oxidized PQS congeners with alkyl chains ranging from C1 to C5 and also *N*-methyl PQS, yielding the corresponding 2-hydroxy-1,2-dihydroquinoline-3,4-diones, but was unable to inactivate the PQS precursor HHQ. This indicates that the hydroxyl group at position 3 in PQS is essential and that *A. xylosoxidans* inactivates PQS via a pathway involving the incorporation of oxygen at C2 of the heterocyclic ring. The conversion of PQS to HHQD also occurred on incubation with 12/17 *A. xylosoxidans* strains recovered from cystic fibrosis patients, with *P. aeruginosa* and with *Arthrobacter*, suggesting that formation of hydroxylated PQS may be a common mechanism of inactivation.

Introduction

Pseudomonas aeruginosa is a nutritionally versatile Gram-negative bacterium commonly found in water, plants, sewage and hospital environments (Ramos, 2004). It is an important member of soil microbial communities capable of degrading aromatic hydrocarbons and producing a variety of secondary metabolites, including rhamnolipid surfactants, iron chelators, hydrogen cyanide and phenazines, many of which have antimicrobial activities. *Pseudomonas aeruginosa* is also an opportunistic pathogen of humans, animals, plants and insects causing both acute and chronic infections.

In soil and water environments as well as in polymicrobial infections, *P. aeruginosa* may act competitively or synergistically with other microbes to gain an adaptive advantage through the production of antimicrobials, signal molecules and type VI effectors, which influence the composition of multi-species microbial communities (Korgaonkar *et al.*, 2013; Tashiro *et al.*, 2013; Murray *et al.*, 2014). Many of these extracellular factors are coordinately regulated at the bacterial population level through 'quorum sensing' (QS). This is a population-dependent adaptive response that depends on the perception and processing of chemical information in the form of self-generated diffusible signal molecules (Williams *et al.*, 2007; Williams and Cámara, 2009). Quorum sensing enables bacteria to determine, by monitoring the concentration of a signal molecule in the surrounding medium, when the number of individuals in the population is sufficient (a quorum) to make a collective 'decision'. Such behavioural decisions impact on bacterial motility, secondary metabolism, virulence and biofilm development (Williams *et al.*, 2007; Williams and Cámara, 2009).

Pseudomonas aeruginosa employs a sophisticated interdependent and overlapping regulatory network employing multiple QS signal molecules and their cognate response regulatory protein receptors (Williams *et al.*, 2007; Williams and Cámara, 2009). These diffusible

Received 16 December, 2014; revised 6 March, 2015; accepted 6 March, 2015. *For correspondence. E-mail paul.williams@nottingham.ac.uk; Tel. +441159515047; Fax +441158467951.

signals include *N*-acyl-L-homoserine lactones (AHLs) and 2-alkyl-4(1*H*)-quinolones (AQs). With respect to the AQs, *P. aeruginosa* generates over 50 different congeners containing saturated or monounsaturated 2-alkyl side chains that vary from 5 to 13 carbons in length, and the presence or absence of a 3-position hydroxyl substituent (Lépine *et al*., 2004). AQs have low water solubility, are mostly associated with the bacterial outer membrane and can be trafficked between cells in membrane vesicles (MVs) (Lépine *et al*., 2003; Mashburn-Warren *et al*., 2008). AQ biosynthesis requires the products of the *pqsABCDE* operon, apart from *pqsE*, which codes for an effector protein of unknown function required for the AQ response (Heeb *et al*., 2011). PqsA, B, C and D are all required for the production of AQs such as the C7 congener, 2-heptyl-4-hydroxyquinoline (HHQ), that are subsequently oxidized to 3-hydroxy congeners such as 2-heptyl-3-hydroxy-4(*1H*)-quinolone (PQS) by the monooxygenase, PqsH (Heeb *et al*., 2011). This confers additional properties as PQS, for example, in contrast to HHQ, is a potent iron chelator (Bredenbruch *et al*., 2006; Diggle *et al*., 2007). PQS, HHQ and their C9 congeners function as auto-inducers by binding to and activating the LysR-type regulator PqsR (MvfR) to drive the transcription of the *pqsABCDE* operon (Ilangovan *et al*., 2013). Apart from the HHQ and PQS series of AQs, the *pqsABCDE* operon is also required, in conjunction with a second monooxygenase, PqsL, for the production of the 2-alkyl-4-hydroxyquinoline *N*-oxides such as 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO), a potent cytochrome inhibitor for both prokaryotes and eukaryotes (Heeb *et al*., 2011).

In complex microbial communities and in the presence of higher organisms, the coordinated QS-dependent behaviour of bacteria may be disrupted through QS signal molecule inactivation, inhibition of QS signal molecule biosynthesis or QS signal transduction. The concept of QS disruption or 'quorum quenching' has attracted considerable interest as a potential strategy for controlling bacterial beneficial or damaging behaviour (Rampioni *et al*., 2014). With respect to the AHLs, the ability to inactivate members of this QS signal molecule family enzymatically is widespread and has been reported for over 20 different bacterial genera as well as fungi, plants and mammalian cells and tissues (Uroz *et al*., 2009). Most AHL-degrading enzymes are either lactonases or acylases that are often produced by bacteria, which do not themselves produce AHLs (Christiaen *et al*., 2011). Consequently, there has been considerable interest in the ability of AHL-degrading microbes to quench QS in both ecological and within host environments (Dong *et al*., 2007). For example, natural or recombinant AHL-lactonase-producing bacteria reduced the ability of *Pectobacterium carotovorum* (formerly, *Erwinia carotovora*) to cause soft-rot in potatoes, while transgenic tobacco or potato plants expressing the bacterial lactonase AiiA were resistant to *P. carotovorum*. In marine microbial assemblages, natural AHL-degrading bacteria interfere with AHL production by other species in the community, which, in turn, impacts on the settlement behaviour of algal zoospores, which are attracted by the AHLs (Tait *et al*., 2009).

Apart from the AHLs, relatively little is known about the inactivation and metabolism of other classes of QS signal molecules, including the AQs. However, the 3-hydroxy-2-methyl-4(1*H*)-quinolone 2,4-dioxygenase (Hod) of *Arthrobacter* sp. strain Rue61a, a cytoplasmic enzyme involved in the pathway of 2-methylquinoline (quinaldine) degradation, is capable of cleaving PQS but not HHQ to form *N*-octanoylanthranilic acid and carbon monoxide (Pustelny *et al*., 2009). When added to *P. aeruginosa* cultures, recombinant Hod quenched AQ signalling with significant downregulation of the *pqsA* promoter, pyocyanin production and reduced *P. aeruginosa* virulence in a plant leaf infection model (Pustelny *et al*., 2009).

Here, we describe the enrichment of a bacterial consortium from a Malaysian rainforest soil sample (which contained *P. aeruginosa*) capable of inactivating PQS. From the consortium, strain Q19, identified as *Achromobacter xylosoxidans*, was shown to inactivate PQS in axenic culture via a novel pathway involving the incorporation of an oxygen atom at C2 of the heterocyclic ring to form 2-heptyl-2-hydroxy-1,2-dihydroquinoline-3,4-dione (HHQD). The conversion of PQS to HHQD also occurred on incubation with 12/17 *A. xylosoxidans* strains recovered from the lungs cystic fibrosis patients, with *P. aeruginosa* PAO1 and with *Arthrobacter* sp. Rue61a, indicating that oxygenation of PQS can occur in both Gram-negative and Gram-positive bacteria.

Results

Enrichment of a bacterial consortium able to grow on PQS as sole carbon source

To enrich for bacterial consortia capable of degrading PQS, a sample of soil collected from a Malaysian rainforest environment that contained AQ-producing *P. aeruginosa* (data not shown) was mixed with KG basal medium containing 10 μM PQS as the sole carbon source and incubated for 7 days at 30°C, as described in the Experimental procedures section. From this culture, a 1% (v/v) transfer was made to fresh basal medium plus PQS and incubated for 7 days and the process was repeated for a third 7-day cycle. Growth was observed after 4 days during the first cycle and within 48 h in the subsequent enrichments (Fig. 1A). No visible growth was observed in the absence of PQS and no turbidity was apparent in the basal PQS-containing medium alone (Fig. 1A). To follow

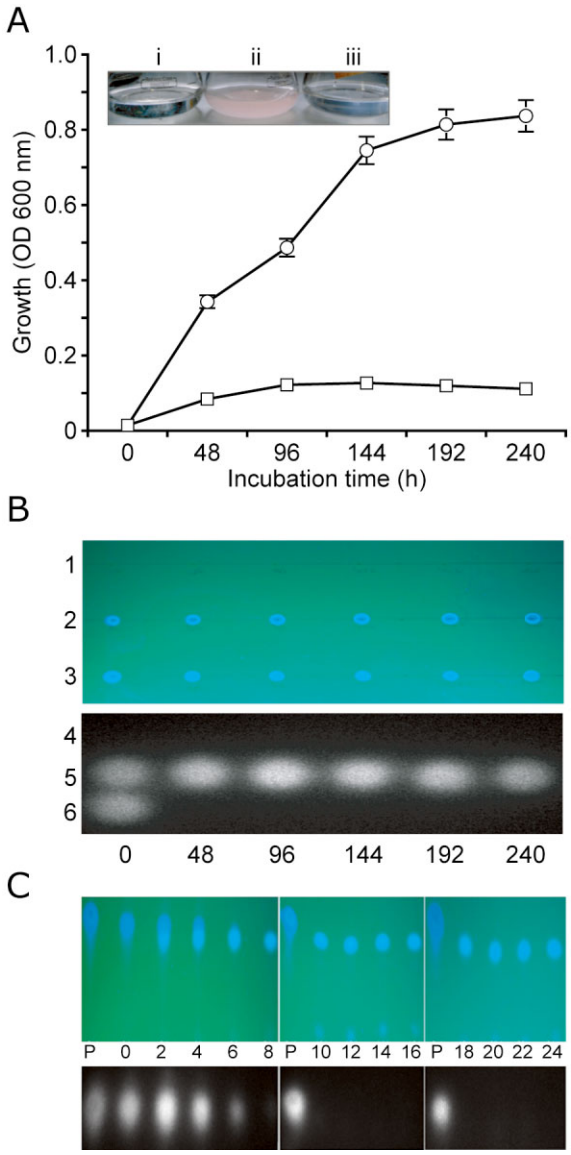

Fig. 1. Growth of the Malaysian soil consortium over 10 days after three cycles of enrichment in a minimal medium containing 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) as the sole carbon source.
A. Growth of the consortium with (circles) or without (squares) PQS. Inset shows cultures where (i) uninoculated medium containing PQS, (ii) inoculated medium containing PQS and (iii) inoculated medium without PQS.
B. Thin layer chromatography (TLC) showing the soil consortium inactivation of PQS. Upper panel, TLC plate spotted with samples taken every 48 h and viewed under UV light. Row 1, samples from un-inoculated medium without PQS, row 2, samples from un-inoculated medium containing PQS and row 3 medium containing PQS inoculated with the soil consortium. Lower panel, TLC plate overlaid with a soft agar containing the PQS-biosensor, *Pseudomonas aeruginosa* PAO1 Δ*pqsA* miniCTX::*pqsA′-lux* and viewed under a photon video camera. Rows 4–6 are as in the upper panel. Note the loss of biosensor activation in row 6.
C. PQS inactivation by *Achromobacter xylosoxidans* Q19 resting cells. Samples taken every 2 h over a 24 h period were subjected to TLC analysis and (i) visualised under UV light (upper panel) or (ii) overlaid with the PQS bioreporter and viewed under a photon video camera (lower panel). P, PQS standard (10 mM, 2 μl). The bioreporter overlay shows that the loss of detectable PQS after 8 h of incubation (lower panel) is accompanied by the appearance of a fluorescent spot that migrates with a slightly lower R_f than PQS (upper panel).

the degradation of PQS, the cultures were sampled at 48 h intervals by removing an aliquot, extracting with acidified ethyl acetate and spotting onto a normal phase thin layer chromatography (TLC) plate, dried and viewed under a UV transilluminator prior to overlaying the TLC plate with soft agar containing the PQS biosensor strain PAO1 Δ*pqsA* miniCTX::*pqsA′-lux* (Fletcher *et al*., 2007). After incubation for 6 h, the plates were imaged with a photon video camera. Figure 1B shows that although fluorescent spots were apparent on the TLC plate at each time point in the presence or absence of the enriched soil consortium, no PQS signal was apparent on the biosensor overlay plates (Fig. 1B, row 6) after 48 h of incubation in the presence of the soil bacteria, confirming inactivation of the QS signal molecule.

PQS inactivation by A. xylosoxidans

To identify individual strains from the consortium capable of inactivating PQS, the enrichment culture was plated on agarose-solidified KG minimal medium containing PQS. A total of 48 individual colonies with different morphologies and pigmentation were isolated by repeated streaking onto the PQS-containing solid medium and onto LB agar. Of these, one isolate, designated as Q19, inactivated PQS as revealed by the loss of bioluminescence when cell-free supernatant from Q19 incubated in PQS enrichment medium was extracted with ethyl acetate, spotted onto a TLC plate, dried and directly overlaid with the PAO1 Δ*pqsA* miniCTX::*pqsA′-lux* biosensor. To gain further insights into PQS inactivation using a 'resting cell assay', Q19 was incubated in a non-growth-supporting basal medium supplemented with PQS (20 μM) and succinate (1% w/v) over a 24 h period. Samples were taken every 2 h, extracted with ethyl acetate and separated by TLC using a dichloromethane : methanol (95:5) mobile phase. Figure 1C shows the presence of a fluorescent compound at the 2 h time point that migrates at a slightly lower R_f value than PQS. When the TLC plate was overlaid with soft agar seeded with the PAO1 Δ*pqsA* miniCTX::*pqsA′-lux* biosensor, there was a corresponding decrease in bioluminescence intensity with complete loss at the 10 h time point (Fig. 1C, lower panel). These data indicate that Q19 appears to inactivate PQS with the concomitant formation of a fluorescent product that is unable to induce the PQS biosensor. Further examination of this strain revealed it to be a Gram-negative,

rod-shaped bacterium that grows optimally under aerobic conditions at 30°C. The 1426-bp 16S ribosomal DNA (rDNA) of Q19 was sequenced (GenBank accession number KP143768) and identified as having 99% identity to the *16S rRNA* gene of *A. xylosoxidans* strain NFRI-A1 (GenBank accession number AB161691). To determine whether PQS was also inactivated by other *A. xylosoxidans* strains, 17 clinical isolates from the lungs of cystic fibrosis patients were incubated with PQS for 24 h, the cultures extracted, spotted onto TLC plates and overlaid with the PAO1 Δ*pqsA* miniCTX::*pqsA′-lux* biosensor. PQS was inactivated by 12 of these isolates (data not shown).

Identification of 'inactivated PQS' (I-PQS)

To identify the putative inactivation product (I-PQS) apparent on TLC after incubation of *Achromobacter* Q19 with PQS, the resulting fluorescent compound was subjected to solid phase extraction (SPE) and high-performance liquid chromatography (HPLC). A major peak that elutes at 16.7 min was detected at λ 312 nm (Fig. S1). Positive electrospray mass spectrometry of this eluate revealed the presence of three main ions at *m/z* 258.1, *m/z* 276.1 and *m/z* 573.3 (Fig. S2). These three ions were subjected to fragmentation via collision-induced dissociation. The ion *m/z* 573.3 fragmented to *m/z* 298.1, whereas the tandem mass spectrometry (MS/MS) spectrum of the ion at *m/z* 276.1 showed a major fragment ion at *m/z* 258.1 (Fig. S2). These analyses indicated that the ion at *m/z* 276.1 was the molecular ion $[M + H]^+$ and that it loses a water molecule, giving rise to an ion at *m/z* 258.1 attributable to $[M + H - H_2O]^+$. The *m/z* 298.1 and *m/z* 573.3 ions are the sodiated singly $[M + Na]^+$ and doubly $[2M + Na]^+$ charged quasi-molecular ion peaks respectively.

We next set out to determine whether the compound with *m/z* 276 $[M + H]^+$ was produced by isolate *A. xylosoxidans* Q19 in response to the presence of PQS in the medium or was truly a by-product of PQS inactivation. To this end, deuterium labelled PQS, 5,6,7,8-tetradeutero-2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS-d_4) instead of PQS, was synthesized and used as the substrate. A cell-free control was included to ensure that there was no abiotic degradation of this substrate within 24 h. At the end of the assay, extracts were subjected to TLC analysis coupled with the PAO1 Δ*pqsA* miniCTX::*pqsA′-lux* PAO1 biosensor overlay as well as to liquid chromatography-tandem mass spectrometry (LC-MS/MS) to detect any new product formed. A fluorescent compound that migrates at a lower R_f than both PQS and PQS-d_4 was detected in the TLC analysis (Fig. 2). This compound did not induce the biosensor, thus demonstrating that PQS-d_4 has been effectively inactivated by *A. xylosoxidans* Q19 (Fig. 2). As before, a molecular ion at *m/z* 280 $[M + H]^+$ that is 16 Da higher than PQS-d_4 was detected, and as expected, the positive MS/MS of this ion showed a major fragment at *m/z* 262.1 $[M + H - H_2O]^+$ (Fig. 2).

The molecular ions of the PQS and PQS-d_4 inactivation products were both 16 Da higher than the substrates, suggesting the incorporation of an additional oxygen atom. This was confirmed by high resolution exact mass spectrometry (LC-HR-MS) of the TLC-purified extract of I-PQS which detected a molecular ion at *m/z* 276.1599, corresponding to the elemental composition of $C_{16}H_{22}O_3N$, along with its prominent fragment ion at *m/z* 258.1491 ($C_{16}H_{20}O_2N$) (Fig. S3). These data confirmed that I-PQS was derived from the parent compound PQS by oxygenation via *A. xylosoxidans* Q19 and that the loss of a water molecule results in formation of the prominent fragment ion.

Elucidation of the structure of I-PQS

There are four primary sites in the structure of PQS that could potentially undergo oxygenation and give rise to hydroxylated products either in the carbocyclic ring, at the C1-position of the heptyl chain, at the 1-NH or C2-positions of the heterocyclic ring respectively (Fig. 3A). In addition, the terminal carbon of the alkyl chain is a less likely possible fifth site for hydroxylation.

The high-field ^{1}H-NMR spectrum of I-PQS ruled out structures **1** and **2** (Fig. 3A and Fig. S4A) as all the carbocyclic and heptyl chain protons were present. Out of structures **3** and **4** in Fig. 3, only structure **4**, 2-heptyl-2-hydroxy-1,2-dihydroxyquinoline-3,4-dione (HHQD), accounts for the observed MS (Figs S2 and S3), ^{1}H (Table S1 and Fig. S4A) and ^{13}C NMR data (Table S1 and Fig. S4B). The observed chemical shifts, integral values, multiplicities and coupling constants for all the protons in ^{1}H spectrum corroborate convincingly with the expected values for all the Hs in HHQD structure (Table S1). Oxygenation at C-2 renders it chiral, thereby causing the neighbouring methylene protons to become asymmetric. Indeed, the 1′-CH_2 protons appear as two sets of ddd instead of a clean triplet as would be expected if they were in the planar configuration. This observation of the asymmetric character of 1′-CH_2 also excludes the plausible ring contracted 2-hydroxy-2-octanoylindolin-3-one structure (Fig. 3A, structure **4b**) that can form via the ring opened tautomer trione structure (Fig. 3A, structure **4a**). Furthermore, in the ^{13}C spectrum, the observed chemical shifts at δ 196.42, 173.65 and 82.48 were assignable to carbonyls at positions 3 and 4 and C2-OH respectively. Other ^{13}C resonances also correlated unambiguously with the assigned structure (Table S1), which was further confirmed as HHQD by the observed fragmentation in the high resolution MS (Fig. 3B).

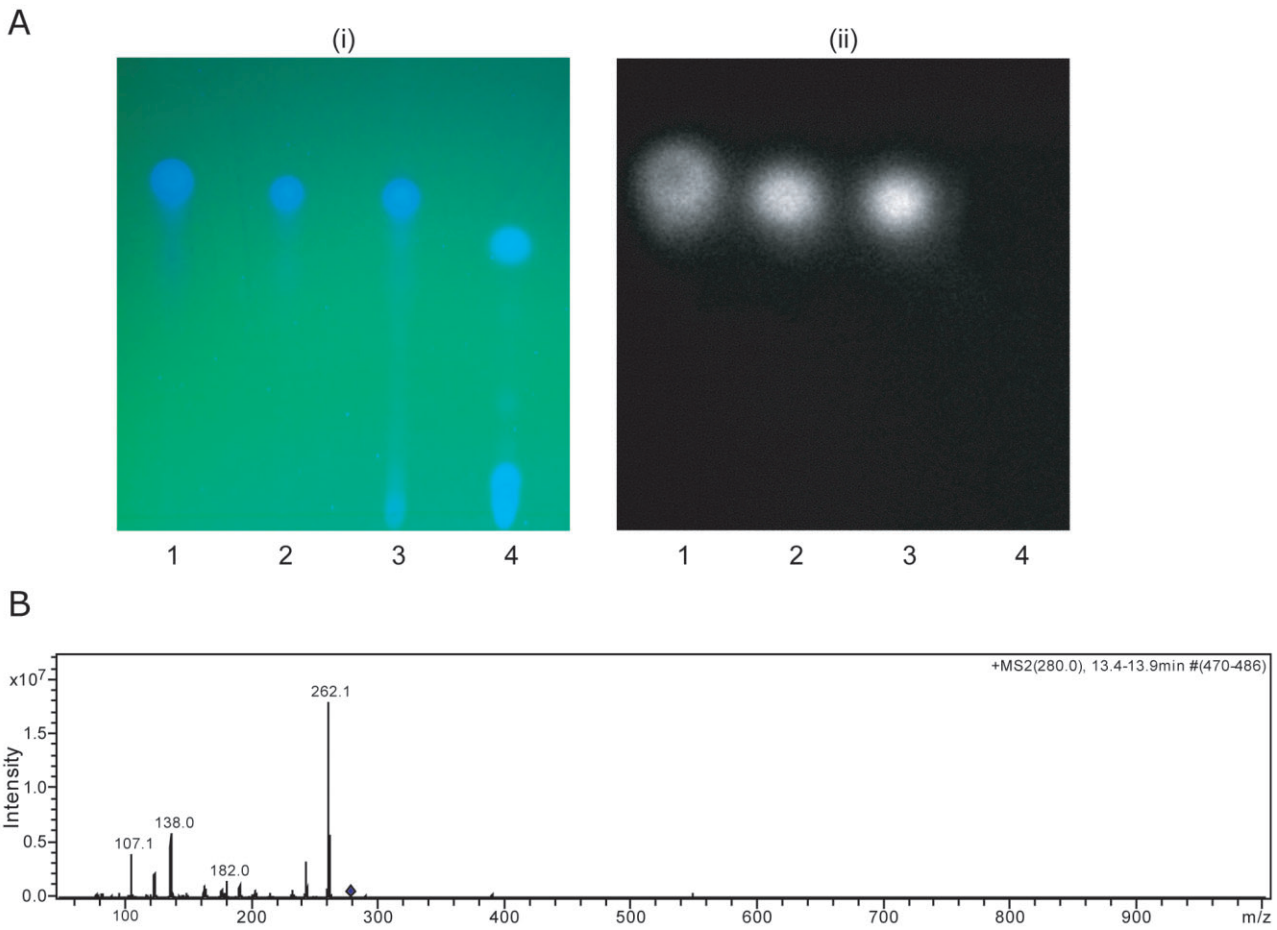

Fig. 2. Inactivated 2-heptyl-3-hydroxy-4(1*H*)-quinolone (I-PQS) is derived from PQS.
A. Inactivation of tetra-deuterated PQS (PQS-d_4) by *Achromobacter xylosoxidans* Q19. (i) Thin layer chromatography (TLC) visualized under UV light and (ii) TLC overlaid with the *Pseudomonas aeruginosa* PAO1 Δ*pqsA* miniCTX::*pqsA′-lux* bioreporter and viewed under a photon video camera. Lane 1, PQS standard (10 mM, 2 μl), lane 2, PQS-d_4 standard (10 mM, 2 μl), lane 3, cell-free control and lane 4 with *A. xylosoxidans* Q19 resting cells.
B. Tandem mass spectrometry spectrum of the $[M + H]^+$molecular ion at *m/z* 280 from the purified fluorescent compound obtained on incubation of *A. xylosoxidans* Q19 with PQS-d_4. The major fragment, $[M + H - H_2O]^+$ observed has an *m/z* 262.1 indicating the presence of hydroxylated PQS-d_4.

Oxygenation of PQS is likely to deliver chirally pure HHQD, although the absolute stereochemistry at C-2 cannot be ascertained from the NMR data.

Oxygenation of PQS derivatives

To gain further insights into the ability of *A. xylosoxidans* Q19 to hydroxylate other PQS analogues, *N*-methyl PQS as well as PQS congeners with alkyl chains ranging from C1 to C5 were evaluated. Each of the substrates provided yielded its corresponding hydroxylated product, which was not detected in the cell-free control. The accurate mass and molecular formula of the respective hydroxylated PQS analogues were confirmed via HR-LC-MS analysis of the TLC purified extracts from the resting cell assays (Table S2 and Fig. S5). This indicated that the side chain length as well as the presence of a methyl group on the N-heteroatom does not affect the hydroxylation capability of *A. xylosoxidans* Q19. However, the PQS precursor, HHQ, which lacks the 3-OH substituent, did not undergo oxygenation. This strongly suggests that the OH group at position 3 is essential for the *Achromobacter*-driven inactivation of 3-hydroxylated AQs such as PQS. It is interesting to note that the UV chromatogram peaks of all the hydroxylated products do not generate the tailing effect that is distinctive of PQS analogues and that is linked to the metal chelating properties of PQS (Ortori *et al.*, 2011 and Fig. S5).

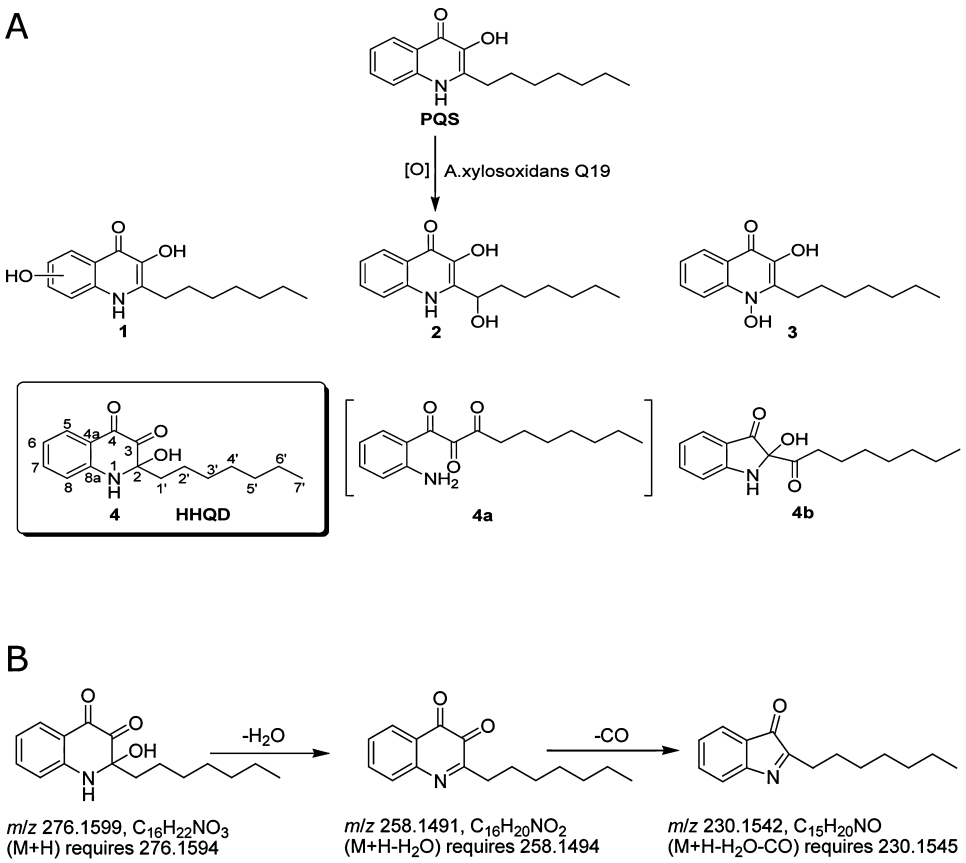

Fig. 3. (A) Plausible structures of inactivated 2-heptyl-3-hydroxy-4(1*H*)-quinolone (I-PQS) and (B) the major fragmentation products in the mass spectrometry (MS) of I-PQS.

HHQD formation by other A. xylosoxidans *isolates*

To determine whether the *A. xylosoxidans* from cystic fibrosis sputum samples could also drive the formation of HHQD from PQS, each of the 17 isolates was subjected to the 'resting cell' assay, and after 24 h of incubation, each culture supernatant was extracted and subjected to LC MS/MS. The approximate fold-difference between I-PQS and PQS levels (I-PQS/PQS) was calculated from the values of the respective peak areas extracted from the MS chromatograms for each isolate. The highest fold-change of > 800 was obtained for the *A. xylosoxidans* Q19 isolate, whereas the PQS-inactivating clinical isolates gave fold-changes ranging from 0 to 176 (Table S3).

Is HHQD formation unique to A. xylosoxidans*?*

Since there is little information on the turnover of PQS in *P. aeruginosa*, the producer organism, we also investigated whether HHQD was formed following incubation of *P. aeruginosa* PAO1 with PQS using the same protocol used for *Achromobacter*, i.e. in a non-growth medium containing succinate. By following the loss of PQS and appearance of HHQD by HPLC (as described in the Experimental procedures section), it is clear that similar amounts of HHQD are formed by both *P. aeruginosa* PAO1 (Fig. 4A) and the isogenic PQS-non-producing *pqsA* mutant (data not shown). Since HHQD does not act as an agonist since it fails to activate the *P. aeruginosa pqsA* reporter gene fusion, we investigated whether it could act as a competitive PQS antagonist using the bioreporter *Pseudomonas putida* KT2440 mini-Tn*7*T::*pqsR*-P_{pqsA}-*lux*. Figure S6 shows that HHQD is unable to inhibit PQS-dependent activation of the reporter. Furthermore, *Arthrobacter* sp. Rue61a, which we previously showed was capable of degrading PQS via the dioxygenase, Hod (Pustelny *et al*., 2009), could also drive the formation of HHQD (Fig. 4B). HHQD formation by *Arthrobacter* did not however depend on Hod as the pAL1-cured strain which lacks the plasmid-encoded *hod* gene retained the ability to convert PQS to HHQD (Fig. 4C). However, Hod-mediated PQS turnover appears to compete with HHQD formation because in Fig. 4B we observe (i) faster PQS elimination and (ii) lower yields of HHQD, compared with Fig. 4C.

To obtain evidence for possible enzyme-driven HHQD formation in *Achromobacter*, we prepared crude cell

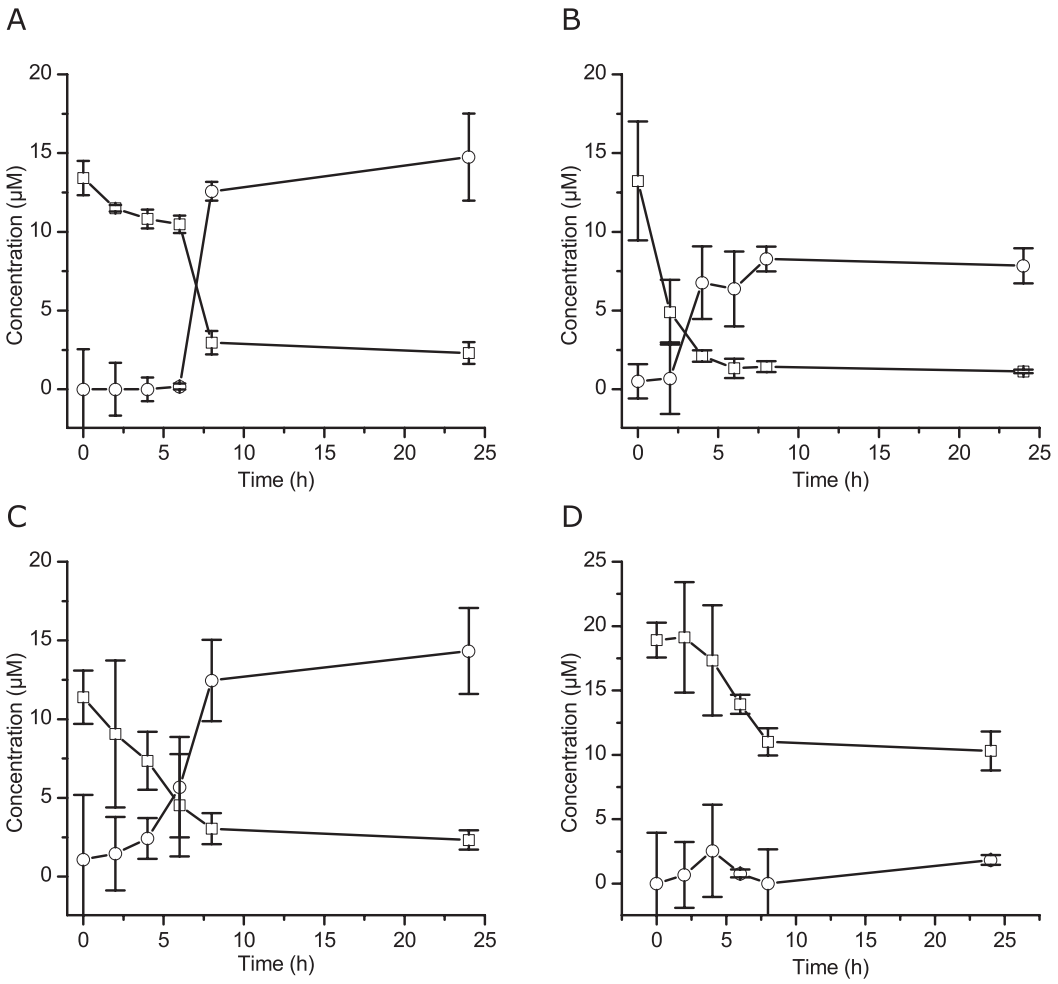

Fig. 4. Conversion of 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) to 2-heptyl-2-hydroxy-1,2-dihydroquinoline-3,4-dione (HHQD) by (A) *Pseudomonas aeruginosa* PAO1, (B) *Arthrobacter* sp. Rue61a, (C) *Arthrobacter* sp Rue61a cured of pAL1 and (D) abiotically in the absence of bacterial cells. Samples were taken over time and the loss of PQS (squares) and appearance of HHQD (circles) determined using high-performance liquid chromatography.

extracts of the soluble protein fraction from strain Q19. Following incubation with 20 μM PQS and 500 μM NADH, the crude protein preparation was extracted with ethyl acetate and subjected to HPLC. However, no HHQD formation was observed. To investigate whether I-PQS could be formed abiotically, we incubated PQS in the same succinate containing incubation medium used above. Preliminary TLC analysis revealed the appearance of HHQD after prolonged incubation in the absence of cells. To quantify the amount of HHQD produced, samples were also subjected to HPLC. Figure 4D shows that after 24 h, less than 15% of the starting PQS concentration was oxidized to HHQD. However, if the incubation medium was prepared without $FeCl_3$ and with 'ultrapure' water from which metal ions had been removed by treatment with Chelex resin, HHQD formation was not detectable (data not shown). We also repeated the PQS-inactivation assay with *P. aeruginosa* incubated in iron-deficient KG medium. Figure S7 shows that PQS is converted to HHQD under these conditions, confirming the biotic nature of the inactivation reaction.

Discussion

Quorum sensing signal molecules need to be produced at the appropriate time and reach a threshold concentration in order to coordinate QS-dependent gene expression at the population level. In turn, such signals must be inactivated to prevent accumulation of inducing concentrations at inappropriate times. This may be achieved by the producer organism, by other organisms within the same consortium or by other organisms competing for the same environment. These last two may inactivate QS signals to gain a survival advantage over, or protect themselves from, the producer. Inactivation or degradation may also depend on the nature of the prevailing physico-chemical

environment. *N*-acyl-L-homoserine lactones, for example, rapidly undergo pH-driven lactonolysis in alkaline conditions (Yates *et al*., 2002).

Relatively little is known about the degradation of AQ signal molecules either by the producer, by other bacteria or in the environment. To discover whether AQs in natural environments can be degraded, we enriched a Malaysian rainforest soil sample containing an AQ-producing *P. aeruginosa* population using a defined minimal medium containing PQS as the sole carbon source. In this medium, growth was only apparent after 4 days of incubation. After further sub-culturing, turbidity was observed after 48 h, suggesting that the enriched microbial consortium had adapted to the low nutrient conditions. These prolonged lag phases are likely to be a consequence of the low solubility of PQS in aqueous media (~1 mg l^{-1} in water at pH 7; Lépine *et al*., 2003) that results in a lack of permeation into the cells, since promotion of bacterial growth generally requires cellular uptake. Furthermore, prolonged lag phases have been observed in bacterial utilization of quinoline and other bicyclic compounds related to the AQs (Sutton *et al*., 1996). In the enrichment experiments, growth, pink pigmentation and biofilm at the air liquid interface were only observed in PQS-containing medium. Under these conditions, no PQS was detectable after 48 h of incubation.

From over 40 cultivatable-enriched soil consortium isolates, only one (strain Q19) inactivated PQS in axenic culture, suggesting that other members may depend on PQS degradation products generated by Q19 in order to grow. 16S rDNA sequencing and phylogenetic analysis identified Q19 as *A. xylosoxidans* (formerly *A. xylosoxidans*). Members of this Gram-negative species can degrade a variety of xenobiotic compounds including endosulfan, bisphenol A and polychlorinated biphenyl congeners (Jencova *et al*., 2004; Zhang *et al*., 2007; Singh and Singh, 2011), and several strains capable of degrading AHLs have also been identified (Christiaen *et al*., 2011).

Several phenomena may contribute to the majority of consortium strains individually appearing not to degrade or inactivate PQS. These include (i) slow PQS degradation rates not apparent using the screening method employed, (ii) a requirement for specific culture conditions or nutrients to promote axenic growth of individual consortium members and (iii) an ability to grow in the enrichment medium only on PQS degradation products released by the primary degraders. These phenomena are routinely observed in the biodegradation of refractory compounds. Previous enrichment experiments have reported the isolation of stable consortia that are more competent at degrading xenobiotic substrates than any of the individual species (Pettigrew *et al*., 1990; Guo *et al*., 2008). For example, Guo and colleagues (2008) isolated two bacterial species from a stable microbial consortium that could degrade carbazole only when both organisms were present. Similarly, from an AHL-degrading consortium, an *Arthrobacter* strain was identified that could utilize the homoserine lactone released by *Variovorax paradoxus* from the same consortium (Flagan *et al*., 2003). In addition, the biodegradation of poorly water-soluble organic compounds may be limited by a lack of bioavailability and may be accelerated by the presence of biosurfactant producers in the consortium (Schwarz *et al*., 1989). In this context, PQS bioavailability is known to be improved by rhamnolipid biosurfactant production by the producer organism, *P. aeruginosa* (Calfee *et al*., 2005). Hence, the presence of *P. aeruginosa* or other biosurfactant producers in the rainforest soil sample may contribute to efficient PQS degradation by the consortium.

PQS was rendered inactive by *A. xylosoxidans* Q19 through hydroxylation at position 2 of the heterocyclic ring. Our data showed that *A. xylosoxidans* Q19 completely inactivated 20 μM PQS within 10 h with the concomitant accumulation of a UV fluorescent by-product. The use of deuterium-labelled PQS enabled us to demonstrate that this by-product was derived from PQS and so was initially termed I-PQS. Based on MS, ^{1}H and ^{13}C NMR spectra, we concluded that I-PQS was HHQD. Possible pathways for enzyme-catalysed as well as abiotic reactions to form HHQD from PQS are presented in Fig. 5.

During the preparation of this manuscript, Müller and colleagues (2014) reported the discovery of the *Rhodococcus erythropolis* strain BG43, which can degrade PQS, its metabolic precursor HHQ and also 2-heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) both of which lack the 3-hydroxy substituent essential for the *Achromobacter*-dependent inactivation of PQS. Interestingly, both HHQ and HQNO degradation are accompanied by the transient formation of PQS, and HHQ hydroxylation by cell extracts requires NADH, indicating that strain BG43 has a HHQ monooxygenase isofunctional to the *P. aeruginosa* biosynthetic enzyme PqsH (Schertzer *et al*., 2010; Müller *et al*., 2014). In this species, however, PQS is degraded to anthranilic acid rather than being oxidized to HHQD (Müller *et al*., 2014).

The structural similarity between 3-hydroxy-2-methyl-4(1*H*)-quinolone (i.e. the C1 congener of PQS, MPQS), the natural substrate for the 2,4-dioxygenase Hod, and PQS previously prompted us to investigate the Hod-dependent inactivation of PQS (Pustelny *et al*., 2009). However, we found that the catalytic activity of Hod towards PQS was relatively weak and only ~ 0.3% of the activity of its natural substrate, MPQS (Pustelny *et al*., 2009). More recently, Thierbach *et al*. (2014) have shed further light on the oxygenolytic cleavage of 2-alkyl-3-hydroxy-4(1*H*)-quinolones by Hod. They reported that

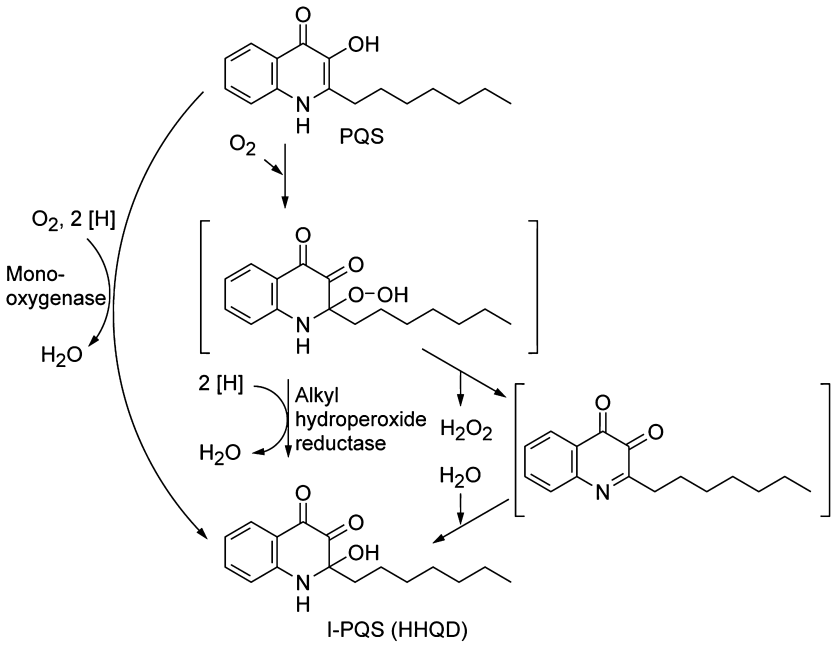

Fig. 5. Possible pathways for the inactivation of 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS) by *Achromobacter xylosoxidans* Q19 to yield inactivated PQS (I-PQS) (HHQD).

oxygen-dependent activation in the HOD active site is substrate-assisted, involving a radical mechanism. Proton abstraction from 3-OH of the substrate promotes single electron transfer from the substrate anion to O_2, yielding a substrate radical – a superoxide anion radical. Incubation of Hod with the C4-congener of PQS, i.e. 2-butyl-3-hydroxy-4(1*H*)-quinolone resulted in the formation of a C2-hydroperoxide, which upon reduction yielded 2-butyl-2-hydroxy-1,2-dihydroquinoline-3,4-dione (Thierbach *et al*., 2014). Consequently, it is possible that *Achromobacter* Q19 possesses an oxygenase with a similar reaction mechanism, which forms a C2-hydroperoxide of PQS (Fig. 5) but does not mediate ring cleavage. This primary product could then be reduced by cellular reductants or by hydroperoxide reductases to HHQD, or decompose with elimination of hydrogen peroxide and subsequent addition of water to also yield HHQD (Fig. 5). Alternatively, a monooxygenase could directly catalyse the hydroxylation of PQS (Fig. 5). However, to date, our attempts to obtain evidence for enzyme-driven HHQD formation in *Achromobacter* cell-free extracts have not been successful.

Apart from the soil, *A. xylosoxidans* can also coexist with *P. aeruginosa* in chronic lung infections in individuals with cystic fibrosis (Steinkamp *et al*., 2005; Razvi *et al*., 2009). Consequently, we evaluated a collection of *A. xylosoxidans* isolates from CF patients for the ability to inactivate PQS. Many but not all of these strains converted PQS to HHQD, albeit with much less efficiency than *A. xylosoxidans* Q19, although the greater activity observed in the latter may be a consequence of the selection and enrichment process. Since we were unable to identify enzymatic activity in crude *Achromobacter* Q19 cell extracts, we evaluated the ability of the PQS producer, *P. aeruginosa* as well as the Gram-positive bacterium *Arthrobacter* sp. Rue61a (from which the PQS-inactivating dioxygenase, Hod was obtained; Pustelny *et al*., 2009) to convert PQS to HHQD. Both organisms generated HHQD from PQS and the mechanism involved was unrelated to the synthesis of PQS or the presence of Hod. This is because a *P. aeruginosa pqsA* mutant and an *Arthrobacter* Rue61a strain cured of the *hod*-containing plasmid both retained the ability to generate HHQD. Consequently, we also investigated whether PQS could be oxidized to HHQD abiotically. The results obtained showed that HHQD can be formed from PQS in the succinate medium used for the resting cell assays albeit at a much lower level than in the presence of bacteria but not when the medium was depleted of Fe(III) and prepared with highly purified water lacking metal ions. Furthermore, PQS was oxidized to HHQD by *P. aeruginosa* incubated in iron-deficient succinate medium confirming the biotic nature of the reaction. However, since PQS is a strong ferric iron chelator (Bredenbruch *et al*., 2006; Diggle *et al*., 2007) and can act as a pro-oxidant promoting free radical generation (Häussler and Becker, 2008), this may conceivably account for some abiotic HHQD formation. Given that not all *Achromobacter* isolates could drive HHQD formation and that *P. aeruginosa* converts PQS to HHQD in iron-deficient conditions, it is possible that the addition of exogenous PQS to certain bacteria induces stress responses, which, in turn, lead to free radical generation. The biochemistry of the Hod reaction (Thierbach *et al*., 2014) as well as model chemistry

(Czaun and Speier, 2002; Frerichs-Deeken *et al*., 2004) indicate that 2-alkyl-3-hydroxy-4(1*H*)-quinolones are susceptible to radical chemistry. In both cases, proton abstraction from 3-OH promotes radical generation at C-2 followed by oxygenation. It is therefore possible that the proposed PQS hydroperoxide intermediate (Fig. 5) results from the reaction of a PQS radical with reactive oxygen. In this context, it is perhaps also noteworthy that PQS induces the upregulation of alkyl hydroperoxide reductases (PA0848 and *ahpC*, *ahpF*) in *P. aeruginosa* (Bredenbruch *et al*., 2006). Alkyl hydroperoxide reductases not only catalyse the reduction of a broad range of organic hydroperoxides to alcohols but also reduce H_2O_2 (Seaver and Imlay, 2001; Parsonage *et al*., 2008). Further work will be required to determine whether reactive oxygen species drive the oxygenation of PQS to form a hydroperoxide whose conversion to the more stable HHQD involves cellular reductases.

Experimental procedures

Bacterial strains, media and growth conditions

The strains used in this study were the *A. xylosoxidans* Q19 (soil isolate, this study) and 17 *A. xylosoxidans* isolates recovered from the sputum of cystic fibrosis patients (provided by Dr T. Bjarnsholt, Department of International Health, Immunology and Microbiology, University of Copenhagen, Copenhagen, Denmark). The *P. aeruginosa* strains PAO1, PAO1 Δ*pqsA* and PAO1 Δ*pqsA* miniCTX::*pqsA′-lux* (Fletcher *et al*., 2007) and *Arthrobacter* sp. Rue61a and its plasmid-cured derivative (Overhage *et al*., 2005) have been described previously. Q19 was identified as *A. xylosoxidans* by 16S ribosomal DNA sequencing.

For construction of the *P. putida* KT2440 mini-Tn*7*T::*pqsR*-P_{pqsA}-*lux* bioreporter strain, the plasmid pUC18-mini-Tn7T-Gm-*lux* (Choi *et al*., 2005) was used. In a first step, the ribosomal binding site (RBS) in front of the *luxC* gene was exchanged by the RBS of pSB401 (Winson *et al*., 1998). For this, a *luxCDA* fragment was amplified by polymerase chain reaction (PCR) from pSB401 with the primer pair luxC-for (5′–GCGCCTCGAGAGGCTTGGAGGATACGTATGACTAAA AAAATTTCATTC–3′, containing the *luxC* RBS, underlined) and luxA-rev (5′–TATAACCGGTGCGCCACCTCTGCTATA CG–3′) and digested with XhoI and AgeI. This fragment was inserted into pUC18-mini-Tn*7*T-Gm-*lux* digested with the same enzymes and *Escherichia coli* DH5α was transformed with the resulting construct termed pUC18-mini-Tn*7*T-RBS-*lux*. In the second step, a 312-bp fragment containing the *pqsA* promoter (P_{pqsA}) was amplified from *P. aeruginosa* PAO1 genomic DNA using primer pqsA-for (5′–ATATCTGC AGTCGAGCAAGGGTTGTAACGG–3′) and pqsA-rev (5′–TA TACTCGAGGACAGAACGTTCCCTCTTCAGC–3′). The PCR product was digested and ligated into the XhoI/PstI sites of pUC18-mini-Tn*7*T-RBS-*lux*. *Escherichia coli* DH5α was transformed with the resulting pUC18-mini-Tn*7*T-P_{pqsA}-*lux*. Finally, the *pqsR* gene of *P. aeruginosa* PAO1 was amplified using primers pqsR-for (5′–ATATCCCGGGCGCCTCTCC CCGCGCGTTG–3′) and pqsR-rev (5′–TATAGAGCTCTTAG TGATGGTGATGGTGATGCTCTGGTGCGGCGCGCTGG–3′). After digestion with SmaI and SacI and ligation in pUC18-mini-Tn*7*T- P_{pqsA}-*lux* digested with the same enzymes, *E. coli* DH5α was transformed with the resulting pUC18-mini-Tn*7*T-*pqsR*-P_{pqsA}-*lux* plasmid. Insertion of the mini-Tn*7*T-*pqsR*-P_{pqsA}-*lux* transposon into the bacterial chromosome of *P. putida* KT2440 was carried out as described elsewhere (Choi and Schweizer, 2006) by co-transformation with the helper plasmid pTNS2 (Choi *et al*., 2005). Integration was verified by PCR using primers Tn7R (5′–CACAGCA TAACTGGACTGATTTC–3′) and glmS-rev (5′–TTACGTGG CCGTGCTAAAGGG–3′).

Lysogeny broth (LB) or LB agar was used for routine bacterial growth and maintenance. For enrichment, isolation and resting cell experiments, we used a modified KG medium (Chan *et al*., 2009). The composition of the basal medium (in g l^{-1} of distilled water) was NaCl, 1.2; KCl, 0.8; Na_2SO_4, 0.25; KH_2PO_4, 0.25; $MgCl_2$, 0.5; $CaCl_2$, 0.25; NH_4Cl, 0.3 and 2-(*N*-morpholino)-ethanesulfonic acid (MES) 1.0. The pH was adjusted to 6.5 with 1 M NaOH before autoclaving. When cooled, $FeCl_3$ (if required) and $MnCl_2$ were added at final concentrations of 12.5 and 6.25 μg l^{-1} of medium respectively. A stock solution of vitamins (Parschat *et al*., 2007) was added at 1 ml l^{-1} of the basal medium. Stock solutions of AQs were prepared at 100 mM by dissolving in 100% methanol and stored at −20°C. For some experiments, metal ions were removed from distilled 'ultrapure' water with 4 g l^{-1} Chelex resin (Serdolit Chelite CHE, SERVA Electrophoresis, Heidelberg, Germany) and used for the preparation of the basal medium.

The following AQs used in this study were synthesized in house by a procedure described previously (Ilangovan *et al*., 2013; Thierbach *et al*., 2014): 2-heptyl-3-hydroxy-4(1*H*)-quinolone (PQS), 2-methyl-3-hydroxy-4(1*H*)-quinolone (C1-PQS), 2-ethyl-3-hydroxy-4(1*H*)-quinolone (C2-PQS), 2-propyl-3-hydroxy-4(1*H*)-quinolone (C3-PQS), 2-butyl-3 -hydroxy-4(1*H*)-quinolone (C4-PQS), 2-pentyl-3-hydroxy-4(1*H*)-quinolone (C5-PQS), *N*-methyl-2-heptyl-3-hydroxy-4(1*H*)-quinolone (N-Me-PQS), 2-heptyl-4-hydroxyquinoline (HHQ) and 5,6,7,8-tetradeutero-2-heptyl-3-hydroxy-4(1H)-quinolone (PQS-d_4). These compounds were added as the sole carbon source at 10 or 20 μM, depending on assay requirements. For cultivation on solid-modified KG medium, agarose (Sigma-Aldrich, Gillingham, UK) was incorporated at a final concentration of 1.5% (w/v). Bacteria were routinely grown at 30°C.

Enrichment and isolation procedures

The soil sample was collected at Rimba Ilmu, University of Malaya (Malaysia). Soil (1 g) was added to 50 ml of the basal medium containing 10 μM of PQS as the sole carbon source and incubated at 30°C with shaking (220 rpm). After 7 days, a 1% (v/v) transfer was made to 50 ml of fresh PQS-supplemented enrichment medium. Cell-free control as well as a PQS-free inoculated control were included in the experiment. To monitor PQS degradation, aliquots were taken every 48 h for extraction and assessment of residual PQS by TLC as described below. A portion of each aliquot was also stored in 25% (v/v) glycerol at −80°C. Growth in the enrichment cultures was observed by the appearance of turbidity

compared with the PQS-free controls. This observation was confirmed by the determination of OD_{600} at 48 h intervals. After three sequential 1% (v/v) transfers at 7 day intervals, the culture was plated onto LB agar and solid enrichment medium. The plates were incubated at 30°C until visible colonies were observed. Colonies with distinct morphologies were re-streaked on LB agar to obtain individual bacterial isolates.

Detection and analysis of PQS by TLC

Samples from enrichment experiments or inactivation assays were extracted three times with equal volumes of acidified ethyl acetate and spotted onto a normal phase silica 60_{F254} (Merck, Nottingham, UK) TLC plates previously soaked for 30 min in 5% (w/v) KH_2PO_4 and activated at 100°C for 1 h. Ethyl acetate extracts from inactivation assays were separated by TLC using a dichloromethane : methanol (95:5) mobile phase. TLC plates were visualized by UV illumination prior to overlaying with 0.3% (w/v) LB soft top agar seeded with a 0.5% (v/v) overnight culture of the *P. aeruginosa* PAO1 Δ*pqsA* mini*CTX*::*pqsA′-lux* PQS bioreporter as described by Fletcher and colleagues (2007). After incubation at 37°C, bioluminescence was detected using a Luminograph LB 980 photon video camera (Berthold Technologies, Bad Wildbad, Germany).

AQ inactivation assay

After overnight growth in LB, bacteria were washed in PBS (pH 6.5) and re-suspended to ~10^9 cells ml^{-1} in 5 or 25 ml of KG enrichment medium containing 1% (w/v) sodium succinate and 20 μM PQS or a related AQ. Cultures were incubated at 30°C, samples collected at timed intervals, extracted with acidified ethyl acetate and the residual AQs analysed by TLC or LC-MS/MS as described by Fletcher and colleagues (2007). In addition, for some experiments, PQS and PQS inactivation product formation were investigated using reverse phase HPLC on a 250 × 4 mm Eurospher II RP-18 column at 35°C. Extracts were separated using a linear gradient (40 min) of 15% (v/v) methanol in water to 100% methanol at a flow rate of 0.5 ml min^{-1}. All eluents were acidified with 1 g l^{-1} citric acid. Light absorption spectra were recorded with a diode array detector (L-2450 LaChrome Elite®, Merck Hitachi, Tokyo, Japan). All experiments were carried out in triplicate.

Extraction and purification of I-PQS

Inactivated PQS (I-PQS) was recovered from PQS-inactivation assays after 24 h of incubation by SPE using Oasis HLB cartridges (Waters, Elstree, UK). Briefly, methanol was added to the cultures to a final concentration of 50% (v/v). After centrifugation, the supernatant was loaded onto an SPE cartridge pre-equilibrated with 50% (v/v) methanol, washed twice with 50% (v/v) methanol and the sample eluted in 100% (v/v) methanol, evaporated to dryness and stored at −20°C.

Elucidation of the structure of I-PQS

Tandem mass spectrometry (MS/MS) and LC-MS/MS analysis was conducted using a Bruker Esquire 4000 HCT plus ion trap mass spectrometer possessing an electrospray ionization interface, in tandem with an Agilent 1200 series LC system (Bruker, Coventry, U.K.). Chromatographic conditions were identical to those described previously. The operating conditions of the MS source were as follows: capillary voltage: 3800V, nebulizer pressure: 40 psi, drying gas flow: 9.0 l min^{-1}, and drying temperature: 365°C. Initial mass scans were from *m/z* 100–400 with ions of interest selected for isolation and fragmentation.

The exact mass of I-PQS was determined using a Thermo Exactive mass spectrometer in conjunction with a Thermo Accela LC system. The chromatographic separation was achieved using a Phenomenex Gemini C18 reversed-phase column (3.0 μm, 100 × 3.0 mm) (Phenomenex, Macclesfield, U.K.) with an appropriate guard column, maintained at 50°C, using a ramped gradient of 0.1% (v/v) formic acid in water (mobile phase A) and 0.1% (v/v) formic acid in MeOH (mobile phase B) at a constant flow rate of 0.45 ml min^{-1}. The mass spectrometer was operated in positive ionization mode with a heated electrospray ionization probe. The electrospray settings were as follows: spray voltage: 4 kV, capillary temperature: 350°C, sheath gas: 40, auxillary gas: 25, sweep gas: 5 and heater temperature: 350°C. Mass scans (*m/z* 100–500) were conducted at a resolution of 100 000 (full width at half maximum). Product ion data were collected using high energy collisional dissociation all ion fragmentation.

^1H NMR spectra were recorded as $CDCl_3$ or DMSO-d_6 sample solutions on Bruker Avance-400 instruments operating at 400 MHz. ^{13}C NMR spectra were recorded in $CDCl_3$ or DMSO-d6 on a Bruker Avance-400 or Bruker Avance(III)-500 MHz instruments operating at 100 or 125 MHz respectively. Chemical shifts were referenced to an internal standard $SiMe_4$ or residual protic solvents, on a broad band decoupled mode.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. HPLC analysis of the major, purified PQS inactivation product (I-PQS) following incubation with *A. xylosoxidans* Q19. Upper panel, I-PQS was detected at λ312 nm and a retention time of 16.7 min. Lower panel – methanol control.
Fig. S2. LC-MS/MS analysis of I-PQS. (A) Positive ESI spectrum for I-PQS showing a molecular ion peak $[M+H]^+$ at *m/z* 276.1. The three most abundant precursor ions are indicated with diamonds. (B) Positive MS/MS mass spectra of the fragment ions of the positive precursor ions *m/z* 298.1 (upper panel), *m/z* 276.1 (middle panel) and *m/z* 258.6 (lower panel). The presence of fragments at *m/z* 258 and *m/z* 240 indicate sequential losses of H_2O.
Fig. S3. Accurate mass and elemental composition of I-PQS. The molecular ion $[M+H]^+$ = *m/z* 276.1599 (upper panel) and its major fragments at *m/z* 258.1491 (middle panel) and *m/z* 230.1542 (lower panel).
Fig. S4. (A) ^1H NMR spectrum for HHQD (I-PQS). (B) ^{13}C NMR spectrum for HHQD (I-PQS).
Fig. S5. Extracted ion chromatogram (EIC) of purified extracts from *A. xylosoxidans* Q19 assays with *N*-methyl PQS provided as the substrate. (A) Cell free control, detected at $[M+H]^+$ = *m/z* 274 and (B) with *A. xylosoxidans* Q19 detected at $[M+H]^+$ = *m/z* 290. The hydroxylated *N*-methyl PQS does not have characteristic tailing effect commonly exhibited by PQS compounds.
Fig. S6. Response of *P. putida* KT2440-mini-Tn7T-*pqsR*-*pqsA*$_P$-*lux* to PQS and I-PQS. Overnight cultures of the reporter strain were diluted to $OD_{600\ nm}$ of 0.05 in LB medium and PQS and I-PQS were added as indicated. Bioreporter luminescence and $OD_{600\ nm}$ was measured every 20 min. Relative luminescence is luminescence (in arbitrary units) divided by $OD_{600\ nm}$. The data are mean values from three technical replicates.
Fig. S7. Conversion of PQS to HHQD by *P. aeruginosa* PAO1 incubated in iron-deficient succinate medium

containing PQS. Samples were taken over time and the loss of PQS (squares) and appearance of HHQD (circles) determined using HPLC.

Table S1. Assignments of observed δ values in ^{1}H and ^{13}C NMR spectra of HHQD.

Table S2. PQS congeners oxidized by *A. xylosoxidans* Q19.

Table S3. Conversion of PQS to HHQD by *A. xylosoxidans* Q19 compared with 17 *A. xylosoxidans* isolates from cystic fibrosis sputum. The fold changes were calculated from peak area values of the respective compounds after incubation of each strain with PQS (10 μM) in minimal medium containing succinate. After 24h incubation, samples were extracted with acidified ethyl acetate and subjected to LC MS/MS. * < 2-fold change.