Chapter Three

Degradation of PQS quorum sensing signal by a novel soil bacterium

3.1 Introduction

Enzyme-mediated inactivation of PQS was recently highlighted as a potential antivirulence strategy against *P. aeruginosa*. Previous studies have shown that inactivation of AQ signalling by mutagenesis of either AQ synthetic genes, such as *pqsA*; or signal transduction genes (i.e. *pqsR* and *pqsE*) hampers virulence gene expression, thus reducing pathogenicity of *P. aeruginosa* in experimental infection models (Diggle et al., 2003, Deziel et al., 2005). Pustelny et al., 2009 recently reported on the use of 3-hydroxy-2-methyl-4(1H)-quinolone 2,4-dioxygenase (Hod, “1H-3-Hydroxy-4-oxoquinaldine 2,4-dioxygenase”) enzyme to inactive the PQS signal. Hod, cytoplasmic enzyme that was purified from *Arthrobacter nitrogalactolicus* strain Rü61a was found to be involved in 2-methylquinoline (qinaldine) utilization, catalyzing the cleavage of 3-hydroxy-2-methyl-4(1H)-quinolone (MPQS, the C1 congener of PQS) to carbon monoxide and *N*-acetylanthranilic acid (Bauer et al., 1994, Bauer et al., 1996, Fischer et al., 1999) (Figure 3.1). Hod-catalyzed conversion of PQS also led to the formation of carbon monoxide, suggesting that it underwent similar 2,4-dioxygenolytic ring cleavage as MPQS (Figure 3.2). The efficacy of Hod as a quorum-quenching enzyme was subsequently evaluated by examining *pqsA::lux* and *lecA::lux* expression
in a pqsA mutant (unable to produce any AQs) supplied with exogenous QS. These experiments demonstrated the significant downregulation of pqsA and lecA expression as well as pyocyanin production (Pustelny et al., 2009). Moreover, wild-type P. aeruginosa cultures treated with Hod also showed a concentration-dependent reduction in PQS levels. This was further extended to a plant infection model, in which Hod reduced both P. aeruginosa virulence and bacterial growth in leaf tissues. These evidences highlight the fact the AQ signalling molecule degradation is indeed a viable target for in vitro and in vivo quorum quenching activity.

There are nonetheless several disadvantages in the application of Hod for quenching PQS-mediated signalling. Upon assaying Hod-catalyzed reactions on 2-alkyl-3-hydroxy-4(1H)-quinolones with alkyl chain lengths ranging from C1-C11, it was apparent that further extension of alkyl chain length to above C3 impeded the enzymatic reaction. The presence of the C7 alkyl chain in PQS substantially reduced Hod activity to around 1200-fold lower than for MPQS, indicating a substantially less catalytic efficiency than its natural substrate. Hod was also found to be susceptible to pseudomonas exoproteases and to competitive inhibition by HHQ (Pustelny et al., 2009). The hod gene as well as other genes involved in 2-methylquinoline conversion to anthranilate was found to be clustered on the linear conjugative plasmid pAL1 of strain Rü61a (Overhage et al., 2005, Parschat et al., 2007). pAL1-like plasmids were also found in other Arthrobacter species that were isolated from soil (Overhage et al., 2005). This observation suggests that soil bacteria with the ability to degrade quinoline compounds, including analogues of PQS signal may not be so uncommon after all.
Figure 3.1 Conversion of quinaldine (2-methylquinoline) by *Arthrobacter nitroguajacolicus* Rü61a. 1. Quinaldine; 2, 1H-4-oxoquinaldine; 3, 1H-3-hydroxy-4-oxoquinaldine (MPQS, the C1 congener of PQS); 4, N-acetylanthranilic acid; 5, anthranilic acid (Overhage et al., 2005).

Figure 3.2 2,4-Dioxygenolytic cleavage of PQS to carbon monoxide and *N*-octanoylanthranilic acid. Catalyzed by Hod from *Arthrobacter nitroguajacolicus* Rü61a (Pustelny et al., 2009).
Figure 3.3 Work flow for the isolation and thorough study of the PQS degrading soil bacterium.
3.2 Results

3.2.1 Screening of AQ producers in soil

A total of 30 bacteria, individually denoted as F1 to F30 were isolated with PIA from the Rimba Ilmu soil sample shown to contain AQs. The PAO1 ΔpqsA CTX-lux::pqsA bioreporter was used for the initial screening of these bacteria for the production of alkyl quinolone compounds. This information is provided via total light output by the bioreporter strain and represents the combination of all AQs in the extracted whole cell culture. Acidified ethyl acetate extracts of each of the soil isolates were prepared and added in microtiter plates as described in the Materials and Methods (Section 2.17.3). Overnight cultures of PAO1 ΔpqsA CTX-lux::pqsA were diluted 1:1000 in fresh LB medium, and 300 µl cultures were grown in the individual wells of the microtiter plates with the dried extracts. The induction of bioluminescence, which corresponds with pqsA expression, is given in relative light units (RLU) divided by OD$_{600}$, measured using an automated luminometer-spectrometer (TECAN). The experiment was carried out in triplicates and the bar chart in Figure 3.4 represents the mean RLU/ OD$_{600}$ for each extract. Figure 3.4 shows that the whole cell culture extracts of eight isolates (F1, F2, F3, F4, F5, F6, F9 and F11) triggered the response of the PAO1 ΔpqsA CTX-lux::pqsA bioreporter due to the presence of AQs, whereas F14 and F17 extracts have no effect in the response of the bioreporter. PAO1 ΔpqsA CTX-lux::pqsA bioreporter culture was also included in the assay as control. To demonstrate that the lack of response of the bioreporter to the extracts from isolates F14 and F17 was not due to growth inhibition, a growth curve was also plotted as shown in Figure 3.5.
Figure 3.4 Screening of AQ producers among the soil isolates. *pqsA* expression in PAO1 Δ*pqsA* CTX-*lux::pqsA* grown in the presence of dried whole cell culture extracts of the soil isolates. Extracts of PAO1 wt and DH5α were added as positive and negative controls respectively.
Figure 3.5 Growth curves of PAO1 ΔpqsA CTX-lux::pqsA in the presence of the different whole cell culture extracts.
3.2.1.1 LC-MS identification of AQs in crude cell culture extracts

It is not possible to discriminate which AQ in a sample of culture is activating the bioreporter in the bioluminescence assay because each AQ activates the bioreporters by a different magnitude. Furthermore, the induction of bioluminescence in the bioreporter may be due to the presence of synergistic effect by the different AQs. Therefore, LC-MS was performed on extracts that showed positive induction of the biosensor (Figure 3.6). The extracts for isolates F14 and F17 were also included in the analysis as negative controls, whilst wildtype PAO1 extracts served as positive control.
Figure 3.6 Identification of AQs by LC-MS analysis. AUC = area under curve.
3.2.2 Identification of AQ producers by 16S rDNA Sequencing

The eight AQ producing soil bacteria were isolated and their 16S rDNA genes (1.5 kb) were amplified by PCR using the 27F and 1525R, as forward and reverse primers respectively, as described in Table 2.3. The amplicons were then purified using the QIAquick® PCR purification Kit (QIAGEN) kit. DNA concentration and purity was determined by a micro-volume spectrophotometer (Thermo Scientific NanoDrop 2000) before subjecting to sequencing analysis. By comparisons to sequences available in the National Center for Biotechnology Information Basic Local Alignment Search Tool (BLAST) database, the isolates were all identified as *Pseudomonas aeruginosa*. 
Figure 3.7 Purified PCR product of 16S rDNA. (A) Agarose gel, visualised under UV showing the 1.5 kb amplified region using primers 27F and 1525R. M – 1kb DNA ladder (Promega, UK).

(B) Purity and concentration of amplicons as determined by a micro-volume spectrophotometer (Thermo Scientific NanoDrop 2000).
3.2.3 Enrichment of PQS degrading consortium

The enrichment procedure was carried out with both uninoculated flasks and flasks without PQS serving as controls. Uninoculated flasks with PQS were used for monitoring any abiotic loss of PQS during the prolonged incubation period. The control flask without PQS served to demonstrate that growth of the consortium should only occur in the presence of PQS. After two successive 1% (v/v) transfers into fresh medium at seven day intervals, a stable consortium that grows in the presence of PQS was obtained. This consortium gave rise to a turbid and pinkish hued culture (Figure 3.8). No growth was observed in the minimal medium devoid of PQS. The pH for the enrichment culture, including both controls was constant throughout the incubation process, suggesting that the culture’s change in colour was not due to pH fluctuations.

Using the stable consortium that was obtained after two enrichment transfers, growth was monitored by obtaining the OD$_{600}$ at a 48 hourly time point (Figure 3.9). Furthermore, 4 ml samples were collected at these time points from each of the three flasks. Whole cell culture extraction (3 ml) was carried out to extract residual PQS, whereas the remaining 1 ml was stored as glycerol stocks. The extracts were spotted on normal phase TLC plates and subsequently overlaid with soft agar seeded with PAO1 ΔpqsA CTX-lux::pqsA. Sterile controls were also analysed at each time interval. The presence of PQS will give rise to a bioluminescent spot when viewed under UV light, which was captured using a light camera. Figure 3.10(B) shows that bioluminescence was undetected from 48 h of incubation onwards for the enrichment culture. Light intensity for the uninoculated control was constant throughout, demonstrating that PQS was being degraded or inactivated by the consortium. Based on Figure 3.10(A), from the 48 h time point, there appeared to be a compound, presumably a PQS degradation metabolite that also fluoresces under UV light. This compound did not induce the bioreporter.
Figure 3.8 Enrichment of PQS degrading consortium. A pink turbid culture was observed when 20 µM PQS was provided as a substrate (B); No observable growth in the uninoculated flask (A) as well as in the flask without PQS (C).

Figure 3.9 Growth curve of consortium in the enrichment medium in the presence or absence of 20 µM PQS.
Figure 3.10 Monitoring of PQS degradation by the stable consortium. Samples were spotted on TLC and viewed under UV light (A) and after being overlaid with agar containing the biosensor for 8 h and viewed using a photon camera (B). Row 1- samples from flask with inoculums only, row 2- samples from uninoculated flask containing PQS only and row 3- enrichment culture. PQS concentration used for the experiment was 20 µM.
3.2.4 Biodegradation of PQS by pure bacterial cultures

In order to identify the particular bacterial strain that was playing the most active role in turning over PQS, individual strains was recovered from the consortium by spread-plating onto solidified PQS enrichment medium (1.5% (w/v) agar). Figure 3.11 shows that the consortium grew better in the presence of PQS compared to the control, which is solid medium devoid of PQS as the carbon source. A total of 48 different types of bacterial colonies from the consortium were isolated and purified. These 48 isolates (denoted as Q1 to Q48) were individually screened to test their ability to degrade PQS (10 µM) by the resting cell. Residual PQS was extracted from the individual cultures after incubation for 96 h and subsequently spotted on a normal phase TLC plate, viewed under UV light before overlaying with soft agar seeded with the PAO1 ΔpqsA CTX-CTX:pqsA bioreporter to detect bioluminescence production. The ability of an isolate to degrade PQS was evaluated qualitatively based on the absence or presence of bioluminescence due to induction of the bioreporter by residual PQS. From one batch of a screening experiment (Figure 3.12 (A)), isolates Q1 and Q26 showed diminished light intensity compared to the control as well as the other non PQS degrading isolates. PQS also appears to have been completely degraded by isolate Q19 as there was no detectable bioluminescence at all. The PQS degradation assay was repeated three times on isolates Q1, Q19 and Q26. Figure 3.12 (B) shows that isolate Q19 consistently degraded PQS. Hence, isolate Q19 was chosen for extensive study of it PQS degradation capacities.
Figure 3.11 Observation of growth for consortium when plated on solid enrichment medium. After 5 days incubation, visible colonies were observed on plates containing 20 µM PQS (A), but no colonies on plate without PQS (B) at both $10^{-4}$ and $10^{-5}$ dilutions.
Figure 3.12 Evaluation of individual isolates for PQS degradation ability. Whole cell culture extract from a resting cell PQS degradation assay was spotted on TLC plates and residual PQS was detected by the PAO1 \( \Delta pqsA \) CTX-\( lux::pqsA \) bioreporter. Bioluminescence production was viewed under a photon camera. (A) Initial screening results for a batch of isolates. (B) Repeat of PQS degradation assay for isolates Q1, Q19 and Q26. PQS concentration that was used in the degradation assays of the individual isolates was set at 10 \( \mu \)M.
3.2.5 Identification and phylogenetic analysis of the PQS degrading strain Q19

The PQS degrading isolate was identified morphologically and genetically. Q19 is a gram-negative, rod-shaped bacterium. The isolate grows in an aerobic condition with an optimum growth temperature of 30 °C. This strain grows slowly on LB agar, with visible colonies after 48 h incubation. BLAST search performed on the 1426 bp 16S rDNA sequence of strain Q19 showed that it is an *Achromobacter xylosoxidans*, with 99% homology. Phylogenetic analyses was carried out with MEGA version 4.0 (Tamura et al., 2007). The phylogenetic tree that was built is shown in Figure 3.13. The tree was rooted with *Bacillus cereus* strain LS25 (GenBank accession number AM110926.1) as outgroup since this taxon is distantly related but still sufficiently conserved or homologous to each of the ingroup taxa considered. This tree was constructed using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1000 replicate was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. There were a total of 1423 positions in the final dataset.

Figure 3.13 Phylogenetic analysis of strain Q19.
3.2.6 PQS-degradation capacities of *Achromobacter xylosoxidans* strain Q19

Time course degradation experiments were carried out with axenic *A. xylosoxidans* Q19 to monitor the disappearance of PQS and the appearance of degraded product of PQS. The inoculum was approximately $10^7$ cells of a pure culture grown in LB, washed twice with PBS and resuspended in minimal medium. Samples were collected at set time points, extracted and subjected to TLC analysis for the separation of the PQS degradation product. The Hod catalyzed conversion of PQS results in the release of carbon monoxide and the formation of N-Octanoylanthranilic acid (Pustelny et al., 2009). Since N-Octanoylanthranilic acid also fluoresces under UV light, a synthetic standard of this compound was also spotted on the TLC plate for the time course analysis to ascertain whether PQS degradation by *A. xylosoxidans* Q19 also led to the formation of this compound. Figure 3.14 demonstrates the disappearance of PQS (10 µM) and emergence of a compound that migrates at a lower $R_f$ than PQS and N-Octanoylanthranilic acid. This compound produced a greenish fluorescent spot when viewed under UV light and was visible from 6 h onwards. It appeared to reach a maximal concentration at 72 h, the time point where PQS is completely depleted.

More detailed investigation of the emergence of this fluorescing compound was carried out on duplicates of *A. xylosoxidans* Q19. In this experiment, 20 µM of PQS, twice the amount that was used in the previous assay was used in an attempt to find out if whether an axenic culture has the capacity to degrade the same concentration of PQS as the consortium did. Periodically, samples collected from the cultures were analyzed using TLC for PQS and PQS degradation products by UV absorbance and overlaid with PAO1 Δ*pqsA* CTX-*lux::pqsA* bioreporter. Cell free controls were also analyzed at each time interval to detect any abiotic changes and to serve as a reference for determining the extent of biodegradation by *A.*
The overlay results show that *A. xylosoxidans* Q19 required 192 h for complete depletion of 20 µM of PQS (Figure 3.15 (B)). Oddly, TLC analysis of the uninoculated control showed apparent abiotic degradation of the PQS substrate from 48 h of incubation onwards, with the emergence of a similar greenish fluorescent compound that migrates at a similar Rf as the one seen in the presence of *A. xylosoxidans* Q19 (Figure 3.15 (A)). In spite of the observable depletion of PQS in the abiotic control at 192 h when viewing the plate under UV light, the intensity of bioluminescence at this time point did not differ much from the two previous time points. The absence of bioluminescence in the duplicates of *A. xylosoxidans* Q19 culture extracts at 192 h further demonstrated that *A. xylosoxidans* Q19 is indeed required to fully degrade 20 µM of PQS. This experiment also proves that the lack of bioluminescence was due to the disappearance of the PQS signal rather than inhibition of detection of PQS by the bioreporter since TLC analysis of the samples separated any possible inhibitors that might be produced by *A. xylosoxidans* Q19.
Figure 3.14 TLC analysis of degradation of PQS by A. xylosoxidans Q19 with the corresponding appearance of an unidentified compound. Samples were viewed under UV light. Lane 1 and lane 7- N-Octanoylanthranilic acid standard (10 mM, 2µl), lane 2 and lane 8- PQS standard (10 mM, 2µl). Lane 3- 0 h, lane 4- 6 h, lane 5- 12 h, lane 6- 24 h, lane 9- 48 h, lane 10- 72 h, lane 11- 96 h, lane 12- 120 h.

Figure 3.15 Time course analysis for the degradation of 20 µM of PQS by A. xylosoxidans Q19. (A) TLC visualised under UV light, (B) TLC overlaid with PAO1 ΔpqsA CTX-lux::pqsA bioreporter and viewed under a photon camera. Lane 1- PQS standard (10 mM, 2 µl), lane 2- abiotic control, lanes 3 and 4- duplicates of A. xylosoxidans Q19 inoculated cultures.
3.2.7 Supplementation of an alternative source of carbon to increase *A. xylosoxidans* Q19 PQS biodegradation efficiency

Different carbon sources were evaluated as a co-substrate to facilitate *A. xylosoxidans* Q19 in complete degradation of 10 µM PQS within 24 h, that is, before the onset of abiotic biodegradation. In this investigation, glycerol, glucose and succinate were tested at 5 % (v/v) and 10 % concentrations. Abiotic controls were included in the experiments to ensure that the additional carbon sources do not affect substrate stability during the 24 h incubation period. Figure 3.16 shows that supplementation of glycerol at 5% assisted in very minimal degradation of PQS (10 µM), with slight accumulation of the green fluorescent metabolite that was observed in the previous degradation experiments. PQS degradation also occurred with 10% (v/v) of glycerol, albeit at a lesser degree. Glucose at both 5% (w/v) and 10% (w/v) did not stimulate PQS degradation at all. An improvement was clearly observed when succinate at both concentrations was tested as a co-substrate. TLC analysis shows a larger accumulation of the degraded compound than with glycerol supplementation (Figure 3.16). Furthermore, overlay results also confirms this observation from the pronounced reduction in bioluminescence.

In view of succinate being the preferred co-substrate in stimulating PQS degradation, a range of concentration was tested to identify the optimum concentration needed. Figure 3.17 shows that less than 4% (w/v) succinate in the culture is ideal for efficient degradation of PQS. When supplemented with 1% succinate, degradation of PQS (10 µM) by *A. xylosoxidans* Q19 was completed within 24 h of incubation as evidenced by the lack of bioluminescence production by the bioreporter. Partial degradation of PQS occurred when supplemented with 4% to 15% succinate, observable in the decreased bioluminescence compared to controls. The lack of degradation observed in the cell free controls show that 24 h is a favourable
incubation duration for the assay. Furthermore, it also confirmed that *A. xylosoxidans* Q19 resting cells indeed played a role in PQS degradation, albeit more efficiently when supplemented with a low concentration of succinate. Based on this result, subsequent PQS degradation assays by axenic *A. xylosoxidans* Q19 were carried with the supplementation of 1% succinate in the culture.
Figure 3.16 Evaluation of other carbon sources to improve the rate of PQS degradation by *A. xylosoxidans* Q19. (A) TLC visualised under UV, (B) TLC overlaid with PAO1 Δ*pqsA* CTX-*lux::pqsA* bioreporter and viewed under a photon camera. Lane 1 and lane 7: PQS standard (10 mM, 2 µl). Lane 2: 5 % glycerol, lane 3: 10 % glycerol, lane 4: control for glycerol, lane 5: 5 % glucose, lane 6: control for 5 % glucose, lane 8: 10 % glucose, lane 9: control for 10 % glucose, lane 10: 5 % succinate, lane 11: control for 5 % succinate, lane 12: 10 % succinate, lane 13: control for 10 % succinate.
Figure 3.17 Supplementation of different concentrations of succinate as a co-substrate. (A) TLC visualised under UV, (B) TLC overlaid with PAO1 ΔpqsA CTX-lux::pqsA bioreporter and viewed under a photon camera. Lane 1 and lane 8: PQS standard (10 mM, 2 µl). Lane 2: 1 %, lane 3: control for 1 %, lane 4: 2 %, lane 5: control for 2 %, lane 6: 3 %, lane 7: control for 3 %, lane 9: 4 %, lane 10: control for 4 %, lane 11: 5 %, lane 12: control for 5 %, lane 13: 15 %, lane 14: control for 15%.
3.2.8 Characterisation of PQS degradation metabolite

3.2.8.1 LC-MS/MS identification of the metabolite

The PQS degradation assay was carried out in triplicates, followed by TLC analysis to separate this compound from other possible compounds that did not fluoresce under UV light. This PQS degraded compound could not be accurately identified by TLC separation alone thus requiring a more detailed analysis using liquid chromatography coupled to mass spectrometry (LC-MS). The prominent fluorescent compound was scraped from the TLC plates, extracted three times with dichloromethane and evaporated in a stream of nitrogen gas. The dried samples were reconstituted in 1 ml methanol and mixed vigorously for thorough resuspension before subjecting to LC-MS analysis. These triplicates were respectively labelled as G1, G2 and G3. The UV chromatograms in Figure 3.18 demonstrated that High Performance Liquid Chromatography (HPLC) analysis on this compound gave rise to a major peak that elutes at a retention time of 16.7 min, when detected at a wavelength of 312 nm. For characterisation of this compound, positive electrospray ionization (+ESI) mass spectrometry in full scan mode in the range of m/z 50 to 1000 was used. The total ion chromatogram (TIC) for this analysis showed a primary peak with similar retention time to the UV chromatograms for each of the triplicates (Figure 3.19). Full scan positive ESI spectra under this prominent peak revealed three positive molecular ions with m/z 276.1, m/z 298.1 and m/z 573.3 (Figure 3.20)

The mass spectral analysis for full scan -ESI was also carried out to test the reliability of the results obtained from +ESI (Figure 3.21). As shown in the TIC, the triplicates yielded similar
peaks at the retention time of 16.7-16.9 min. Analysis of the spectra under this peak for each of the three samples revealed two negative ions with \( m/z \) 273.8 and \( m/z \) 571.1, corresponding respectively to the positive ions with \( m/z \) 276.1 and \( m/z \) 573.3. These peaks were not observed in neither positive nor negative ESI analysis of the MeOH control (figure 3.22).

For unequivocal characterisation of this compound, sample G3 was selected for auto LC-MS/MS experiments in positive ion mode to identify the fragment ions. The TIC from auto MS/MS analysis showed a peak that also elutes at the retention time of 16.8 min (Figure 3.23). Figure 3.24 (A) shows the mass spectra under this peak (RT 16.8 min) revealing the three most abundant ions at \( m/z \) 258.1, \( m/z \) 276.1 and \( m/z \) 573.3. These three ions were subjected to fragmentation via Collision Induced Dissociation (CID). The positive ion with \( m/z \) 573.3 gave rise to a main fragment ion at \( m/z \) 298.1, whereas the MS/MS spectra of the positive ion with \( m/z \) 276.1 had a major fragment ion occurring at \( m/z \) 258.1 (Figure 3.24 (B)).
Figure 3.18 UV chromatograms of the triplicates (A) G1, (B) G2 and (C) G3 from HPLC analysis. The compound was detected at 312 nm, producing a major peak with the retention time of 16.7 min. MeOH was introduced as a negative control (D).
Figure 3.19 Total ion chromatograms of the triplicates obtained in positive ion mode MS. (A) G1, (B) G2 and (C) G3. MeOH (D) was included as a negative control.
Figure 3.20 Positive full scan ESI spectra under the major peak at retention time 16.8 min. from each of the total ion chromatograms for samples (A) G1, (B) G2 and (C) G3. Two major positive ions with \( m/z \) 276.1 and \( m/z \) 573.3 were detected for all triplicates. MeOH (D) was included as a negative control. The x-axis denotes the mass to charge ratio \( (m/z) \) whereas the y-axis denotes the intensity of the spectra.
Figure 3.21  Total ion chromatograms of the triplicates obtained in negative ion mode MS. (A) G1, (B) G2 and (C) G3. MeOH (D) was included as a negative control.
Figure 3.22 Negative ion mode full scan ESI spectra under the peak at retention time 16.7-16.9 min from each of the total ion chromatograms for samples (A) G1, (B) G2 and (C) G3. Two major negative ions with m/z 273.8 and m/z 571.1 were detected for all triplicates. MeOH (D) was included as a negative control.
Figure 3.23 Total ion chromatograms (TIC) of sample G3 obtained in positive ion mode MS. (A) TIC from MS, (B) TIC from MS/MS.
Figure 3.24 MS/MS analysis of sample G3. (A) Positive full scan ESI spectra under the major peak at retention time 16.8 min from the TIC of sample G3. The three most abundant precursor ions that are auto selected by the Smartfrag option in the software are indicated with diamonds. (B) Mass spectra of the fragment ions of the auto selected positive precursor ions with m/z 573.3 (i), m/z 276.1 (ii) and m/z 258.6 (iii).
Table 3.1 Summary of positive precursor ions and the respective fragment ions that were detected under MS/MS analysis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor ion (m/z)</th>
<th>Fragment ions (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PQS</td>
<td>260</td>
<td>175</td>
</tr>
<tr>
<td>Unidentified metabolite</td>
<td>573.3</td>
<td>298.1</td>
</tr>
<tr>
<td></td>
<td>276.3</td>
<td>258.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>134.0</td>
</tr>
<tr>
<td></td>
<td>258.6</td>
<td>134.0</td>
</tr>
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3.2.8.2 D-C7-PQS degradation

The next crucial step is to ascertain whether the compound with \( m/z \) 276 was produced by isolate \( A. \ xylosoxidans \) Q19 in response to the presence of PQS in the media or truly a product that is derived from PQS degradation. In this investigation, a deuterium labelled PQS, 5,6,7,8-tetradeutero-4,3-dihydroxy-2-heptylquinoline (D-C7-PQS) instead of PQS was used as the carbon source at 20 µM final concentration in a degradation assay with \( A. \ xylosoxidans \) Q19 resting cells. A cell-free control was included to ensure that there was no abiotic degradation of this substrate in 24 h. At the end of the assay, extractions were carried out with acidified ethyl acetate to obtain the degradation products. The PAO1 \( \Delta pqsA \) CTX-lux::\( pqsA \) biosensor is able to detect D-C7-PQS at the same sensitivity level as PQS. Hence, TLC separation coupled with biosensor overlay was carried out on aliquots of the extracts to confirm the biodegradation of labelled PQS. Figure 3.25 (A) shows a fluorescing compound that migrates at a lower \( R_f \) than both PQS and D-C7-PQS. This compound did not induce the biosensor, thus proving that D-C7-PQS has been effectively inactivated by isolate \( A. \ xylosoxidans \) Q19. The said products formed from the degradation assay were then isolated by preparative TLC and subsequently subjected to LC-MS/MS analysis.

Positive ion mode MS/MS was set to monitor D-C7-PQS and its fragment ions at \( m/z \) 264 and \( m/z \) 179 respectively, which are 4 Da higher than the PQS ions. The Electrospray Ion Chromatograms (EIC) from this analysis showed the presence of several peaks from the triplicate’s extracts (denoted as DH1, DH2, DH3), appearing at similar retention times as the single peak from the cell-free extract (Figure 3.26 (A)). By overlaying the EIC of the control with the triplicates (Figure 3.26 (B)), it is demonstrated that PQS has been reduced drastically, as shown by the more intense peak from the control. Upon closer inspection of
the spectra under these peaks, the \textit{m/z} 179 fragment ion was not present in the samples, proving that PQS was no longer available (Figure 3.27).
Figure 3.25 Degradation of D-C7-PQS by strain A. xylosoxidans Q19. (A) TLC visualised under UV light, (B) TLC overlaid with PAO1 ΔpqsA CTX-lux::pqsA bioreporter and viewed under a photon camera. Lane 1- PQS standard (10 mM, 2 µl), lane 2- D-C7-PQS standard (10 mM, 2 µl), Lane 3- Cell-free control, Lane 4, 5 and 6- Triplicates with A. xylosoxidans Q19 resting cells, denoted respectively as DH1, DH2 and DH3.
Figure 3.26 Electrospray Ion Chromatograms (EIC) from +MS/MS detection of D-C7-PQS, m/z 179 (264.3). (A) Individual EIC from analysis, D-C7-PQS synthetic standard, (10 µl, 10 µM) (i); cell-free control (ii); triplicates, DH1 (iii), DH2 (iv) and DH3 (v). (B) Overlay of (A)(ii), (iii), (iv) and (v) EIC peaks. The purple peak represents the cell-free control whereas all the red peaks represent the triplicates. The x-axis denotes the retention time (RT) whereas the y-axis denotes the intensity of the peaks.
Figure 3.27 +MS/MS full scan ESI spectra under the prominent peak(s) at retention time 12.5-14 min. from each of the EIC in figure 3.24 (A). D-C7-PQS synthetic standard, (10 µl, 10 µM)(A); triplicates, DH1 (B), DH2 (C) and DH3 (D).
3.2.9 Elucidation of the molecular formula for the PQS degradation metabolite

The molecular weights of the PQS and D-C7-PQS degradation metabolite were both 16 Da higher than the substrates, suggesting the presence of an additional oxygen atom. To confirm this hypothesis, we set out to identify the molecular formula of this compound. This was carried by measuring the accurate mass of the degraded compound up to five decimal points with use of Fourier transform mass spectrometry (FTMS). Extraction and purification of the PQS degradation metabolite prior to accurate mass analysis was carried out similarly to the LC-MS/MS experiment described in section 3.2.8.

Table 3.2 summarises the results that were obtained from the calculated peak profile (Figure 3.28) for the PQS degradation metabolite. The accurate mass was obtained at 276.16123 Da, yielding an elemental composition of $C_{16}H_{22}O_3N$. There was indeed an additional oxygen atom in the degraded metabolite when compared with the molecular formula of the PQS substrate. Mass measurement error is typically expressed as ppm (parts per million). In our experiment, MS measurements for the compound gave rise to an error of 6.53661 ppm (i.e., $6.53661 \times 10^{-6}$), sufficient by the given standards for unambiguous determination of a chemical formula of that mass. Using the given formula:

$$
\text{ppm} = \frac{\text{mass error}}{\text{accurate mass}}
$$

$$
6.53661 \text{ ppm} = \frac{\text{mass error}}{276.16123}
$$

$$
6.53661 \times 10^{-6} = \frac{\text{mass error}}{276.16123}
$$

Mass error = $(6.53661 \times 10^{-6}) \times (276.16123) = 0.0018$

Hence, peak mass for the PQS degradation metabolite = $276.16123 \pm 0.0018$
### Table 3.2 Accurate mass measurement of the PQS degradation metabolite.

<table>
<thead>
<tr>
<th>Mass</th>
<th>Formula</th>
<th>Error (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>276.16123</td>
<td>C_{16}H_{22}O_{3}N</td>
<td>6.53661</td>
</tr>
</tbody>
</table>

![Figure 3.28 Calculated peak profile for the PQS degradation metabolite.](image-url)
3.2.10 Time course analysis of PQS degradation

A 24 h time course PQS degradation assay, with succinate as co-substrate was carried out with *A. xylosoxidans* Q19 to monitor the disappearance of PQS (20 µM) and the appearance of the degraded products. Two sets of samples, one for TLC separation coupled with biosensor overlay; and another for LC-MS/MS analysis were obtained at every 2 h. In Figure 3.29, TLC separation show the degradation product first appearing at 2 h and its rapid accumulation in the following time points. Overlay results demonstrated that PQS was not detectable by the biosensor after 8 h, indicating that PQS was completely inactivated by *A. xylosoxidans* Q19 within the 24 h time frame.

LC-MS-MS analyses were also performed on the extracts to elucidate relative loss of PQS and accumulation of the degraded product. The ion trap was set to isolate the specific precursor ion of PQS, with *m/z* 259 and after fragmentation, scan for the subsequent fragment ions. The stacked Extracted Ion Chromatograms (EIC) of the PQS fragment ion *m/z* 175 in Figure 3.30 illustrated the decrease in PQS concentration, observable in the reduction of peak height from the 0 h sample (most forefront chromatogram) to the 24 h sample (final peak at background). A reverse of this result was observed in the stacked EIC of the degradation product, which was monitored at +MS2 *m/z* 258(276) (Figure 3.31). The peak area of each curve were recorded and tabulated and a graph with values for the area under curve (AUC) on the y-axis was plotted against time (h). Corresponding well with the TLC analysis results, PQS concentration remained at a relatively low level beyond 8 h (Figure 3.32). The degradation product on the other hand reached maximal accumulation at 10 h (Figure 3.33).
Figure 3.29 TLC analysis of PQS degradation over a 24 h time course. (A) TLC visualised under UV light, (B) TLC overlaid with PAO1 ΔpqsA CTX-lux::pqsA bioreporter and viewed under a photon camera. Std: Synthetic PQS (10 mM, 2 µl)
Figure 3.30 LC-MS-MS detection of PQS ($m/z$ 260) from the 24 h degradation assay that was carried out using 20 µM of PQS. The extracted ion chromatograms (EIC) for the signature fragment ion with $m/z$ 175 were stacked to illustrate the decreased substrate concentration.
Figure 3.31 LC-MS-MS detection of PQS degradation product (m/z 276) from the 24 h degradation assay. Extracted ion chromatograms (EIC) for the signature fragment ion with m/z 258 were stacked to illustrate the increased product concentration.
Figure 3.32 Degradation of PQS by isolate Q 19. AUC = area under curve.

Figure 3.33 Accumulation of the PQS degradation product. AUC= area under curve.
3.2.11 Degradation capacity of *A. xylosoxidans* Q19

To test whether the C7 alkyl chain was a critical recognition point for degradation by *A. xylosoxidans* Q19, C7-PQS was substituted with C1-PQS as the main carbon source, at 20 µM in a degradation assay, with succinate as co-substrate. TLC analysis in Figure 3.34 shows the depletion of C1-PQS for the triplicates after 24 h incubation. No abiotic degradation of the substrate was detected in the cell-free control. C1-PQS is less polar than PQS, thus migrating at a lower R$_f$.

Subsequently, the precursor of PQS, namely HHQ was also provided as a carbon source to *A. xylosoxidans* Q19. TLC analysis coupled with overlay with the bioreporter illustrated the fact that *A. xylosoxidans* Q19 was unable to degrade HHQ (Figure 3.35), even with prolonged incubation in the presence of succinate as the co-substrate.
Figure 3.34 TLC analysis of C1-PQS biodegradation samples. Lane 1- PQS standard (10mM, 2 µl), lane 2- C1-PQS standard (10mM, 2 µl), lane 3- cell-free control, lane 4, 5 and 6- triplicates with *A. xylosoxidans* Q19 resting cells.

Figure 3.35 TLC analysis of HHQ degraded samples. Samples were viewed under UV light (A), or after being overlaid with PAO1 Δ*pqsA* CTX-*lux::pqsA* bioreporter and viewed under a photon camera (B). Lane 1- HHQ standard (10 mM, 2 µl), lane 2- cell-free control, lane 3- incubated with Q19 resting cells.
3.3 Discussion

3.3.1 Enrichment of PQS degraders from rain forest soil

The successful isolation of PQS and HHQ producers from rain forest soil indicates the presence of PQS-degrading bacteria in this environment. Otherwise, PQS and HHQ molecules can only rely on non-biological degradation. The AQ signal producing strains were isolated and identified by 16S rDNA sequencing. These sequences were compared with available sequences in the BLAST database. All the AQ positive isolates that were obtained in this study were *Pseudomonas aeruginosa*, which is a species of bacteria well known to produce the complete spectrum of quinolone signalling compounds. LC-MS results (Figure 3.6) showed that all eight *P. aeruginosa* isolates had similar AQ profiles compared with wildtype PAO1. The results corresponds with the finding which stated that major AQs found in *P. aeruginosa* cultures are the C7 and C9 congeners of PQS and HHQ, together with the N-oxides of HHQ (Fletcher et al., 2007).

TLC analysis used in conjunction with biosensor overlay with synthetic standards for PQS and HHQ only provides tentative identification of AQs in *P. aeruginosa* cultures. Therefore MS analysis is needed to unequivocally identify the AQ compounds. It is not always possible to predict the quorum sensing (QS) signal profile of a new isolate even though the species is known. For example, two different strains of *Rahnella aquatilis*, a known AHL producer was previously demonstrated to have different AHL profiles (Steidle et al., 2001). Numerous bacterial strains that degrade AHL compounds have
been successfully isolated from soil. Some of these AHL degraders were able to grow on AHLs as the sole source of energy and nitrogen (Huang et al., 2003, Dong et al., 2002, Leadbetter and Greenberg, 2000). It is hypothesized that bacteria capable of degrading PQS are present in this soil environment.

A defined medium was developed for the enrichment of bacteria with the ability to degrade PQS. Phosphate buffers strongly precipitate metals. In some microorganisms, phosphate production confers protection from certain metals. Non-metal-binding buffers, including the sulfonic acids such as MES (2-(N-morpholino)ethanesulfonic acid), $pK_a=6.15$, and PIPES (1,4-piperazinediethanesulfonic acid), $pK_a=6.80$, can optimize metal bioavailability in culture media. Furthermore, pH also strongly influences metal bioavailability since metals readily precipitate as carbonic salts at pH>7.0. Therefore, the pH in this enrichment medium was kept slightly acidic (~pH 6.3) with MES buffer to maintain metal solubility and its bioavailability to the bacteria (Maier et al., 2009).

At the beginning of the enrichment experiment, the consortium took 7 days for complete degradation of 20 µM PQS. After two subsequent 1% v/v transfers to fresh medium, the time required for degrading the same amount of PQS was reduced to two days. This was because the consortium have acclimatized to the culture conditions and required less time for the induction of PQS degradation. Incubation period that amounts to several days is a very common phenomenon in the degradation of quinoline compounds. Previous work on the enrichment of quinoline/methylquinoline derivative-degrading bacteria usually entails long incubation periods, ranging from two to eight days (Sun et al., 2009, Sutton et al., 1996, Bott and Lingens, 1991, Röger et al., 1990).
The fact that substituted quinoline compounds such as methylquinolines are particularly resistant to microbial attack was also emphasized repeatedly when explaining for the long lag period (Rothenburger and Atlas, 1993). The PQS degrading ability of this consortium was evident from the primary fact that the consortium was enriched based on the utilization of PQS as sole carbon source. No other co-substrate was supplemented in the enrichment medium. Secondly, growth, accompanied by a light pink pigment production, was readily observed in enrichment cultures. This was not observable in the control flask devoid of a carbon source. Third, biosensor overlay indicates the disappearance of PQS from the enrichment medium from 48 h onwards. In the cell-free control, PQS concentration was maintained throughout the whole incubation period, indicated by the same bioluminescence intensity from the biosensor overlay for all time points.

Pink pigment production during quinoline biodegradation has been highlighted previously for other organisms (Zhu et al., 2008, Aislabie et al., 1990, Shukla, 1986). It appears that biodegradation of this type of bicyclic compound is almost always accompanied by pigment formation, indicating the presence of intermediates that are produced. However, there are several variables that can affect this phenomenon, such as the type of substrate derivatives used, species of degrader involved, as well as incubation conditions (O'Loughlin et al., 1996). The pH of the enrichment medium for all three flasks was recorded throughout the enrichment experiment. No significant pH difference between the controls as well as before and after incubation was observed, suggesting that the pink pigment was not produced due to pH changes in the medium.
3.3.2 Inactivation of PQS by pure bacterial cultures

Due to prolonged exposure to low-nutrient conditions of the enrichment medium, there is a possibility that some of the bacteria from the consortium may not have the metabolic capacity to respond to sudden nutrient increase in a rich medium. This condition is also known as nutrient shock (Jensen et al., 1996). In our investigation, the consortium was diluted and spread-plated onto both LBA and solid minimal medium with 20 μM PQS as the sole carbon source. This latter medium was essentially a solidified version of the enrichment medium used in the previous enrichment experiments. Very slow growth was observed on the minimal medium plates, requiring up to 5 days before visible colonies were formed. Colonies with different morphologies and colours were retained from both types of plates. These isolates were routinely grown as well as maintained in LB medium as it provided a nutrient rich environment to support better and faster growth. Some strains still required at least two days incubation before sizable colonies were formed on LBA.

A screening method was subsequently developed to evaluate the PQS degradation capability of each isolate. Precultures, or quinoline- adapted cells were routinely used to reduce the long lag phase in substrate utilization assays of quinoline or methyl quinoline degrading bacteria (Brockman et al., 1989, Zhu et al., 2008). In the initial PQS degradation assays, precultures were introduced by inoculating the isolate in minimal medium with 5-10 μM PQS, including additional growth factors such as glucose or succinate to acclimatize the strain prior to subjecting it to the harsh and limiting conditions of the minimal medium with PQS as the sole carbon source. However, no
obvious growth, determined by lack of turbidity was observed. Only some isolates exhibited very slight growth after incubating for more than 100 h, but with no noticeable degradation of PQS.

The extended lag phase, signifying a lack of growth could be attributed to two main factors. Firstly, PQS is a highly insoluble compound compared to quinolines and methylquinolines, with a solubility of only 1 mg/l in water at pH 7, and of 5 mg/l in LB (Lépine et al., 2003). Most substrates that promote microbial growth have to undergo either cellular uptake or attachment in order to be available to the cell’s catabolic mechanism (Wentzel et al., 2007). The non-utilization of PQS could be due to its poor solubility in aqueous medium as well as the lack of permeation into the cell (Shukla, 1986, Schwarz et al., 1989). Prolonged lag phase in the degradation of quinoline compounds is largely due to its refractory nature. Sutton et al., (1996) observed a lag period of up to 100 h for the degrading strain, in the presence of 4-methylquinoline. The long lag phase of the preculture, coupled with the uncertainty as to whether a particular isolate would grow to sufficient density rendered this method overly time consuming and inefficient for screening potential PQS degraders. Furthermore, there might be insufficient enzymes in the small inocula of an axenic culture for PQS metabolism. Hence, resting cells harvested from a 5 ml overnight LB culture grown to approximately $10^7$ c.f.u. ml$^{-1}$ for the in vivo PQS inactivation assay.

Of the 48 isolates that were purified from the consortium, only one (A. xylosoxidans, Q19) was shown to degrade PQS. A few factors can contribute to the majority of the strains appearing to not degrade PQS. One primary explanation might be that the other isolates exhibit very slow degradation that was not detected in the present screening
method. Different isolates may have varied culture requirements when grown as an axenic culture, thus necessitating further optimization to rule out this factor. The second reason may be that the other strains in the consortium grew on the PQS degradation products that are generated and released into the media by the primary degraders. For AHL signal degradation, *Arthrobacter* was reported to utilize HSL, the product of AHL degradation that is released by *Variovorax paradoxus*, both of which were from the original consortium (Flagan et al., 2003). This type of bacterial population is able to co-exist in the enrichment culture. Thirdly, the bacterial strains may need to exist in a consortium in order to degrade PQS. Guo et al., (2007) isolated a stable microbial consortium consisting of two types of bacteria that was able to degrade carbazole. However, none of the two species of bacteria could utilize carbazole when incubated singly in a minimal medium with carbazole as the sole carbon source.

Surprisingly, *A. xylosoxidans* Q19 was able to degrade PQS in pure, monoculture. However, the degradative capability of this axenic culture was not as efficient as the consortium. *A. xylosoxidans* Q19 required approximately 96 h for complete depletion of 10 µM PQS if concentrated cells were used (Figure 3.14). When 20 µM PQS was used in the inactivation assay, it took *A. xylosoxidans* Q19 close to 192 h to completely degrade it. This demonstrates that axenic cultures were less efficient than the consortium in PQS degradation as it only took 48 h for the acclimatized consortium to degrade 20 µM of PQS. This phenomenon was routinely observed in the biodegradation of refractory compounds. Previous enrichment works have reported on the isolation of stable consortia that were more competent at degrading xenobiotic substrates than any of the individual species from it (Pettigrew et al., 1990, Guo et al., 2008). This work shows that there may be only one or a few strains from the consortium that were truly degrading PQS. Biodegradation of organic compounds with limited water solubility is often slow due to lack of bioavailability of these compounds to microbial cells (Schwarz
et al., 1989). Plausibly, in a consortium, PQS degraders may work collaboratively with
biosurfactant producers which will synergistically increase the bioavailability of
insoluble PQS to be degraded by *A. xylosoxidans* Q19.

### 3.3.3 PQS-degradation capacity of *A. xylosoxidans* Q19

BLAST search performed on the 16S rDNA sequence of isolate Q19 showed that it was
*Achromobacter xylosoxidans*. This motile Gram-negative bacillus was previously
named *Alcaligenes xylosoxidans* (Saiman et al., 2001). *A. xylosoxidans* was reported to
have the ability to degrade bisphenol A as well as to utilize carbazole. Carbazole is an
aromatic heterocyclic compound with a tricyclic structure, consisting of two six-
membered benzene ring fused on either side of a nitrogen-containing ring (Zhang et al.,
2007, Guo et al., 2008). Previously, Pustelny et al. (2009) reported on the capability of
the 3-hydroxy-2-methyl-4(1H)-quinolone 2,4-dioxygenase (Hod) in inactivating PQS.
Hod is capable of catalyzing the conversion of PQS to *N*-octanoylanthranilic acid and
carbon monoxide (Figure 3.2). *N*-octanoylanthranilic acid has an intact chromophore
and is able to fluoresce under the similar UV wavelength as PQS. The primary objective
of TLC analysis was to ascertain whether *A. xylosoxidans* Q19 degrades PQS in a
similar fashion as Hod. This was done by comparing the *R*<sub>f</sub> values of the separated
metabolites from the assay with the *N*-octanoylanthranilic acid standard.

We demonstrated that *A. xylosoxidans* Q19 was not degrading PQS with the same mode
as Hod, indicated by the lack of a metabolite migrating with the same *R*<sub>f</sub> as the *N-
octanoylanthranilic* acid standard. TLC analysis in the time course assay for PQS
biodegradation by *A. xylosoxidans* Q19 showed the emergence and accumulation of a
greenish fluorescence compound after 6 h incubation. PQS was completely absent after
the 72 h time point. The unidentified compound migrated at a lower $R_f$ than both N-octanoylanthranilic acid and PQS, indicating that it is more polar. Hence, it is considered to be a novel PQS degradation product from an inactivation pathway dissimilar to Hod.

Interestingly, during the investigation of the degradation product, it was noticed that PQS appears to be abiotically degraded when incubated for more than 48 h. This peculiar phenomenon was detected from the TLC separation of the cell-free control extracts from the PQS degradation assay using 20 µM PQS. There was an observable accumulation of the same greenish compound that was also present in the extracts from the *A. xylosoxidans* Q19 resting cells incubated with PQS. Thus far, sample extracts from enrichment experiments as well as from the *in vivo* PQS degradation assays were only spotted on TLC plates and qualitatively evaluated for the presence of residual PQS by visualisation of bioluminescence production from the bioreporter overlay. Consequently, the abiotic effect on PQS was overlooked, especially since the cell-free control from the enrichment assay maintained the same bioluminescence intensity for samples from all time points (Figure 3.10).

Without TLC separation, abiotic degradation of PQS was not noted because the enrichment extract from the 240 h incubation time point was also inducing the bioreporter at a similar intensity as the 0 h samples. We suspect that in spite of the unavoidable abiotic degradation of PQS due to prolonged incubation, it was actually occurring at a minimal rate, thus retaining a suitably high concentration that is still sufficient for maximal induction of the PAO1 $\Delta pqsA$ CTX-lux:*$pqsA$ bioreporter. Furthermore, the results shown in Figure 3.15 (B) also proved that *A. xylosoxidans* Q19 was indeed playing an active role in PQS degradation since the PAO1 $\Delta pqsA$ CTX-
lux:pqsA bioreporter overlay results showed complete depletion of PQS at 192 h for the duplicates, whereas there was still ample amount of residual PQS in the cell-free control. It is crucial to note that TLC separation coupled with biosensor overlay confirms that the lack of bioluminescence was due to the disappearance of PQS, and not from the inhibition of the biosensor. TLC analysis of the extracts aids in the separation of possible inhibiting compounds that may be produced by A. xylosoxidans Q19.

Chemical conversion of a signalling molecule due to extended incubation is a natural phenomenon that was also highlighted by Uroz et al., 2005. In their case, the AHLs provided were converted to the corresponding \( N \)-acylhomoserine (AH) through lactonolysis upon prolonged incubation. They subsequently proved that this observation was not affecting their results by showing that the isolated \( Rhodococcus \) erythropolis strain W2 degraded AHLS during short-term incubations which did not permit the chemical conversion of AHL to AH. Furthermore, the two enzymic activities reported in their work were found to specifically degrade or modify bona fide AHL molecules only (Uroz et al., 2005). Abiotic degradation of PQS was not reported in any literature since most work carried out with PQS never exceeded 48 h incubation. We theorized that this observation in our investigations could be due to oxidation, resulting from prolonged incubation in a highly aerated environment.

### 3.3.4 Supplementation of co-substrates

Co-metabolism has been described as an important technique for the biodegradation of recalcitrant compounds. It was reported that an organophosphate pesticide, known as demeton-s-methyl could be degraded by \( Corynebacterium \) glutamicum in the presence of fructose as a co-substrate (Girbal et al., 2000). There are two ways a growth substrate
can act to enhance the metabolic process: firstly, it may stimulate cell growth and improve the transformation of the non-growth substrate (Grant and Betts, 2004); secondly, it could perform as a co-substrate in xenobiotic metabolism by inducing certain enzymatic pathways that are shared by both growth and non-growth substrate (Girbal et al., 2000). Co-substrates can sometimes act as inducing agents for biodegradative enzymes as well (Chaudhuri and Wiesmann, 1995).

In this enrichment experiment, PQS is considered a growth substrate because the consortium was able to grow using PQS as the sole carbon source. However, axenic *A. xylosoxidans* Q19 grew on PQS at an extremely slow rate, only achieving slight turbidity after a week of incubation. *A. xylosoxidans* Q19 was capable of degrading up to 20 µM PQS only when high concentrations of resting cells were provided, though still at a much slower rate than the consortium. From these observations, we presumed that PQS was not an ideal growth substrate for *A. xylosoxidans* Q19 although this strain possesses the required enzymes to transform PQS. A suitable growth substrate is able to induce enzyme cofactors and metabolites required for the xenobiotic transformation and/or have the capability to support cell growth via energy and carbon generation. Three kinds of conventional carbon sources (glycerol, glucose and succinate) were investigated to identify the most ideal co-substrate that will aid in enhancing PQS degradation by *A. xylosoxidans* Q19. It is ideal for *A. xylosoxidans* Q19 to achieve complete turnover of 20 µM PQS within 24 h, before the onset of abiotic biodegradation.
Among the co-substrates tested, succinate greatly accelerated the degradation of PQS, followed by glycerol. Glucose on the other hand appeared to slow down PQS degradation. This observation contrasts with the degradation of a known bicyclic compound, 1H-4-oxoquinoline (Bauer et al., 1996). The authors discovered that compared with pyruvate and succinate, growth of the degrader, *P. putida* 33/1 and 1H-4-oxoquinoline degradation was accelerated when glucose was supplemented as an additional carbon source. Glucose has been known to cause catabolic repression with respect to other substrates. It is believed that retardation of PQS degradation in the presence of glucose occurred because glucose was being utilized as a primary growth substrate.

While exploring the optimum succinate concentration for future PQS degradation experiments, it has been confirmed that low concentrations of succinate exerted increased PQS degradation. The supplementation of less than 3% (w/v) succinate was sufficient to achieve 100% degradation of 20 µM PQS within 24 h. This minimal co-substrate required was in accordance to a report, stating that when in the presence of a preferred growth substrate, increased degradation of a compound normally occurs by co-metabolism. It is especially so when the concentration of the preferred substrate is growth limiting (Harder et al., 1982, Grant and Betts, 2004). Instead of co-metabolism, sequential utilization occurs when the co-substrate is present in high concentrations and not growth limiting, thus delaying the metabolism of the refractory substrate (Harder et al., 1982). This may explain the lack of PQS degradation in the 24 h timeframe when a higher concentration of succinate is supplemented, because the consortium may be selectively utilizing succinate but not PQS.
3.3.5 LC-MS/MS analysis

For unequivocal determination of the PQS degradation compound, mass spectral analysis is a preferred technique owing to its specificity, sensitivity and high degree of certainty in the identification of compounds. We have used LC-MS/MS with both positive and negative electrospray ionization (ESI) and collision induced dissociation (CID) to identify the compound that was produced from PQS degradation.

TLC analysis demonstrated that this compound fluoresces at a similar UV wavelength as PQS. Hence, the same UV wavelength (312 nm) normally used for PQS and HHQ detection was applied in the HPLC analysis of this compound. The compound produces a prominent peak with a retention time of 16.7 min. Synchronous peaks were generated in the total ion chromatograms from both positive and negative ESI. The positive ions with \( m/z \) 276 and \( m/z \) 573 were most abundant in the positive ESI-MS spectra, with the former being the base peak in all three samples. The protonated ion \([M+H]^{+}\) at \( m/z \) 276 was 16 Da higher than PQS, presumably corresponding to the addition of an oxygen atom onto the PQS molecular structure. We deduce that the positive ion at \( m/z \) 573 is a sodium adduct of the dimer product \([2M+Na]^{+}\). In addition to that, a low intensity sodium adduct of the monomer \([M+Na]^{+}\) at \( m/z \) 298 was also present in the positive ESI-MS spectra of G1 and G3. The lower abundance of the monomer may be attributed to a lack in stability compared to the dimerized molecule. A corresponding deprotonated ion \([M-H]^{-}\) with \( m/z \) 273.8 was observed in the negative ESI-MS, thus confirming the significance of the positive MS results.
Subsequently, MS/MS analysis was carried out to gain better structural information of the detected molecules. In MS/MS analysis, the \([M+H]^+\) parent ions formed from the electrospray source were fragmented by adding extra collisional energy. In this work, auto MS/MS was carried out, in which ions were selected above an intensity threshold as the instrument was acquiring in full scan mode. The instrument isolates and fragments these ions up to a set maximum number and then returns to full scan mode. Isolation and fragmentation of the \(m/z\) 276 precursor ion gave rise to a high abundance product ion at \(m/z\) 258.1 and to a lesser extent, another fragment at \(m/z\) 240.1. This observation may be attributed to sequential losses of two water (18 Da) molecules.

Considering the results obtained from LC-MS/MS analysis, the obvious question that arose was whether the compound at \(m/z\) 276 was derived from PQS or produced by *A. xylosoxidans* Q19 in response to the presence of PQS in the medium. Hence, a subsequent degradation assay was performed using the analogous 5,6,7,8-tetradeutero-4,3-dihydroxy-2-heptylquinoline (D-C\(_7\)-PQS) as a carbon source. This deuterium labelled PQS has \([M+H]^+\) ions that are 4 Da higher than PQS, at \(m/z\) 264 instead of the \(m/z\) 260 of PQS. The resulting extracts from the assay was subjected to similar TLC and LC-MS/MS analysis as when PQS was utilized as the substrate. Similar enzymatic alteration on this labelled substrate will give rise to the compound which will be at 4 Da higher, at \(m/z\) 280. This can easily be determined via targeted LC-MS/MS analysis.

A total of seven samples were subjected to targeted analysis in multiple reactions monitoring (MRM) mode. The first sample in the analysis was the D-C\(_7\)-PQS synthetic standards, followed by extracts from the cell-free control (negative control) and the triplicates from the degradation assay. Initial experiments were carried out to acquire
MRM data for the D-C$_7$-PQS positive ion at $m/z$ 179 (264). EIC of the standard as well as the negative control under this analysis showed a single prominent chromatographic peak that eluted between 13 and 14 min. The triplicates however showed multiple peaks at this retention time. As expected, the fragment ion at $m/z$ 179 was present in the MS/MS spectra of the standards as well as the control, but absent in the triplicates. This indicates that the original D-C$_7$-PQS is no longer present in these samples, most likely inactivated by *A. xylosoxidans* Q19. The subsequent positive MRM analysis was set to monitor $m/z$ 262(280) which were detected in the triplicate samples. This confirms that the unidentified compound is indeed derived from PQS substrate after being acted upon by *A. xylosoxidans* Q19.

Accurate mass measurements were carried out to obtain the molecular formula of the unidentified PQS degradation compound. The mass of the compound was given up to five decimal points, at $m/z$ 276.16123. The molecular formula for this compound is C$_{16}$H$_{22}$O$_3$N, with an acceptable error of 6.5 ppm. There is an additional oxygen atom in the molecular formula, confirming our postulation that an oxygen atom has been added to PQS. This is most likely due to an oxygenation reaction elicited by *A. xylosoxidans* Q19.

### 3.3.6 Elucidation of the structure of the unidentified metabolite

LC-MS-MS and FTMS analyses identified a hydroxylated PQS as the degraded product, but due to the lack of authentic standards, we were unable to identify the specific sites of hydroxylation. The catabolic pathways for \(N\)-heteroaromatic compounds, similar to PQS in terms of the presence of the bicyclic ring structure have been investigated. Enzyme catalyzed oxygenation of the \(N\)-heterocyclic compound by a
water molecule is a common phenomenon, and is normally initiated by the hydroxylation of the position adjacent to the heteroatom (Schach et al., 1993, Fetzner, 2000). This finding supports the earlier reports on the biodegradation of quinolines and methylquinolines, demonstrating that these bicyclic ring structures generally undergo microbial hydroxylation (Aislabie et al., 1990, Rothenburger and Atlas, 1993). Quinoline degradation typically involves hydroxylation at the number 2 position, via the formation of 2-hydroxyquinoline (Sun et al., 2009, Zhu et al., 2008, Pereira et al., 1988, Shukla, 1986, Grant and Al-Najjar, 1976). Methylquinolines, particularly those with substitutions at position 4 and 6 also hydroxylates at position 2, forming monohydroxymethylquinolines (Sutton et al., 1996, Rothenburger and Atlas, 1993, Aislabie et al., 1990). The respective pathways are shown in Figure 3.36. These monohydroxylated compounds are known to be primary metabolites formed from microbial metabolism of substituted and unsubstituted quinolines under aerobic and anaerobic conditions. $^{18}$O$_2$/$^{16}$O$_2$ incorporation analysis has proven that the $^{1H}$-3-hydroxy-4-oxoquinoline 2,4-dioxygenase (Qdo) from P. putida 33/1 and purified $^{1H}$-3-hydroxy-4-oxoquinaldine 2,4-dioxygenase (Hod) from A. Ilicis Rü61a catalyzes the insertion of a single molecule of oxygen at position 2 and 4 of the $^{1H}$-3-hydroxy-4-oxoquinoline and $^{1H}$-3-hydroxy-4-oxoquinaldine respectively. Hod is a dioxygenase, and it proceeds after the initial monooxygenase action. Since there is no breakdown of PQS ring structure from our degradation assays with A. xylosoxidans Q19, we may possibly be looking at PQS inactivation by a monooxygenase reaction.

The above examples convinced us that the hydroxylated PQS may indeed be a primary metabolite of PQS degradation. But it is important to bear in mind that the compounds mentioned above are merely similar to PQS in terms of having the bicyclic ring backbone structure. The degrader we have isolated is of a completely different species
from those well documented quinoline degraders. In addition to that, PQS is much less soluble than those quinoline compounds due to the presence of the long heptyl chain on position 2. Nevertheless, the given examples can still serve as a reference when predicting the position on PQS where oxygenation is most likely to occur. Figure 3.37 shows the putative structures that are suggested for this unidentified compound. We presume that the most possible scenario would be to have the oxygenation occur on the first carbon of the alkyl side chain (Figure 3.37 (A)). In view of the tendency for quinoline compounds and its derivatives to undergo hydroxylation at position 2, having the oxygen atom in position 2 of the PQS ring structure is also very likely to occur (Figure 3.37 (B). This second hypothetical compound is a regio-isomer of the series 6, 3-alkyl-2,3-dihydroxy-4-quinolones that was highlighted previously as a type of quinolone produced by *P. aeruginosa* (Lépine et al., 2004). However, introduction of an oxygen atom at position 2 gives rise to an aminal compound, which is not a very stable structure. The next structure postulated would be oxygenation on the nitrogen atom (Figure 3.37 (C)). This molecular structure can tautomerize into an N-oxide form, in which the hydrogen atom migrates to the ketone at C4 (Figure 3.37 (D). Oxygenation could also occur on the carbon ring, either on the 5th, 6th, 7th or 8th position, though this structure is still considered less likely because the carbon atoms on the ring are usually very stable and not susceptible to any conversion (Figure 3.37(D)). In addition, quinoline compounds are usually catabolised by preferential attack on the pyridine nucleus (Rüger et al., 1993, Shukla, 1989).

Pereira et al., (1988) investigates the source of the oxygen for the initial oxidation of quinoline to 2(1H)quinolinone with labelled H2O (18O) to elucidate the mechanism of this hydroxylation reaction. Their results demonstrate the microbially mediated incorporation of the oxygen atom from water, as hydroxyl, into position 2 of the quinoline ring. The accurate mass measurement also indicated no participation of
atmospheric oxygen in the reaction. Additionally, the review by Fetzner 2000 also mentions that oxygen incorporated into a 2-methylquinoline (quinaldine) by hydroxylases is derived from a water molecule. It is thus quite likely that the oxygen in the present unidentified PQS degraded compound was derived from water as well. However, since the present finding may be dealing with an entirely different enzyme, further work using $^{18}\text{O}_2$ needs to be carried out to confirm the source of the incorporated oxygen in the hydroxylated PQS compound.

Samples that are analyzed via HPLC or direct infusion faces problems in distinguishing between isomers and isobaric compounds that have an identical exact mass, even when using high resolution mass spectrometry (HR-MS) (Mullen et al., 2009). It is important to bear in mind that the sum formulas can be associated with more than a single structure, thus requiring the need for further experimental evaluation by NMR-based analysis (Giavalisco et al., 2008).
Figure 3.36 Metabolism of quinoline and methylquinoline compounds. Proposed pathway for quinoline (A) and 4-methylquinoline (B) biodegradation (Sun et al., 2009, Sutton et al., 1996).
Figure 3.37 Putative structures of the PQS degradation compound (m/z 276). Substitution of oxygen atom at (A) C1 of the heptyl chain, (B) position 2 on the pyridine ring, (C) nitrogen moiety on ring, (D) tautomer of structure (C), (E) positions 5, 6, 7, or 8 on the benzene ring.
3.3.7 Time course analysis of PQS degradation

Quantitation of a compound can be accomplished using HPLC and UV detection. HPLC analysis relied on; retention time, peak area and UV spectra character. Though more sensitive than TLC analysis, the HPLC assay still suffered from lack of sensitivity and specificity. MS is able to detect many more components in the same sample, including UV transparent components. The ideal way of identifying or differentiating a compound is by using a mass spectrometer capable of MS/MS fragmentation. This is due to the fact that many compounds have similar intact mass, of which cannot be distinguished with MS analysis alone. The second dimension of MS fragmentation provides a unique fragment. Based on the acquired LC/MS/MS data from the PQS degradation compound, this time course experiment was carried out with MS/MS set to selectively monitor specific ions. For monitoring of PQS level, the specific parent mass, with \( m/z \) 259 and its unique fragment ion at \( m/z \) 175 was used. The degradation product (\( m/z \) 276) was monitored with detection of its unique fragment ion at \( m/z \) 258. A total of 13 samples, acquired at a 2-h interval was analysed with this setting. By calculating peak area from the EIC of the targeted ions, it was able to semi-quantify the loss and accumulation of substrates and products, respectively.

The most rapid reduction in substrate concentration occurred in the first 8 h of the experiment, in which the bulk of it was quickly metabolised. By 10 h, PQS was already reduced to a concentration that was no longer detectable by the bioreporter. There appeared to be no lag period prior to degradation of PQS because LC-MS analysis showed that substrate levels dropped quickly at 4 h. Sterile controls for this experiment...
showed no substrate loss. Interestingly, LC-MS/MS analysis showed that the hydroxylated metabolite was already present, even at 0 h. Nonetheless, we were able to demonstrate that *A. xylosoxidans* Q19 was responsible for accelerating the oxygenation of PQS since the hydroxylated compound increased exponentially until the 6 h time point, corresponding to the loss of PQS. From 4 h onwards, we also observed the formation and eventual thickening of a biofilm layer at the air liquid interface in the incubation flasks. Synonymous to the enrichment assays, this biofilm rim was not present in the cell-free and PQS-free controls. However, no further colour changes that were initially seen in the consortium cultures was observed. This suggests that there might be other bacteria in the consortium that plays a role in transforming the initial metabolite, leading to the formation of pink pigments in the enrichment cultures.

The data reported in this investigation unambiguously demonstrated that PQS was specifically degraded by *A. xylosoxidans* Q19. This was due to the fact that the resting cells were shown to degrade PQS within 24 h incubation, a time frame that did not permit the chemical conversion of PQS.

### 3.3.8 Metabolism of other PQS analogues by *A. xylosoxidans* Q19

Other PQS analogues, such as C1-PQS and HHQ were tested as substrates for metabolism by *A. xylosoxidans* Q19. C1-PQS (20 µM) was effectively degraded within 24 h, even without the addition of succinate. TLC separation of the assay extracts showed diminished C1-PQS compared to the intact substrate from the control. Unlike PQS degradation, no breakdown product that fluoresces under UV light was observed. No prominent breakdown product was detected when the extracts were subjected to LC-MS analysis. Previous report mentioned that AQs with alkyl chains smaller than C5 are
more problematic in LC/MS detection due to increasing interferences with solvent impurities (Lépine et al., 2004). We believe that even in the absence of a co-substrate, C1-PQS was degraded efficiently because it is more soluble, thus having higher cell permeability than PQS. This investigation demonstrated that chain length does not seem to affect the degradation capability of *A. xylosoxidans* Q19 towards AQ compounds.

Even though PQS and HHQ have very similar chemical structures, *A. xylosoxidans* Q19 was not able to degrade HHQ when the resting cells were provided with HHQ as the sole source of carbon. No degradation was observed even with the supplementation of various concentrations of succinate. This suggests that the -OH group at position 3 may be essential for substrate binding and/or transformation. The lack of this substituent could sterically hinder access of HHQ to the active site of the yet to be identified enzyme.
3.5 Conclusion

The main purpose of quorum quenching research is to uncover alternatives to the traditional mode of fighting bacterial infection. It is believed that nature has a rich collection of anti-pathogenic compounds or enzymes that can interfere with microbial activity, rather than inhibiting growth. The ability to control P. aeruginosa infections in animal models have demonstrated the usefulness of quorum sensing as a promising drug target. Moreover, the successful attenuation of virulence by plant pathogens expressing the AHL degrading enzyme, AiiA (Dong et al., 2000) demonstrates the need to investigate the existence of indigenous PQS degrading bacteria from rain forest soil samples.

This project demonstrated the capability of selectively enriched soil bacteria from soil samples containing AQ producers to degrade the PQS quorum sensing signal. This work represents the first report on a bacterium that was specifically enriched by its ability to degrade the PQS quorum sensing signal. This soil isolate, A. xylosoxidans Q19 degraded PQS with the subsequent formation of a monooxygenated metabolite. This work has confirmed via accurate mass analysis the chemical formula of the unidentified compound, indicating the addition of an oxygen atom on PQS. Nonetheless, unambiguous elucidation of a chemical structure to a chemical formula is important to elucidate the precise position on PQS where oxygenation has occurred, thus aiding in the identification of previously undiscovered novel pathways. We are currently in the midst of developing a more efficient preparative method for the large scale purification of the oxygenated compound for NMR analysis. This finding will help lay the
foundation in the discovery of putative enzymes responsible for the degradation of PQS to the oxygenated form. Subsequent work will include the analysis of mutants impaired in the candidate gene that encodes the putative enzyme involved.

It is still unknown whether ring hydroxylation is an absolute prerequisite to ring fission by degraders. However, it is believed that this compound is not the final product of PQS degradation, but rather an intermediate compound. This theory was based on observations of the TLC analysis of degradation culture aliquots from time points of beyond 24 h; showing the decreased fluorescing intensity of the oxygenated compound, coupled with the emergence of other mildly fluorescing compounds or perhaps non-fluorescing compounds. These additional compounds migrate at a higher Rf than PQS, indicating that they are less polar. Further work need to be carried out to identify the remaining metabolites of PQS degradation by A. xylosoxidans. Knowing the identity of these compounds will then allow us to propose a more comprehensive degradation pathway.