Characterization of wetland quorum quenching *Pseudomonas aeruginosa* strain 2SW8 and its 2-heptyl-3-hydroxy-4-quinolone production

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SUMMARY

Most *Proteobacteria* produce *N*-acylhomoserine lactones for bacterial cell-to-cell communication, a process called quorum sensing. Interference of quorum sensing, commonly known as quorum quenching, represents an important way to control quorum sensing. This work reports the isolation of quorum quenching bacterium strain 2WS8 from Malaysia tropical wetland water (2°11'8"N, 102°15'2"E, in 2007) by using a modified version of a previously reported KG medium. Strain 2WS8 was isolated based on its ability to utilize *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) as the sole source of energy. This bacterium clustered closely to *Pseudomonas aeruginosa* PAO1. Strain 2SW8 possesses both *quiP* and *pvdQ* homologue acylase genes. Rapid Resolution Liquid Chromatography analysis confirmed that strain 2SW8 preferentially degraded *N*-acylhomoserine lactones with 3-oxo group substitution but not those with unsubstituted groups at C3 position in the acyl side chain. Strain 2SW8 also showed 2-heptyl-3-hydroxy-4-quinolone production.

KEY WORDS: 2-heptyl-3-hydroxy-4-quinolone (PQS), *N*-acylhomoserine lactone, *Pseudomonas aeruginosa*, Quorum sensing, Quorum quenching, Wetland

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INTRODUCTION

Gram negative bacterial cell-to-cell communication regulates gene expression in a population density-dependent manner, a process known as "quorum sensing" (QS). Typically, gram negative bacteria produce *N*-acyl homoserine lactones (AHLs) by AHL synthase (luxI homologue), and once AHLs reach threshold level AHLs will bind to its cognate receptor (luxR homologue) to regulate gene expression (Fuqua *et al*., 2001; Miller and Bassler 2001).

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AHLs vary in their *N*-acyl side chain number, ranging from C4 to C18, and state of oxidation of C3 that either belongs to the *N*-acyl, *N*-(3-oxoacyl) or *N*-(3-hydroxyacyl) classes (Chhabra *et al*., 2005; Williams *et al*., 2007). It is well documented that QS regulates diverse bacterial physiological processes, including virulence determinants, bioluminescence, swarming, antibiotic biosynthesis, biofilm differentiation, and *Agrobacterium* plasmid conjugal transfer (Chan *et al*., 2010a; Miller and Bassler 2001, Dunny and Winans, 1999; Fuqua *et al*., 1996; Hardman *et al*., 1998; Salmond *et al*., 1995; Schauder and Bassler 2001). There are several ways to interrupt QS, i.e. targeting the AHLs inhibits activity of luxI synthase or luxR receptor protein, and all these are collectively known as 'quorum quenching' (QQ) (Chan *et al*., 2007; Chan *et al*., 2009; Chan *et al*., 2010b). For the scope of this work, QQ refers to inactivation of AHL molecules by enzymatic turnover

(Chan *et al*., 2007; Chan *et al*., 2009; Chan *et al*., 2011). Thus far, lactonase and acylase have been reported as the enzymes that degrade AHL (Zhang, 2003). The first lactonase was isolated from soil bacillus (Dong *et al*., 2001). Recently lactonase activity is also found in *Acinetobacter* sp. (Chan *et al*., 2011). Acylase from *Ralstonia* sp. has been isolated from a biofilm surface (Lin *et al*., 2003). Clinical and soil *P. aeruginosa* was found to produce another amidase termed PvdQ (Huang *et al*., 2003) and another acylase QuiP has been reported (Huang *et al*., 2006).

It is anticipated that AHLs produced by quorum sensing bacteria could be inactivated in a polymicrobial environment such as the tropical wetland. The present work describes for the first time the characterization of Malaysia tropical wetland *Pseudomonas* sp. that showed QQ activity and its acylase genes were PCR identified.

MATERIALS AND METHODS

Modified Luria-Bertani medium (LBm)

To facilitate isolation of wetland quorum quenching bacteria, we designed LBm by modifying Luria-Bertani medium (Sambrook *et al*., 1989) whereby LBm consists of (in 100 ml sterile deionised water) 1.0 g tryptone, 0.5 g yeast extract, and 2.5 g NaCl. LBm agar was solidified using bacteriological agar (1.5% w/v).

Bacteria strain, media and culture conditions

Wetland bacteria were grown in LBm medium at 28°C. *E. coli* DH5 was grown at 37°C in LB media. Where necessary, growth media were supplemented with appropriate antibiotics and solidified with bacteriological agar (1.5% w/v).

KGm medium

KGm medium was designed based on the KG medium as reported previously (Chan *et al*., 2009) with minor modifications. KGm medium consisted of 0.25 g of $CaCl_2$, 0.75 g of KCl, 7.5 g of KH_2PO_4, 0.5 g of $MgCl_2$, 1.25 g of NaCl, 0.25 g of Na_2SO_4, 0.3 g of NH_4Cl and 1.0 g of 2-(*N*-morpholino)-ethanesulfonic acid (MES), in 1000 ml sterile deionised water. This basal medium was buffered by MES to pH 5.5 before autoclaving. Filter-sterilized (0.22- m pore size) trace elements (5 mg of $FeCl_3$, 2.5 mg of $MnCl_2$, and 0.6 g $ZnCl_2$) were added in to this cooled basal medium aseptically. *N*-(3-oxohexanoyl)-L-homoserine lactone (3-oxo-C6-HSL) was added into the basal medium as the sole carbon source (0.05 mg/ml, final concentration).

Enrichment and isolation procedures

Wetland water was collected in a sterile plastic container at subsurface level (5 cm beneath water level) in 2007 at Malacca (2°11'8"N, 102°15'2"E). The wetland water sample was briefly spun to pellet any large particles. Then 1 ml of the wetland water sample was inoculated into 3 ml of KGm medium supplemented with 3-oxo-C6-HSL. The mixture was incubated at 28°C with shaking (220 rpm). Sub-inoculation of this suspension into fresh KGm medium was made at 48-h intervals by transferring the suspension (5% v/v) into 3 ml of fresh KGm medium. At the fifth enrichment cycle, pure colonies were obtained by several passages on LBm agar and a plate of 3-oxo-C6-HSL-containing KGm agar.

Molecular and phylogenetic studies of quorum quenching genes and 16S rDNA gene

16S rDNA gene was PCR amplified using 27F (5'-AGAGTTTGATC(M)TGGCTCAG-3') and 1525R (5'-AAGGAGGTG(W)TCCA(R)-CC-3') as the forward and reverse primers, respectively, as previously described (Chan *et al*., 2007). QQ acylase genes (*quiP* and *pvdQ*) were PCR amplified as previously described (Huang *et al*., 2003, Huang *et al*., 2006) with the following PCR condition: 94ºC for 10 min, followed by 35 cycles of denaturation at 94ºC (30 s), annealing at 57ºC (30 s), elongation at 72ºC (1 min); and finally by primer extension at 72ºC for 5 min and kept at 4ºC. For *pvdQ* gene, the forward and reverse primers used were *pvdQ*F (5'-AGGCCAAGCTTATGGGGGATGCGTACCGTACTG-3') and *pvdQ*R (5'-GTTATATAGCGGCCGCTAGGATTGCTTATCATTCG-3'). For *quiP* gene, the forward and reverse primers used were *quiP*F (5'-ATTAGAAGCTTATGGCCTCGCCAGCCTTC-3') and *quiP*R (5'-ATTACTCTAGATCAGCGAGCGGGAGTG-3'). Phylogenetic analysis of nucleotides sequences and molecular evolutionary analyses were conducted using published criteria (Chan *et al*., 2007; Chan *et al*., 2009).

N-acylhomoserine lactone (AHL) inactivation assays

AHL inactivation assay involving bacterial cells was performed as previously described (Chan *et al.*, 2009). Resting cell suspension was used to rehydrate the AHL to a final concentration of 0.5 mg/ml. The mixture of bacterial cells and AHLs were reacted for 24 h at room temperature with gentle shaking. To extract residual AHL, the reactions were stopped at appropriate time points by the addition of equal volume of ethyl acetate and extracted twice. Dissolved AHLs was evaporated to dryness and resuspended in appropriate volume of acetonitrile.

Reverse-phase Rapid Resolution Liquid Chromatography (RRLC) analysis of AHL degradation

Extracted AHL was detected by RRLC as previously described (Chan *et al.*, 2009; Chan *et al.*, 2010b) using an Agilent Technologies 1200 Series Rapid Resolution LC system (Agilent Technologies, Germany). Extracts (10 µl) from AHL inactivation assay were applied onto an analytical C18 reverse-phase column (Agilent ZORBAX Eclipse® XDB-C18, 4.6 mm × 50 mm, particle size of 1.8 µm). RRLC was run on isocratic profile of acetonitrile-water (35:65, v/v) with a constant flow rate (0.7 ml/min) and spectrum monitored at 210 nm with a diode-array detector. Data were collected and analysed by using Agilent Chemstation (version B.04.01). Both the spectral properties and retention time were compared to synthetic AHL standards obtained from Sigma-Aldrich. Negative controls were AHLs incubated with washed *E. coli* DH5 cells and PBS buffer.

Thin layer chromatography (TLC) analysis of 2-heptyl-3-hydroxy-4-quinolone (PQS)

Extraction of PQS from supernatants was performed as previously described (Shaw *et al.*, 1997; Diggle *et al.*, 2003). Spent supernatant (100 ml) was obtained from stationary phase cultures by centrifugation at 7,000 × g for 10 min, followed by mixing with an equal volume of acidified ethyl acetate (0.01% v/v glacial acetic acid in ethyl acetate) and shaken vigorously. The extraction mixture separated for 10 min. The top organic layer was collected and dried by adding excessive amount of anhydrous $MgSO_4$. The organic phase was subsequently filtered through a Whatmann 3MM paper into a round bottomed flask and rotary evaporated. The residue was resuspended in 500 µl absolute methanol and stored at -20°C. TLC was performed as described (Shaw *et al.*, 1997; Diggle *et al.*, 2003). Synthetic PQS (gift from Paul Williams) was included as standard for comparison.

Nucleotide sequence accession numbers

The 16S rDNA gene sequence for strain 2WS8 has been deposited at GenBank under the GenBank accession number GQ180119. All other sequences were from the GenBank database.

RESULTS

Enrichment and isolation of bacteria from Malaysia wetland water

KGm medium containing 3-oxo-C6-HSL was inoculated with wetland water samples. The pH of the wetland water sample was pH 7.8. The medium became turbid after incubation for 48 h suggesting growth of bacteria. No obvious turbidity was observed in a control tube in depleted of 3-oxo-C6-HSL. When grown on grown on LBm plates for 24 h at 28°C, white, transparent, convex colonies with colony diameter of 5 mm were obtained in pure culture and named as strain 2WS8. Strain 2WS8 was stained Gram-negative and appeared as long rod-shaped (approximately 2.0 µm, data not shown).

Based on its 16S rDNA genes nucleotide sequences of the strain 2WS8 (1,479 nucleotides each), it belonged to the genus *Pseudomonas*, sharing 99.8% sequence identities with the 16S rDNA gene of *Pseudomonas aeruginosa* PAO1 (Figure 1).

Degradation of various AHLs

We first aimed to confirm whether the *Pseudomonas* strain 2SW8 isolated from KGm medium supplemented with 3-oxo-C6-HSL showed degradation of this AHL molecules. Strain 2SW8 was able to degrade 3-oxo-C6-HSL molecules within 24 h (Figure 2A) as compared to the control (Figures 2B and 2C). Approximately 45% of 3-oxo-C6-HSL was degraded after 24 h incubated with *P. aeruginosa* strain 2WS8, as analysed by RRLC (Figure 2A). A slight decreased

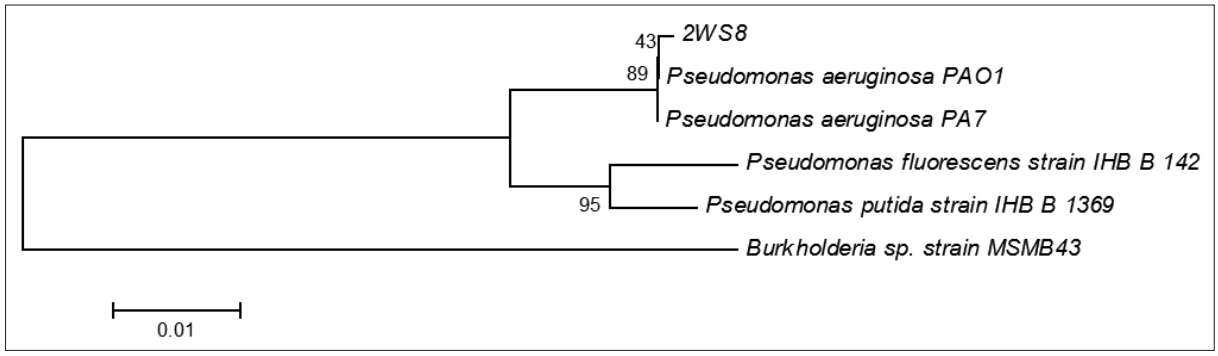

FIGURE 1 - *Phylogenetic analysis of strain strain 2WS8. Phylogenetic analysis of 2SW8 with its nearest relatives generated using Neighbour-Joining algorithm. Bar represents evolutionary distance as 0.01 changes per nucleotide position. Bootstrap support values express as percentages of 1,000 replications and given to support tree topology. Burkholderia sp. strain MSMB43 was used as outgroup. GenBank accession number (in parentheses): Pseudomonas aeruginosa PA7 (CP000744 [rrs 807093 to 808589]), Pseudomonas aeruginosa PAO1 (AE004091 [rrs722096 to 723631]), Pseudomonas fluorescens strain IHB B 142 (GU186124), Pseudomonas putida strain IHB B 1369 (GU186116), Burkholderia sp. strain MSMB43 (EF114404).*

of AHL was detected in the controls (Figures 2B and 2C) due to insignificant loss of AHLs during extraction.

Interestingly, after incubating *P. aeruginosa* strain 2WS8 with C6-HSL for 24 h, RRLC showed that 65% of the C6-HSL was degraded (Figure 3A). The degradation activity of C8-HSL was slightly less efficient as compared to degradation of C6-HSL, with only about 58% was degraded by *P. aeruginosa* strain 2WS8 (Figure 4A).

Molecular identification of *pvdQ* and *quiP* genes

QQ genes (*pvdQ* and *quiP*) were PCR amplified using genomic DNA of *P. aeruginosa* strain 2WS8 as template. The expected PCR product size for these two genes is ~2.5 kb (Figure 5). Web-based similarity searches against the GenBank database using the partial nucleotide sequences of the *pvdQ* gene (626 nucleotides) of *P. aeruginosa* strain 2WS8 suggested that these nucleotide se-

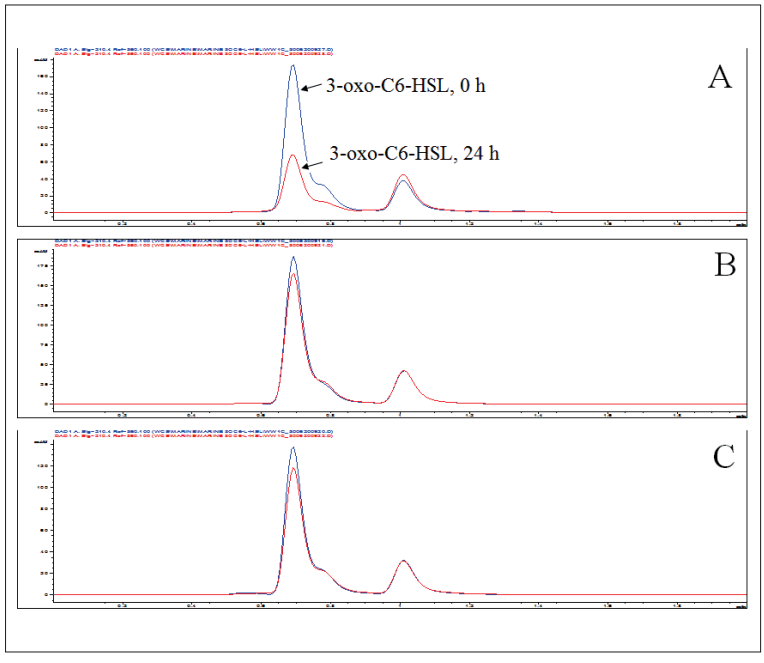

FIGURE 2 - *RRLC analysis of degradation of 3-oxo-C6-HSL (marked by arrows) after 0-h (blue) and 24-h (red) incubation with (A) P. aeruginosa strain 2WS8, (B) E. coli DH5 , and (C) PBS buffer (100mM, pH 6.5).*

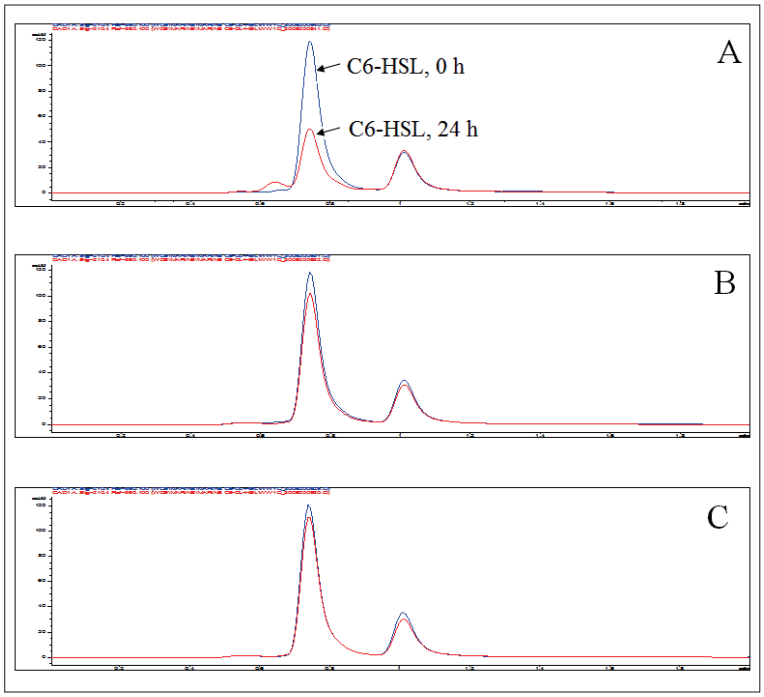

FIGURE 3 - *RRLC analysis of degradation of C6-HSL (marked by arrows) after 0-h (blue) and 24-h (red) incubation with (A) P. aeruginosa strain 2WS8, (B) E. coli DH5 , and (C) PBS buffer (100 mM, pH 6.5).*

quences were highly similar to a gene encoding penicillin acylase in *Pseudomonas aeruginosa* UCBPP-PA14 (with 99.4% sequence identity). Phylogenetic analysis of *pvdQ* homologue genes cloned from *P. aeruginosa* strain 2WS8 was highly conserved as compared with *P. aeruginosa pvdQ* genes (Figure 6). This gene is also clustered closely to the acylase in *P. aeruginosa* strain MW3A, a strain we previously isolated from Malacca marine water sample.

In a similar approach, *quiP* gene of *P. aeruginosa* strain 2WS8 (1459 nucleotides) was found highly similar to *quiP* in *Pseudomonas aeruginosa* UCBPP-PA14 (>99.0% sequence identity).

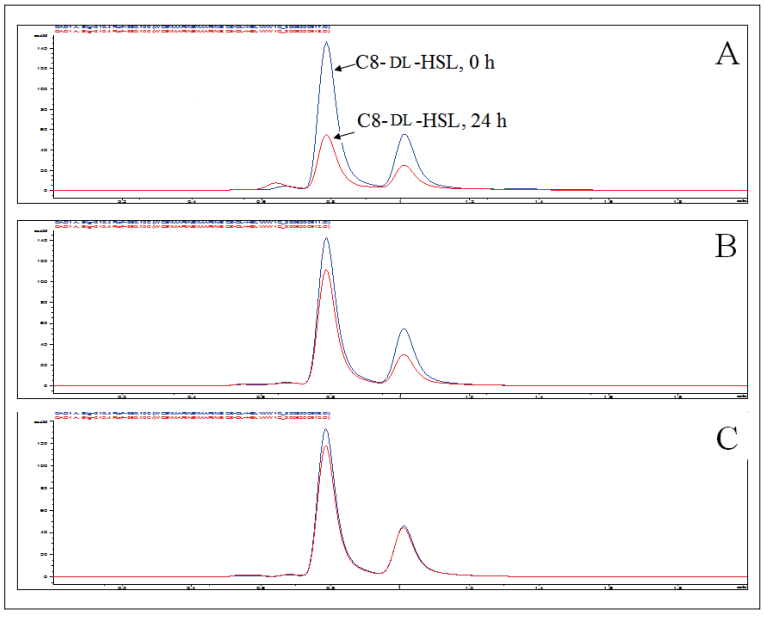

FIGURE 4 - *RRLC analysis of degradation of C8-DL-HSL after 0-h (blue) and 24-h (red) incubation with (A) P. aeruginosa strain 2WS8, (.B) E. coli DH5 , and (C) PBS buffer (100 mM, pH 6.5).*

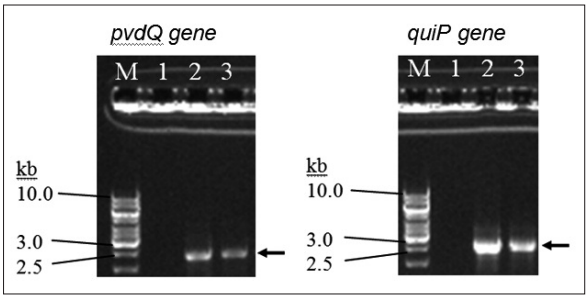

FIGURE 5 - *PCR detection of pvdQ and quiP genes in P. aeruginosa strain 2WS8. pvdQ (left panel) and quiP (right panel) genes were PCR amplified (approximately 2.5 kb, arrows) from purified genomic DNA of strain 2WS8 (lane 2). Negative control was PCR using water to replaced DNA template (lane 1) and positive control (lane 3) involved Pseudomonas aeruginosa PAO1 genomic DNA as template. M: DNA Ladders.*

Phylogenetic analysis indicated that *quiP* homologue gene of *P. aeruginosa* strain 2WS8 was highly conserved (Figure 7).

TLC analysis of PQS

When spent supernatant extract of *P. aeruginosa* 2WS8 was analysed by TLC and visualized under UV at 302 nm, a blue fluorescence spot under UV light was observed that migrated with a characteristic R_f value similar to the synthetic standard PQS (Figure 8). Based on their respective R_f values as compared to the synthetic PQS, tentative PQS spots were identified on TLC plate in supernatant extracts of *P. aeruginosa* 2WS8.

DISCUSSION

Wetlands have been regarded as the most biologically diverse of all ecosystems (Mitsch *et al.*, 2009). In this work, we isolated AHLs degrading bacterium from the Malaysia tropical tidal area. Although it is well documented that in freshwater wetlands soil bacteria are important in nutrient cycle regulation and have major impact on water quality (Richardson and Marshall, 1986) and global carbon cycling (Roulet, 2000), little is known about tropical wetland bacterial cell-to-cell communication in this unique environment. Our results indicate QQ bacteria can be isolated from the surface or subsurface of water in tropical wetland. By modifying our previously reported KG medium, this medium was supplemented with extra NaCl to resemble osmotic pressure in tropical wetland water. Initial inoculation of a tropical wetland water sample into the KGm medium turned turbid after 48 h post-inoculation, indicating bacterial growth. Bacterial cul-

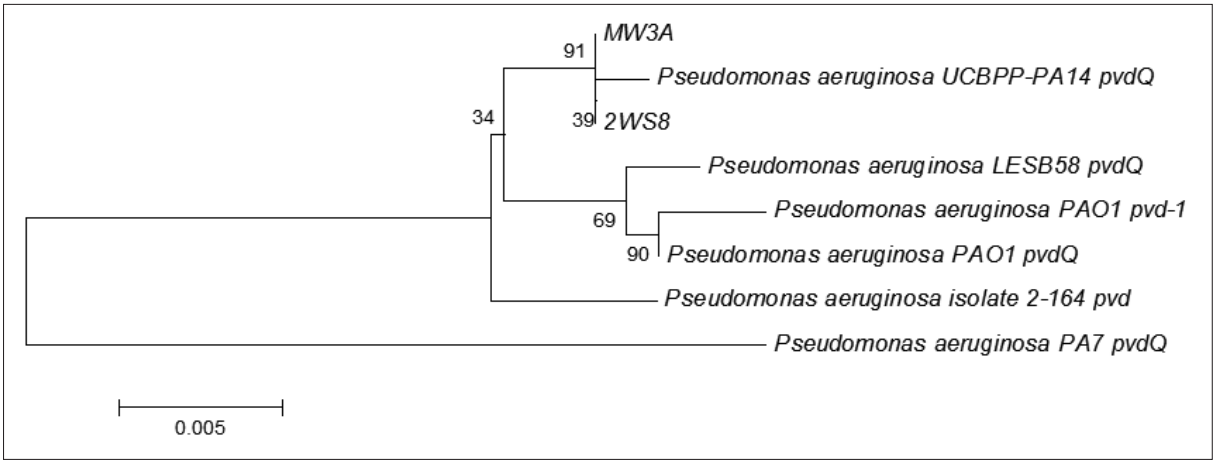

FIGURE 6 - *Phylogenetic analysis of P. aeruginosa strain 2WS8 pvdQ gene. Phylogenetic analysis of pvdQ gene of P. aeruginosa strain 2WS8 generated using Neighbour-Joining algorithm. The horizontal bar at the bottom represents evolutionary distance as 0.005 changes per nucleotide position. GenBank accession number: Pseudomonas aeruginosa UCBPP-PA14 pvdQ (115583796:3004988-3007276), Pseudomonas aeruginosa LESB58 pvdQ (218768969:3206318-3208606), Pseudomonas aeruginosa PAO1 pvdQ (110227054:2636517-2638805), Pseudomonas aeruginosa PAO1 pvd-1 gene (1129155), Pseudomonas aeruginosa isolate 2-164 pyd (AF540993), Pseudomonas aeruginosa PA7 pvdQ (150958624:2955521-2957803). MW3A is P. aeruginosa strain MW3A that we have previously isolated from Malacca marine water.*

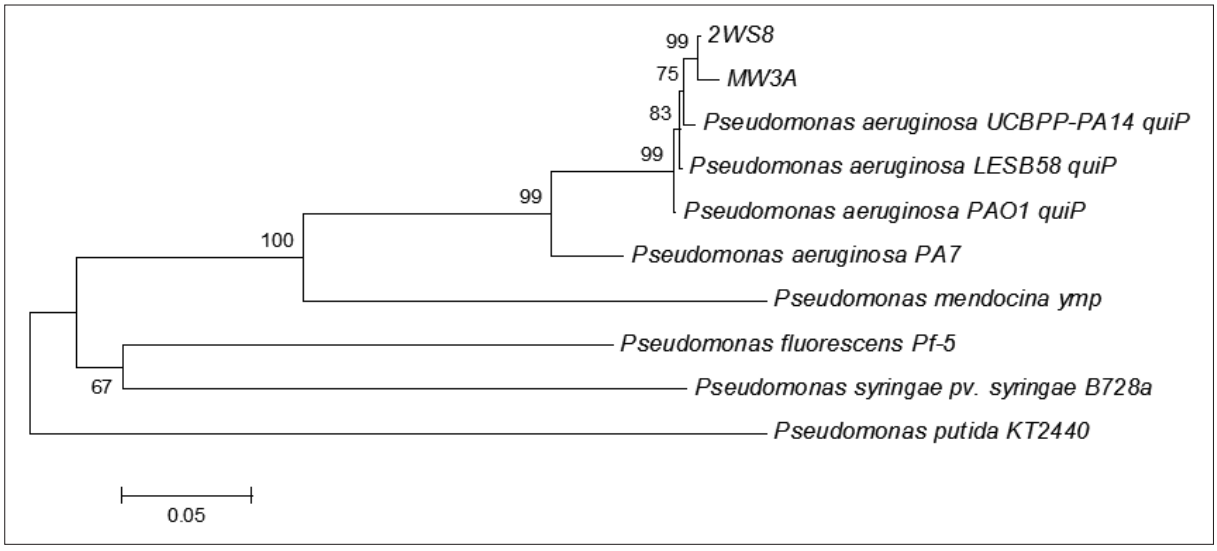

FIGURE 7 - *Phylogenetic analysis of P. aeruginosa strain 2WS8 quiP gene. Phylogenetic analysis of quiP gene of P. aeruginosa strain 2WS8 generated using Neighbour-Joining algorithm. Bar represents evolutionary distance as 0.05 changes per nucleotide position. GenBank accession number: Pseudomonas aeruginosa UCBPP-PA14 quiP (115583796:4527711-4530254), Pseudomonas aeruginosa LESB58 quiP (218768969:4725132-4727675), Pseudomonas aeruginosa PAO1 quiP (110227054:1119674-1122217), Pseudomonas aeruginosa PA7 (150958624:4478165-4480708), Pseudomonas mendocina ymp (145573243:1577468-1579981), Pseudomonas fluorescens Pf-5 (68342549:1438348-1440777), Pseudomonas syringae pv. syringae B728a (63253978:4609176-4611650), Pseudomonas putida KT2440 (24987239:1265544-1267985).*

ture was sub-inoculated in successive cycles to ensure efficient enrichment of AHL-degrading bacteria. Pure colony strain 2SW8 which later confirmed as *Pseudomonas* strain was selected for further analysis due to its ability to survive in the KGm medium.

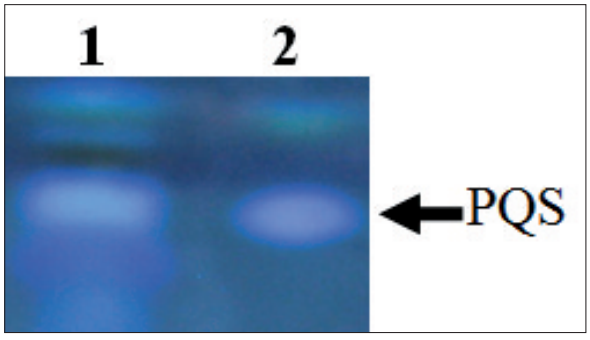

FIGURE 8 - *Thin layer chromatography of 2-heptyl-3-hydroxy-4 quinolone (PQS) extracted from P. aeruginosa 2WS8 spent supernatant. PQS were visualized on a 302 nm UV transilluminator (TFM-20V Transilluminator, UVP Inc. USA). The image was digitally photographed with DigiDoc-IT Imaging System (UVP Inc. USA). Lane 1: 2WS8 supernatant extract (10 l); lane 2: PQS synthetic standard (2.5 g) (arrow).*

P. aeruginosa produces a quinolone signalling molecule namely PQS which provides regulatory link of the *las* and *rhl* systems and HHQ is the direct precursor of the PQS signaling molecule (Pesci *et al.*, 1999). PQS belongs to the family of 4-hydroxy-2-alkylquinolines, which has been reported to show antimicrobial activity (Deziel *et al.*, 2004) although PQS itself has no such activity. It has been suggested that PQS signalling plays an important role in *P. aeruginosa* pathogenesis because it regulates virulence factors production (Pesci *et al.*, 1999; Diggle *et al.*, 2003; Deziel *et al.*, 2004). *P. aeruginosa* kills competing bacteria to acquire their iron stores, and PQS is required for such activity (Schertzer *et al.*, 2009) whereas other bacteria use siderophore for iron uptake (Chan *et al.*, 2009). It is therefore speculated that PQS production in *Pseudomonas* strain 2SW8 may be important for iron acquisition in the wetland water which is scarcely available.

Using our previously reported QQ assay, we confirmed that *Pseudomonas* strain 2SW8 showed rapid AHL turn over *in vivo* (data not shown). *Pseudomonas* strain 2SW8 was then subjected to detailed RRLC analysis of its ability to degrade

two types of AHLs namely 3-oxo-acylhomoersine lactone and 3-unsubstituted-acylhomoersine lactone. This bacterium does not show preferential AHL substrate specificity and it efficiently degrades all the AHLs tested within 24 h.

Although soil and clinical pseudomonads are able to metabolize AHLs has been reported (Huang *et al*., 2003), however, to the best of our knowledge, this is the first documentation of tropical wetland QQ pseudomonad. Contrary to the finding of Huang *et al*., (2003) who reports on pseudomonads that did not degrade AHLs with *N*-acyl side chains shorter than eight carbons, the pseudomonad in this study, strain 2SW8 efficiently degraded AHLs with *N*-acyl side chains of six and eight carbons.

It has been shown that *Variovorax* and *Ralstonia* isolates use AHL acylase for AHL degradation (Leadbetter and Greenberg, 2000; Lin *et al*., 2003). *Ralstonia* strain XJ12B degrades and grow rapidly on both short- and long-chain AHLs (Lin *et al*., 2003) but *V. paradoxus* utilizes the entire range of short- and long chain AHLs, and growing most rapidly on 3-oxo-C6-HSL. Similar to *V. paradoxus*, *Pseudomonas* strain 2SW8 degraded 3-oxo-C6-HSL rapidly as well.

In *P. aeruginosa*, the acylase *pvdQ* was identified as being a late responder to the 3-oxo-C12-HSL QS circuit (Whiteley *et al.*, 1999). Subsequently, another gene *quiP* (gene PA1032) encoding acylase was identified, but it was not required for AHL utilization (Huang *et al.*, 2006). Our result showed that *Pseudomonas* strain 2SW8 possessed both acylase genes of *pvdQ* and *quiP*. This leads us to speculate that *Pseudomonas* strain 2SW8 degrades AHL via acylase activity. Acylases degrade the AHL molecules by releasing homoserine lactone, which can be reacted by a homoserine lactone-lactonase to generate homoserine (Huang *et al*., 2003). It has been reported that *Pseudomonas* spp. can metabolize AHLs with acyl side chains of at least 8 carbons for growth (Huang *et al*., 2003). Hence, acylases in *Pseudomonas* strain 2SW8 may confer a competitive advantage by utilizing AHLs as a growth substrate in the tropical wetland environment.

QS was first described in the bioluminescent marine bacterium, *Vibrio fischeri* and *Vibrio harveyi* (Eberhard, 1972; Nealson *et al.*, 1970) and subsequently, *Roseobacter* spp. associated with marine snow have been reported to show QS activity (Gram *et al*., 2002). Our finding of QQ *Pseudomonas* strain 2SW8 may play an important role for biological signal decay in the wetland environment. Biological turnover of AHL such as that performed by *Pseudomonas* strain 2SW8 may be important to regulate AHL concentration in the tropical wetland habitat.

Zoospores of the marine green seaweed *Enteromorpha* have been reported to respond to bacterial AHL for the selection of surface sites for permanent attachment. While wild type *V. anguillarum* NB10 was a strong attractor of zoospores, inactivation of AHL production in *V. anguillarum* NB10 abolished zoospores attraction. Because bacterial biofilms play an important role in the development of algal communities (Joint *et al*., 2002), therefore tropical wetland QQ bacteria such as strain 2SW8 may influence and disrupt the selection of attachment sites of marine alga and may be important to its marine ecology.

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