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ORIGINAL PAPER

Coexistence of quorum-quenching and quorum-sensing in tropical marine *Pseudomonas aeruginosa* strain MW3A

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Abstract A chemically defined medium called KGm medium was used to isolate from a sample of sea water a bacterial strain, MW3A, capable of using *N*-3-oxohexanoyl-L-homoserine lactone as the sole carbon source. MW3A was clustered closely to *Pseudomonas aeruginosa* by 16S ribosomal DNA sequence analysis. It degraded both *N*-acylhomoserine lactones (AHLs) with a 3-oxo group substitution and, less preferably, AHLs with unsubstituted groups at C3 position in the acyl side chain, as determined by Rapid Resolution Liquid Chromatography. Its *quiP* and *pvdQ* homologue gene sequences showed high similarities to those of known acylases. Spent supernatant of MW3A harvested at 8-h post inoculation was shown to contain long-chain AHLs when assayed with the biosensor *Escherichia coli* [pSB1075], and specifically *N*-dodecanoyl-L-homoserine lactone and *N*-3-oxotetradecanoyl-L-homoserine lactone by high resolution mass spectrometry. Hence, we report here a novel marine *P. aeruginosa* strain MW3A possessing both quorum-quenching and quorum-sensing properties.

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Introduction

Although unicellular, bacteria are highly interactive and are able to communicate with each other to regulate gene expression in a concerted way. The term "quorum-sensing" (QS) refers to the ability of a population of unicellular bacteria to act as a multicellular organism in a cell-density-dependent manner, that is, a way to sense ''how many are out there'' (Fuqua et al. 1996, 2001; Miller and Bassler 2001; Schauder and Bassler 2001).

QS involves production, release, detection, and response to small molecules termed autoinducers by bacteria. Thus far the most well studied gram negative bacterial QS molecules are *N*-acyl homoserine lactones (AHLs) (Chhabra et al. 2005; Williams et al. 2007). The concentration of these signalling molecules can act as a measure of population density, allowing whole bacterial communities to regulate gene expression in a concerted way. QS regulates diverse bacterial physiological processes, which include, but are not limited to, bioluminescence, swarming, swimming and twitching motility, antibiotic biosynthesis, biofilm differentiation, plasmid conjugal transfer, and the production of virulence determinants in animal, fish, and plant pathogens (Dunny and Winans 1999; Fuqua et al. 1996; Hardman et al.1998; Salmond et al. 1995).

AHL molecules are highly conserved. Each AHL consists of a homoserine lactone ring unsubstituted in the β- and γ-positions but *N*-acylated with a fatty acyl group at the α-position (Chhabra et al. 2005). The acyl side-chain varies from 4 to 18 carbons in its lengths, mostly even-numbered,

with different saturation levels and oxidation states which belong to the *N*-acyl, *N*-3-oxoacyl, or *N*-3-hydroxyacyl class. Stereochemistry at the α-centre of the homoserine lactone (HSL) ring has been unequivocally established to be the L-isomer of *N*-3-oxohexanoyl-L-homoserine lactone (3-oxo-C_6-HSL) produced by *Erwinia carotovora* (Bainton et al. 1992) and by analogy it is deduced that all other natural AHLs have the same configuration (Bainton et al. 1992; Williams et al. 2007). It has been reported that synthetic D-isomers lack autoinduction activity (Chhabra et al. 1993, 2005).

The AHL signalling system has been regarded as a promising target for developing novel approaches to controlling bacterial infections by regulating the virulence of the population as a whole without affecting the viability of the individual cells. This means that there is less selection pressure for the evolution of resistance than in the case in, for example, antibiotic therapy (Hentzer and Givskov 2003). The disruption of bacterial QS is known as 'quorum-quenching' (QQ).

The bacterial QS system can be grouped into three key functional components: signal generation, signal perception, and signal transportation, which links the first two components. Therefore QQ can be achieved by targeting one or more of these components, and the straightforward way is to degrade the AHL molecules, hence reducing the 'quorum' and preventing QS-mediated gene expression.

Two QQ genes, *pvdQ* and *quiP* that encode AHL acylases, have been identified from *Pseudomonas aeruginosa* by Huang et al. (2003, 2006) and Sio et al. (2006). Huang et al. (2006) suggested that these two AHL acylases play a critical role in the degradation and utilization of AHLs, including the *P. aeruginosa* native *N*-3-oxododecanoyl-L-homoserine lactone (3-oxo-C_{12}-HSL), to control different ratios of long- and short-chain AHL signalling molecules during different phenotypic stages, for example, biofilm and planktonic states.

Although the marine ecosystem is rich in microbial diversity, roseobacters seem to be the dominant QS bacteria in the marine environment and they produce various types of AHLs (Cicirelli et al. 2008; MacLeod 1965; Wagner-Döbler et al. 2005). Recently, Romero et al. (2011) reported 15 cultivable bacterial strains with QQ activity from three different marine samples. None of the 15 strains belonged to the genus *Pseudomonas*. Here we describe the characterisation of a marine *P. aeruginosa* with QS and QQ activities and its QQ genes.

Materials and methods

Modified Luria–Bertani (LBm) medium

LBm medium was modified from LB broth (Sambrook et al. 1989). It consisted of 1.0% w/v tryptone, 0.5% w/v yeast extract, and 2.5% w/v NaCl. For extraction of AHLs, cells were grown in LB buffered with 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) to pH 6.5 (LB_{MOPS}) to prevent alkaline lactonolysis (Yates et al. 2002).

Bacterial strains, media and culture conditions

Marine bacteria were grown in LBm medium at 28°C. *Escherichia coli* DH5α and *lux*-based biosensor *E. coli* [pSB1075] were grown at 37°C in LB and LB supplemented with tetracycline (10 mg l^{-1}), respectively. *E. coli* [pSB1075] responds to exogenous long-chain AHLs by emitting light (Winson et al. 1998). Broth culture was incubated with shaking (220 rev min^{-1}).

Modified KG (KGm) medium

KGm medium was modified from KG medium (Chan et al. 2009). It comprised a basal medium containing NaCl (1.25 g l^{-1}), KCl (0.75 g l^{-1}), Na_2SO_4 (0.25 g l^{-1}), KH_2PO_4 (7.5 g l^{-1}), $MgCl_2$ (0.5 g l^{-1}), $CaCl_2$ (0.25 g l^{-1}), NH_4Cl (0.3 g l^{-1}) (supplied unless otherwise stated), and 2-(*N*-morpholino)-ethanesulfonic acid (MES) (1.0 g l^{-1}). After the basal medium was autoclaved and cooled, filter-sterilized (0.22-μm pore size) $FeCl_3$, $MnCl_2$, and $ZnCl_2$ solutions were added to it to final concentrations of 5 mg l^{-1}, 2.5 mg l^{-1}, and 0.6 g l^{-1}, respectively. Finally, 3-oxo-C_6-HSL was added to 50 mg l^{-1} as the sole carbon source.

Enrichment and isolation procedures

Seawater was collected at subsurface level (5 cm beneath water level) in November 2007 in Malacca (2°11′12″N, 102°15′12″E), Malaysia. The sample was collected in a sterile plastic container, transported to the laboratory and processed immediately. The marine water sample (1 ml) was added to 3 ml of KGm medium supplemented with ammonium chloride (300 mg l^{-1}) and 3-oxo-C_6-HSL (50 mg l^{-1}) as sole sources of nitrogen and carbon, respectively. This mixture was incubated at 28°C with shaking (220 rev min^{-1}). After 48 h, 150 μl of the suspension was inoculated into 3 ml of fresh enrichment medium. The same procedure was repeated four times. At the fifth enrichment cycle, a diluted suspension was plated onto LBm agar and a plate of 3-oxo-C_6-HSL-containing KGm agar to isolate individual colonies.

PCR amplification of *pvdQ* and *quiP* homologue genes and 16S rDNA genes

For PCR of both *quiP* and *pvdQ* genes, the thermal cycling programs for PCR consisted of an initial denaturation at

94°C for 10 min, followed by 35 cycles of 94°C (30 s), 57°C (30 s), 72°C (1 min), and a final primer extension at 72°C for 5 min. For *quiP* gene, the forward and reverse primers used were *quiP*F (5′-ATTAGAAGCTTATGGCCT CGCCAGCCTTC-3′) and *quiP*R (5′-ATTACTCTAGATC AGCGAGCGGGAGTG-3′), respectively (Huang et al. 2006). For *pvdQ* gene, the forward and reverse primers used were *pvdQ*F (5′-AGGCCAAGCTTATGGGGGATGC GTACCGTACTG-3′) and *pvdQ*R (5′-GTTATATAGCG GCCGCTAGGATTGCTTATCATTCG-3′), respectively (Huang et al. 2003).

PCR of 16S rDNA genes was carried out as described previously (Chan et al. 2007), using 27F (5′-AGAGTTTG ATCMTGGCTCAG-3′) and 1525R (5′-AAGGAGGTGW TCCARCC-3′) as the forward and reverse primers, respectively. For all PCR, negative controls were included by replacing the DNA template with sterile deionized water. PCR products were gel excised, column-cleaned, and ligated into the pGEM-T Easy vector (Promega, USA) as per the manufacturer's instructions, followed by transformation into *E. coli* DH5α (Sambrook et al. 1989).

Phylogenetic analysis

Sequences were analysed using Chromas Lite version 2.01 (Technelysium Pte Ltd, Australia) to exclude primer-binding sites. Phylogenetic and molecular evolutionary analyses were conducted with *MEGA* version 4.0 (Tamura et al. 2007) as described previously (Chan et al. 2007, 2009). Bootstrap analyses for 1,000 re-samplings were always performed to provide confidence estimates for tree topologies.

Whole-cell AHL inactivation assays

Washed overnight-grown cells suspended in PBS buffer (pH 6.5) were used as direct source of whole-cell inactivation assay as described previously (Chan et al. 2009, 2011a). Aliquots of AHL in absolute ethanol were dispensed into sterile tubes and the solvent was evaporated to dryness under sterile conditions. A resting cell suspension was used to rehydrate the AHL to a final concentration of 0.5 μg μl^{-1}. The mixtures were incubated at room temperature for 24 h with shaking (220 rev min^{-1}). The reactions were stopped at appropriate intervals by the addition of ethyl acetate, which also served to extract any remaining AHL. Ethyl acetate containing the residual AHL was removed and evaporated to dryness. The residue was reconstituted in an appropriate volume of acetonitrile. Extracted AHL was detected by Rapid Resolution Liquid Chromatography (RRLC).

Reverse-phase RRLC analysis of AHL degradation

Reverse-phase RRLC analysis of AHL was carried out as reported (Chan et al. 2010) using an Agilent Technologies 1200 Series Rapid Resolution LC system (Agilent Technologies, Germany) equipped with a vacuum degasser, a binary pump, and a diode-array detector. Ten microlitres of extracted AHL from AHL inactivation assay was applied onto an analytical C_{18} reverse-phase column (Agilent ZORBAX Eclipse XDB-C18, 4.6 mm × 50 mm, 1.8 μm particle size). RRLC was run on isocratic profile of acetonitrile–water (35:65, v/v, for short-chain AHL; 60:40, v/v, for long-chain AHL) for 3 min at a constant flow rate of 0.7 ml min^{-1} and the spectrum was monitored at 210 nm. Agilent Chemstation (version B.04.01) was used for data collection and analysis. The resultant retention time and spectral properties were compared to those of a series of synthetic AHL standards obtained from Sigma–Aldrich. AHLs incubated with washed *E. coli* DH5α cells and PBS were used as negative controls. The percentage of AHL inactivated and the specific activity were determined by estimating the amount of AHL (by comparison of the reduction in peak areas for a given retention time) with respect to AHL solutions of known concentrations (Chan et al. 2010).

Extraction of AHL

For extraction of AHL, spent supernatant of *P. aeruginosa* strain MW3A (200 ml) was collected by centrifugation (7,000×*g*, 10 min) at 8-h post inoculation, extracted twice with an equal volume of acidified ethyl acetate (0.01% v/v glacial acetic acid in ethyl acetate). The organic layer was collected, dried by adding an excess of anhydrous $MgSO_4$, filtered through a Whatmann 3MM paper, and rotary evaporated. The extract residue was resuspended in 200 μl of acetonitrile and analysed by luminometer–spectrophotometer used in conjunction with *lux*-based biosensor *E. coli* [pSB1075] and liquid chromatography mass spectrometry (LC–MS).

Measurement of bioluminescence

Bioluminescence was measured as a function of cell density using a combined automated Tecan luminometer–spectrophotometer (Infinite, Tecan). An overnight culture of *E. coli* [pSB1075] was diluted with LB to OD_{600} 0.01 and 0.2 ml of this dilution was added to each well of a 96-well optical bottom microtitre plate. *E. coli* [pSB1075] cells were grown at 37°C for 24 h in luminometer–spectrophotometer. When required, AHL solvent (ethyl acetate), AHL extracted from *P. aeruginosa* strain MW3A, and synthetic *N*-3-dodecanoyl-L-homoserine lactone (C_{12}-HSL) (100 mM, Sigma Aldrich) were added. Growth measurements and bioluminescence

were the means of triplicate experiments. Data were plotted as RLU (Relative Light Units)/OD against time.

Mass spectrometry analysis of AHLs

The Agilent RRLC 1200 system was used as the LC delivery system. The column used in the LC analysis was Agilent ZORBAX Rapid Resolution HT column (100 mm × 2.1 mm, 1.8 μm particle size). Analysis was carried out at 60°C with flow rate of 0.3 ml min^{-1}. The volume injected was 20 μl. Mobile phases A and B were 0.1% v/v formic acid in water and 0.1% v/v formic acid in acetonitrile, respectively. The gradient profiles were as follows (time: mobile phase A: mobile phase B): 0 min: 60:40, 5 min: 20:80, 7 and 10 min: 5:95, and 11 and 13 min: 60:40.

The high-resolution electrospray ionization mass spectrometry (ESI–MS) and electrospray ionization tandem mass spectrometry (ESI–MS/MS) analyses were performed with the Agilent 6500 Q-TOF LC/MS system. The MS experiment was performed in the ESI-positive mode. The probe capillary voltage was set at 3 kV, desolvation temperature at 350°C, sheath gas at 11 ml h^{-1}, and nebulizer pressure at 50 psi. Nitrogen gas was used as the collision gas in the collisionally induced dissociation mode for the MS/MS analysis, with collision energy set at 20 eV. The MS data were analysed using the Agilent MassHunter software.

Nucleotide sequence accession numbers

The 16S rDNA, *pvdQ*, and *quiP* nucleotide sequences of MW3A were deposited at GenBank under the GenBank accession numbers GQ180117, GQ423677, and GQ423680, respectively. All other sequences were from the GenBank database.

Results

Enrichment and isolation of bacteria from marine water

Within 48 h after inoculation with a marine water sample, 3-oxo-C_6-HSL-containing KGm medium became turbid. The pH of the marine water sample was 7.88. No obvious turbidity was visible in a control tube without 3-oxo-C_6-HSL. Subsequently, a bacterial isolate, designated as MW3A and grown on 3-oxo-C_6-HSL-containing KGm agar, was re-streaked on LBm plate. It yielded white, transparent, convex colonies with colony diameter of 5 mm. Each colony was essentially round with entire edges and appeared smooth and shiny. MW3A appeared as long rod-shaped cells with cell length of approximately 2.0 μm and its cells were stained Gram-negative (data not shown). The ability of MW3A to degrade 3-oxo-C_6-HSL was verified by RRLC analysis, which suggested that approximately 40% of 3-oxo-C_6-HSL was degraded by MW3A after 24 h of incubation (Fig. 1). MW3A was selected for further analysis.

Web-based similarity searches against GenBank using the 16S rDNA nucleotide sequence (1,479 nucleotides) of MW3A indicated its similarity to the genus *Pseudomonas*, sharing at least 99.8% sequence identities with the 16S rDNA of *P. aeruginosa* PAO1. Further phylogenetic analysis supported the conclusion that MW3A was a species of *Pseudomonas* clustered closely to *P. aeruginosa* PAO1 (Fig. 2).

Degradation of various AHLs

Further studies were carried out to determine degradation of AHLs other than 3-oxo-C_6-HSL (Fig. 1) by *P. aeruginosa* strain MW3A. RRLC analyses showed that ~55 and ~40% of *N*-hexanoyl-L-homoserine lactone (C_6-HSL) (Fig. 3a) and *N*-octanoyl-DL-homoserine lactone (C_8-DL-HSL) (Fig. 3b), respectively, were degraded after 24 h of incubation with MW3A. It seems that MW3A preferentially degraded unsubstituted AHLs with shorter *N*-acyl side chain. In all AHL-degradation analyses, no obvious degradation of AHLs was observed when the AHL-inactivation assays were repeated with *E. coli* DH5α and PBS buffer (100 mM, pH 6.5) (data not shown).

Molecular cloning of the *pvdQ* and *quiP* genes

PCR of the *pvdQ* and *quiP* homologue genes of MW3A yielded amplicons of ~2.5 kb (Fig. 4). Web-based BLAST similarity searches against the GenBank using the partial nucleotide sequence of the *pvdQ* gene (671 nucleotides) of MW3A indicated that the nucleotide sequence was highly similar to that of a gene encoding penicillin acylase in *P. aeruginosa* UCBPP-PA14 (Fig. 5). Similarly, phylogenetic analysis of the *quiP* homologue gene (1,459 nucleotides) of MW3A indicated that it was highly similar to *quiP* of *P. aeruginosa* UCBPP-PA14 (Fig. 6).

Detection of AHLs produced by *P. aeruginosa* MW3A

When *E. coli* [pSB1075] was incubated with ethyl acetate, the AHL solvent, no bioluminescence was detected (Fig. 7). However, when it was incubated with the AHL extract of *P. aeruginosa* MW3A, bioluminescence was detected (Fig. 7), suggesting the presence of long chain AHLs that activated the expression of *luxCDABE*

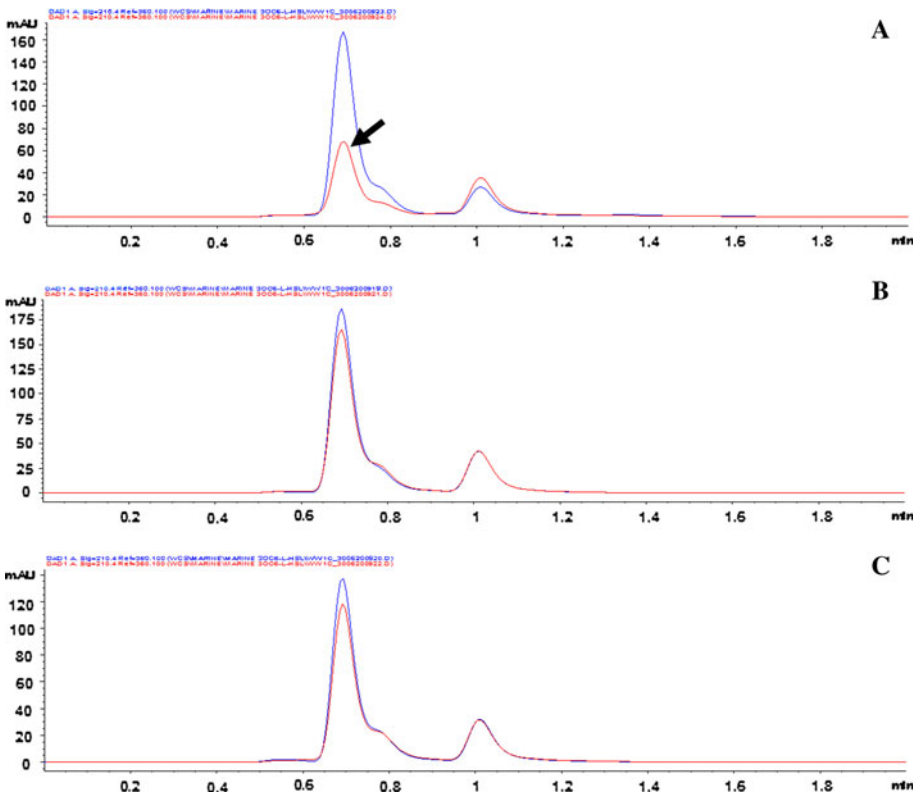

Fig. 1 RRLC analysis of 3-oxo-C_6-HSL after 0 (*blue*) and 24 h (*red*) incubation with **a** MW3A, **b** *E. coli* DH5α, and **c** PBS buffer (100 mM, pH 6.5); *arrow* shows 3-oxo-C_6-HSL degradation by MW3A after 24 h of incubation

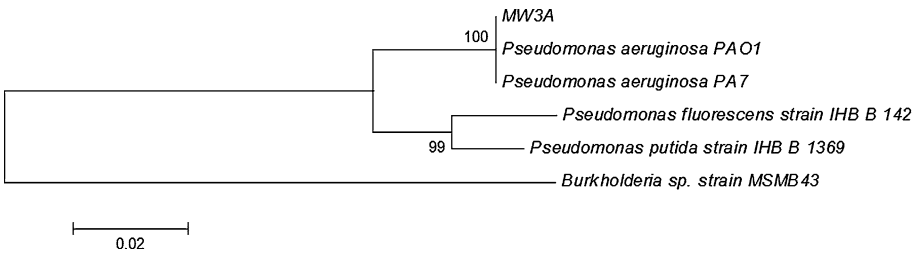

Fig. 2 16S rDNA gene-based phylogenetic tree, generated using Neighbour-Joining algorithm, showing the phylogenetic position of strain MW3A; the *horizontal bar* at the bottom represents evolutionary distance as 0.02 change per nucleotide position, determined by measuring the lengths of the *horizontal lines* connecting the species; the *numbers* (bootstrap values as percentages of 1,000 replications) provide support for the robustness of the adjacent nodes; *Burkholderia* sp. strain MSMB43 was used as outgroup; GenBank accession numbers (in parentheses): *P. aeruginosa* PAO1 (AE004091 [*rrs*722096–723631]), *P. aeruginosa* PA7 (CP000744 [*rrs* 807093–808589]), *P. fluorescens* strain IHB B 142 (GU186124), *P. putida* strain IHB B 1369 (GU186116), and *Burkholderia* sp. strain MSMB43 (EF114404)

transcriptional fusion in *E. coli* [pSB1075] (Winson et al. 1998).

Mass spectrometry analysis of AHL

Results of mass spectrometry confirmed the presence of *N*-3-oxotetradecanoyl-L-homoserine lactone (3-oxo-C_{14}-HSL) (*m/z* 326.2339) (Fig. 8a) and C_{12}-HSL (*m/z* 284.2221) (Fig. 8b) in the spent supernatant of MW3A processed at 8-h post inoculation. The ESI–MS/MS spectrum of C_{12}-HSL (*m/z* 284.2221, Fig. 8b) shows fragments (*m/z* 95.0880, 102.0946, and 109.1025, Fig. 8c) typical of an AHL-moiety (Ortori et al. 2007).

Discussion

KG medium has been successfully used to select and enrich QQ bacteria from different environments (Chan et al. 2009, 2010). Here, we successfully used a modified KG medium to enrich QQ bacteria from a sample of seawater. In KGm medium, the concentrations of all salts were increased to produce higher salinity to adjust for the osmotic pressure for halophilic bacteria in the marine environment. KGm medium was buffered to pH 6.5 with MES to prevent lactonolysis of AHLs under alkaline conditions. The isolation of the marine *P. aeruginosa* strain MW3A, possessing both QQ and QS properties, confirmed that KG

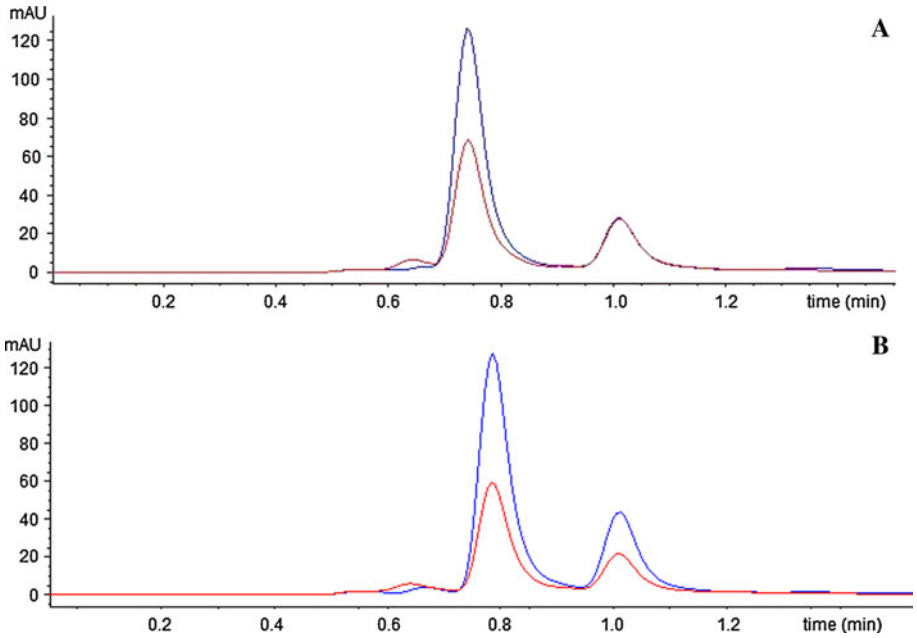

Fig. 3 RRLC analysis of **a** C_6-HSL and **b** C_8-DL-HSL after 0 (*blue*) and 24 h (*red*) of incubation with MW3A; a drop of peak shows degradation of signalling molecules

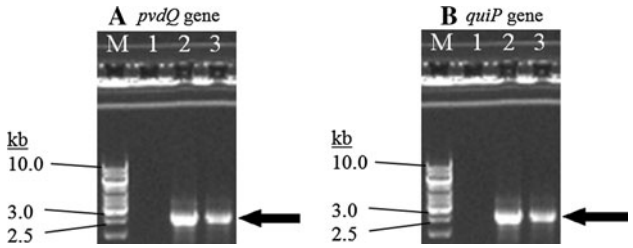

Fig. 4 PCR-amplified **a** *pvdQ* and **b** *quiP* homologue genes of MW3A (*lane 2*) and *P. aeruginosa* PAO1 (*lane 3*); *arrow* shows the 2.5 kb amplicons; *M* = 1-kb DNA ladder; *lane 1* = negative control

medium, with or without modification, was effective in selecting QQ bacteria from various environments including the marine ecosystem.

P. aeruginosa is a common bacterium found in diverse environmental conditions owing to its metabolic versatility. Consequently, it is ubiquitous in the soil and water ecosystems including the marine ecosystem. In this study, the marine *P. aeruginosa* strain MW3A was shown to degrade 3-oxo-C_6-HSL, C_6-HSL, and C_8-HSL with different efficiencies. The ability to degrade AHLs with a C_6 acyl side chain, with or without a 3-oxo substituent, is additional to the reported capability of *P. aeruginosa* PAO1 to degrade AHLs with acyl chains of eight or more carbons. Spent supernatants of *P. aeruginosa* strain MW3A were assayed for AHLs at 24-h post inoculation, but neither 3-oxo-C_{14}-HSL nor C_{12}-HSL was detected by ESI–MS (data not shown). This led us to speculate that AHLs produced by *P. aeruginosa* strain MW3A were self-degraded. Hence, the extraction of AHLs from the supernatants was performed at 8-h post inoculation. Homologues of both *pvdQ* and *quiP* genes, showing high nucleotide sequence similarities to those of known acylase genes, were detected in *P. aeruginosa* strain MW3A.

Currently, relatively little information is available on the AHL-based QS systems of marine bacteria compared to

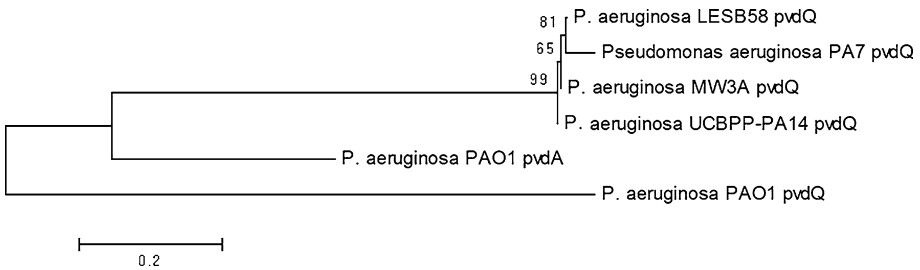

Fig. 5 Phylogenetic tree, generated using Neighbour-Joining algorithm, showing the phylogenetic position of *pvdQ* homologue gene of *P. aeruginosa* strain MW3A; the *horizontal bar* at the bottom represents evolutionary distance as 0.2 change per nucleotide position, determined by measuring the lengths of the *horizontal lines* connecting the species; GenBank accession number: *P. aeruginosa* LESB58 *pvdQ* (218768969:3206318–3208606), *P. aeruginosa* PA7 *pvdQ* (150958624:2955521–2957803), *P. aeruginosa* UCBPP-PA14 *pvdQ* (115583796:3004988–3007276), *P. aeruginosa* PAO1 *pvdA* gene (Z25465:1129155), *P. aeruginosa* PAO1 *pvdQ* (110227054: 2636517–2638805)

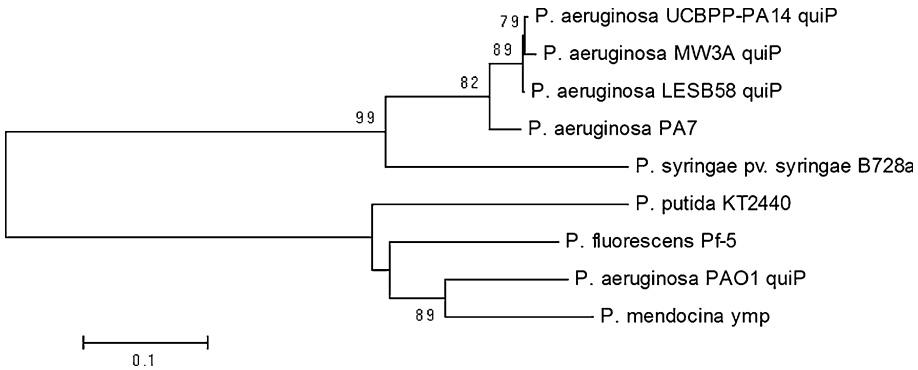

Fig. 6 Phylogenetic tree generated using Neighbour-Joining algorithm, showing the phylogenetic position of *quiP* homologue gene of *P. aeruginosa* strain MW3A; the *horizontal bar* at the bottom represents evolutionary distance as 0.1 change per nucleotide position, determined by measuring the lengths of the *horizontal lines* connecting the species; GenBank accession number: *P. aeruginosa* UCBPP-PA14 *quiP* (115583796:4527711–4530254), *P. aeruginosa* LESB58 *quiP* (218768969:4725132–4727675), *P. aeruginosa* PA7 (150958624:4478165–4480708), *Pseudomonas syringae* pv. *syringae* B728a (63253978:4609176–4611650), *P. putida* KT2440 (24987239:1265544–1267985); *P. fluorescens* Pf-5 (68342549:1438348–1440777), *P. aeruginosa* PAO1 *quiP* (110227054:1119674–1122217), *Pseudomonas mendocina* ymp (145573243:1577468–1579981)

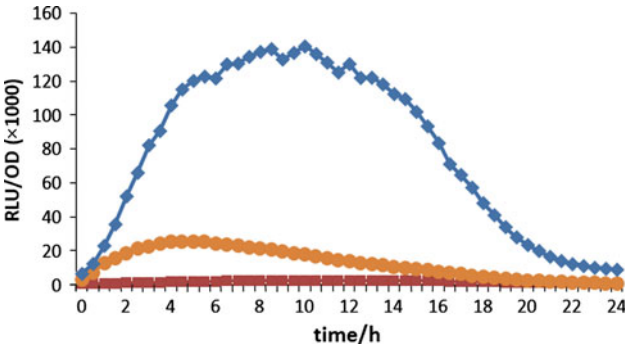

Fig. 7 Bioluminescence and OD were measured throughout growth for 24 h at 37°C in the presence of C_{12}-HSL (*diamond*) (positive control), ethyl acetate (*square*) (negative control), and AHL extracted from *P. aeruginosa* strain MW3A (*circle*); in all experiments, RLU and OD were determined as described in Sect. "Materials and methods"; the *graphs* represent assays of two independent experiments, each with triplicate sets of similar results; RLU, relative light units

those of bacteria from the terrestrial environment, although the marine *Vibrio fischeri* is the first bacterium that provides the evidence of an AHL-based QS mechanism (Eberhard 1972). From marine snow, different AHL-producing *Roseobacter* strains have been isolated (Gram et al. 2002). Synthesis of a novel AHL molecule with a C_{14} *N*-acyl side chain and a double bond at C-7, namely 7-*cis*-*N*-(tetradecenoyl)-HSL, has been reported in the free-living marine bacterium *Rhodobacter sphaeroides* (Puskas et al. 1997). A marine *Mesorhizobium* sp. that produces structurally novel AHLs, i.e., 5-*cis*-3-oxo-C_{12}-homoserine lactone (5-*cis*-3-oxo-C_{12}-HSL) and 5-*cis*-C_{12}-HSL, has been reported (Krick et al. 2007). In addition, a diverse range of AHLs (with *N*-acyl side chains from C_4 to C_{14}) detected in *Proteobacteria* associated with marine sponge are mostly produced by α-proteobacterial isolates mainly from the *Silicibacter–Ruegeria* subgroup of the *Roseobacter* clade (Mohamed et al. 2008).

Recently, Romero et al. (2011) reported that QQ is a common feature among cultivable bacteria isolated from dense marine bacterial communities. Fifteen isolates, capable of inactivating AHLs, were characterized and they belong to nine different genera. However, none of them belong to the genus *Pseudomonas*.

The presence of *P. aeruginosa* strain MW3A in the marine environment may be important in inactivating AHLs secreted in marine waters, thereby controlling AHL-regulated phenotypes in marine QS bacteria such as *Vibrio* sp., *Roseobacter* sp., and *Mesorhizobium* sp. (Eberhard 1972; Gram et al. 2002; Krick et al. 2007; Mohamed et al. 2008). This QQ activity may have an impact on aquatic fish and human opportunistic pathogens such as *Aeromonas* spp. (Chan et al. 2011b; Swift et al. 1996). Since the supernatant incubated for 24 h showed no detectable AHLs as compared to that incubated for 8 h, this led us to speculate that the QQ activity of *P. aeruginosa* strain MW3A may be important in the self-perception and regulation of the AHLs it produces. Once the QS-mediated phenotypes have been expressed, the AHLs that it produces are degraded.

Two major AHLs produced by *P. aeruginosa* strain MW3A were characterized by mass spectrometry. The data obtained are interesting because *P. aeruginosa* has been reported to produce two major AHLs, namely 3-oxo-C_{12}-HSL and *N*-butanoylhomoserine lactone (C_4-HSL) (Latifi et al. 1996; Schuster and Greenberg 2006), and two minor AHLs, C_6-HSL and 3-oxo-C_6-HSL (Rumbaugha et al. 2000; Winson et al. 1995). In contrast, *P. aeruginosa* strain MW3A was shown to produce 3-oxo-C_{14}-HSL and

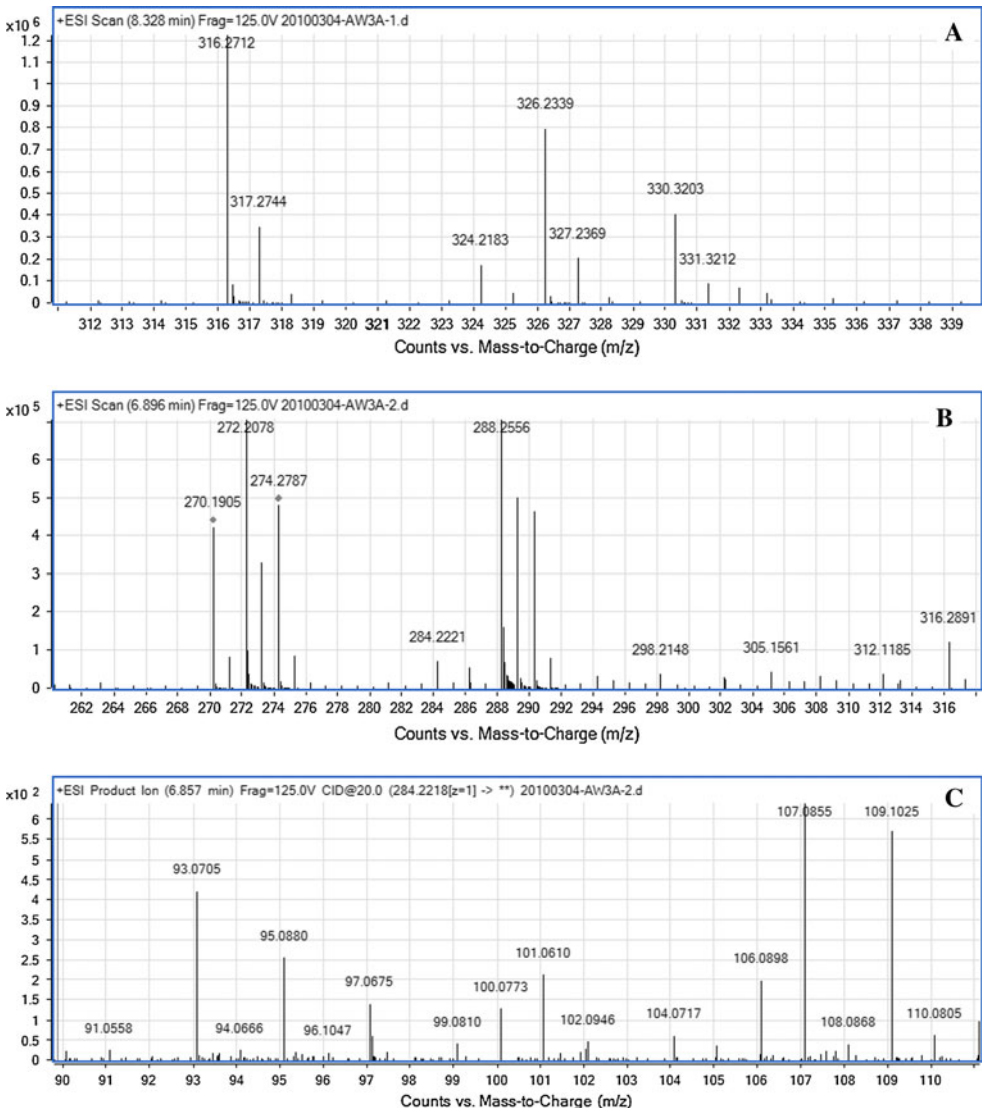

Fig. 8 Mass spectrometric analysis of AHLs: **a** ESI–MS spectrum of 3-oxo-C_{14}-HSL (326.2339, 8.33 min), **b** ESI–MS spectrum of C_{12}-HSL (284.2221, 6.90 min), and **c** ESI–MS/MS spectrum of C_{12}-HSL (284.2221, 6.90 min)

C_{12}-HSL, which have not been reported previously. No C_4-HSL was detected in this study and this may be because AHLs were extracted at 8-h post inoculation when hardly any or very little C_4-HSL was produced. In addition to the AHL-based QS system, *P. aeruginosa* is known to produce 2-alkyl-4-quinolone signal molecules called *Pseudomonas* quinolone signal (PQS; 2-heptyl-3-hydroxy-4-quinolone) that functions as a link between the *las* and *rhl* QS systems (Pesci et al. 1999). Indeed, we have detected PQS from *P. aeruginosa* strain MW3A (data not shown).

Further investigation is required to clarify the role of *P. aeruginosa* strain MW3A, possessing both QS and QQ properties, in the marine environment.

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