MICROBIAL QUORUM QUENCHING ACTIVITIES IN MARINE WATER, WETLAND WATER, AND RAINFOREST SOIL

WONG CHENG SIANG

FACULTY OF SCIENCE
UNIVERSITI MALAYA
KUALA LUMPUR
2010

MICROBIAL QUORUM QUENCHING ACTIVITIES IN MARINE WATER, WETLAND WATER, AND RAINFOREST SOIL

WONG CHENG SIANG

DISSERTATION SUBMITTED IN FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE

INSTITUTE OF GRADUATE STUDIES
UNIVERSITY OF MALAYA
KUALA LUMPUR

2010

Publications

Part of this study has been accepted for publication and presented in:

Journal publication:

Kok-Gan Chan, Cheng-Siang Wong, Wai-Fong Yin, Choon-Kook Sam and Chong-Lek Koh. (2010) "**Rapid degradation of N-3-oxo-acylhomoserine lactones by a *Bacillus cereus* isolate from Malaysian rainforest soil.**" Antonie van Leeuwenhoek DOI 10.1007/s10482-010-9438-0 [Journal impact factor 1.637 as of year 2008].

Oral presentation:

Cheng-Siang Wong and Kok-Gan Chan. "**Enzymatic Degradation of Quorum Sensing Signal Molecules by *Bacillus* sp. Enriched from Malaysian Rain-Forest Soil using Novel Selection KG Medium.**" Second International Conference and Workshops on Basic and Applied Sciences and Regional Annual Fundamental Science Seminar 2009 (ICORAFSS 2009), 03 Jun 2009 to 04 Jun 2009, Universiti Teknologi Malaysia, Johor (International).

Poster presentation:

Cheng-Siang Wong and Kok-Gan Chan. "**Degradation of N-acyl-homoserine lactone quorum-sensing molecules by yeast.**" 30th Symposium Malaysian Society For Microbiology, 16 Aug 2008 to 19 Aug 2008, Kuantan, Pahang (National).

Other publications (accepted for publication):

Kok-Gan Chan, Savithri D Puthucheary, Wai-Fong Yin, Cheng-Siang Wong, Wah-Seng See Too, Kek-Heng Chua. (2010) "**Quorum Sensing in *Aeromonas* Species Isolated from Patients in Malaysia.**" Current Microbiology. (ISI/SCOPUS Cited Publication)

Kok-Gan Chan, Cheng-Siang Wong, Zi-Bin Low Joel, Wai-Fong Yin, Choon-Kook Sam and Chong-Lek Koh. (2009) "**Degradation of N-acylhomoserine Lactone by *Enterobacter* sp. sw39 Isolated from Sewage.**" Asia Pacific Journal of Molecular Biology and Biotechnology. (SCOPUS-Cited Publication)

ABSTRACT

Quorum sensing refers to the bacterial cell-to-cell communication phenomenon that is employed to regulate a wide range of activities in a variety of bacteria. One of the most extensively studied mechanisms is that of Gram-negative bacteria utilizing *N*-acyl homoserine lactone as the signaling molecule. Quorum quenching on the other hand refers to the interference of quorum sensing, that can be achieved by, inter alia, directly inactivating the signaling molecule, and thus preventing the expression of certain genes, notably those regulating virulence.

This study aimed to isolate *N*-acyl homoserine lactone-degrading microorganisms from Malaysian marine water, wetland water, and rainforest soil using a defined enrichment medium. The present work successfully yielded three bacterial strains and one fungal strain from three environmental samples. The major finding generated by this study was the characterization of a basidiomycetous yeast strain isolated from wetland water, which was capable of degrading a variety of *N*-acyl homoserine lactones and growing on 3-oxo-C6-HSL. To the best of our knowledge, this is the first report of such activities in the fungal strain *Trichosporon*.

The *N*-acyl homoserine lactone-degrading bacterial strains include *Bacillus cereus* isolated from rainforest soil and two *Pseudomonas aeruginosa* strains isolated from marine water. *Bacillus cereus* strain KM1S was able to degrade rapidly 3-oxo-C6-HSL and 3-oxo-C8-HSL *in vitro*. The kinetic of the AHL turnover was characterized using RRLC. The *aiiA* homologue in this isolate was found to contain the motif $_{106}$HXDH-59 amino acids-H$_{169}$-21 amino acids-D$_{191}$, essentially needed for the AHL degradation activity. The AHL degrading activities were confirmed in the marine pseudomonads using RRLC and the quorum quenching homologue genes *quiP* and *pvdQ* were successfully PCR amplified.

ABSTRAK

Pengesanan kuorum adalah fenomena komunikasi sel-sel yang digunakan oleh bakteria untuk mengawalaturkan pelbagai aktiviti. Di kalangannya, satu mekanisme yang paling dikaji ialah yang melibatkan bakteria Gram-negative yang menggunakan *N*-acyl homoserine lactone sebagai molekul pengesan. Sebaliknya, perencatan kuorum merujuk kepada perencatan pengesanan kuorum, yang boleh dicapai dengan merencatkan molekul pengesan secara langsung, dan seterusnya menghentikan ekspresi gen-gen tertentu, terutamanya yang mengawalatur virulen.

Kerja ini bertujuan mengasingkan mikroorganisma yang boleh menguraikan *N*-acyl homoserine lactone dari air lautan, air wetland dan tanah hutan Malaysia dengan menggunakan media defined enrichment. Kerja ini berjaya menghasilkan tiga strain bakteria dan satu strain kulat dari tiga sampel persekitaran tersebut. Satu hasil yang penting daripada kerja kajian ini adalah penghuraian satu basidiomycetous yis yang diasingkan dari air wetland, yang berupaya menguraikan pelbagai jenis *N*-acyl homoserine lactone dan bertumbuh atas 3-oxo-C6-HSL. Ini merupakan laporan pertama tentang aktiviti yang sebagaimana ini dalam kulat strain *Trichosporon*.

Bakteria pengurai *N*-acyl homoserine lactone termasuk *Bacillus cereus* yang diasingkan dari tanah hutan dan dua strain *Pseudomonas aeruginosa* yang diasingkan dari air lautan. *Bacillus cereus* strain KM1S berupaya menguraikan 3-oxo-C6-HSL dan 3-oxo-C8-HSL *in vitro* dengan cepat. Kinetik penguraian AHL telah dikaji dengan menggunakan RRLC. Homolog *aiiA* dalam bakteria ini didapati mengandungi motif $_{106}$HXDH-59 amino acids-H$_{169}$-21 amino acids-D$_{191}$, yang penting untuk aktiviti penguraian AHL. Aktiviti penguraian AHL telah dipastikan dalam marine pseudomonads dengan menggunakan RRLC dan homolog gen perencatan kuorum *quip* dan *pvdQ* telah Berjaya diamplifikasikan dengan menggunakan PCR.

ACKNOWLEDGEMENTS

My sincere gratitude and appreciation to my supervisor, Dr. Chan Kok Gan, for his invaluable guidance and supervision. To Professor Koh Chong Lek and Dr Sam Chook Kook, thank you for your advice and support. Thank you to Miss Yin Wai Fong, who always lends an ear and shares her knowledge. I am truly honored and blessed to be under their care over this period of time.

My gratitude to all lab members, all of whom were always willing to lend a helping hand and to share their knowledge and experience with me. Thank you for making the lab such a warm and friendly place to be. I enjoyed the conversations, the time and joy we spent together in the lab. I really value the friendships that blossomed within this duration.

Finally, I would like to express my love and gratitude to my dearest parents and sisters for their unconditional support, care and love, for always being a warm shelter for me whenever I faced difficulty. Thanks to my dearest girlfriend, for her durable love and support in countless ways throughout the study.

I truly cherish all the faith that all of you have put in me. This momentous journey would not be possible without all of you.

TABLE OF CONTENTS

CHAPTER THREE: RESULTS

ISOLATION OF QUORUM QUENCHING YEAST

CHAPTER FOUR: RESULTS

ISOLATION OF QUORUM QUENCHING BACTERIA

CHAPTER FIVE: DISCUSSION

LIST OF FIGURES

LIST OF TABLES

List of abbreviations

°C	:	Degree Celsius
4Q	:	4-quinolone
ACP	:	Acyl carrier protein
AHL	:	*N*-acyl homoserine lactone
Amp	:	Ampicillin
BLAST	:	Basic Local Alignment and Search Tool
bp	:	Basepair
cfu	:	Colony-forming unit
Da	:	Dalton
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxynucleotide triphosphate
df	:	Degree of freedom
EDTA	:	Ethylenediamine tetraacetic acid
et al.	:	*et alia* (and others)
g	:	Gram
g	:	Gravity (relative centrifugal force)
Gen	:	Gentamycin
h	:	Hour
HSL	:	Homoserine lactone
i.e.	:	That is
IPTG	:	Isopropyl-β-$_D$-thiogalactopyranoside
ITS	:	Internal transcribed spacer
Kan	:	Kanamycin
kb	:	Kilobase pair
kV	:	Kilovolt
LB	:	Luria-Bertani
m	:	Mole(s)
M	:	Molar
MES	:	2-(*N*-morpholino)-ethanesulfonic acid
min	:	Minute
ml	:	Millilitre

mM	:	millimolar
ms	:	Millisecond
OD	:	Optical density
PBS	:	Phosphate buffer saline
PCR	:	Polymerase chain reaction
PQS	:	*Pseudomonas* quinolone signal
psi	:	Pound per square inch
rDNA	:	Ribosomal deoxyribonucleic acid
R_f	:	Retention factor
rpm	:	Revolutions per minute
RRLC	:	Rapid Resolution Liquid Chromatography
s	:	Second
SAM	:	S-adenosylmethionine
sdH_2O	:	Sterile distilled water
sp.	:	Species
Str	:	Streptomycin
TAE	:	Tris acetate EDTA
TBE	:	Tris borate EDTA
Tet	:	Tetracycline
TLC	:	Thin layer chromatography
UV	:	Ultraviolet
v/v	:	Volume per volume
w/v	:	Weight per volume
X-gal	:	5-bromo-4-chloro-3-indolyl-β-$_D$-thiogalactopyranoside
µg	:	Microgram
µl	:	Microlitre
µm	:	Micrometer

CHAPTER ONE: INTRODUCTION

1.1 Quorum sensing

The discovery of quorum sensing over 30 years ago has changed our general perception of bacteria as single and simple organisms. It is becoming increasingly clear that 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" has been coined to describe 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'' (Schauder and Bassler, 2001; Miller and Bassler, 2001; Fuqua *et al.*, 2001).

Quorum sensing involves producing, releasing, detecting, and responding to small molecules termed autoinducers. Bacteria use these small diffusible molecules to exchange information amongst themselves. Most quorum sensing signalling molecules are either small (<1000 Da) organic molecules or peptides with 5–20 amino acids (Lazazzera, 2001; 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, according to the size of the population.

Initially, quorum sensing was thought to control only a limited number of bacterial phenotypes, but it is now clear that a wide range of microorganisms regulate gene expression according to population density, possibly representing a primitive form of multicellularity (Barnard and Salmond, 2005). Examples of quorum sensing-modulated physiological processes include 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 (for reviews, see Dunny and Winans, 1999; Fuqua *et al.*, 1996; Hardman *et al.*, 1998; Salmond *et al.*, 1995).

Many different types of signalling molecule have now been identified. Based on the types of autoinducers and signaling pathway, quorum sensing systems can be divided into two paradigmatic classes: LuxI/LuxR-type quorum sensing systems in Gram-negative bacteria and oligopeptide/two component-type quorum sensing circuits in Gram-positive bacteria (Waters and Bassler, 2005). In Gram-negative bacteria, the most extensively studied signalling molecules are the *N*-acyl homoserine lactones (AHLs) (Fuqua *et al.*, 2001).

1.2 The *Vibrio fischeri* LuxI/LuxR bioluminescence system

The first described quorum sensing system is that of the bioluminescent marine bacterial species, *Vibrio fischeri*, and it is considered the paradigm for quorum sensing in most Gram-negative bacteria (Nealson and Hastings, 1979). This marine bacterium is maintained in a symbiotic relationship in specialized light organ of a number of eukaryotic hosts, one example is the Hawaiian squid *Eupryma scolopes* (McFall-Ngai, 2000).

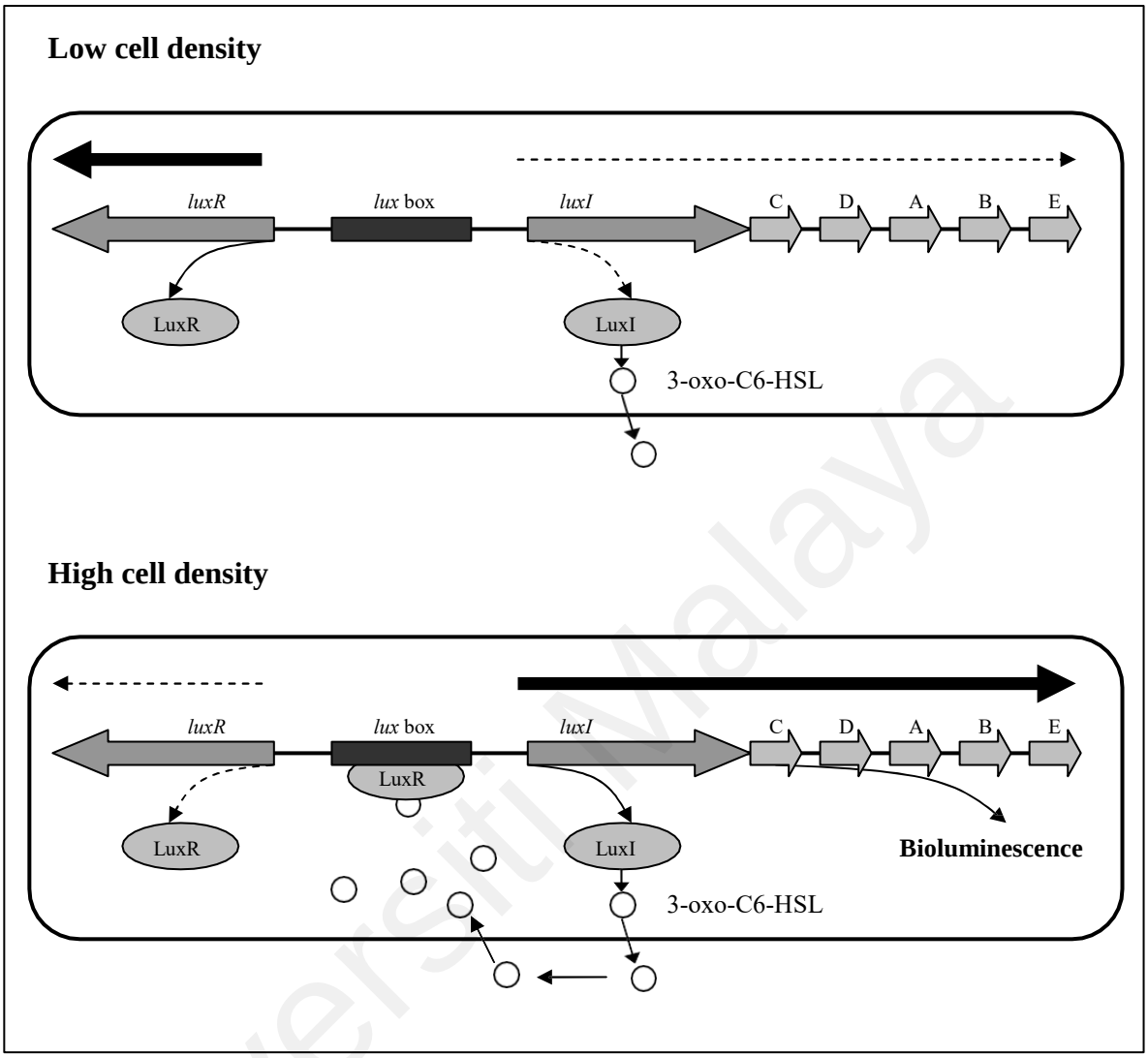
The role of *V. fischeri* is to provide the host with light. In the specialized light organ, the marine bacteria grow to high cell density (10^{11} cells per ml) and induce the expression of genes required for bioluminescence. Each eukaryotic host then uses the light provided by the bacteria for a specific purpose, for example counter-illumination. The bacteria benefit because the light organ is rich in nutrients and allows proliferation in numbers unachievable in seawater (Visick and Ruby, 2006).

The *V. fischeri* quorum sensing regulatory elements consist of five luciferase structural genes (*luxCDABE*) and two regulatory genes (*luxR* and *luxI*), which play central roles in regulation of the *lux* operon as shown in Figure 1.1 (Engebrecht *et al.*,

1983). The genes are arranged in two adjacent but divergently transcribed units, *luxR* is transcribed to the left, and the *luxICDABE* operon is transcribed to the right. The *luxR* gene encodes LuxR, a DNA-binding transcriptional regulator. The *luxI* gene encodes the autoinducer 3-oxo-C6-HSL synthase and the *luxCDABE* structural genes encode the luciferase enzymes required for light production.

Following the production of 3-oxo-C6-HSL by the LuxI AHL synthase enzyme, this signalling molecule is freely diffusible across the cell membrane, so the concentration of 3-oxo-C6-HSL in the extracellular environment is the same as the intracellular concentration of 3-oxo-C6-HSL. At low cell density, the *luxICDABE* operon is transcribed at a low basal level (Kaplan and Greenberg 1985), but when 3-oxo-C6-HSL accumulates to a threshold level (approximately 1–10 μg/ml) that is sufficient for detection and binding by the cytoplasmic LuxR protein (Eberhard *et al.*, 1981). Activated LuxR is then able to bind as a dimer to a 20-bp palindromic DNA sequence termed the *lux* box, located upstream of the target *luxICDABE* bioluminescence operon and activate its transcription (Engebrecht and Silverman, 1984; Devine *et al.*, 1989; Egland and Greenberg, 1999). The LuxR-3-oxo-C6-HSL complex also induces expression of *luxI* because it is encoded in the luciferase operon. This regulatory configuration floods the environment with the signalling molecule, creating a positive feedback loop, resulting in an exponential increase in both autoinducer production and light emission.

Figure 1.1 Bioluminescence in *V. fischeri*
(modified from http://www.nottingham.ac.uk/quorum/fischeri3.htm)



At low cell densities, the *luxICDABE* genes (*luxCDABE* genes are responsible for bioluminescence) are transcribed at a low level and the small amounts of 3-oxo-C6-HSL produced diffuse out of the cell and disappear in the environment. At high cell densities, 3-oxo-C6-HSL accumulates in the local environment and therefore also inside the cell. Transcription of *luxICDABE* appears to be increased by a complex of the LuxR protein and 3-oxo-C6-HSL, binding to a region of DNA called the *lux* box.

1.3 Quorum sensing in Gram-negative bacteria

Since the discovery of the LuxI/LuxR type quorum sensing system in *V. fischeri*, similar systems utilizing homologues of the LuxI and LuxR regulatory proteins have been identified in a wide range of Gram-negative bacteria, as shown in Table 1.1. Many of these bacteria produce multiple AHLs and possess more than one AHL synthase.

The AHL molecules are highly conserved, which consists of a homoserine lactone ring unsubstituted in the β- and γ-positions which is *N*-acylated with a fatty acyl group at the α-position (Chhabra *et al.*, 2005). The acyl side-chain varies from 4-18 carbons in its lengths, mostly even-numbered, with different saturation levels and oxidation states which belong to either the *N*-acyl, *N*-(3-oxoacyl) or *N*-(3-hydroxyacyl) classes. Stereochemistry at the α-centre of the homoserine lactone (HSL) ring has been unequivocally established to be $_{L}$-isomer for the 3-oxo-C6-HSL produced by *Erwinia carotovora* (Bainton *et al.*, 1992a) and by analogy it is deduced that all other natural AHLs have the same configuration (Williams, 2007). It has been reported that synthetic $_{D}$-isomers lack of autoinduction activity (Chhabra *et al.*, 1993; Chhabra *et al.*, 2005). Shown in Figure 1.2 are structures of some AHL molecules.

The biosysthesis of AHL depends on the LuxI family proteins that use S-adenosylmethionine (SAM) and an acyl carrier protein (ACP) charged with the appropriate fatty acids as precursor for the homoserine lactone (HSL) and acyl chain, respectively (More *et al.*, 1996; Fuqua and Greenberg, 2002). Two chemical events occur in LuxI-mediated AHL biosynthesis. The LuxI proteins couple a specific acyl-ACP to SAM via amide bond formation between the acyl group of the acyl-ACP and the amino group of the homocysteine moiety of SAM. The subsequent lactonization of the ligated intermediate in the reaction, along with the release of methylthioadenosine, results in the formation of the AHL (Hanzelka and Greenberg, 1996; More *et al.*, 1996; Val and Cronan Jr., 1998).

Table 1.1 Examples of AHL-dependent quorum sensing systems and the phenotypes controlled

Organism	Autoinducer Identity	Target Genes and Functions	Reference
Acidithiobacillus ferrooxidans	C12-HSL, C14-HSL, 3-oxo-C14-HSL, 3-hydroxy-C8-HSL, 3-hydroxy-C10-HSL, 3-hydroxy-C12-HSL, 3-hydroxy-C14-HSL, 3-hydroxy-C16-HSL	Unknown	Farah *et al.*, 2005; Rivas *et al.* 2005; Rivas *et al.* 2007; Ruiz *et al.*, 2008
Acinetobacter baumannii	3-hydroxy-C12-HSL	Biofilm maturation	Niu *et al.*, 2008
Aeromonas hydrophila	C4-HSL, C6-HSL	Serine protease and metalloprotease production, biofilms	Swift *et al.*, 1997; Lynch *et al.*, 2002
Aeromona salmonicida	C4-HSL, C6-HSL	*aspA* (exoprotease)	Swift *et al.*, 1999
Agrobacterium tumefaciens	3-oxo-C8-HSL	*tra*, *trb* (Ti plasmid conjugal transfer)	Piper *et al.*, 1993; Zhang *et al.*, 1993
Agrobacterium vitis	C14:1-HSL, 3-oxo-C16:1-HSL	Virulence	Zheng *et al.*, 2003; Hao *et al.*, 2005; Hao and Burr, 2006; Wang *et al.*, 2008
Burkholderia cepacia	C6-HSL, C8-HSL	Protease and siderophore production	Lewenza *et al.*, 1999
Burkholderia cenocepacia	C6-HSL, C8-HSL	Exoenzymes, biofilm formation, swarming motility, siderophore, virulence	Lewenza *et al.*, 1999; Sokol *et al.*, 2003; Malott *et al.*, 2005
Burkholderia pseudomallei	C8-HSL, C10-HSL, 3-hydroxy-C8-HSL, 3-hydroxy-C10-HSL, 3-hydroxy-C14-HSL	Virulence, exoproteases	Ulrich *et al.*, 2004a; Valade *et al.*, 2004; Song *et al.*, 2005
Burkholderia mallei	C8-HSL, C10-HSL	Virulence	Ulrich *et al.*, 2004b
Chromobacterium violaceum	C6-HSL	Production of violacein pigment, hydrogen cyanide, antibiotics, exoproteases and chitino-lytic enzymes	Chernin *et al.*, 1998; McClean *et al.*, 1997

Table 1.1 *continued*

Enterobacter agglomerans	3-oxo-C6-HSL	Unknown	Swift *et al.*, 1993
Erwinia carotovora	3-oxo-C6-HSL	Carbapenem, exoenzymes, virulence	Jones *et al.*, 1993; Pirhonen *et al.*, 1993 Bainton *et al.*, 1992a
Erwinia chrysanthemi	3-oxo-C6-HSL	*pecS* (regulator of pectinase synthesis)	Nasser *et al.*, 1998; Reverchon *et al.*, 1998
Methylobacterium extorquens	C6-HSL, C8-HSL, C14:1-HSL, C14:2-HSL	Extracellular polysaccharide production	Nieto Penalver *et al.*, 2006a; Nieto Penalver *et al.*, 2006b
Pantoea (Erwinia) stewartii	3-oxo-C6-HSL	Capsular polysaccharide biosynthesis, virulence	von Bodman and Farrand, 1995; von Bodman *et al.*, 2003
Pseudomonas aureofaciens	C6-HSL	phz (phenazine antibiotic biosynthesis)	Pierson *et al.*, 1994; Wood *et al.*, 1997; Zhang and Pierson, 2001
Pseudomonas aeruginosa	C4-HSL, C6-HSL, 3-oxo-C12-HSL	Exoenzymes, exotoxins, protein secretion, biofilms, swarming motility, secondary metabolites, 4-quinolone signalling, virulence	Winzer and Williams, 2001; Schuster *et al.*, 2003; Smith and Iglewski, 2003; Wagner *et al.*, 2003; Juhas *et al.*, 2005
Pseudomonas chlororaphis	C6-HSL	Phenazine-1-carboxamide	Chin *et al.*, 2001; Chin *et al.*, 2005
Pseudomonas putida	3-oxo-C10-HSL, 3-oxo-C12-HSL	Biofilm development	Bertani and Venturi, 2004; Steidle *et al.*, 2002
Pseudomonas fluorescens	3-oxo-C10-HSL, 3-hydroxy-C14:1-HSL	Mupirocin	Laue *et al.*, 2000; El-Sayed *et al.*, 2001
Pseudomonas syringae	3-oxo-C6-HSL	Exopolysaccharide, swimming motility, virulence	Dumenyo *et al.*, 1998; Quinones *et al.*, 2004
Ralstonia solanacearum	C6-HSL, C8-HSL	Unknown	Flavier *et al.*, 1997; Flavier *et al.*, 1998
Rhizobium etli	Multiple, unconfirmed	Restriction of nodule number	Rosemeyer *et al.*, 1998

Table 1.1 *continued*

Rhizobium leguminosarum bv. *viciae*	C14:1-HSL, C6-HSL, C7-HSL, C8-HSL, 3-oxo-C8-HSL, 3-hydroxy-C8-HSL	Root nodulation/symbiosis, plasmid transfer, growth inhibition; stationary phase adaptation	Cubo *et al.*, 1992; Gray *et al.*, 1996; Schripsema *et al.*, 1996; Rodelas *et al.*, 1999; Lithgow *et al.*, 2000 Wisniewski-Dyé and Downie, 2002
Rhodobacter sphaeroides	7-cis-C14-HSL	Prevents bacterial aggregation	Puskas *et al.*, 1997
Roseobacter sp.	C6-HSL, 3-oxo-C6-HSL	Chemotaxis functions	Gram *et al.*, 2002
Serratia liquefaciens	C4-HSL, C6-HSL	Swarming motility, exoprotease, biofilm development, biosurfactant	Eberl *et al.*, 1996; Givskov *et al.*, 1997; Labbate *et al.*, 2004;
Serratia marcescens	C6-HSL, 3-oxo-C6-HSL, C7-HSL, C8-HSL	Sliding motility, biosurfactant, pigment, nuclease, transposition frequency	Horng *et al.*, 2002; Rice *et al.*, 2005; Van Houdt *et al.*, 2006; Wei *et al.*, 2006
Serratia proteamaculans	3-oxo-C6-HSL	Exoenzymes	Gram *et al.*, 1999; Christensen *et al.*, 2003
Sinorhizobium meliloti	C8-HSL, C12-HSL, 3-oxo-C14-HSL, 3-oxo-C16:1-HSL, C16:1-HSL, C18-HSL	Nodulation efficiency, symbiosis, exopolysaccharide	Marketon and Gonzalez, 2002; Marketon *et al.*, 2002
Vibrio anguillarum	C6-HSL, 3-hydroxy-C6-HSL, 3-oxo-C10-HSL	Unknown	Milton *et al.*, 1997; Milton *et al.*, 2001
Vibrio fischeri	3-oxo-C6-HSL	*luxICDABE* (bioluminescence)	Eberhard *et al.*, 1981; Engebrecht *et al.*, 1983
Vibrio harveyi	3-hydroxy-C4-HSL	Bioluminescence, polyhydroxybutyrate metabolism	Eberhard *et al.*, 1981
Yersinia enterocolitica	C6-HSL, 3-oxo-C6-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL, 3-oxo-C14-HSL	Swimming and swarming motility	Throup *et al.*, 1995; Jacobi *et al.*, 2003; Atkinson *et al.*, 2006
Yersinia pseudotuberculosis	C6-HSL, 3-oxo-C6-HSL, C8-HSL	Motility, aggregation	Atkinson *et al.*, 1999; Yates *et al.*, 2002; Atkinson *et al.*, 2008

Figure 1.2 Structures of several *N*-acyl homoserine lactone molecules (adapted from Fuqua *et al.*, 1996; Schaefer *et al.*, 2008)

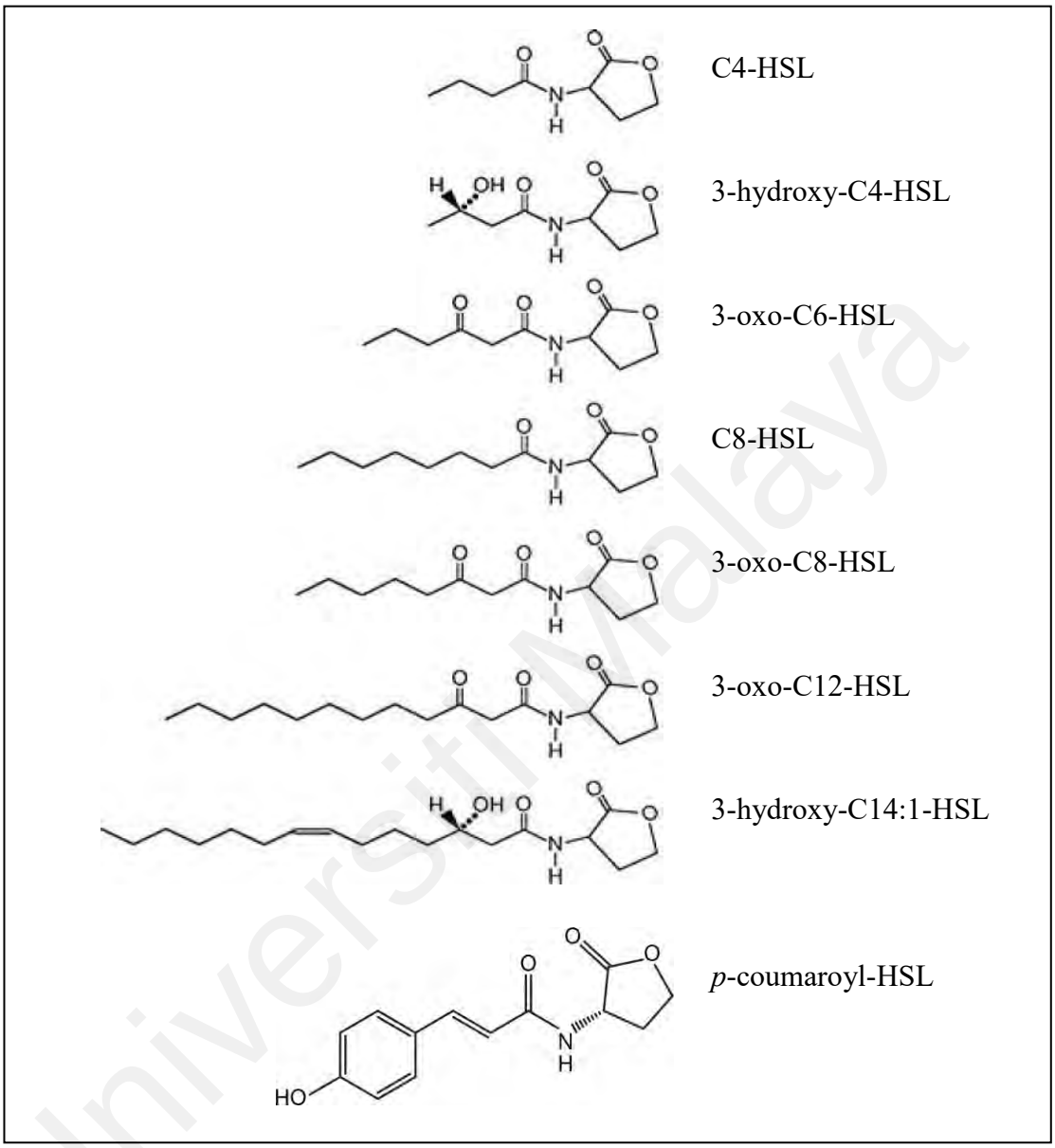
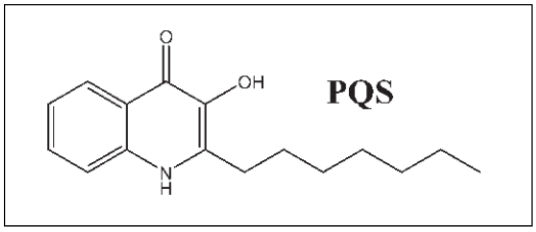


Figure 1.3 Structures of *Pseudomonas* quinolone signal molecule (adapted from Williams, 2007)



Recently, Schaefer *et al.* (2008) reported the discovery of a new class of quorum sensing signalling molecule, *p*-coumaroyl-HSL, in photosynthetic bacterium *Rhodopseudomonas palustris* (Figure 1.2). This extends the range of the possibilities of quorum sensing signalling molecules. *R. palustris* degrades a wide variety of aromatic compounds in the soil. The signalling molecule *p*-coumaroyl-HSL is produced from *p*-coumaric acid (i.e. *p*-hydroxycinnamate), a major constituent of the lignin polymers abundant in plant cell walls. The LuxI/LuxR homologue, RpaI/RpaR system in *R. palustris* constitutes a novel quorum sensing circuit that depends on an exogenous substrate produced by a different organism (Schaefer *et al.*, 2008).

Quorum sensing in *Pseudomonas aeruginosa*

A wide range of virulence determinants and secondary metabolites are regulated via the action of a hierarchical quorum-sensing system which integrates two chemically distinct classes of signal molecules, i.e. the *N*-acyl homoserine lactones (AHLs) and the 4-quinolones (4Qs) (Diggle *et al.*, 2006). Interestingly, two AHL quorum sensing systems that are analogous to the *V. fischeri* LuxI/LuxR system are present in *Pseudomonas aeruginosa*, the LasI/LasR and RhlI/RhlR system. Both LasI and RhlI are AHL synthases that catalyze the formation of 3-oxo-C12-HSL and C4-HSL, respectively, that activates the respective transcriptional activator protein LasR and RhlR (Pearson *et al.*, 1994; Pearson *et al.*, 1995). However, the *las* and *rhl* systems are not compatible, meaning that the RhlI-produced C4-HSL cannot activate LasR, and that LasI-produced 3-oxo-C12-HSL cannot activate RhlR (Pearson *et al.*, 1995; Pearson *et al.*, 1997).

The *rhl* system regulates the production of rhamnolipid, elastase, LasA protease, hydrogen cyanide, pyocyanin, siderophores, and the cytotoxic lectins PA-I and PA-II (reviewed in Venturi, 2006). The *las* system regulates the production of virulence

factors including elastase, the LasA protease, alkaline protease, exotoxin A, Xcp secretion, and biofilm maturation (reviewed in Venturi, 2006). In addition, the LasR-3-oxo-C12-HSL complex activates *rhlR* expression to initiate the second signaling cascade. However, the LasR-3-oxo-C12-HSL also prevents the binding of the C4-HSL to its cognate regulator RhlR (Pesci *et al.*, 1997). Presumably, this second level of control of RhlI/RhlR autoinduction by the LasI/LasR system ensures that the two systems initiate their cascades sequentially and in the appropriate order.

Pesci *et al.* (1999) discovered the presence of a chemically distinct class of quorum sensing signalling molecule present in spent *P. aeruginosa* culture supernatants, which belongs to the 4-quinolone (4Q) family, chemically characterised as 2-heptyl-3-hydroxy-4-quinolone and termed the *Pseudomonas* quinolone signal (PQS) (Figure 1.3). PQS is synthesized from anthranilate via the action of the *pqsABCD* and *pqsH* gene products. Production of PQS appears to be finely controlled by the opposing actions of the *las* and *rhl* quorum sensing systems (Calfee *et al.*, 2001; Gallagher *et al.*, 2002; McGrath *et al.*, 2004). PQS induces the expression of the *lasB* elastase, as well as the expression of *rhlI* and *rhlR* and thus PQS acts as an additional regulatory link between the *las* and *rhl* quorum sensing circuits (Pesci *et al.*, 1999; McKnight *et al.*, 2000).

1.4 AHL Biosensors

The screening of Gram-negative bacteria for putative AHL producers has been greatly aided by the development of simple AHL biosensors based on *lux*, *lacZ* or *gfp* reporter gene fusions (Bainton *et al.*, 1992b; Winson *et al.*, 1998; Andersen *et al.*, 2001; Shaw *et al.*, 1997) or pigment induction (McClean *et al.*, 1997). These biosensors, coupled with Thin Layer Chromatography (TLC), provide a simple and rapid technique for assessing the minimum number of different AHL species produced by a given organism (Shaw *et al.*, 1997).

Bioluminescent biosensors *Escherichia coli* [pSB401] and [pSB1075]

The *lux*-based sensor strain *E. coli* [pSB401] and *E. coli* [pSB1075] emits bioluminescence when a source of exogenously supplied AHL is provided due to the bioluminescent monitor plasmid pSB401 and pSB1075. The recombinant plasmids pSB401 and pSB1075 were constructed by fusing the *V. fischeri luxI/luxR* or *P. aeruginosa lasI/lasR* promoter region to *Photorhabdus luminescens lux* structural operon (*luxCDABE* cassette), respectively (Winson *et al.*, 1998). Hence, the presence of exogenously 3-oxo-C6-HSL or other middle ranged-acyl chain AHL will activate *luxR* and subsequently *luxCDABE*.

***Chromobacterium violaceum* CV026**

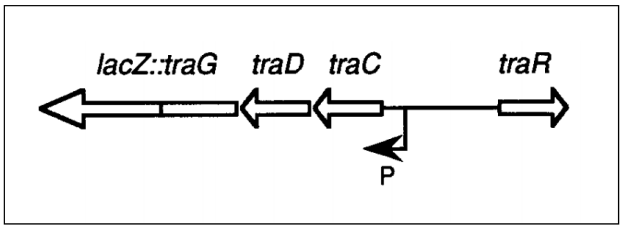
Chromobacterium violaceum is a versatile Gram-negative β-proteobacterium (Dewhirst *et al.*, 1989) that produces the violet non-diffusible water-insoluble antibiotic pigment violacein (Lichstein and Van de Sand, 1945; Antonio and Creczynski-Pasa, 2004). Since the production of violacein is quorum sensing-driven, it has been exploited for AHL bioassays. By subjecting the *C. violaceum* to mini-Tn*5* transposon mutagenesis, a white violacein-negative mutant, CV026 was obtained, which is defective in the

production of the AHL, C6-HSL (McClean *et al.*, 1997). Exposure to exogenous AHLs (*N*-acyl side chains from C4 to C8) will induce violacein production in CV026, where the white mutants will turn purple. CV026 can also be employed to detect AHLs with acyl chains between C10 and C14 by their ability to inhibit short-chain AHL-mediated activation of violacein. This ability indicates that the CV026 can be used as a simple biosensor for the detection of a broad range of AHLs (McClean *et al.*, 1997).

Agrobacterium tumefaciens NTL4(pZLR4)

Agrobacterium tumefaciens strain NTL4 is a Ti plasmid-cured derivative of the nopaline-agrocinopine type strain C58 in which the *tetAR* locus was removed by deletion (Luo *et al.*, 2003). The indicator strain *A. tumefaciens* NTL4(pZLR4) contains inserts from pTiC58 encoding a *traR* and TraR-activable *traG*::*lacZ* fusion, and does not produce detectable levels of AHL, 3-oxo-C8-HSL. The insert was derived from pTHB58*traG*::Tn*3lac1*, which contains a transcriptional fusion of *lacZYA* from Tn*3*HoHo1 to *traG*, as shown in Figure 1.4 (Farrand *et al.*, 1996). Exposure to exogenous AHLs (3-oxo-acyl-HSLs with side chains of 4 to 12 carbons, 3-unsubstituted-acyl-HSLs with side chains of 6 to 12 carbons) will induce the expression of β-galactosidase from the *traG*::*lacZ* fusion, that will cause the formation of a blue zone resulting from hydrolysis of the X-Gal in the medium (Shaw *et al.*, 1997, Cha *et al.*, 1998).

Figure 1.4 Structures of *traG*::*lacZ* reporter fusions in plasmid pZLR4 (Cha *et al.*, 1998)



1.5 Quorum quenching

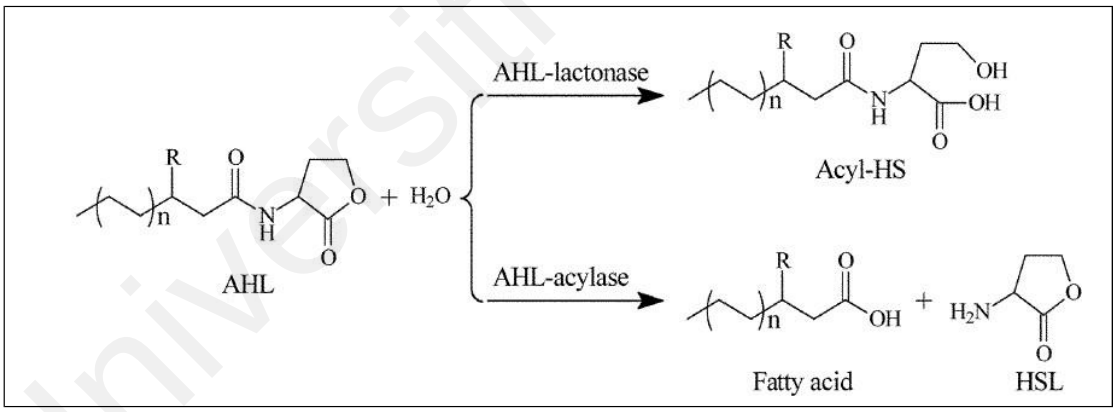
AHL signaling system has been regarded as a promising target for developing novel approaches to control bacterial infections without affecting the viability of the individual cells, but rather the virulence of the population as a whole. This means that there is less selection pressure for the evolution of resistance than in the case in, for example, antibiotic therapy (Hentzer and Givskov, 2003). The disruption of bacterial quorum sensing was termed 'quorum quenching'.

As discussed earlier, bacterial quorum sensing system can be grouped into several key functional components: signal generation, signal perception and the signal transportation (which links the first two components). Therefore quorum quenching can be achieved by targeting one or more of these components. Firstly, the inhibition of AHL biosysthesis. For instance, triclosan, an inhibitor of the enoyl-ACP reductase FabI. FabI involves in reduction of enoyl-ACP to acyl-ACP, which reacts with S-adenosylmethionine, catalysed by AHL synthase, to produce AHL (Schaefer *et al.*, 1996; Moré *et al.*, 1996). Triclosan has been shown to reduce AHL production *in vitro* (Hoang and Schweizer, 1999).

Secondly, by preventing the signalling molecules from being perceived by the bacteria, by either blockage or destruction of the receptor protein – the LuxR homologue. The halogenated furanone compounds produced by seaweed *Delisea pulchra* was found to accelerate the turnover of LuxR up to 100 times by an unknown mechanism (Givskov *et al.*, 1996; Manefield *et al.*, 2002). Another way is by competitive inhibition by AHL signal-mimic compounds as shown in some plants (Teplitski *et al.*, 2000). The AHL analogues can be also be constructed synthetically, by introduction of substitutions in the acyl side chain or introduction of substitutions and alterations in the lactone ring or both. Persson *et al.* (2005) created AHL analogues that were able to block expression in both LuxR- and LasR-controlled QS reporters.

Thirdly, inactivation of the signalling molecules. This can be achieved by different methods: chemical degradation, enzymic degradation, and metabolism of the AHL. An example of chemical degradation is lactonolysis, the opening of the lactone ring of the AHL molecules at pH above 7 (Yates *et al.*, 2002). For enzymatic degradation of AHL molecules, generally two groups of enzymes have so far been identified in a range of bacterial species, i.e. AHL-lactonase and AHL-acylase, as shown iin Figure 1.5 (reviewed in Dong and Zhang, 2005). However, it would also be theoretically possible to enzymically alter AHL molecules by optical inversion from the L to the D form, which has been shown to be less active in *Erwinia carotovora* (Bainton *et al.*, 1992). This activity has not been found in the screens carried out so far.

Figure 1.5 The general structure of AHL molecule and enzymatic degradation products



1.6 Quorum quenching in prokaryotes

Quorum quenching enzyme activity has so far been documented in a numbers of bacterial species. The first such isolate was *Bacillus* sp. 240B1, which was found to produce AHL-lactonase, AiiA (Dong *et al.*, 2000, 2001). AHL-lactonase inactivates AHL molecules by hydrolysing the ester bond of the lactone ring to give acylhomoserine. AiiA homologues were later found in many subspecies of *Bacillus thuringiensis* and closely related *Bacillus* species, including *Bacillus cereus*, *Bacillus mycoides*, and *Bacillus antracis* (Dong *et al.*, 2002; Lee *et al.*, 2002; Ulrich, 2004). Sequence alignments with known proteins and the subsequent site-directed mutagenesis indicated that AiiA contains a $HxDHx_{59}Hx_{21}D$ motif that resembles the zinc-binding motif of several enzymes in the metallohydrolase superfamily (Dong *et al.*, 2000, 2004).

Quorum quenching activity was also found in a strain of *Ralstonia*; in this case the gene encoding the acylase, aiiD, was cloned and found to show homology to a number of other acylases and N-terminal hydrolases of the Ntn-hydrolase superfamily. The acylase acts by cleaving the amide bond connecting the HSL ring to the acyl chain, releasing homoserine lactone and fatty acid, which is further metabolized. Expression of AiiD in *P. aeruginosa* PAO1 quenched the bacterial quorum sensing, decreasing its ability to swarm, produce elastase and pyocyanin and to paralyse nematodes (Lin *et al.*, 2003).

Shortly after the discovery of AiiA, a strain of *Variovorax paradoxus* (VAI-C) was reported to be capable of using AHL molecules as the sole sources of energy and nitrogen (Leadbetter and Greenberg, 2000). This is in contrast to *Bacillus* sp. 240B1, which did not further degrade AHL. *V. paradoxus* VAI-C was found to degrade AHL through an acylase activity. However, the gene encoding for the AHL-acylase remains to be cloned and characterized.

AHL degrading activity has also been discovered in bacteria that actually produce AHL. The first such enzyme was found in *Agrobacterium tumefaciens*. Gene *attM*, which encodes a homologue of the AiiA, was expressed at the stationary phase of *A. tumefaciens* (Zhang *et al.*, 2002). The AttM system of *A. tumefaciens* helps the bacterium to exit from a quorum-sensing mode upon reaching stationary-phase conditions. This results in shutting off the energetically expensive conjugation and possibly other such quorum-sensing-regulated phenotypes in the bacterium. Later, a second enzyme, AiiB, which is homologous to AttM, was identified in *A. tumefaciens* and was shown to degrade AHL molecules (Carlier *et al.*, 2003).

Subsequently, Flagan *et al.* (2003) reported of a strain of *Arthrobacter* (VAI-A) capable of utilizing HSL and acyl-homoserine, the two known nitrogenous breakdown products of AHL molecules, as energy and nitrogen sources, respectively. At the same time, Park *et al.* (2003) reported of the isolation of another strain of *Arthrobacter* (IBN110) having the same AHL molecules metabolic capablility and cloned the gene encoding the AHL-lactonase, which was dubbed *ahlD*. Interestingly, AhlD was found to contain the conserved motif HXDH≈H≈D, suggesting that this conserved motif is essential for AHL degradation activity (Park *et al.*, 2003).

Interestingly, a homologue of an AHL acylase, *pvdQ* (PA2385) was identified in another quorum sensing bacterium, *P. aeruginosa* PAO1 (Huang *et al.*, 2003). The enzyme was found to efficiently degrade long-chain-acyl AHL and constitutive expression prevented accumulation of the native 3-oxo-C12-HSL signalling molecule, but not the short-chain-acyl C4-HSL signalling molecule. However, the ability of the bacteria to grow on AHL as a sole carbon source was not affected when *pvdQ* was inactivated, which may suggest that other enzymes are involved in this activity. Furthermore, growth on AHL was characterized by very long lag phases and doubling

times, suggesting that the bacteria are not adapted for AHL metabolism in these growth conditions (Huang *et al.*, 2003).

A homologue of PvdQ, the product of gene PA1032, named QuiP was later identified (Huang *et al.*, 2006). Transposon mutants of *quiP* were defective for growth when *P. aeruginosa* was cultured in medium containing C10-HSL as a sole carbon and energy source. Complementation with a functional copy of *quiP* restored the AHL degrading potential. It was also observed that constitutive *quiP* expression in *P. aeruginosa* resulted in a remarkable decrease of 3-oxo-C12-HSL accumulation (Huang *et al.*, 2006). However, it is not yet known under which physiological conditions both this AHL-acylases are expressed and what are their main function.

Uroz and colleagues have reported that *Rhodococcus erythropolis* strain W2 is capable of degrading and using various AHL as the sole carbon and energy source. Further analysis revealed that *R. erthropolis* strain W2 is capable of modifying and degrading AHL signal molecules through both oxidoreductase, which converts 3-oxo-AHLs to their corresponding 3-hydroxy derivatives, and acylase activities (Uroz *et al.*, 2005). Park and colleagues have reported of the third type of AHL degradation activity in *R. erythropolis*, i.e. AHL-lactonase (Park *et al.*, 2006). Recently, a gene involved in the AHL degradation was identified by screening the genomic library of *R. erthropolis* strain W2. This gene *qsdA* encodes an AHL-lactonase, belonging to the PTE family of zinc-dependent metalloproteins, unrelated to the AHL-lactonase AiiA (Uroz, 2008).

Subsequently, it was reported that a strain of *Streptomyces* sp. (M664) was found to secrete an AHL-degrading enzyme into a culture medium (Park *et al.*, 2005). The *ahlM* gene encoding the AHL-acylase was cloned from *Streptomyces* sp. strain M664 and expressed in *Streptomyces lividans*. The addition of AhlM to the growth medium reduced the accumulation of AHL and decreased the production of virulence factors, including elastase, total protease, and LasA, in *P. aeruginosa* (Park *et al.*, 2005).

Later, Uroz and colleagues reported of *Comamonas* sp. strain D1 capable of enzymatically inactivating AHL through an amidohydrolase activity. *Comamonas* sp. D1 exhibits the broadest inactivation range of known bacteria, capable of degrading AHL with acyl-side chains ranging from 4 to 16 carbons, with or without 3-oxo or 3-hydroxy substitutions. *Comamonas* strain D1 efficiently quenches various quorum sensing-dependent functions in other bacteria, such as violacein production by *Chromobacterium violaceum* and pathogenicity and antibiotic production in *Pectobacterium* (Uroz *et al.*, 2007).

Another acylase-type AHL degradation activity has been found in the biomass of the filamentous nitrogen-fixing cyanobacterium *Anabaena* (*Nostoc*) sp. PCC 7120. The gene *all3924* has been identified and cloned whose product, named AiiC, exhibits homology to the acylase QuiP of *P. aeruginosa* PAO1. It is suggested that the AiiC could be involved in the self-regulation of the AHL levels in the diazotrophic filaments, the cyanobacterium could also use the enzyme as a defence barrier in order to avoid the interference of external signals with its own signalling system (Romero *et al.*, 2008).

A summary of the various types of quorum quenching bacteria and enzymes is given in Table 1.2.

Table 1.2 Summary of quorum quenching bacteria and enzymes

Species	Gene name	Activity	Notes	Reference
Acinetobacter sp. C1010	ND	ND	Degrades C6-HSL and C8-HSL	Kang *et al.*, 2004
Agrobacterium tumefaciens	*attM*, *aiiB*	AHL lactonase	Degrades 3-oxo-C8-HSL, C6-HSL; aiiB less active	Zhang *et al.*, 2002; Carlier *et al.*, 2003
Arthrobacter sp. IBN110	*ahlD*	AHL lactonase	Degrades 3-oxo-C6-HSL, 3-oxo-C12-HSL, C4-HSL, C6-HSL, C8-HSL, C10-HSL	Park *et al.*, 2003
Anabaena (Nostoc) sp. PCC 7120	*all3924* (AiiC)	AHL acylase	Degrade AHLs with acyl chain from C4 to C14, with or without 3-oxo or 3-hydroxy substitutions, with preference for long-chain AHLs	Romero *et al.*, 2008
Bacillus sp. 240B1	*aiiA*	AHL lactonase	Degrades 3-oxo-C6-HSL, 3-oxo-C8-HSL, 3-oxo-C10-HSL	Dong *et al.*, 2000
Bacillus strain COT1, strains of *Bacillus thuringiensis*, *B. cereus*, *B. mycoides*	*aiiA* homologues	AHL lactonase	Degrade 3-oxo-C6-HSL with differing efficiencies	Dong *et al.*, 2002
Bacillus thuringiensis (various subspecies)	*aiiA* homologues	AHL lactonase	Degrade 3-oxo-C6-HSL with differing efficiencies	Lee *et al.*, 2002
Bacillus megaterium CYP102 A1	P450BM-3 (mono-oxygenase)	AHL lactonase	Degrades long-chain AHLs	Chowdhary *et al.*, 2007
Comamonas sp. strain D1	ND	AHL acylase	Degrades AHLs with acyl chain from C4 to C16, with or without 3-oxo or 3-hydroxy substitutions	Uroz *et al.*, 2007
Delftia acidovorans	ND	ND	Degrades C6-HSL and C8-HSL	Jafra *et al.*, 2006
Klebsiella pneumoniae KCTC2241	*ahlK*	AHL lactonase	Degrades C6-HSL and 3-oxo-C6-HSL	Park *et al.*, 2003
Ochrobactrum sp.	ND	ND	Degrades C6-HSL and C8-HSL	Jafra *et al.*, 2006

Table 1.2 *continued*

Pseudomonas strain PAI-A, *Pseudomonas aeruginosa* PAO1	*pvdQ* (PA2385)	AHL acylase	Both strains can degrade long-chain AHLs (>C6)	Huang *et al.*, 2003; Sio *et al.*, 2006
Pseudomonas aeruginosa PAO1	*quiP*, PA1032	AHL acylase	Degrades long-chain AHLs (>C6)	Huang *et al.*, 2006
Rhodococcus erthropolis strain W2	*qsdA*	AHL lactonase, AHL acylase, Oxidoreductase	Degrades AHLs with acyl chain from C6 to C14, with preference for short-chain AHLs with oxo- substitution	Uroz *et al.*, 2005; Park *et al.*, 2006; Uroz *et al.*, 2008
Ralstonia strain XJ12B	*aiiD*	AHL acylase	Degrades 3-oxo-C6-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL, C4-HSL	Lin *et al.*, 2003
Shewanella sp. strain MIB015	*aac*	AHL acylase	Degrades broad range of AHLs, with preference for long-chain AHLs	Morohoshi *et al.*, 2005; Tait *et al.*, 2009
Streptomyces sp. strain M664	*ahlM*	AHL acylase	Degrades AHLs (>C8) with different acyl chain substitution	Park *et al.*, 2005
Variovorax paradoxus strain VAI-C	ND	AHL acylase	Grows on a wide range of AHLs, with differing efficiencies	Leadbetter and Greenberg, 2000

ND: Not determined

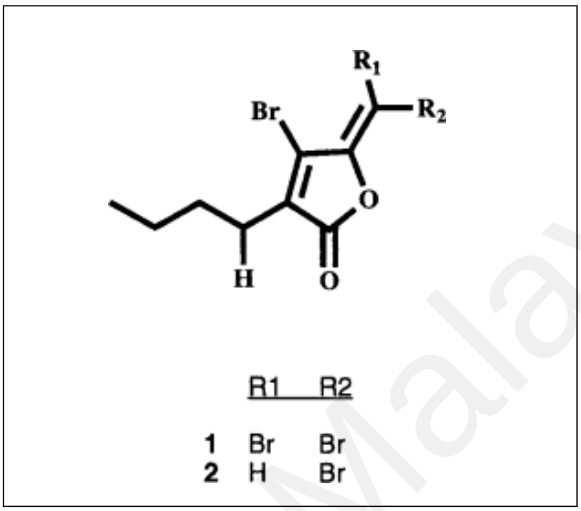
1.7 Quorum quenching in eukaryotes

As eukaryotic hosts have frequent encounters with microbial pathogens, it may not be surprising that higher organisms have also evolved or exploited existing mechanisms to disarm the quorum sensing signalling systems of pathogenic invaders. Evidence is beginning to accumulate showing quorum quenching activity in eukaryotes. Some eukaryotes, such as plants and fungi, lacking active immune systems, have to rely on physical and chemical defences. This implies that quorum sensing interference may also be one of innate defence mechanisms of higher organisms against microbial infection.

The first quorum sensing interference in eukaryotes was reported by Givskov *et al.* (1996) of the production of halogenated furanone compounds by the Australian marine red alga *Delisea pulchra* (Figure 1.6). This species produces the compounds in the central vesicle of gland cells, from which they are released to the surface of the plant (Dworjanyn *et al.*, 1999), where they prevent extensive surface growth by bacteria and higher fouling organisms (Steinberg *et al.*, 1997; Maximilien *et al.*, 1998). The furanones are structurally similar to AHLs, and they inhibit the induction of AHL-stimulated behaviors by binding competitively to the AHL receptor protein (Manefield *et al.*, 1999). The halogenated furanones have been shown to inhibit several quorum sensing-controlled phenotypes, including swarming motility of *Serratia liquefaciens*, toxin production of *Vibrio harveyi* and bioluminescence of *Vibrio fischeri* (Givskov *et al.*, 1996; Kjelleberg *et al.*, 1997; Manefield *et al.*, 2000; Rasmussen *et al.*, 2000). In a more clinical context, a synthetic derivative of the furanones (C-30) was found to downregulate expression of more than 80% of the quorum sensing-regulated genes in *P. aeruginosa*, many of which encode known virulence factors. This effect is not limited to planktonic bacteria: it also applies to biofilm-dwelling *P. aeruginosa*. Biofilms

developed in the presence of furanone compounds become more susceptible to treatments with antibiotics and disinfectants (Hentzer *et al.*, 2002, 2003).

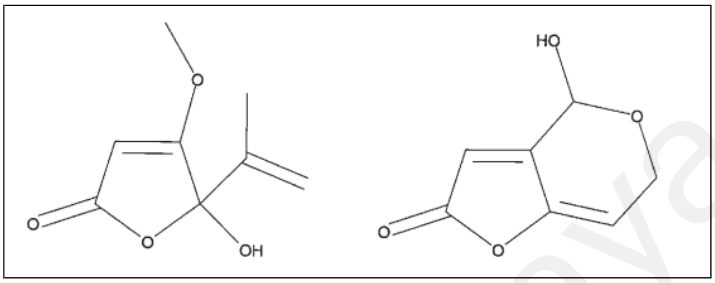
Figure 1.6 Structure of two furanones produced by *D. pulchra*. (Adapted from Givskov *et al.*, 1996)



Subsequently, similar quorum sensing interference mechanism has been reported in a range of eukaryotic organisms. Teplitski *et al.* (2000) have reported that the secretion of AHL signal-mimic compounds is indeed common among higher plants. Pea (*Pisum sativum*) and crown vetch (*Coronilla varia*) were found to secrete compounds that inhibited violacein synthesis in *C. violaceum* CV026. Later, Peters *et al.* (2003) reported of a marine bryozoan *Flustra foliacea* producing brominated alkaloids that exhibit antagonistic effect on the quorum sensing system. The metabolite compounds significantly reduced the production of extracellular proteases in *P. aeruginosa* PAO1. Shortly after that, *Chlamydomonas reinhardtii*, a unicellular soil-fresh water alga, was also found to secrete substances that interfered with bacterial quorum sensing (Teplitski *et al.*, 2004). In fungi, the first report of quorum sensing interference was of the filamentous fungal genus *Penicillium* (Rasmussen *et al.*, 2005). Further analysis on the identification of the active compounds suggested that the active compounds from

Penicillium radicicola and *Penicillium coprobium* were penicillic acid and patulin respectively (Figure 1.7).

Figure 1.7 Structures of penicillic acid (left) and patulin (right) (Adapted from Rasmussen *et al.*, 2005)



Other than competitive inhibition of quorum sensing, quorum quenching activities via enzymatic degradation of AHLs in eukaryotes have been reported. Delalande *et al.* (2005) reported of rapid disappearance of AHL at the root system of legume plants such as clover or *Lotus*. Further investigation revealed that the inactivating ability is temperature-dependent, suggesting that the degradation of AHL by germinating *Lotus corniculatus* seedlings may depend on one or more enzymatic activities. However, the type of degradation activity was not reported.

Later, Uroz and Heinonsalo (2008) reported of forest root-associated fungi capable of degrading AHL signal molecules. This was the first report of degradation of AHL signal molecules by enzymatic means. The four fungal isolates were identified as *Philocephala fortinii*, a Mycorrhizal basidiomycote isolate, an Ascomycete isolate, and *Meliniomyces variabilis*, all of them belonging to the *Ascomycota* and *Basidiomycota* lineages. The AHL degradation was found to be of lactonase activity.

Quorum quenching activities have also been demonstrated in higher organisms. Xu *et al.* (2003) reported that the commercial porcine kidney acylase I (EC 3.5.14) is able to deacylate C4-HSL and C8-HSL to produce L-homoserine. Its biological

effectiveness against AHL may be questionable as the enzyme has a very low activity under both acidic and neutral pH conditions against C4-HSL (Xu *et al.*, 2003). Nevertheless, a BLAST search shows that porcine kidney acylase I is widely conserved in eukaryotes, such as mice, rats and zebrafish, but its role as a quorum quenching enzyme in these organisms remains to be examined.

Interestingly, Chun and colleagues have reported of enzymatic degradation of AHL by the cell membrane of differentiated human airway epithelia (Chun *et al.*, 2004). The enzyme was able to inactivate C6-HSL and 3-oxo-C12-HSL. The ability to inactivate 3-oxo-C12-HSL varied significantly among different cell types, with tissues likely to be exposed to pathogens showing the highest inactivation of the quorum sensing signal, such as A549 cells from human lungs and CaCo-2 cells from human colon. More recent studies have shown that the 3-oxo-C12-HSL degradation activity is most likely due to the lactonase activity of the paraoxonases encoded by the PON genes (Greenberg *et al.*, 2004). It appears that inactivation of quorum sensing signals has now become a new index to the diverse spectrum of the recognized biological functions of PON enzymes.

Shortly after that, Yang *et al.* (2005) have reported the presence of lactonase activity against a range of AHL signal molecules in the serum samples from 6 mammalian species i.e. human, rabbit, mouse, horse, goat, and bovine. The enzyme characteristics of the serum lactonases were highly reminiscent to those of PONs.

The above description of the rapidly increasing number of quorum quenching activity in eukaryotes holds promise for the advancement of therapeutic medicine and pharmacology. In addition, the presence of such activity in eukaryote that can effectively attenuate the virulence of a variety of bacterial strains reflects on the complexity of coevolution and suggests the existence of intricate molecular signalling in eukaryote-bacterial relationships.

1.8 Objectives

The primary objectives were:

- To isolate microbial quorum quencher(s) from various environments i.e. rainforest soil, wetland water, and marine water.
- To study and characterize the AHL-degradation activity of the microorganism(s).
- To study the capability of the microbial quorum quencher(s) to utilize AHL molecules as the sole sources of energy and nitrogen
- To isolate and characterize the gene(s) conferring the quorum quenching activity.
- To investigate the quorum sensing activity in the quorum quenching bacteria.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Bacterial strains and plasmids

Table 2.1 Bacterial strains used in this study

Name	Genotype/Description	Source/Reference
C. violaceum CV026	Mini-Tn*5* mutant derived from *C. violaceum* ATCC 31532, Hg^R, *cviI*::Tn*5 xylE* Kan^R plus spontaneous Str^R, AHL biosensor producing violacein pigment only in the presence of exogenous AHL	McClean *et al.*, 1997
E. coli DH5*α*	F^- Φ80*d lacZΔM*15 Δ(lacZYA-*argF*) *U169 deoR recA1 endA1 hsdR17* (r_k^- m_k^+)	Sambrook *et al.*, 1989
E. coli [pSB401]	*luxRluxI*' (*Photobacterium fischeri* [ATCC 7744])::*luxCDABE* (*Photorhabdus luminescens* [ATCC 29999]) fusion; pACYC184-derived, Tet^R, short chain AHL biosensor producing bioluminescence	Winson *et al.*, 1998
E. coli [pSB1075]	*lasRlasI'* (*P. aeruginosa* PAO1)::*luxCDABE* (*Photorhabdus luminescens* [ATCC 29999]) fusion in pUC18, Amp^R, long chain AHL biosensor producing bioluminescence	Winson *et al.*, 1998
A. tumefaciens NTL4(pZLR4)	pTiC58 encoding a *traR* and TraR-activable *traG*::*lacZ* fusion cloned into pBBR1MCS5; pTHB58*traG*::Tn*3lac1*-derived, Amp^R and Gen^R, broad range AHL biosensor	Luo *et al.*, 2003

Table 2.2 Plasmids used in this study

Name	Description	Source/Reference
pGEM®-T Easy Vector	f1 ori, Amp^R, TA cloning vector	Promega

Table 2.3 Oligonucleotide used in this study

Name	Sequence	Length (-mer), Reference
16S rDNA forward primer 27F	5'-AGAGTTTGATCMTGGCTCAG-3'	20, Otto *et al.*, 2004; Weisburg *et al.*, 1991
16S rDNA forward primer 338F	5'-ACTCCTACGGGAGGCAGCAG-3'	20, Rivas *et al.*, 2002
16S rDNA forward primer 515F	5'-GTGCCAGCAGCCGCGGTAA-3'	19, Kotilainen *et al.*, 1998; Lane *et al.*, 1985
16S rDNA forward primer 783F	5'-AGGATTAGATACCCTGGTAGTCCA-3'	24, Foulon *et al.*, 2003
16S rDNA forward primer 1174F	5'-GAGGAAGGTGGGGATGACGT-3'	20, Wilck *et al.*, 2001
16S rDNA reverse primer 1525R	5'-AAGGAGGTGWTCCARCC-3'	17, Dewhirst *et al.*, 1999
ITS region forward primer ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	19, White *et al.*, 1990
ITS region reverse primer ITS4	5'-TCCTCCGCTTATTGATATGC-3'	20, White *et al.*, 1990

Table 2.3 *continued*

18S rDNA forward primer NS1	5’-GTAGTCATATGCTTGTCTC-3’	19, White *et al.*, 1990
18S rDNA forward primer NS2	5’-GGCTGCTGGCACCAGACTTGC-3’	21, White *et al.*, 1990
18S rDNA forward primer NS3	5’-GCAAGTCTGGTGCCAGCAGCC-3’	21, White *et al.*, 1990
18S rDNA forward primer NS7	5’-GAGGCAATAACAGGTCTGTGATGC-3’	24, White *et al.*, 1990
18S rDNA forward primer NS8	5’-TCCGCAGGTTCACCTACGGA-3’	20, White *et al.*, 1990
M13F forward primer	5’-GTAAAACGACGGCCAGT-3’	17, Universal primer
M13R reverse primer	5’-CAGGAAACAGCTATGACC-3’	18, Universal primer
T7	5’-TAATACGACTCACTATAGGG-3’	20, Universal primer
SP6	5’-GATTTAGGTGACACTATAG-3’	19, Universal primer
aiiA gene forward primer	5'-ATGGGATCCATGACAGTAAAGAAG CTTTAT-3'	30, Dong *et al.*, 2002
aiiA gene reverse primer	5'-GTCGAATTCCTCAACAAGATACTCC TAATG-3'	30, Dong *et al.*, 2002
pvdQ gene forward primer	5'-AGGCCAAGCTTATGGGGGATGCGTA CCGTACTG-3'	33, Huang *et al.*, 2003
pvdQ gene reverse primer	5'-GTTATATAGCGGCCGCTAGGATTGC TTATCATTCG-3'	35, Huang *et al.*, 2003
pvdQ gene forward primer PvdQ713F	5'-CGCACTTCCCCTGGAACG-3'	18, This work

Table 2.3 *continued*

quiP gene forward primer 1032F	5'-ATTAGAAGCTTATGGCCTCGCCAG CCTTC-3'	29, Huang *et al.*, 2006
quiP gene reverse primer 1032R	5'-ATTACTCTAGATCAGCGAGCGGGA GTG-3'	27, Huang *et al.*, 2006
quiP gene forward primer QuiP664F	5'-GAAAACCTGCCGTTCGACGA-3'	20, This work
quiP gene forward primer QuiP1410F	5'-ACGCTATCCGAACCGCAAG-3'	19, This work

Legend: M = A + C; W = A + T; R = A + G

2.2 Chemical reagents

All chemical reagents used were of highest grade obtained from Amresco®, (USA); BDH Ltd., (England); BD, Difco™ Laboratories, (USA); Merck, (Germany); Sigma®, (USA); Fisher-Scientific, (UK); Ajax Finechem, (UK); Cambrex Bio Science Rockland, (USA); and J. T. Baker, (USA).

2.3 Commercial kits

The commercial kits used for Gram staining, genomic DNA isolation, DNA purification, and plasmid DNA isolation were as below:

(i) BD™ Gram Stain Kit (Becton, Dickinson and Company, USA)

(ii) QIAamp® DNA Mini Kit (Qiagen Pty. Ltd., Germany)

(iii) QIAquick® Gel Extraction Kit (Qiagen Pty. Ltd., Germany)

(iv) QIAquick® PCR Purification Kit (Qiagen Pty. Ltd., Germany)

(v) QIAquick® Spin Miniprep Kit (Qiagen Pty. Ltd., Germany)

2.4 Growth media

All media were sterilized by autoclaving at 121°C, 15 psi for 20 min, unless stated otherwise. Heat-labile solutions were sterilized by filtration through sterile syringe membrane filters with 0.22 μm pore size (Sartorius Minisart).

Luria-Bertani (LB) medium

LB broth was prepared as described by Sambrook *et al.* (1989) and consisted of 1.0% w/v tryptone, 0.5% w/v yeast extract, and 1.0% w/v NaCl, in 1,000 ml sdH_2O. All ingredients were dissolved in distilled water and autoclaved. 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 ensure that the supernatant did not rise above pH 7.0 and promote lactonolysis (Yates *et al.*, 2002). LB agar was prepared by addition of Bacto™ Agar (BD) to a final concentration of 1.5% w/v.

Modified Luria-Bertani (LBm) medium was identical to LB medium except that it had higher salinity (2.5% w/v NaCl).

SOB medium

SOB medium was prepared as described by Sambrook *et al.* (1989) and consisted of 2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, and 2.5 mM KCl, in 1,000 ml sdH_2O. After sterilization by autoclaving, 5 ml of filter-sterilized (0.22 µm pore size) 2 M $MgSO_4$ was added aseptically to the medium before use.

SOC medium

SOC medium was prepared by addition of 10 ml of filter-sterilized (0.22 µm pore size) D-glucose solution (20 mM) to 1,000 ml of SOB medium.

KG medium

KG medium consisted of 1.0 g of NaCl, 0.5 g of KCl, 0.15 g of Na_2SO_4, 5.0 g of KH_2PO_4, 0.4 g of $MgCl_2$, 0.1 g of $CaCl_2$, 0.3 g of NH_4Cl (unless stated otherwise), and 1.0 g of 2-(*N*-morpholino)-ethanesulfonic acid (MES), in 1,000 ml sdH_2O. This basal medium was autoclaved, and after cooling, filter-sterilized (0.22 µm pore size) trace elements (1 mg of $FeCl_3$, 0.1 mg of $MnCl_2$, and 0.46 g $ZnCl_2$) were added. AHL was used as the sole carbon source, with the final concentration of 50 µg/ml.

KGm medium

KGm medium consisted of 1.25 g of NaCl, 0.75 g of KCl, 0.25 g of Na_2SO_4, 7.5 g of KH_2PO_4, 0.5 g of $MgCl_2$, 0.25 g of $CaCl_2$, 0.3 g of NH_4Cl (unless stated otherwise), and 1.0 g of 2-(*N*-morpholino)-ethanesulfonic acid (MES), in 1,000 ml sdH_2O. This basal medium was autoclaved, and after cooling, filter-sterilized (0.22 µm pore size) trace elements (5 mg of $FeCl_3$, 2.5 mg of $MnCl_2$, and 0.6 g $ZnCl_2$) were added. AHL was used as the sole carbon source, with the final concentration of 50 µg/ml.

Antibiotics

Growth media were supplemented when required, with the following antibiotics: ampicillin (Amp) at 100 µg/ml, and tetracycline (Tet) at 20 µg/ml. All antibiotics stocks were obtained from Bioshop® (Canada). Stock solutions were prepared as according to Sambrook *et al.* (1989).

2.5 Synthetic acyl-homoserine lactones

The AHLs were obtained from Sigma-Aldrich©.

2.6 Stock solutions and buffers

Solutions for DNA extraction of yeast

Phenol/chloroform

Equal volumes of phenol and chloroform (1:1) were mixed and shaken vigorously for 5 min. The resulting mixture was placed at 4°C overnight prior to use. Stock solution was stored at 4°C in a glass bottle wrapped in aluminum foil.

Solutions for agarose gel electrophoresis

5× Tris borate EDTA buffer (TBE)

The 5× TBE stock solution was prepared as follows and consisted of 54.0 g Tris base, 27.5 g boric acid, 3.72 g $Na_2EDTA•2H_2O$, in 1,000 ml sdH_2O. Stock solution was stored at room temperature. To prepare 1× TBE, the 5× TBE stock was diluted at 1/5 with sdH_2O.

5× Tris acetate EDTA buffer (TAE)

The 5× TAE stock solution was prepared as follows and consisted of 24.2 g Tris base, 5.75 ml glacial acetic acid, 100 ml EDTA, in 1,000 ml sdH_2O. Stock solution was stored at room temperature. To prepare 1× TAE, the 5× TAE stock was diluted at 1/5 with sdH_2O.

6× Gel loading dye

The 6× GLD was obtained from Fermentas.

Ethidium bromide (EtBr) solution (10 mg/ml)

The EtBr solution was obtained from Sigma®.

Solutions for AHL inactivation assay

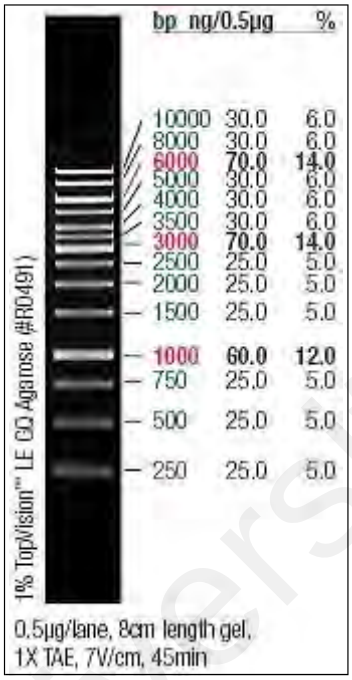
Phosphate buffer saline (PBS)

PBS solution consisted of 80.0 g NaCl, 2.0 g KCl, 11.5 g $NaHPO_4$, and 2.0 g KH_2PO_4, in 1,000 ml sdH_2O. The solution pH was adjusted to pH 6.5 before autoclaving and stored at room temperature.

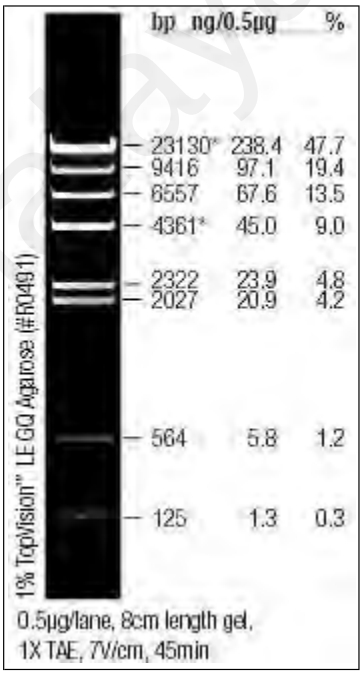
2.7 DNA size reference marker

The DNA ladder used in this study was Lambda DNA/HindIII Marker, 2 and GeneRuler^{TM} 1 kb DNA ladder (Fermentas International Inc., Canada). The molecular sizes of various fragments in the DNA ladder are shown in Figure 2.1.

Figure 2.1 Molecular sizes of DNA size reference ladder



Lambda DNA/HindIII Marker, 2



GeneRuler^{TM} 1 kb DNA ladder

2.8 Bacteria strain, media and culture conditions

All strains were grown in LB medium at 28°C except *E. coli* strains, which were grown at 37°C. *Trichosporon loubieri* was grown in LBm medium. Broth culture was incubated with shaking (220 rpm). Where necessary, growth media were supplemented with appropriate antibiotics and solidified with bacto-agar (15 g/l).

2.9 Enrichment and isolation procedures of environmental bacteria

Marine water, wetland water, and wetland soil were collected on 13 November 2007 at Malacca, Malaysia. The rainforest soil was collected on 17 November 2007 at Templer's Park, Selangor state, Malaysia. The samples were collected in sterile plastic container, transported to the laboratory and processed immediately.

Wetland water and marine water

One milliliter of the water sample or one gram of the soil sample was added to 3 ml of KGm medium supplemented with ammonium chloride and 3-oxo-C6-HSL (final concentration of 300 µg/ml and 50 µg/ml, respectively) as sole nitrogen and carbon source, respectively, and incubated at 28°C with shaking (220 rpm). 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-C6-HSL-containing KGm agar to isolate individual colonies.

Rainforest soil

One gram of the soil sample was added to 3 ml of KGm medium supplemented with ammonium chloride and 150 µg 3-oxo-C6-HSL (i.e. 50 µg/ml final concentration)

as sole nitrogen and carbon source, respectively, and incubated for 48 h at 28°C with shaking (220 rpm). After 48 h, 150 µl of the suspension was inoculated into 3 ml of fresh enrichment medium. The same procedure was repeated five times. At the sixth enrichment cycle, a diluted suspension was plated onto LBm agar and a plate of 3-oxo-C6-HSL-containing KGm agar to isolate individual colonies.

2.10 Gram staining

Gram staining was performed by using BD™ Gram Stain Kit as per the manufacturer's instructions. Briefly, bacterial colony grown overnight on agar plate was picked and transferred to a glass slide to make a thin smear and heat-fixed on the slide. The smear was covered with Gram Crystal Violet solution for 1 min and washed with sdH_2O to remove the excessive stains. Next, Stabilized Gram Iodine solution was added to the smear for 1 min and then rinsed off with sdH_2O. After that, the smear was decolorized with acetone for 2 to 3 seconds until the solvent running from the slide was colorless, then immediately rinsed off with sdH_2O. Finally, the smear was counterstained with Gram Safranin solution for 1 min. The stained smear was then air-dried and observed under Olympus™ IX71 inverted research microscope. Micrographs of the results of Gram staining were taken using Image Analysis Software analySIS® version 3.2.

2.11 Preparation and analysis of DNA

Preparation of genomic DNA

Genomic DNA was isolated either by using Qiagen™ DNA mini kit as per the manufacturer's instructions or by means of conventional phenol-chloroform method.

For the phenol-chloroform method, 20 ml of overnight yeast cells were harvested by centrifugation at 5,500 × *g* for 20 min and resuspended in 1 ml of lysis

buffer (100 mM Tris, pH 8.0, 50 mM EDTA, 3% w/v SDS, 100 µg/ml Proteinase K, 1% v/v β-mercaptoethanol). The suspension was vortexed and incubated at 60°C for 1 h. Then the suspension was centrifuged at 3,400 × *g* for 5 min. The supernatant was transferred to a new microcentrifuge tube and equal volume of phenol-chloroform (1:1) was added. The mixture was inverted 20 times and centrifuged at 11,000 × *g* for 3 min. The aqueous layer was transferred to a new microcentrifuge tube and the phenol-chloroform extraction and centrifugation step was repeated. The aqueous layer was transferred to a new microcentrifuge tube and equal volume of chloroform was added. The mixture was inverted 20 times and centrifuged at 11,000 × *g* for 3 min. Next, the supernatant was transferred to a new microcentrifuge tube and 0.6 volume of 7.5 M NH_4OAc was added to a final concentration of 2.0 to 2.5 M. The DNA was recovered by adding 2 volume of ice-cold absolute ethanol and the mixture was incubated on ice for 30 min. The tube was then centrifuged at 11,000 × *g* for 3 min at 4°C. The DNA pellet was washed in 1 ml ice-cold 70% v/v ethanol and then centrifuged at 11,000 × *g* for 10 min at 4°C. The supernatant was discarded and the DNA pellet was dried before resuspended in appropriate amount of sdH_2O.

Analysis of DNA by agarose gel electrophoresis (AGE)

DNA samples were analyzed by electrophoretic examination in a submerged horizontal agarose gel (0.8 to 1.0% w/v) containing EtBr (0.5 µg/ml), flooded with 1× TBE buffer. The DNA sample was mixed with 6× gel loading dye (Fermentas) at 1/5 dilution before loading the entire mixture into a well in an agarose gel.

Electrophoresis was carried out at 100V until the gel loading dye reached about 1.0 cm from the edge of the gel. DNA fragments complexed with EtBr 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).

The size of the amplified DNA fragment was estimated with reference to DNA size reference marker, GeneRuler^{TM} 1 kb DNA ladder (Fermentas).

2.12 Polymerase chain reaction

Primers

Oligonucleotide primers were purchased from Operon® Biotechnologies (Singapore) and Eurogentec (Singapore). Nucleotide sequences of the primers used in this study are shown in Table 2.3.

Amplification of targeted regions by PCR

PCR amplification of targeted regions for cloning or sequencing was carried out using purified genomic DNA as template in an automated DNA thermocycler (2720 Thermal Cycler, Applied Biosystems). The volume of the reaction mixture was 15 µl (Table 2.4). For each PCR run, a negative control was always included, by substituting the template DNA with 0.5 µl of sdH_2O.

The amplified regions and optimized cycling conditions are shown in Table 2.5. A small volume of the PCR products (3 µl) were then analyzed by agarose gel electrophoresis and the remainder was purified for cloning and sequencing.

Table 2.4 PCR set up for amplification of targeted regions

PCR master mix component	Volume (µl) per sample
sdH_2O	9.0
10× Optimized Buffer (iNtRON Biotechnology, Korea)	1.5
dNTP (250 µM each)	1.5
i-Taq™ Plus DNA Polymerase, iNtRON Biotechnology (5 Unit/ml)	0.5
Forward primer (4 µM)	1.0
Reverse primer (4 µM)	1.0
DNA template	0.5
Total volume	**15.0**

Table 2.5 Amplified regions and optimized cycling conditions

No.	Gene/ amplified region (size)	Primer pairs	Cycling conditions
1.	16S rDNA (~1,500 bp)	27F and 1525R	94°C for 3 min, 94°C for 30s, 61°C for 30s, 72°C for 60s, } 25 cycle 72°C for 7 min
2.	18S rDNA (~1,700 bp)	NS1 and NS8	95°C for 6 min, 95°C for 30s, 50°C for 30s, 72°C for 120s, } 25 cycle 72°C for 7 min
3.	ITS gene (~500 bp)	ITS1 and ITS4	94°C for 5 min, 94°C for 30s, 45°C for 30s, 72°C for 45s, } 25 cycle 72°C for 7 min
4.	*aiiA* gene (~750 bp)	*aiiA* gene forward and reverse primer	94°C for 4 min, 94°C for 30s, 50°C for 30s, 72°C for 90s, } 25 cycle 72°C for 7 min
5.	*pvdQ* gene (~2,500 bp)	*pvdQ* gene forward and reverse primer	96°C for 5 min, 96°C for 45s, 60°C for 30s, 72°C for 120s, } 25 cycle 72°C for 7 min
6.	*quiP* gene (~2,500 bp)	1032F and 1032R	95°C for 5 min, 95°C for 45s, 63°C for 30s, 72°C for 120s, } 25 cycle 72°C for 7 min

Purification of PCR products

PCR products were purified by either direct column purification or size-fractionation in agarose gel followed by column purification. For direct column purification, the QIAquick® PCR purification Kit (Qiagen) was used as per the manufacturer's instructions. Briefly, 5 volumes of PB buffer was added to 1 volume of PCR products and the mixture was applied into a column provided and centrifuged at 13,000 × g for 1 min and the flow-through was discarded. The column was then washed with 0.75 ml of PE buffer and centrifuged for 1 min. The flow-through was discarded and the column was re-centrifuged at 13,000 × g for 1 min. The column was placed in a sterile microcentrifuge tube and DNA was eluted with 50 µl sdH_2O by centrifuging for 1 min.

For purification of DNA by gel excision, the QIAquick® Gel Extraction Kit (Qiagen) was used as per the manufacturer's instructions. Briefly, the DNA samples were resolved by AGE in which 1× TBE buffer was replaced by 1× TAE buffer. After electrophoresis, the DNA fragment of interest was excised using a sterile cover slip on a low wavelength UV transilluminator (TFM-20V Transilluminator, UVP Inc. USA) and transferred to a sterile microcentrifuge tube. Then 3 volumes of QG buffer was added to 1 volume of gel slice containing the DNA. The mixture was incubated at 50°C for 10 min (or until the gel slice has completely dissolved). The solution was applied to a column provided and centrifuged at 13,000 × g for 1 min and the flow-through was discarded. The washing and elution of DNA was carried out as described above. After purification, 3 µl of the eluted DNA sample was analyzed by AGE.

2.13 TA cloning of PCR products

The purified PCR product was cloned into pGEM®-T Easy vector supplied by the pGEM®-T Easy Cloning Kit (Promega) as per the manufacturer's instructions. Briefly, PCR products were mixed with pGEM®-T Easy vector as according to the ligation reaction recipe shown in Table 2.6. The ligation reactions were incubated overnight at 4°C.

Table 2.6 Ligation mixture using the pGEM®-T Easy vector system

Ligation mixture component	Volume (µl) per sample
2× Rapid Ligation Buffer	5.0
pGEM®-T Easy Vector (50ng)	1.0
PCR product	X*
T4 DNA Ligase (3 Weiss units/µl)	1.0
sdH_2O to final volume of	10.0

*Adjusted to a molar ratio of PCR product to vector (3:1) after quantitative estimation of DNA concentration using spectrophotometer.

2.14 Transformation

Preparation, storage and transformation of calcium chloride competent cells

Competent cells were prepared as described by Sambrook *et al.* (1989). Briefly, 5 ml of LB medium was inoculated with single pure colony of overnight plate culture of *E. coli* DH5α and grown at 37°C with vigorous shaking (300 rpm) for 2 to 3 h. The cells

were transferred into 100 ml fresh LB medium and grown with vigorous shaking for another 2 to 3 h until an OD_{600} of 0.4 was achieved and immediately placed on ice for 15 min. After harvesting by centrifugation at 1,500 × g for 10 min at 4°C, cells were resuspended with 20 ml of ice-cold 0.1 M $CaCl_2$ and left on ice for 20 min. Cells were centrifuged at 1,500 × g for 10 min at 4°C and finally resuspended in 4 ml of ice-cold 0.1 M $CaCl_2$. The cell suspension was stored at 4°C for 16 h for increased transformation efficiency. Using a chilled sterile pipette tip, ice-cold sterile glycerol was added to a final concentration of 10% (v/v) and left on ice for 30 min. The cell suspension was then dispensed in 40 µl aliquots into sterile 1.5 ml microcentrifuge tubes and used directly for transformation or snap frozen in cold ethanol bath and stored at -80°C for future use.

For transformation, 1 to 2 µl of plasmid or ligation reaction was added to each tube and mixed by flicking gently. The cells were incubated on ice for 30 min and heat-shocked for 45 to 50 s in a water bath at 42°C. The tubes were immediately placed on ice for 2 min. Transformed cells were recovered by adding 960 µl of room temperature SOC medium and incubated for 1.5 h at 37°C with shaking (150 rpm). Subsequently, 100 µl of each transformation culture was plated on appropriate selective LB plates.

Preparation, storage and transformation of electrocompetent cells

Electrocompetent cells were prepared as described by Sambrook *et al.* (1989). Briefly, 500 ml of LB medium was inoculated with 1 ml of overnight culture of *E. coli* DH5α. The broth was grown at 37°C with vigorous shaking (300 rpm) until an OD_{600} of 0.6 was achieved and immediately placed on ice for 30 min. After harvesting by centrifugation at 8,000 × g for 15 min at 4°C, cells were washed sequentially with 500 ml, 250 ml of sterile ice-cold 10% v/v glycerol. Cells were finally resuspended in 1 ml of sterile ice-cold 10% v/v glycerol. Using a chilled sterile pipette tip, cell suspension

was then dispensed in 40 µl aliquots into sterile 1.5 ml microcentrifuge tubes and used directly for transformation or snap frozen in cold ethanol bath and stored at -80°C for future use.

For electroporation, 2 µl of plasmid or ligation reaction was added to 40 µl of competent cells and the mixture was transferred into a chilled 0.1 cm gap Gene Pulser® Cuvette (Bio-rad, USA). Electroporation was carried out by using the Gene Pulser System (Bio-rad, USA), set to the following conditions: 2.5 kV, 125 µF, and 200 Ω. Electroporated cells were recovered by adding 500 µl of SOC medium and incubated for 1 h at 37°C with shaking (150 rpm). Subsequently, 100 µl of each transformation culture was plated on appropriate selective LB plates.

2.15 Selection of transformant based on Blue/white selection

Recombinant plasmids containing DNA fragment inserted into the *E. coli lacZ* multicloning sites were screened by blue/white selection using X-Gal/IPTG plates. Using α–complementation (blue-white selection), the β-galactosidase enzyme will not be produced when the *lacZ* gene in the plasmid vector is disrupted, but a plasmid without an insert will still produce β-galactosidase because the *lacZ* gene is still intact. β-Galactosidase cleaves a synthetic sugar, X-gal, that is similar to lactose, into two sugar components, galactose and glucose, one of which is blue. Therefore, colonies containing plasmid vectors without insert will turn blue; while on the other hand colonies containing plasmid vectors with insert will remain white.

2.16 Sequencing of DNA

Sequencing of DNA was carried out by Macrogen Inc. (Seoul, Korea) using an Applied Biosystems ABI3730xl automated sequencer. Nucleotide sequences were analyzed with LASERGENE software package (DNASTAR, Inc.) in combination with the BLAST programs available from the NCBI web site (www.ncbi.nlm.nih.gov). The sequences were trimmed to exclude the PCR primer binding sites and aligned with MegAlign (Version 5.05; DNASTAR, Inc.).

For identification of closest relatives, newly determined sequences were compared to those available in GenBank databases (www.ncbi.nlm.nih.gov) using the standard nucleotide-nucleotide BLAST program (www.ncbi.nlm.nih.gov).

2.17 Phylogenetic analysis

Sequences were analyzed using Chromas Lite version 2.01 (Technelysium Pty Ltd, Australia) and aligned using ClustalX version 2 (Thompson *et al.*, 1997). Phylogenetic and molecular evolutionary analyses were conducted with *MEGA* version 4.0 obtained from website (http://www.megasoftware.net). Trees were calculated with gene sequences using the Neighbour-Joining algorithm. Reliability was measured as the probability that the members of a given clade are always member of that clade. Bootstrap analyses for 1,000 resamplings were always performed to provide confidence estimates for tree topologies. The tree was rooted with appropriate outgroup because unrooted tree showed only phylogenetic relationships, but not the direction of evolution. An outgroup is a taxon that is more distantly related but sufficiently conserved or homologous to each of the ingroup taxa being considered.

2.18 *N*-acyl homoserine lactones inactivation assays

Preparation of resting cells

Bacterial cells or yeast cells grown overnight with shaking (220 rpm) in appropriate growth medium were harvested. The optical density at 600 nm (OD_{600}) of the cultures were standardized to approximately OD 1.0 by diluting with fresh culture medium. A 100 ml of the standardized culture was centrifuged at 7,000 × g for 10 min at 4°C. The cells were resuspended in 100 ml PBS (pH 6.5) and washed twice in the same buffer. The final cell pellet was resuspended in 1 ml of PBS (pH 6.5) and used directly as a source of resting cells for *in vivo* AHL inactivation assays.

AHL inactivation assay

Aliquots of AHL in absolute ethanol were dispensed into sterile tubes and the solvent evaporated to dryness under sterile conditions. Resting cell suspension was used to rehydrate the AHL to a final concentration of 0.5 µg/µl. The mixtures were incubated at room temperature for 24 h with shaking (220 rpm). 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 before reconstituted in appropriate volume of acetonitrile. Extracted AHL was detected using the AHL bioreporters and Rapid Resolution Liquid Chromatography (RRLC).

To detect the extracted AHL using bioluminescence bioreporters, a 10-fold diluted culture of *E. coli* [pSB401] or *E. coli* [pSB1075] (grown to log phase) was used to rehydrate the AHL and incubated at 37°C with shaking 220 rpm. Extracted AHL were measured as bioluminescence and expressed as Relative Light Unit (RLU) using Berthold Junior LB9509 Luminometer (Berthold Technologies GmbH & Co KG). To detect the extracted AHL using bioreporter CV026, the reaction was stopped by heating

at 95°C for 5 min as described by Park *et al.* (2003). After cooling, the reaction mixture was spotted onto LB agar plate seeded with overnight culture of CV026 and incubated at 28°C. Extracted AHL was detected by the production of purple pigmentation. Incubation buffer (PBS, pH 6.5) and *E. coli* DH5α were used as negative control.

Determination of AHL degradation by lactonase activity

For the detection of a possible lactonase activity, i.e. the formation of corresponding *N*-acyl homoserine, was detected using the method described by Yates *et al.* (2002). Briefly, samples withdrawn at the end of AHL inactivation assay were divided into 2 aliquots of equal volumes, where one was acidified with 100 mM HCl to pH 2.0 to induce recyclization of the lactone ring and the other untreated aliquot was used as control. Acidified sample was incubated at room temperature for 24 h before adjusting to pH 6.5 with 0.5 M MOPS buffer. All samples were extracted twice with equal volume of ethyl acetate and detected using appropriate AHL bioreporters.

2.19 Analysis of PQS molecules

Extraction of PQS molecules

PQS were extracted from culture supernatants as described by Camara *et al.* (1998) and Shaw *et al.* (2007). All glasswares were washed with acetone and air-dried before use. Cell-free supernatant (100 ml) was obtained from stationary phase cultures by centrifugation at 7,000 × g for 10 min. Equal volume of acidified ethyl acetate (0.01% v/v glacial acetic acid in ethyl acetate) was added to the supernatant and shaken vigorously. The extraction mixture was transferred into a separation funnel and the two phases were allowed to separate 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.

Thin layer chromatography (TLC)

Thin layer chromatography was performed as described by Shaw *et al.* (1997) and Fletcher *et al.* (2007). The reconstituted PQS sample extracts were spotted on a line drawn at 2.5 cm from the bottom of the normal phase silica 60_{F254} (Merck) TLC plates, which had been activated by previously soaking for 30 min in 5% w/v KH_2PO_4, and air-dried. The TLC plate was placed in a developing tank and chromatographed by using a mixture of dicholoromethane:methanol (95:5) as the mobile phase until the solvent front is 1–2 cm from the top of the plate. The TLC plate was then air-dried and visualized by using UV Transilluminator and spots corresponding to the synthetic PQS were marked to measure the migration distance. Migratory distances of substances on TLC are generally fixed as R_f values.

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

Reverse-phase RRLC analysis of AHL was carried out as reported (Swift *et al.*, 1996) with slight modification. Analysis was performed using an Agilent Technologies 1200 Series Rapid Resolution LC system (Agilent Technologies, Germany) equipped with a vacuum degasser, a binary pump SL, an autosampler, and a diode-array detector (DAD). Ten microliters of extracted AHL from AHL inactivation assay were applied onto an analytical C18 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 and spectrum monitored at 210 nm. Data

collection and analysis were performed using Agilent Chemstation (version B.04.01). Both the retention time and spectral properties were compared to a series of synthetic AHL standards obtained from Sigma-Aldrich©. 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 concentration.

2.21 Quantitative analysis of AHL degradation by using RRLC

To estimate the amount of residual 3-oxo-C8-HSL extracted from the AHL inactivation assay were analysed using RRLC using the build in software (Agilent ChemStation B.04.01). Known amount of synthetic 3-oxo-C8-HSL (0.025, 0.05, 0.075, 0.1 to 0.15 µg/µl) analysed in the same RRLC conditions were used to construct standard curve for estimation of residual 3-oxo-C8-HSL concentration calculated from the areas below the curve of the RRLC chromatograms. Degradation kinetics was determined from the linear portion of semi-log curve of the residual AHLs concentration against time. The square of correlation coefficient (R^2) were determined by using Microsoft Excel®.

CHAPTER THREE: RESULTS

Isolation of quorum quenching yeast

3.1 Enrichment and isolation of strain WW1C

Enrichment was carried out as describe in Section 2.9, using a defined medium supplemented with 3-oxo-C6-HSL as the sole carbon source and ammonium chloride as the nitrogen source. The medium became turbid within 48 h after inoculation with wetland water. The pH of the wetland water sample was pH 6.93 as measured with pH meter. No obvious turbidity was observed in a control tube in the absence of 3-oxo-C6-HSL. Cell suspensions were diluted and plated onto both LBm agar and plates of 3-oxo-C6-HSL-containing KGm medium. Pure cultures were obtained after several successive streaks from single colony picks. Four individual colonies with distinctively different morphologies and colors were retained, and designated WW1A, WW1C, WW2, and WW3 (Table 3.1). Amongst them, only one isolate, strain WW1C induced a disappearance of the 3-oxo-C6-HSL signal after 24h cultivation, using AHL inactivation assay.

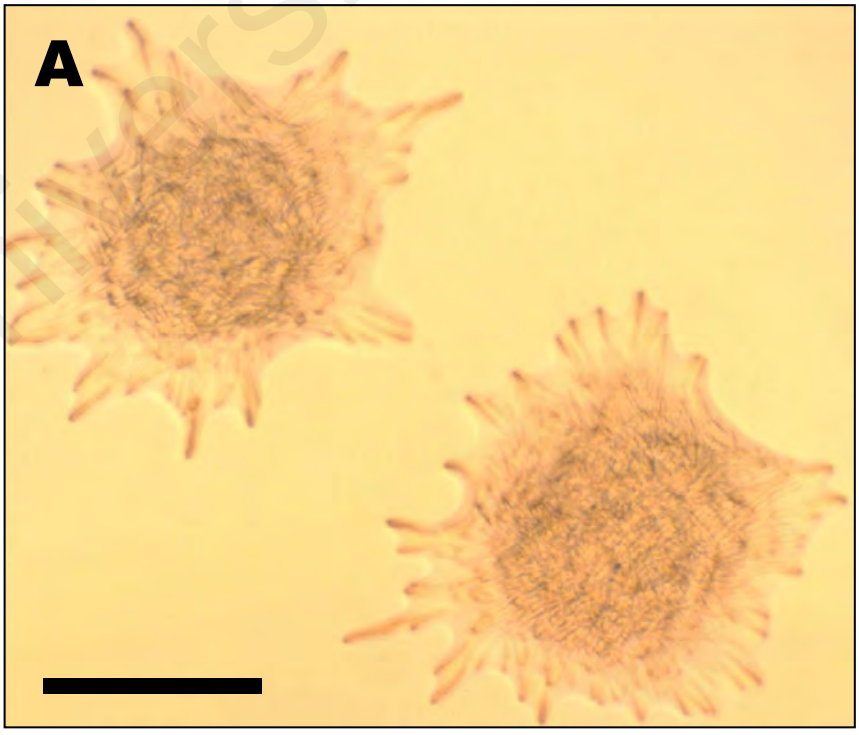
Table 3.1 Different types of bacteria enriched from wetland water based on colony morphology

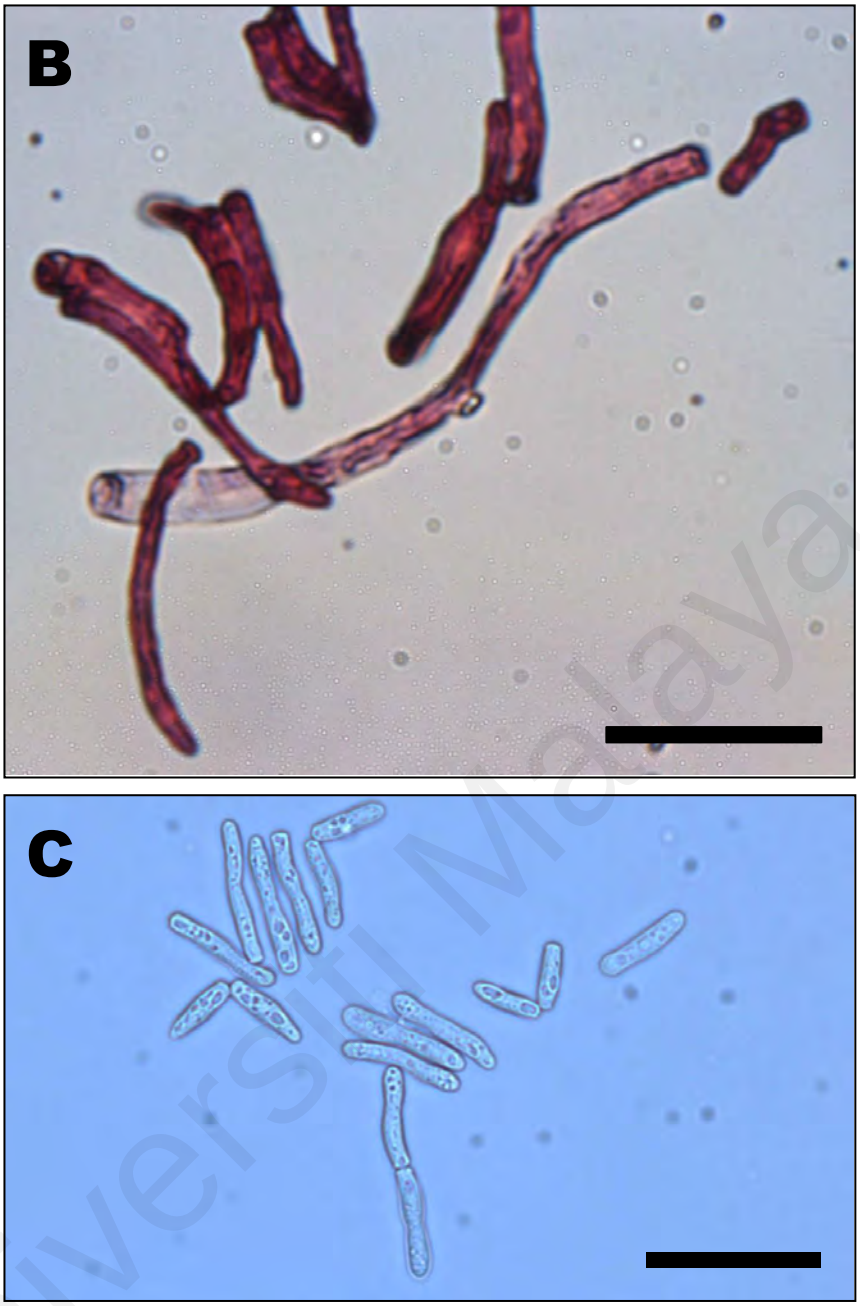
No.	Name of isolates	Colony morphology	AHL degradation
1	WW1A	Round, entire, convex, white, transparent	Negative
2	WW1C	Round, filamentous, doom, white, opaque	Positive
3	WW2	Irregular, entire, raised, white, opaque	Negative
4	WW3	Round, entire, convex, white, opaque	Negative

3.2 Cell and colony morphology

Strain WW1C grown on LBm plates for 48 h at 28°C yielded white, opaque, domed-shaped colonies with colony diameter of 5 mm. Colonies were essentially round with filamentous edges and appeared dry and powdery (Figure 3.1A). The wrinkled appearance became more prominent with prolonged incubation. Microscopically, pleomorphic yeast-like cells with cell length ranged from approximately 30 to 150 µm were observed (Figure 3.1B). Well-developed hyphal structures and barrel-shaped arthroconidia were observed (Figure 3.1C). Based on morphological features, strain WW1C was provisionally suggested to be of a member of the kingdom Fungi.

Figure 3.1 Colonial and cell morphology of strain WW1C on LBm.





Colonial and cell morphology of strain WW1C. (A) Photograph of colonies grown on LBm plates. (B) Light micrograph of Gram-stained cells of strain WW1C grown on LBm plates. (C) Light micrograph of a droplet of overnight LBm broth culture of strain WW1C stained with crystal violet. The bars represent 500 µm (A), 50 µm (B), and 100 µm (C).

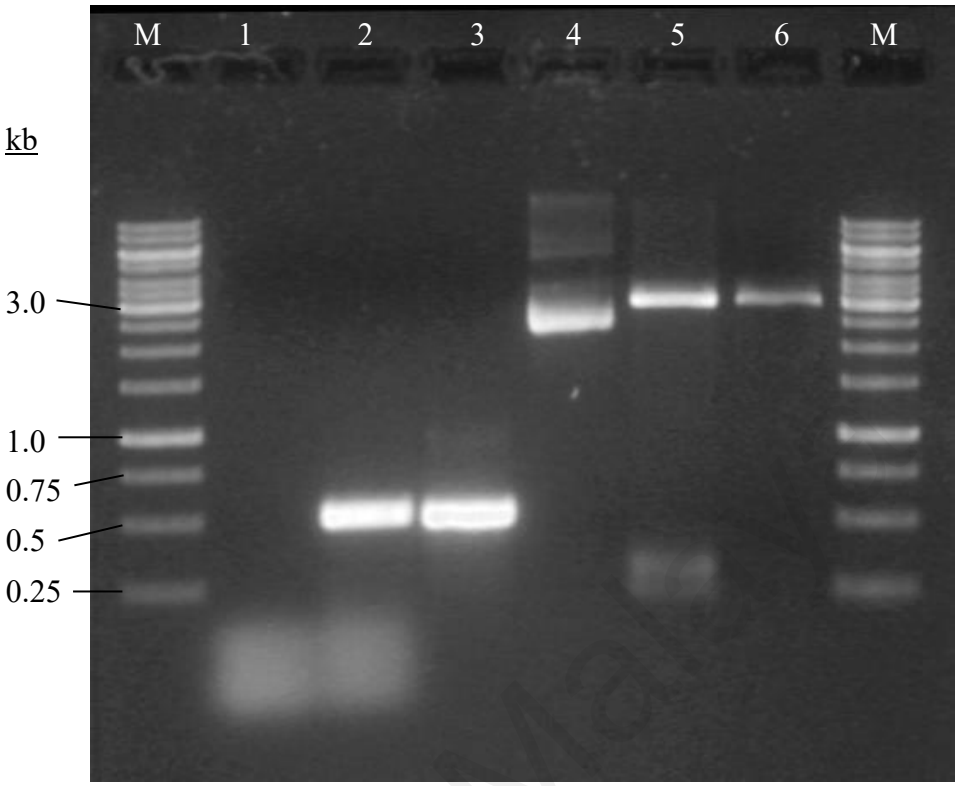
3.3 Internal transcribed spacer region and 18S rDNA gene amplification

To identify strain WW1C, the internal transcribed spacer (ITS) region and 18S rDNA gene (approximately 0.5 kb and 1.7 kb, respectively) were amplified by PCR using the genomic DNA isolated as described in Section 2.11. Amplified ITS region and 18S rDNA gene of approximately 0.5 kb and 1.7 kb, respectively, are shown in Figure 3.2 and Figure 3.3. The amplicons were subsequently gel-excised, column-purified and ligated into pGEM®-T Easy cloning vector (Section 2.13). Clones with the correct insert were confirmed by colony PCR using universal primers (T7 and SP6) and plasmids were isolated for sequencing analysis. Sequencing was performed using vector-specific universal primers (T7 and SP6) followed by internal primers (NS2, NS3, and NS7) listed in Table 2.3. The fragments were then aligned and assembled to generate the complete nucleotide sequence. The sequences obtained are shown in Appendix 1.

3.4 Nucleotide sequence accession number

The ITS region and 18S rDNA gene sequences have been deposited at GenBank under the GenBank accession number FJ383170 and FJ426276. All other sequences were from the GenBank database.

Figure 3.2 ITS region PCR amplification



Legends:

M: GeneRuler^{TM} 1kb DNA Ladders (Fermentas)

1: Negative control (PCR mixture where DNA was replaced with sdH_2O)

2: ITS region PCR products

3: Purified ITS region PCR products

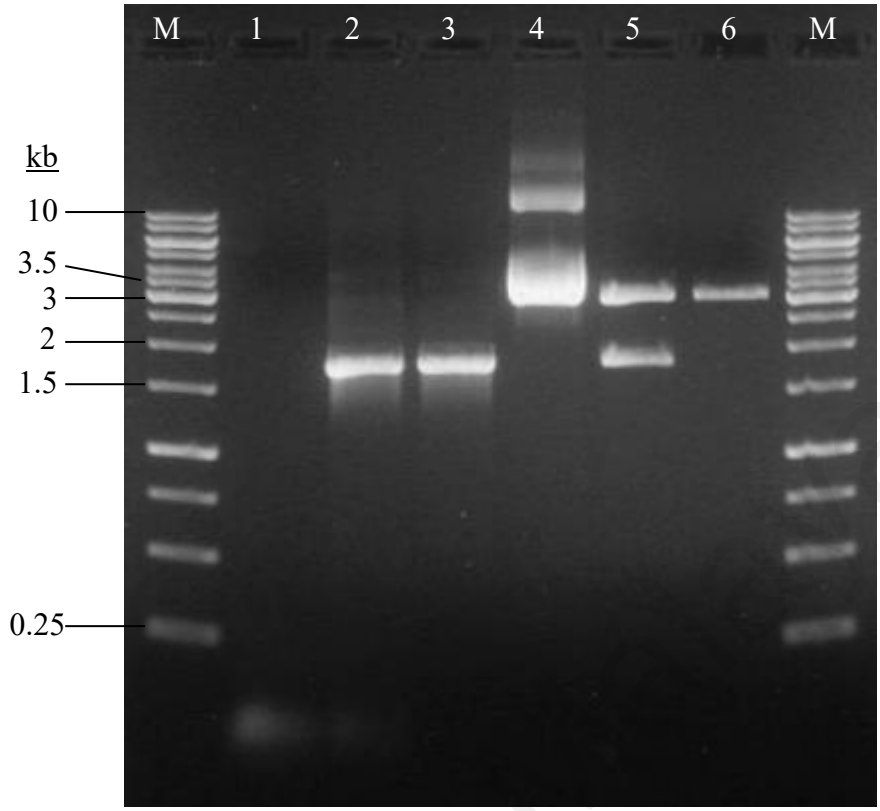
4: pGEM®-T Easy Vector containing ITS region

5: *EcoR*I digested recombinant plasmid

6: pGEM®-T Easy Vector fragment

ITS region (0.5 kb) was amplified from purified total DNA extracted from strain WW1C. The PCR primers ITS1 and ITS4, the PCR conditions were as described in Materials and Methods (Section 2.12). The PCR product was subsequently gel-excised, column-purified and ligated into pGEM®-T Easy cloning vector (Section 2.13). DNA was analyzed by electrophoretic examination in a submerged horizontal agarose gel (0.8 to 1.0% w/v) containing EtBr (0.5 µg/ml), flooded with 1× TBE buffer. DNA was 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). Note that insert containing *EcoR*I site was digested into two fragments of approximately 0.25 kb.

Figure 3.3 18S rDNA gene PCR amplification



Legends:

M: GeneRuler™ 1kb DNA Ladders (Fermentas)

1: Negative control (PCR mixture where DNA was replaced with sdH_2O)

2: 18S rDNA gene PCR products

3: Purified 18S rDNA gene PCR products

4: pGEM®-T Easy Vector containing 18S rDNA gene

5: *EcoR*I digested recombinant plasmid

6: pGEM®-T Easy Vector fragment

18S rDNA gene (1.7 kb) was amplified from purified total DNA extracted from strain WW1C. The PCR primers NS1 and NS8, the PCR conditions were as described in Materials and Methods (Section 2.12). DNA was analyzed by electrophoretic examination in a submerged horizontal agarose gel (0.8 to 1.0% w/v) containing EtBr (0.5 µg/ml), flooded with 1× TBE buffer. DNA was 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).

3.5 Identification of strain WW1C

The resulting complete nucleotide sequences of the ITS region and 18S rDNA gene of strain WW1C were compared to those deposited in the GenBank database by using the BLAST (Basic Local Alignment Search Tool) search algorithm available on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST) to identify homologues. Web-based similarity searches against the GenBank using ITS region nucleotide sequence (492 nucleotides) suggested that strain WW1C belonged to the phylum Basidiomycota, sharing 100% sequence identities with the ITS region of two strains of *Trichosporon loubieri*, i.e. strain CBS 7065 (492 out of 492 nucleotides specifically aligned; GenBank accession number AF444438; last updated on 4th January 2010) and strain ATCC MYA-2615 (491 out of 491 nucleotides specifically aligned; GenBank accession number AY101607; last updated on 4th January 2010). Similar result was obtained in web-based similarity searches against the GenBank using 18S rDNA gene nucleotide sequence (1,774 nucleotides), that strain WW1C shared 99.9% sequence identities with the 18S rDNA gene of *Trichosporon loubieri* (1,756 out of 1,758 nucleotides specifically aligned; GenBank accession number AB001759; last updated on 4th January 2010), indicating the most possible identity of this yeast.

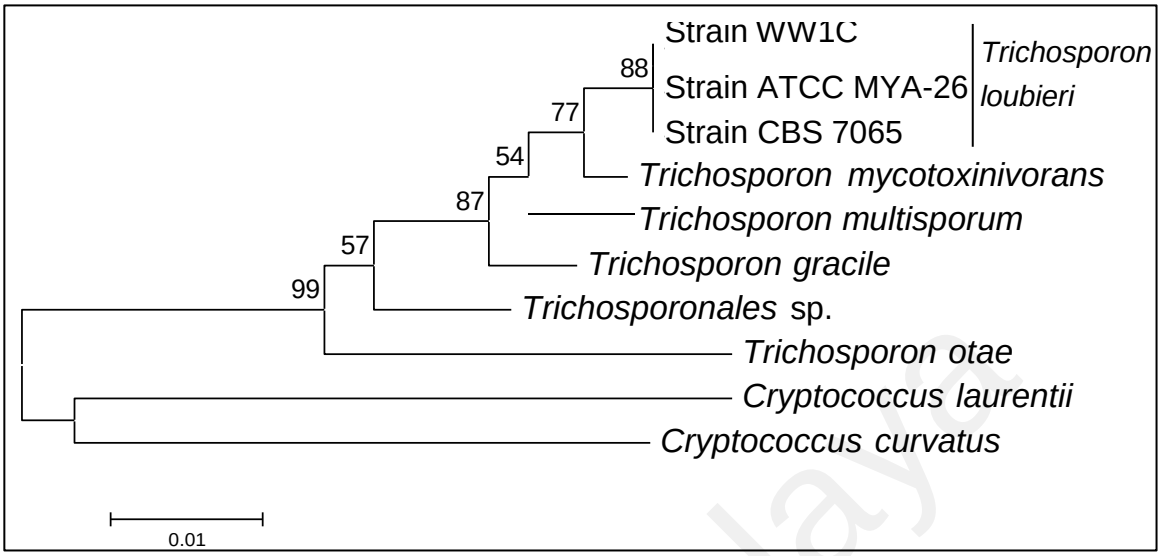
It has been reported that *Trichosporon* showed certain level of tolerance to cycloheximide (Padhye *et al.*, 2003). In addition, Gueho and colleagues have reported that *T. loubieri* can be distinguished from other species of *Trichosporon* by its ability to grow at 42°C (Gueho *et al.*, 1992). Further testing demonstrated growth of strain WW1C at 37°C, 42°C, and in LBm broth supplemented with 0.01 µg/ml cycloheximide. Hence, in agreement with the ITS region and 18S rDNA gene identification of strain WW1C, the physiological characteristics confirmed strain WW1C to be a strain of *T. loubieri.*

3.6 Phylogenetic analysis of strain WW1C

The ITS region-based phylogenetic tree (Figure 3.4) was rooted with *Cryptococcus laurentii* strain WM 601 (GenBank accession number EF568051) and *Cryptococcus curvatus* strain ATCC 10567 (GenBank accession number EU266558) as outgroup whereas the 18S rDNA gene-based phylogenetic tree (Figure 3.5) was rooted with *Kockovaella calophylli* (GenBank accession number AB042222) as outgroup, since they are taxa that are more distantly related but sufficiently conserved or homologous to each of the ingroup taxa being considered.

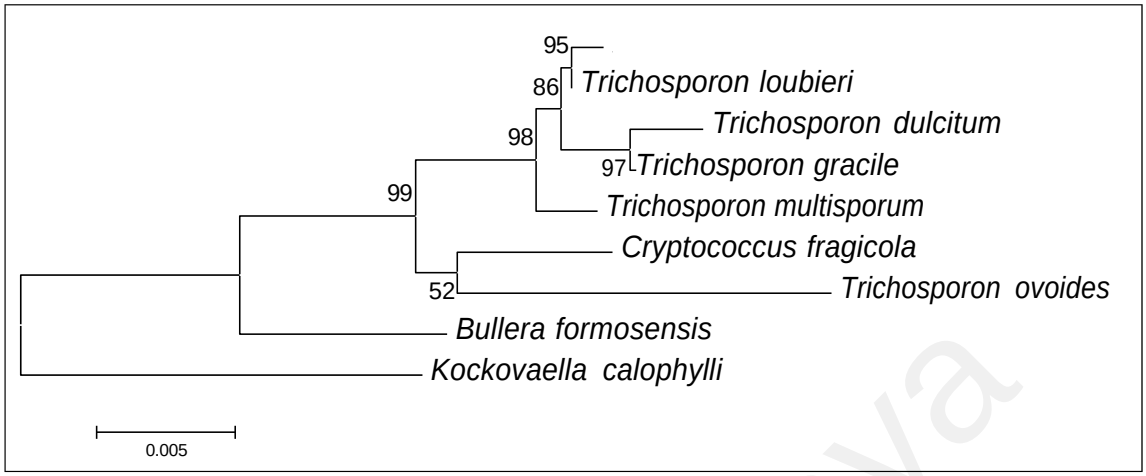
Phylogenetic analysis using both complete nucleotide sequences of the ITS region and 18S rDNA gene supported the conclusion that WW1C is a strain of *Trichosporon loubieri*. Therefore, the AHL-degrading isolate WW1C was named *T. loubieri* strain WW1C.

Figure 3.4 ITS region-based phylogenetic analysis of strain WW1C



ITS region-based phylogenetic tree showing the phylogenetic position of strain WW1C generated using Neighbour-Joining algorithm. The horizontal bar at the bottom represents evolutionary distance as 0.01 changes 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. *Cryptococcus laurentii* and *Cryptococcus curvatus* were used as outgroup. GenBank accession number (in parentheses): *Trichosporon loubieri* strain ATCC MYA-26 (AY101607), *Trichosporon loubieri* strain CBS 7065 (AF444438), *Trichosporon mycotoxinivorans* (AJ601389), *Trichosporon multisporum* strain CBS2495 (AF414695), *Trichosporon gracile* strain CBS 8519 (AF444456), *Trichosporonales* sp. LM117 (EF060484), *Trichosporon otae* (AB180196), *Cryptococcus laurentii* strain WM 601 (EF568051), *Cryptococcus curvatus* strain ATCC 10567 (EU266558).

Figure 3.5 18S rDNA gene-based phylogenetic analysis of strain WW1C

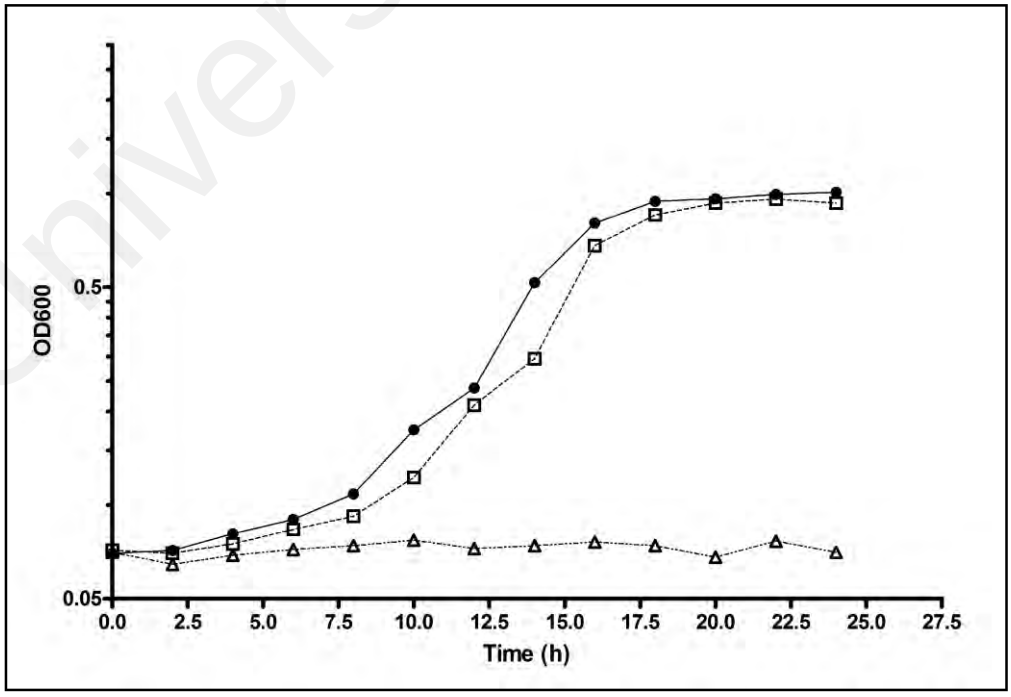


18S rDNA gene-based phylogenetic tree showing the phylogenetic position of strain WW1C generated using Neighbour-Joining algorithm. The horizontal bar at the bottom represents evolutionary distance as 0.005 changes per nucleotide position, determined by measuring the lengths of the horizontal lines connecting the species. The numbers (bootstrap values as percentages of 1,000 replications) provide support for the robustness of the adjacent nodes. *Kockovaella calophylli* was used as outgroup. GenBank accession number (in parentheses): *Trichosporon loubieri* (AB001759), *Trichosporon dulcitum* (AB001755), *Trichosporon gracile* (AB001756), *Trichosporon multisporum* (AB001764), *Cryptococcus fragicola* (AB035588), *Trichosporon ovoides* (AB001765), *Bullera formosensis* (AB072235), *Kockovaella calophylli* (AB042222).

3.7 3-oxo-C6-HSL as energy sources for growth

The ability of *T. loubieri* strain WW1C to grow on 3-oxo-C6-HSL was investigated by examining growth in a defined medium supplemented with 3-oxo-C6-HSL as the sole carbon, or carbon and nitrogen source. Strain WW1C was able to grow in minimal medium supplemented with 3-oxo-C6-HSL (1 µg/ml), with doubling time of approximately 2.5 h, estimated from the linear portion of the semi-log plot of the OD_{600} against time as shown in Appendix 3. When assessed, the presence or absence of NH_4Cl in the medium did not affect the growth capability of the strain. No statistically significant difference was found between the ammonium chloride-replete and ammonium chloride-deplete growth curve using a two-tailed paired t-test ($t = 3.0192$, $df = 12$, $p = 0.01$). No growth was observed in a minimal medium devoid of 3-oxo-C6-HSL (Figure 3.6).

Figure 3.6 Growth of *T. loubieri* strain WW1C on with 3-oxo-C6-HSL as the sole energy source.



OD_{600} was determined over 24 h in ammonium chloride-replete (●) and deplete (□) minimal medium supplemented with 3-oxo-C6-HSL. Residual growth without any carbon or nitrogen source is also shown (Δ). The error bars are too small to be shown.

3.8 Degradation of various AHLs

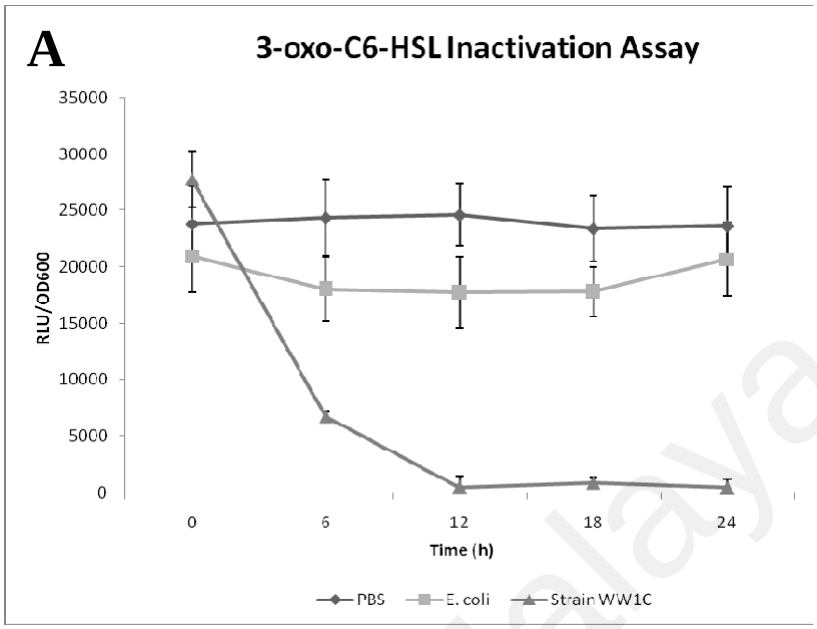
The AHL substrate range of *T. loubieri* strain WW1C was assessed using whole-cell assay by reacting harvested yeast cells with AHLs with acyl chains ranging from 4 to 14 carbons. The isolate degrade both 3-oxo-C6-HSL and 3-oxo-C8-HSL within 12h, as detected by short-chain *lux*-based biosensor *E. coli* [pSB401] (Figure 3.7). Further RRLC analysis showed that it effectively degraded C4-HSL (Figure 5.1), C6-HSL (Figure 5.2), 3-oxo-C6-HSL (Figure 5.3), C7-HSL (Figure 5.4), C8-HSL (Figure 5.5), 3-oxo-C8-HSL (Figure 5.6), and 3-oxo-C10-HSL (Figure 5.8), which have different lengths and 3-oxo side chain substitution at C3 position, showing a preference for AHLs with *N*-acyl side chains up to C10 (Table 3.2). In particular, C8-HSL was the most efficiently degraded molecule i.e. with an estimated activity of 4.04 µg AHL degraded h^{-1} $(10^5$ CFU/ml$)^{-1}$. No significant degradation was detected in C10-HSL (Figure 5.7), C12-HSL (Figure 5.9), 3-oxo-C12-HSL (Figure 5.10), C14-HSL (Figure 5.11), and 3-oxo-C14-HSL (Figure 5.12). No degradation of AHL was observed in the negative controls *E. coli* DH5α cells and PBS buffer (100mM, pH 6.5). The estimated activity was calculated from the linear semi-log plot of the residual AHL concentrations against time (Figure 3.10).

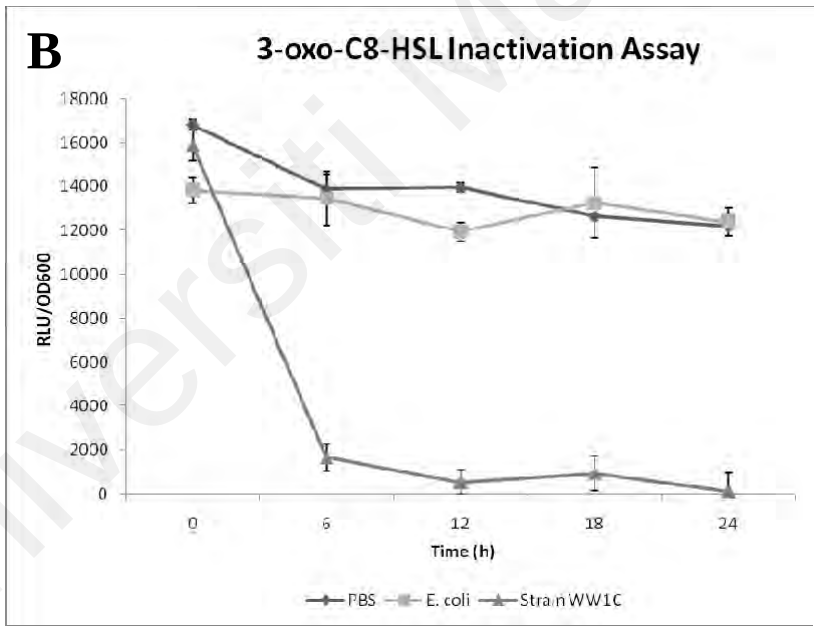
Boiled resting cells of *T. loubieri* strain WW1C failed to degrade C6-HSL, suggesting that the AHL degradation occurs by an enzymatic inactivation mechanism (Figure 3.8).

3.9 Identification of AHL degradation products

Using the method based on the acidification of the reaction medium using HCl (after completion of the AHL inactivation assay) as described in Section 2.18, the acidified C6-HSL degradation media incubated with the resting cells of *T. loubieri* strain WW1C was able to induce violacein production in the biosensor CV026, suggesting the recyclization of lactone ring by acidification (Figure 3.9).

Figure 3.7 Degradation of (A) 3-oxo-C6-HSL and (B) 3-oxo-C8-HSL by *T. loubieri* strain WW1C over a 24-h period.



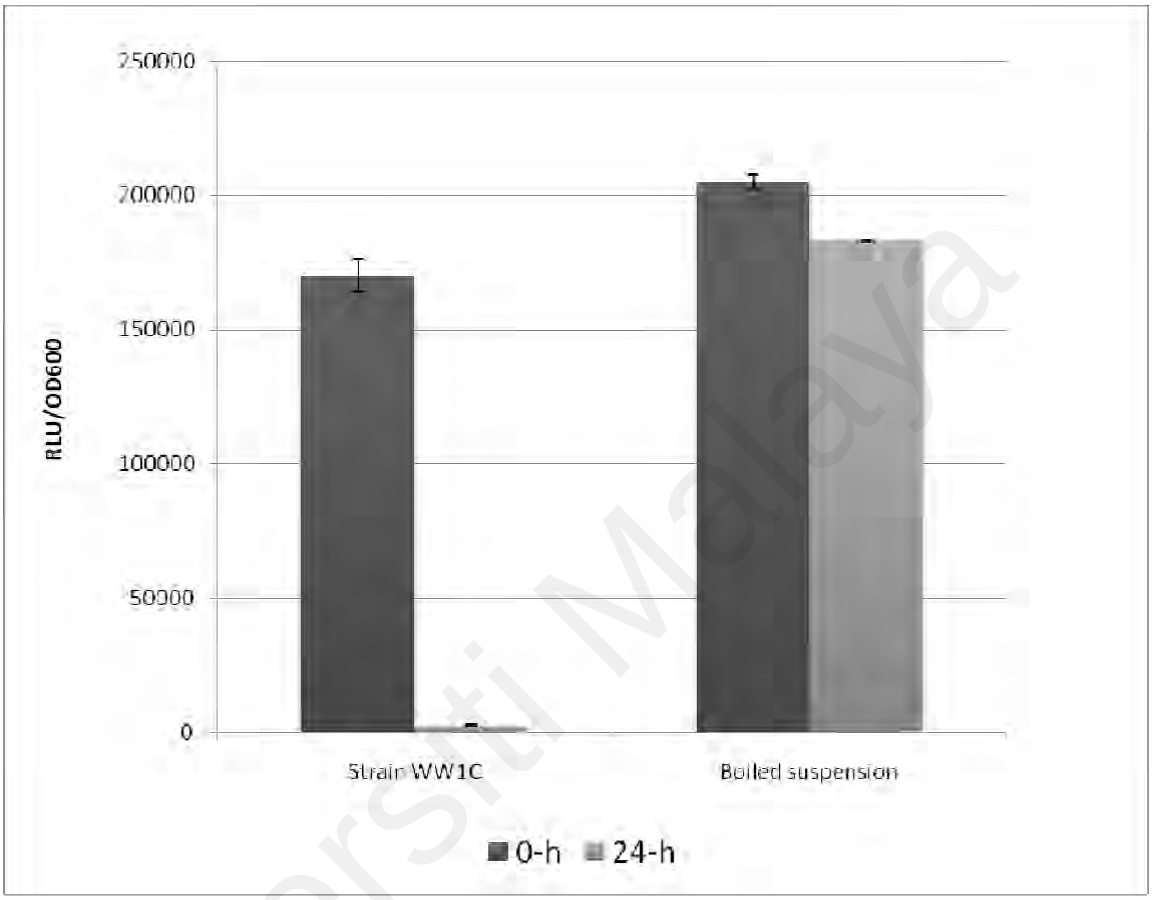


Residual AHLs as measured with the bioluminescent sensor strain *E. coli* [pSB401]. *E. coli* DH5α and PBS Buffer (100mM, pH 6.5) were used as the negative control. The experiments were repeated twice; the results are means ± SD. RLU: relative light units.

Legends:

PBS	:	Incubation with PBS buffer (100mM, pH 6.5)
E. coli	:	Incubation with *E. coli* DH5α resting cells
Strain WW1C	:	Incubation with *T. loubieri* strain WW1C resting cells

Figure 3.8 Analysis of C6-HSL after 0- and 24-h incubation with *T. loubieri* strain WW1C and boiled resting cells of *T. loubieri* strain WW1C



Legends:

Strain WW1C: C6-HSL incubated with resting cells of *T. loubieri* strain WW1C

Boiled suspension: C6-HSL incubated with boiled resting cells of *T. loubieri* strain WW1C

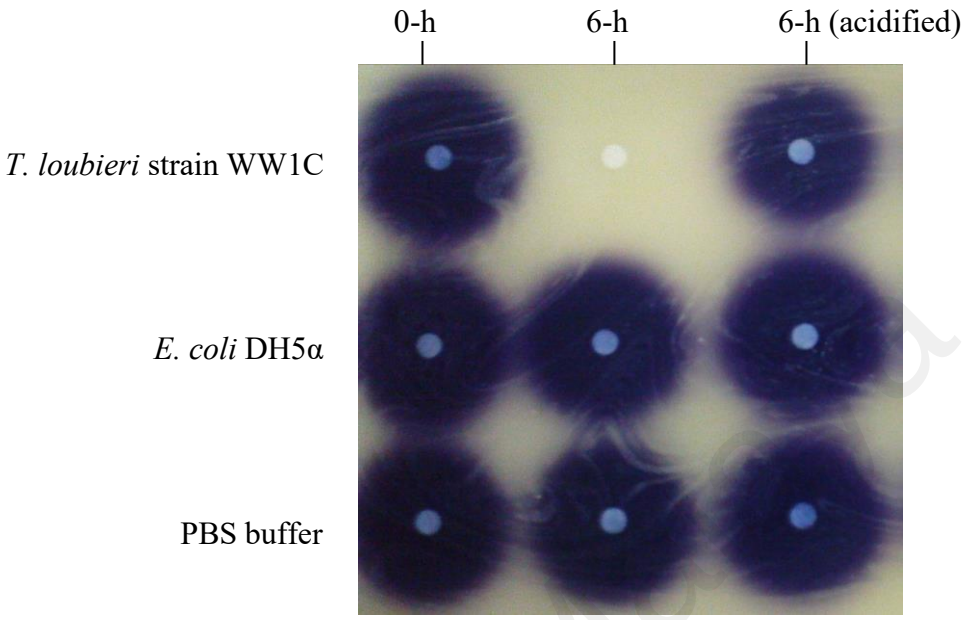
0-h: Samples withdrawn at 0 h

24-h: Samples withdrawn at 24 h

C6-HSL is detected by using the bioluminescent sensor strain *E. coli* [pSB401].

Boiled resting cells of *T. loubieri* strain WW1C failed to degrade AHL, suggesting that the AHL degradation occurs by an enzymatic inactivation mechanism.

Figure 3.9 Identification of degradation products of C6-HSL incubated with *T. loubieri* strain WW1C



Legends:

0-h: Samples withdrawn at 0 h

6-h: Samples withdrawn at 6 h

6-h (acidified): Samples withdrawn at 6 h, acidified at pH 2 for 24 h to promote lactone ring closure

Bacterial names were labeled on the left of the figure.

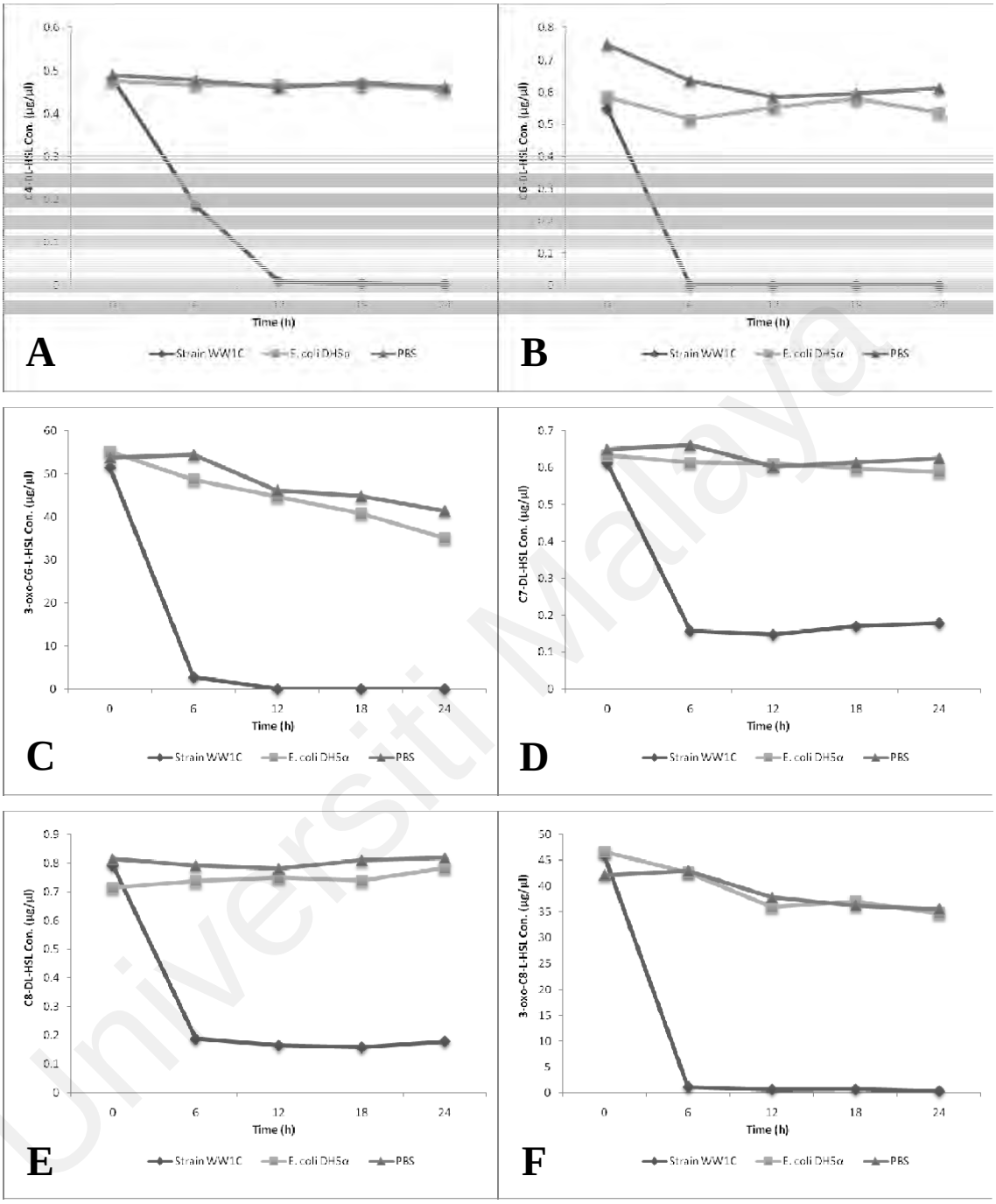
C6-HSL was detected by using CV026.

Table 3.2 Degradation of various AHLs by *T. loubieri* strain WW1C

Type of AHL	EA*
C4-HSL	1.23
C6-HSL	3.67
3-oxo-C6-HSL	3.25
C7-HSL	3.04
C8-HSL	4.04
3-oxo-C8-HSL	2.99
C10-HSL	0.24
3-oxo-C10-HSL	0.76
C12-HSL	0
3-oxo-C12-HSL	0
C14-HSL	0
3-oxo-C14-HSL	0

*EA, estimated activity, expressed as µg AHL degraded h^{-1} (10^5 CFU/ml)$^{-1}$.

Figure 3.10 Degradation of (A) C4-HSL, (B) C6-HSL, (C) 3-oxo-C6-HSL, (D) C7-HSL, (E) C8-HSL, and (F) 3-oxo-C8-HSL by *T. loubieri* strain WW1C over a 24 h period.

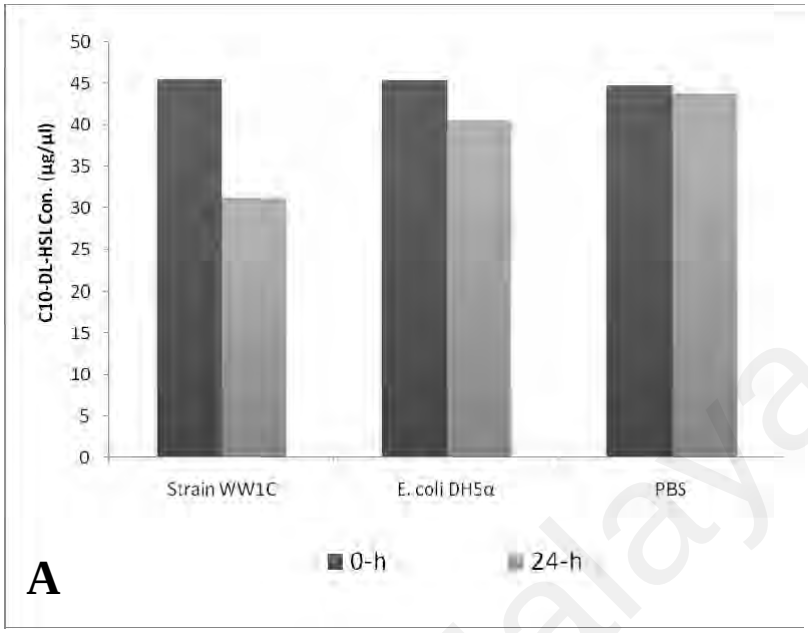


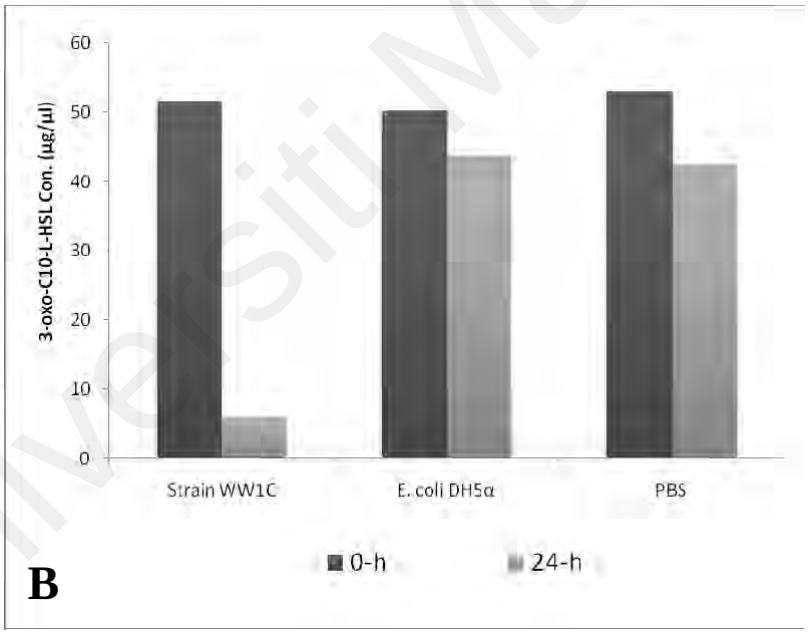
Degradation of (A) C4-HSL, (B) C6-HSL, (C) 3-oxo-C6-HSL, (D) C7-HSL, (E) C8-HSL, and (F) 3-oxo-C8-HSL by *T. loubieri* strain WW1C (■). Residual AHLs as measured based on calibration curve derived from calibration standards ranging from 0.025 to 0.15 µg/µl plotted as described in Section 2.21. *E. coli* DH5α (○) and extraction buffer (PBS 100 mM, pH 6.5) (Δ) were used as the negative controls.

Legends:

Strain WW1C	:	AHL incubated with *T. loubieri* strain WW1C resting cells
E. coli DH5α	:	AHL incubated with *E. coli* DH5α resting cells
PBS	:	AHL incubated with PBS buffer (100mM, pH 6.5)

Figure 3.11 Degradation of (A) C10-HSL and (B) 3-oxo-C10-HSL by *T. loubieri* strain WW1C over a 24 h period.





Degradation of (A) C10-HSL and (B) 3-oxo-C10-HSL by *T. loubieri* strain WW1C. Residual AHLs as measured based on calibration curve derived from calibration standards ranging from 0.025 to 0.15 µg/µl plotted as described in Section 2.21. *E. coli* DH5α and extraction buffer (PBS 100 mM, pH 6.5) were used as the negative controls.

Legends:

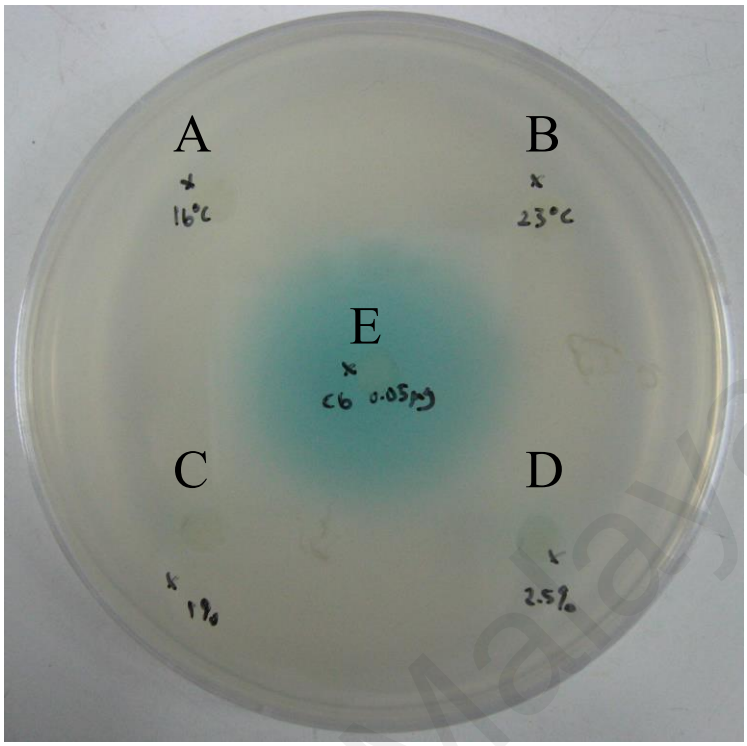
Strain WW1C	:	AHL incubated with *T. loubieri* strain WW1C resting cells
E. coli DH5α	:	AHL incubated with *E. coli* DH5α resting cells
PBS	:	AHL incubated with PBS buffer (100mM, pH 6.5)
0-h:		Samples withdrawn at 0 h
24-h:		Samples withdrawn at 24 h

3.10 Detection of AHL production in *T. loubieri* strain WW1C

To check for the presence of compounds in cultures of *T. loubieri* strain WW1C that can activate quorum sensing response, organic phase extraction was performed on culture medium of *T. loubieri* strain WW1C grown in various environmental parameters, i.e. different salinity and temperature. *T. loubieri* strain WW1C was grown in LB (1% w/v NaCl) at 16°C and 23°C for 7 days, and in LB and LBm (2.5% w/v NaCl) at 28°C for 2 days. Extraction of spent supernatant of *T. loubieri* strain WW1C was carried out as described in Section 2.19. The sample extract (10 µl) was spotted directly onto paper disk placed on the surface of AB agar seeded with *A. tumefaciens* NTL4(pLZR4).

No induction of blue zone formation was observed using *A. tumefaciens* NTL4(pLZR4) in all the sample extracts, showing that *T. loubieri* strain WW1C did not produce any detectable long chain AHL molecules (Figure 3.12).

Figure 3.12 Testing for production of AHLs in *T. loubieri* strain WW1C



Extration of AHL from cultures of *T. loubieri* strain WW1C was carried out as described in Section 2.19. The sample extract (10 µl) of *T. loubieri* strain WW1C grown in (A) LB at 16°C for 7 days, (B) LB at 23°C for 7 days, (C) LB at 28°C for 2 days, (D) LBm at 28°C for 2 days, was spotted directly onto paper disk placed on the surface of AB agar seeded with *A. tumefaciens* NTL4(pLZR4). (E) Synthetic C6-HSL standard of 0.05 µg.

CHAPTER FOUR: RESULTS

Isolation of quorum quenching bacteria

4.1 Enrichment and isolation of bacteria from rainforest soil and marine water

Enrichment was carried out as described in Section 2.9, KG medium and KGm medium containing 3-oxo-C6-HSL were inoculated with rainforest soil sample and marine water sample, respectively. The pH of the rainforest soil and marine water sample was pH 6.74 and pH 7.88, respectively, as measured with pH meter. The media became turbid within 48 h after inoculation. No obvious turbidity was observed in a control tube in the absence of 3-oxo-C6-HSL. Cell suspensions were diluted and plated onto both LB agar and plates of 3-oxo-C6-HSL-supplemented KG or KGm medium agar. Pure cultures were obtained in similar manner as described in Section 3.1.

In the enrichment with rainforest soil sample, only one isolate, designated strain KM1S induced a disappearance of the 3-oxo-C6-HSL signal after 24h cultivation, using AHL inactivation assay. In the marine water sample, two strains were able to inactivate 3-oxo-C6-HSL, they were designated as MW3A and 2WS8.

4.2 Cell and colony morphology

Rainforest soil bacterium strain KM1S grown on LB plates for 48 h at 28°C yielded white, opaque, raised colonies with colony diameter of 5 mm. Colonies were essentially circular with undulate margin and appeared dull (Figure 4.1). Microscopically, long rod-shaped cells with cell length of approximately 2.0 µm were observed (Figure 4.2). Cells were stained Gram-positive.

Marine bacterial strains MW3A and 2WS8 were grown on LBm plates for 24 h at 28°C where both yielded white, transparent, convex colonies with colony diameter of 5 mm. Colonies were essentially round with entire edges and appeared smooth and shiny (Figure 4.3). Under the microscope, these isolates appeared as long rod-shaped cells with cell length of approximately 2.0 µm (Figure 4.4). Cells were stained Gram-negative in these isolates.

Figure 4.1 Colony morphology of strain KM1S grown on LB plates for 48 h at 28°C.

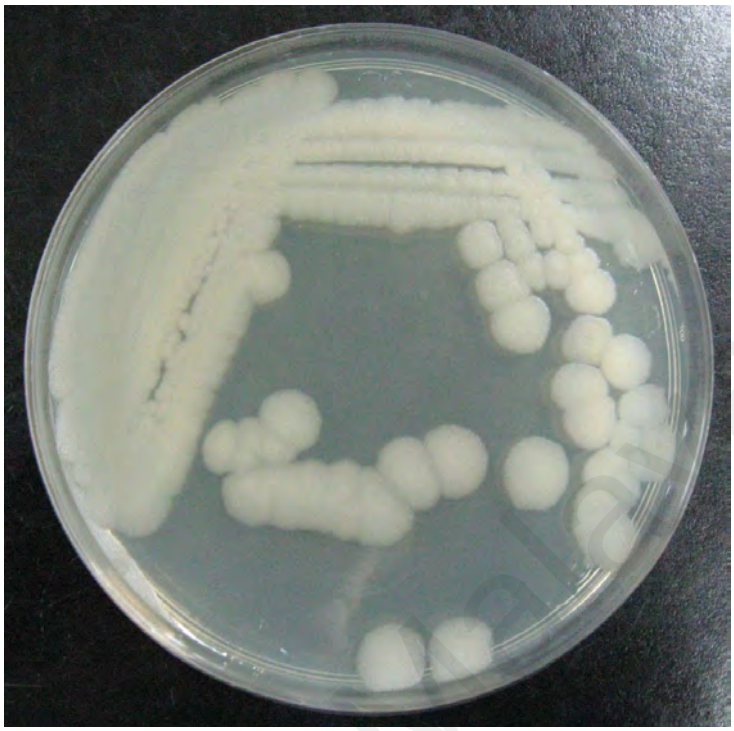


Figure 4.2 Light micrograph of Gram-stained cells of strain KM1S grown overnight on LB plates at 28°C. The bar represents 10 µm.

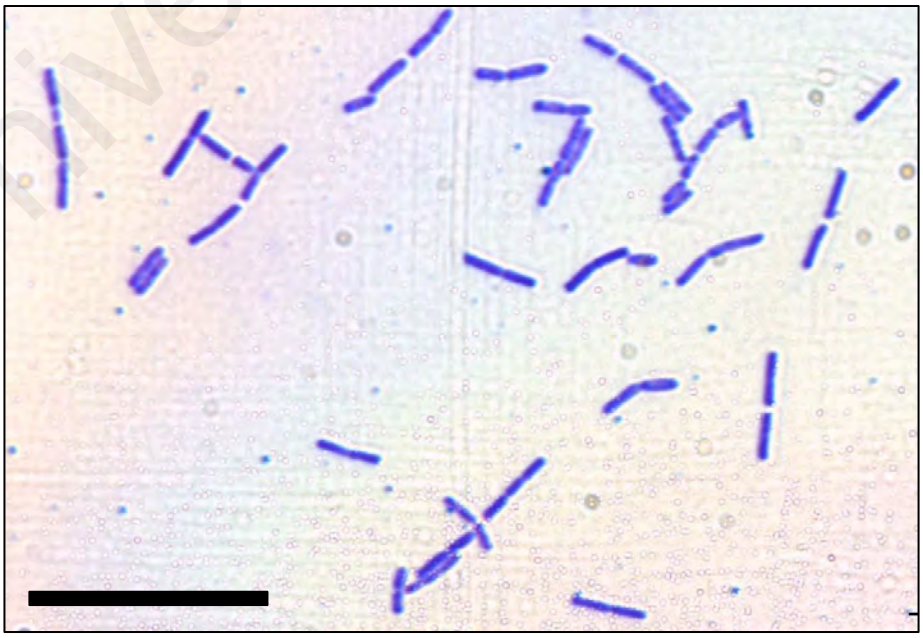


Figure 4.3 Colony morphology of (A) strain MW3A and (B) strain 2WS8 grown on LBm plates for 24 h at 28°C.

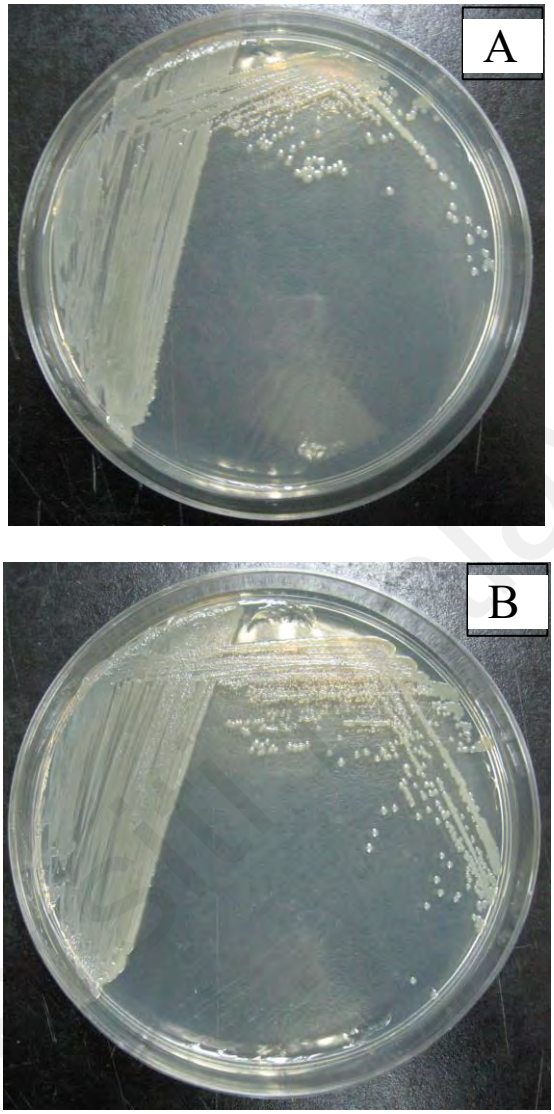
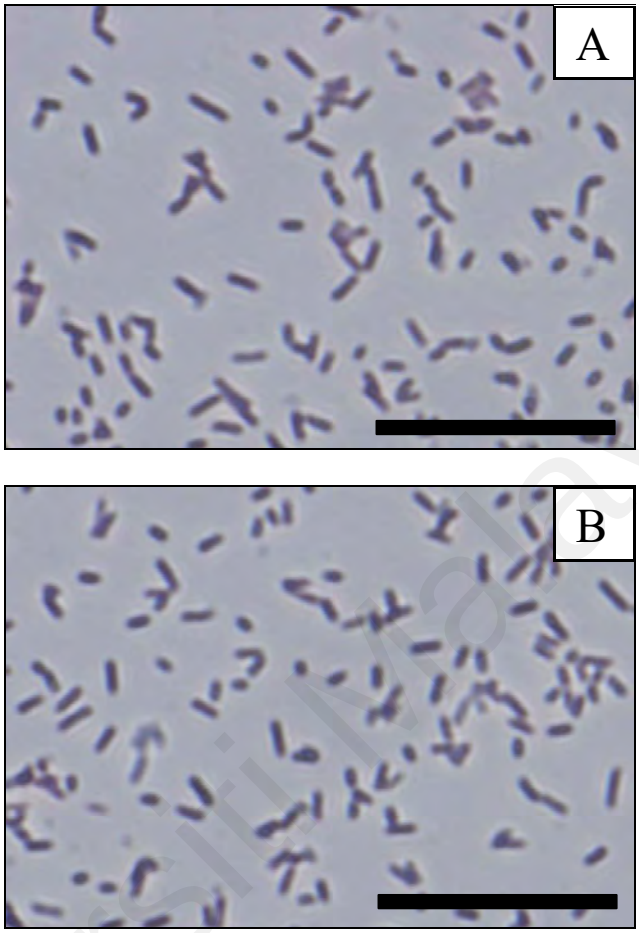


Figure 4.4 Light micrograph of Gram-stained cells of (A) strain MW3A and (B) strain 2WS8 grown on LBm plates. The bars represent 100 µm.



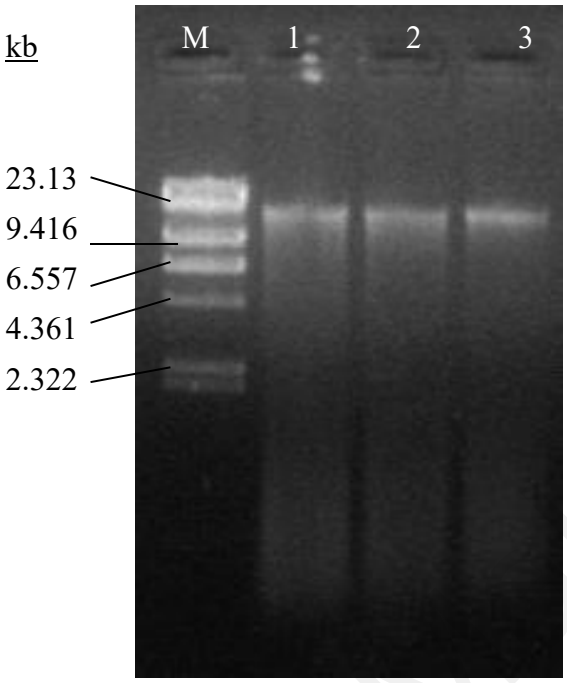
4.3 16S rDNA gene amplification

To identify the strains isolated from rainforest soil and marine water samples, 16S rDNA genes (approximately 1.5 kb) were amplified by PCR using the genomic DNA as template (Section 2.11) (Figure 4.5). Amplified 16S rDNA genes of approximately 1.5 kb are shown in Figure 4.6. The amplicons were subsequently gel-excised, column-purified (Figure 4.7) and ligated into pGEM®-T Easy cloning vector (Figure 4.8). Clones with the correct insert were confirmed by colony PCR using universal primers and plasmids were isolated for sequencing analysis. Sequencing was performed using vector-specific universal primers T7 and SP6 followed by internal primers (338F, 515F, 783F, and 1174F) listed in Table 2.3. The nucleotide sequences were then aligned and assembled to generate the complete nucleotide sequence. The sequences obtained are shown in Appendix 1.

4.4 Nucleotide sequence accession number

The 16S rDNA gene sequences for strain KM1S, strain MW3A, and strain 2WS8 have been deposited at GenBank under the GenBank accession number FJ827748, GQ180117, and GQ180119, respectively. All other sequences were from the GenBank database.

Figure 4.5 Genomic DNA isolation



Legends:

M: Lambda DNA/HindIII Marker, 2 (Fermentas)

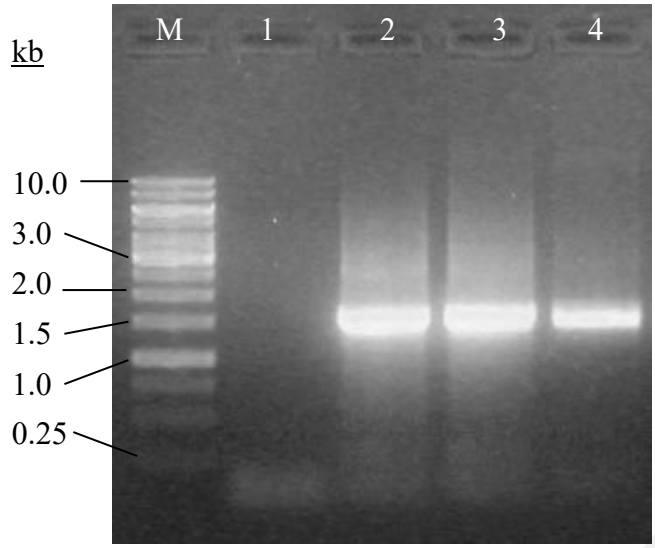
1: Strain MW3A

2: Strain 2WS8

3: Strain KM1S

Genomic DNA was isolated from bacterial cells using Qiagen™ DNA mini kit as described in Section 2.11. The genomic DNA was analyzed by electrophoretic examination in a submerged horizontal agarose gel (0.8 w/v) containing EtBr (0.5 µg/ml), flooded with 1× TBE buffer. DNA was 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).

Figure 4.6 16S rDNA gene PCR amplification



Legends:

M: GeneRuler^{TM} 1kb DNA Ladders (Fermentas)

1: Negative control (PCR mixture where DNA was replaced with sdH_2O)

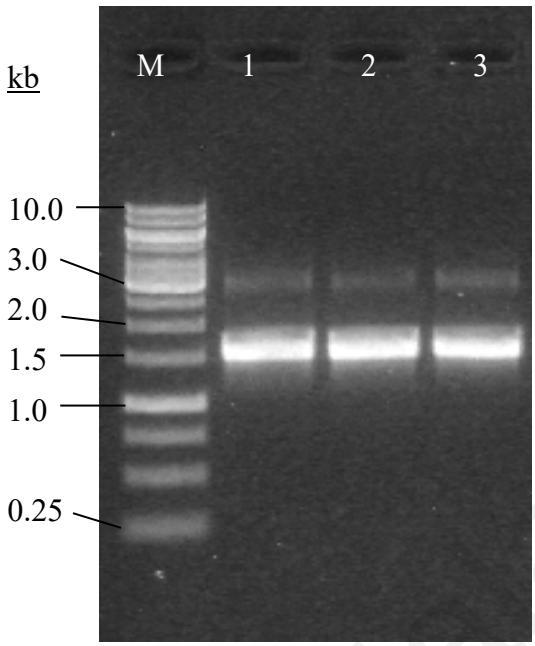
2: Strain MW3A

3: Strain 2WS8

4: Strain KM1S

16S rDNA gene (1.5 kb) was amplified from genomic DNA isolated from bacterial cells. The PCR primers 27F and 1525R, the PCR conditions were as described in Section 2.12. The PCR products were analyzed by electrophoretic examination in a submerged horizontal agarose gel (1.0% w/v) containing EtBr (0.5 µg/ml), flooded with 1× TBE buffer. DNA was 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).

Figure 4.7 16S rDNA gene PCR product purification



Legends:

M: GeneRuler^{TM} 1kb DNA Ladders (Fermentas)

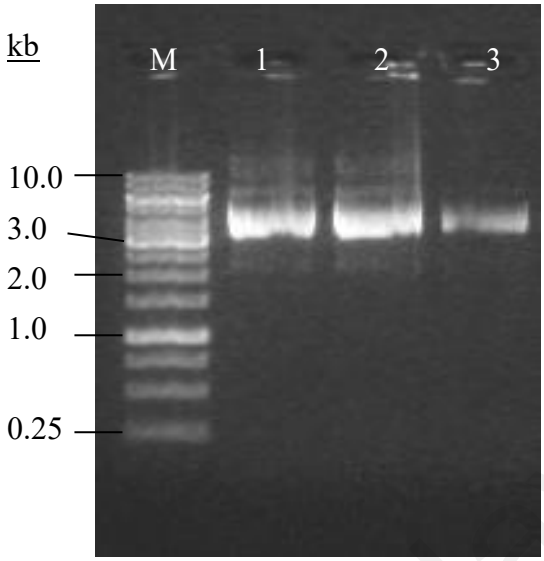
1: Strain MW3A

2: Strain 2WS8

3: Strain KM1S

16S rDNA gene PCR products were gel-excised and column-purified as described in Section 2.12. The purified PCR products were analyzed by electrophoretic examination in a submerged horizontal agarose gel (0.8 to 1.0% w/v) containing EtBr (0.5 μg/ml), flooded with 1× TBE buffer. DNA was 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).

Figure 4.8 Amplified 16S rDNA gene ligated into pGEM®-T Easy cloning vector



Legends:

M: GeneRuler™ 1kb DNA Ladders (Fermentas)

1: Strain MW3A

2: Strain 2WS8

3: Strain KM1S

Purified 16S rDNA gene PCR products were ligated into pGEM®-T Easy Cloning Kit (Promega) as per the manufacturer's instructions (Section 2.13). The recombinant plasmid vectors inserted with 16S rDNA genes were analyzed by electrophoretic examination in a submerged horizontal agarose gel (0.8 to 1.0% w/v) containing EtBr (0.5 µg/ml), flooded with 1× TBE buffer. DNA was 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).

4.5 Strains identification

The resulting complete nucleotide sequences of the 16S rDNA gene of strain KM1S were compared to those deposited in the GenBank database by using the BLAST (Basic Local Alignment Search Tool) search algorithm available on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST) to identify homologues. Web-based similarity searches against the GenBank using 16S rDNA gene nucleotide sequence (1,544 nucleotides) suggested that strain WW1C belonged to the genus *Bacillus*, sharing 99.8% sequence identities with the 16S rDNA gene of *Bacillus cereus* strain G8639 (1,541 out of 1,544 aligned nucleotides; GenBank accession number AY138271; last updated on 4th January 2010), indicating the most possible identity of this bacteria. Web-based similarity searches against the GenBank using 16S rDNA genes nucleotide sequences of strain MW3A and 2WS8 isolated from marine water sample (1,479 nucleotides) suggested that these 2 strains belonged to the genus *Pseudomonas*, sharing at least 99.8% sequence identities with the 16S rDNA gene of *Pseudomonas aeruginosa* strain PAO1 (1,525 out of 1,528 nucleotides specifically aligned; GenBank accession number AE004091; last updated on 4th January 2010), indicating the most possible identities of the bacteria.

4.6 Phylogenetic analysis

Strain KM1S

The 16S rDNA gene-based phylogenetic tree (Figure 4.9) was rooted with *Staphylococcus aureus* strain MPU99 (GenBank accession number AB353073) as outgroup.

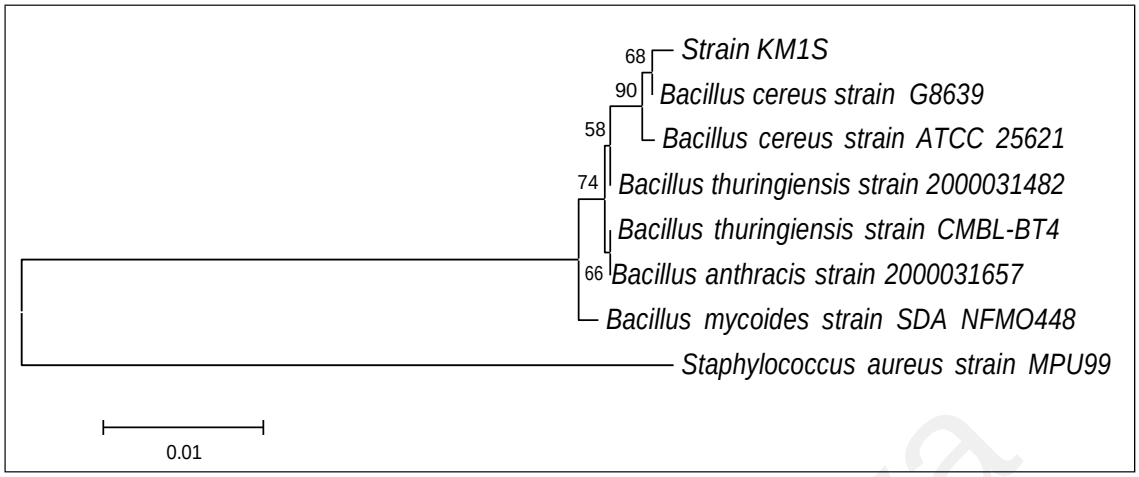
Phylogenetic analysis using complete nucleotide sequences of the 16S rDNA gene supported the conclusion that KM1S is a strain of *Bacillus cereus*. Therefore, the AHL-degrading isolate KM1S was named *B. cereus* strain KM1S.

Strain MW3A and 2WS8

The 16S rDNA gene-based phylogenetic tree (Figure 4.10) was rooted with *Burkholderia* sp. strain MSMB43 (GenBank accession number EF114404) as outgroup since it is a taxon that is more distantly related but sufficiently conserved or homologous to each of the ingroup taxa being considered.

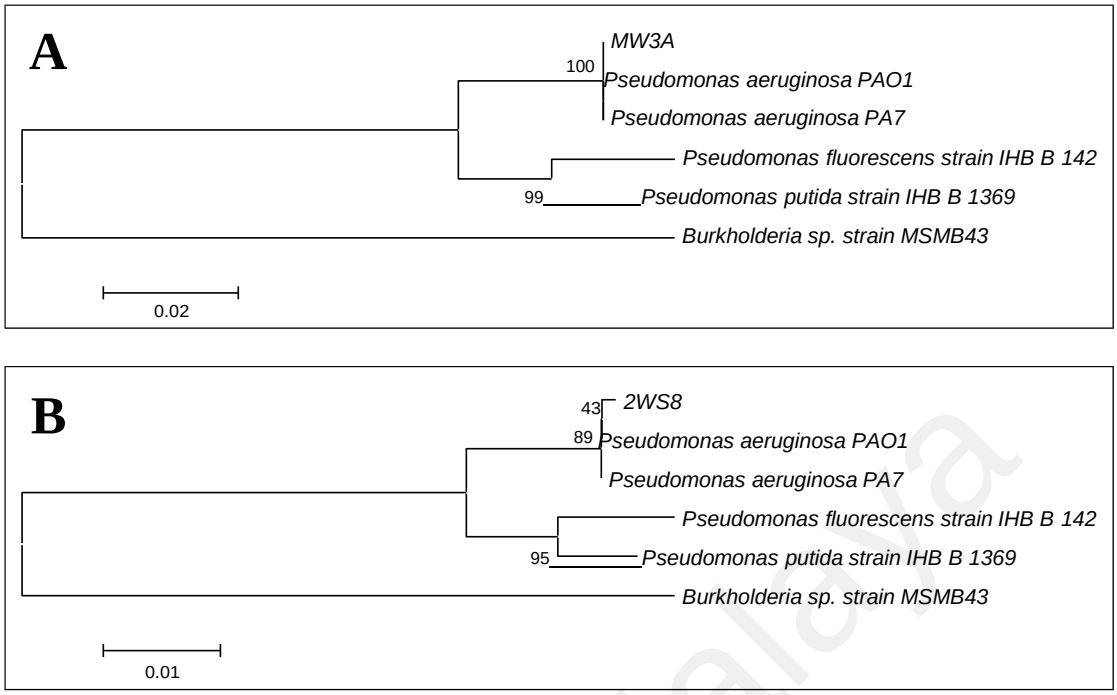
Phylogenetic analysis using complete nucleotide sequences of the 16S rDNA gene supported the conclusion that strain MW3A and strain 2WS8 isolated from marine water sample are strains of *Pseudomonas aeruginosa*.

Figure 4.9 Phylogenetic analysis of strain KM1S



16S rDNA gene-based phylogenetic tree showing the phylogenetic position of strain KM1S generated using Neighbour-Joining algorithm. The horizontal bar at the bottom represents evolutionary distance as 0.01 changes 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. *Staphylococcus aureus* strain MPU99 was used as outgroup. GenBank accession number (in parentheses): *Bacillus cereus* strain G8639 (AY138271), *Bacillus cereus* strain ATCC 25621 (AY795568), *Bacillus thuringiensis* strain 2000031482 (AY138290), *Bacillus thuringiensis* strain CMBL-BT4 (AM778997), *Bacillus anthracis* strain 2000031657 (AY138382), *Bacillus mycoides* strain SDA NFMO448 (AM747228), *Staphylococcus aureus* strain MPU99 (AB353073).

Figure 4.10 Phylogenetic analysis of (A) strain MW3A and (B) strain 2WS8



16S rDNA gene-based phylogenetic tree showing the phylogenetic position of strain MW3A and strain 2WS8 generated using Neighbour-Joining algorithm. The horizontal bar at the bottom represents evolutionary distance as (A) 0.02 and (B) 0.01 changes 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 number (in parentheses): *Pseudomonas aeruginosa* PA7 (CP000744: 807093 to 808589), *Pseudomonas aeruginosa* PAO1 (AE004091: 722096 to 723631), *Pseudomonas fluorescens* strain IHB B 1427 (GU186124), *Pseudomonas putida* strain IHB B 1369 (GU186116), *Burkholderia* sp. strain MSMB43 (EF114404).

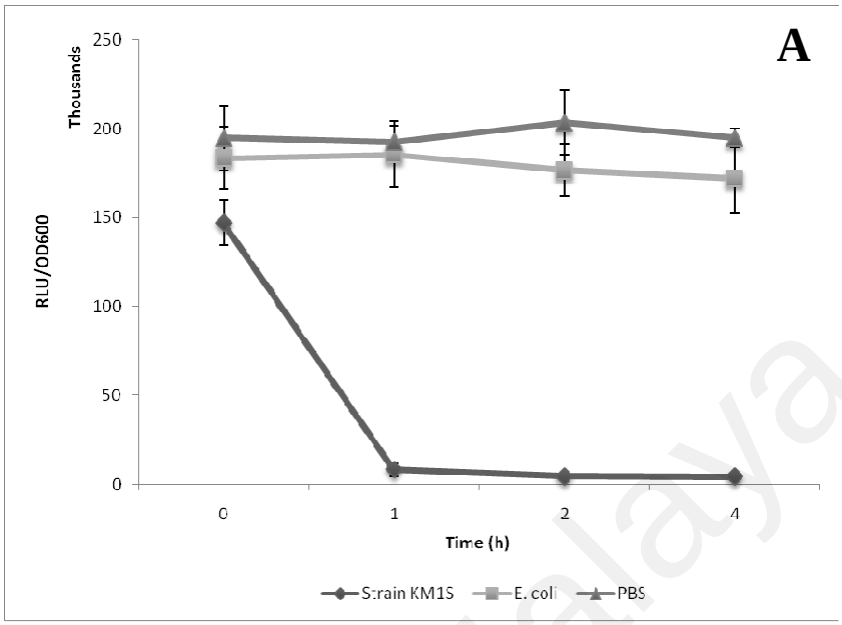
4.7 Degradation of various AHLs

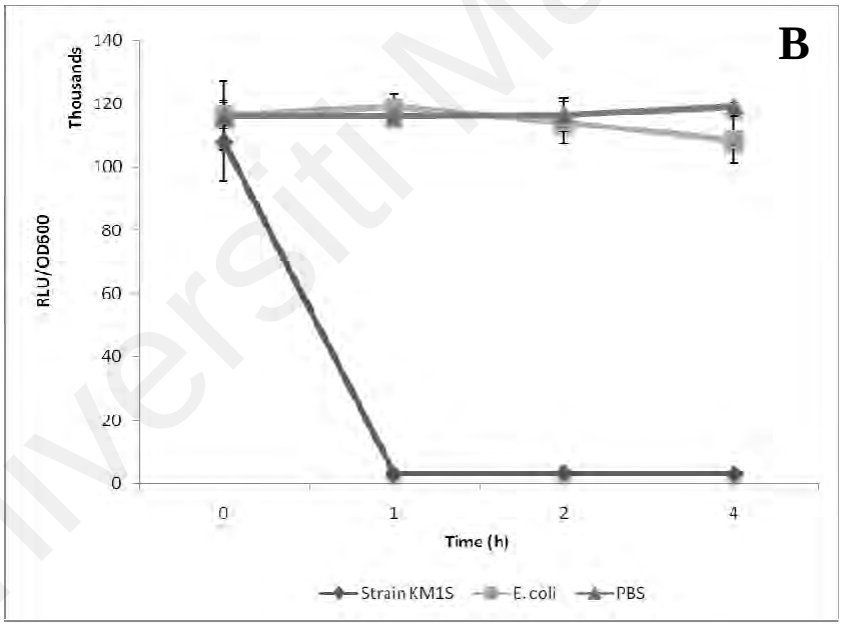
Strain KM1S

As KG medium was supplemented with 3-oxo-C6-HSL as sole carbon source, *B. cereus* strain KM1S ability to degrade 3-oxo-C6-HSL was first verified. *B. cereus* strain KM1S was able to degrade both 3-oxo-C6-HSL (Figure 4.11A) and 3-oxo-C8-HSL molecules (Figure 4.11B) within 1 h, suggesting a rapid turnover of both AHL molecules under our experimental condition. No degradation of AHL molecules was obvious when the AHL-inactivation assays were repeated with *E. coli* DH5α cells and PBS buffer (100mM, pH 6.5) (Figure 4.11). We analyzed AHL degradation by *B. cereus* strain KM1S over a period of 0 to 120 min using Rapid Resolution Liquid Chromatography (RRLC). It was found that degradation of 3-oxo-C6-HSL (Figure 4.12) and 3-oxo-C8-HSL (Figure 4.13) by this isolate occurred within 15 min. No AHL-degradation was evident in negative control involving *E. coli* DH5α and PBS buffer (100mM, pH 6.5) (Figure 4.12, Figure 4.13). This indicated our isolate showed very high affinity towards degrading these AHLs.

RRLC analysis showed that under the same experimental conditions, about 60% of the 3-oxo-C8-HSL incubated with the *B. cereus* strain KM1S resting cells was degraded after 15 min, approximately 95% after 60 min and almost completely after 120 min (Figure 4.14B), with an estimated activity of about 6.56 µg 3-oxo-C8-HSL h^{-1} (10^9 CFU/ml)$^{-1}$. The degradation activity of 3-oxo-C6-HSL was slower as compared to 3-oxo-C8-HSL, with about 40% degraded after 15 min, 70% after 60 min and approximately 80% after 120 min (Figure 4.14A). The estimated degradation activity of 3-oxo-C6-HSL was 4.98 µg 3-oxo-C6-HSL h^{-1} (10^9 CFU/ml)$^{-1}$. The estimated activity was calculated from the linear semi-log plot of the residual AHL concentrations against time (Figure 4.15).

Figure 4.11 Degradation of (A) 3-oxo-C6-HSL and (B) 3-oxo-C8-HSL by *B. cereus* strain KM1S over a 4-h period.



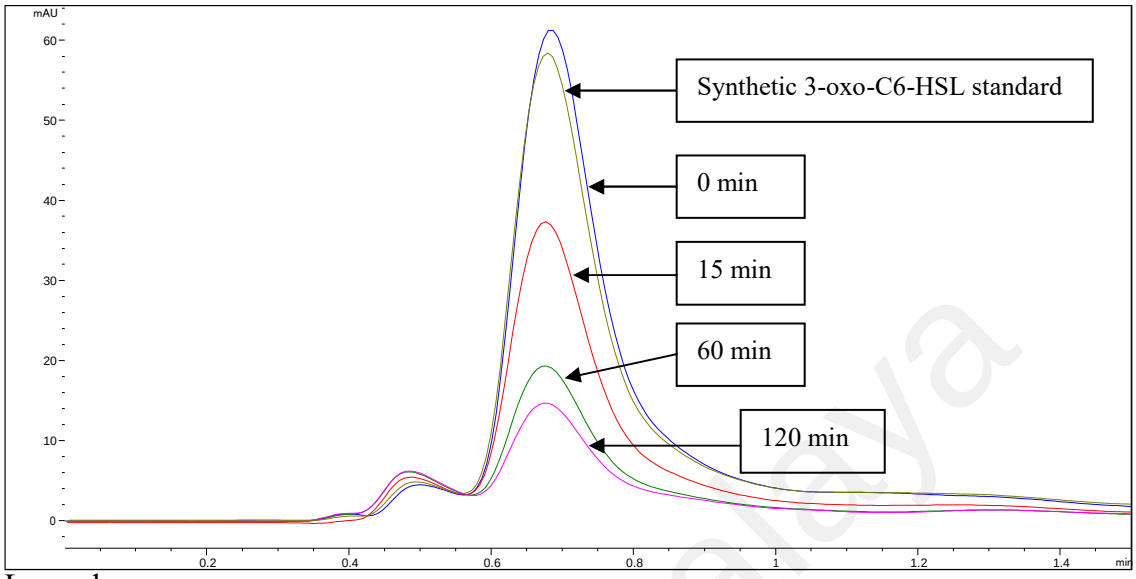


Degradation of (A) 3-oxo-C6-HSL and (B) 3-oxo-C8-HSL by *B. cereus* strain KM1S (◊). Residual AHLs as measured with the bioluminescent sensor strain *E. coli* [pSB401]. *E. coli* DH5α (□) and extraction buffer (PBS 100 mM, pH 6.5) (Δ) were used as negative controls. The experiments were carried out twice; the results are means ± SD. RLU: relative light units.

Legends:

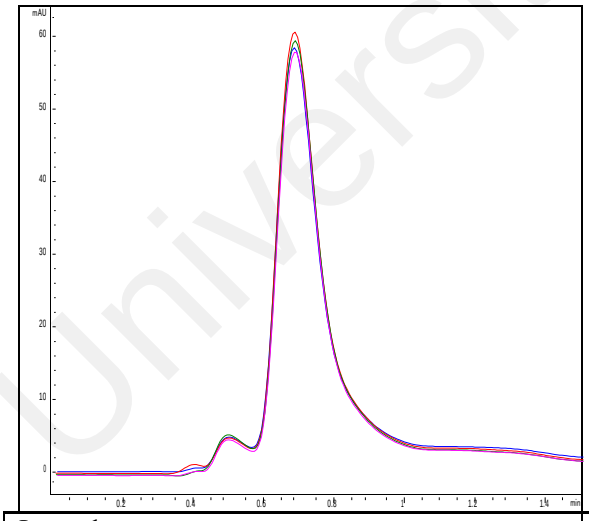
PBS	:	AHL incubated with PBS buffer (100mM, pH 6.5)
E. coli	:	AHL incubated with *E. coli* DH5α resting cells
Strain KM1S	:	AHL incubated with *B. cereus* strain KM1S resting cells

Figure 4.12 RRLC analysis of 3-oxo-C6-HSL after 0-, 15-, 60-, and 120-min incubation with *B. cereus* strain KM1S, *E. coli* DH5α, and PBS buffer (100mM, pH 6.5)



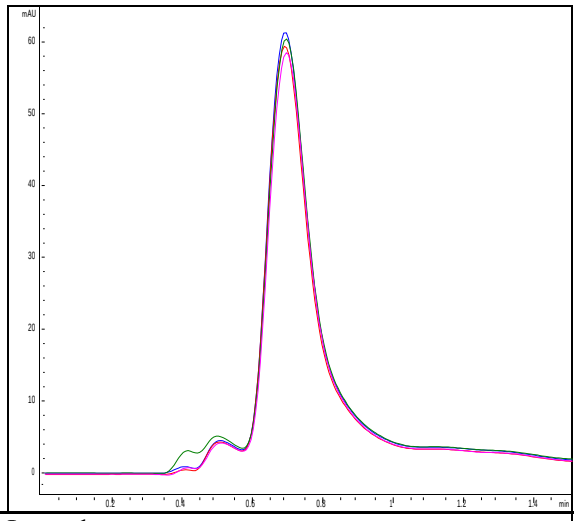
Legends:

- 3-oxo-C6-HSL incubated with *B. cereus* strain KM1S resting cell for 0-min
- 3-oxo-C6-HSL incubated with *B. cereus* strain KM1S resting cell for 15-min
- 3-oxo-C6-HSL incubated with *B. cereus* strain KM1S resting cell for 60-min
- 3-oxo-C6-HSL incubated with *B. cereus* strain KM1S resting cell for 120-min
- Synthetic 3-oxo-C6-HSL standard (5 µg)



Legends:

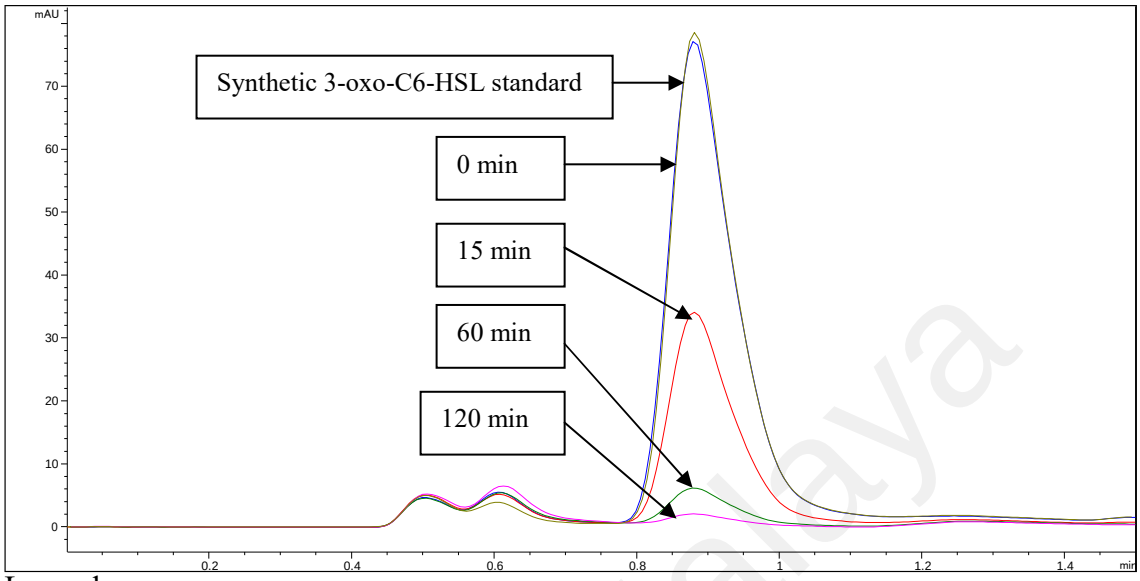
- 3-oxo-C6-HSL incubated with *E. coli* DH5α resting cell for 0-min
- 3-oxo-C6-HSL incubated with *E. coli* DH5α resting cell for 15-min
- 3-oxo-C6-HSL incubated with *E. coli* DH5α resting cell for 60-min
- 3-oxo-C6-HSL incubated with *E. coli* DH5α resting cell for 120-min



Legends:

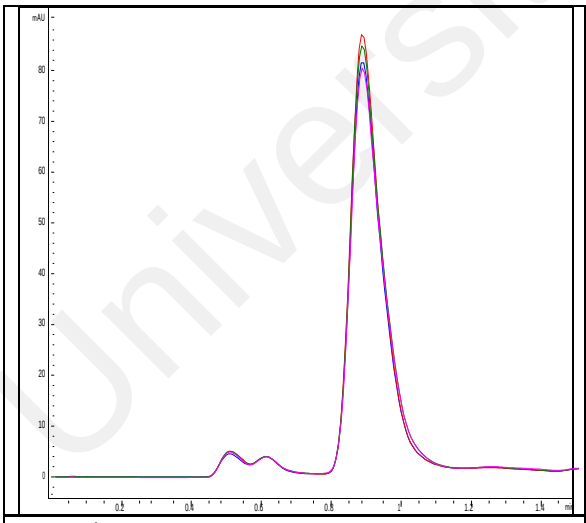
- 3-oxo-C6-HSL incubated with PBS buffer (100mM, pH 6.5) for 0-min
- 3-oxo-C6-HSL incubated with PBS buffer (100mM, pH 6.5) for 15-min
- 3-oxo-C6-HSL incubated with PBS buffer (100mM, pH 6.5) for 60-min
- 3-oxo-C6-HSL incubated with PBS buffer (100mM, pH 6.5) for 120-min

Figure 4.13 RRLC analysis of 3-oxo-C8-HSL after 0, 15, 60, and 120 m incubation with *B. cereus* strain KM1S, *E. coli* DH5α, and PBS buffer (100mM, pH 6.5)



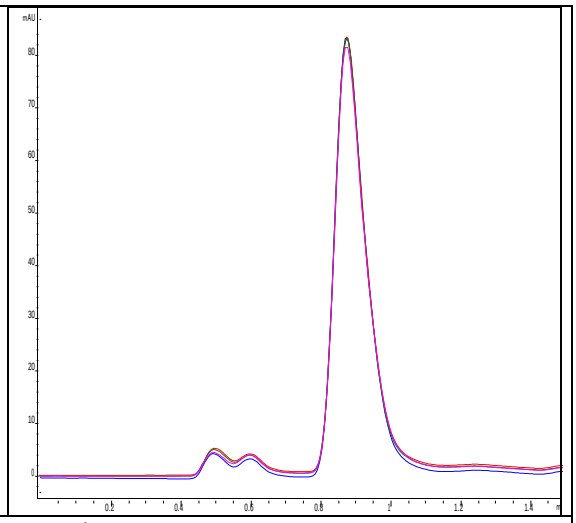
Legends:

- 3-oxo-C8-HSL incubated with *B. cereus* strain KM1S resting cell for 0-min
- 3-oxo-C8-HSL incubated with *B. cereus* strain KM1S resting cell for 15-min
- 3-oxo-C8-HSL incubated with *B. cereus* strain KM1S resting cell for 60-min
- 3-oxo-C8-HSL incubated with *B. cereus* strain KM1S resting cell for 120-min
- Synthetic 3-oxo-C8-HSL standard (5 µg)



Legends:

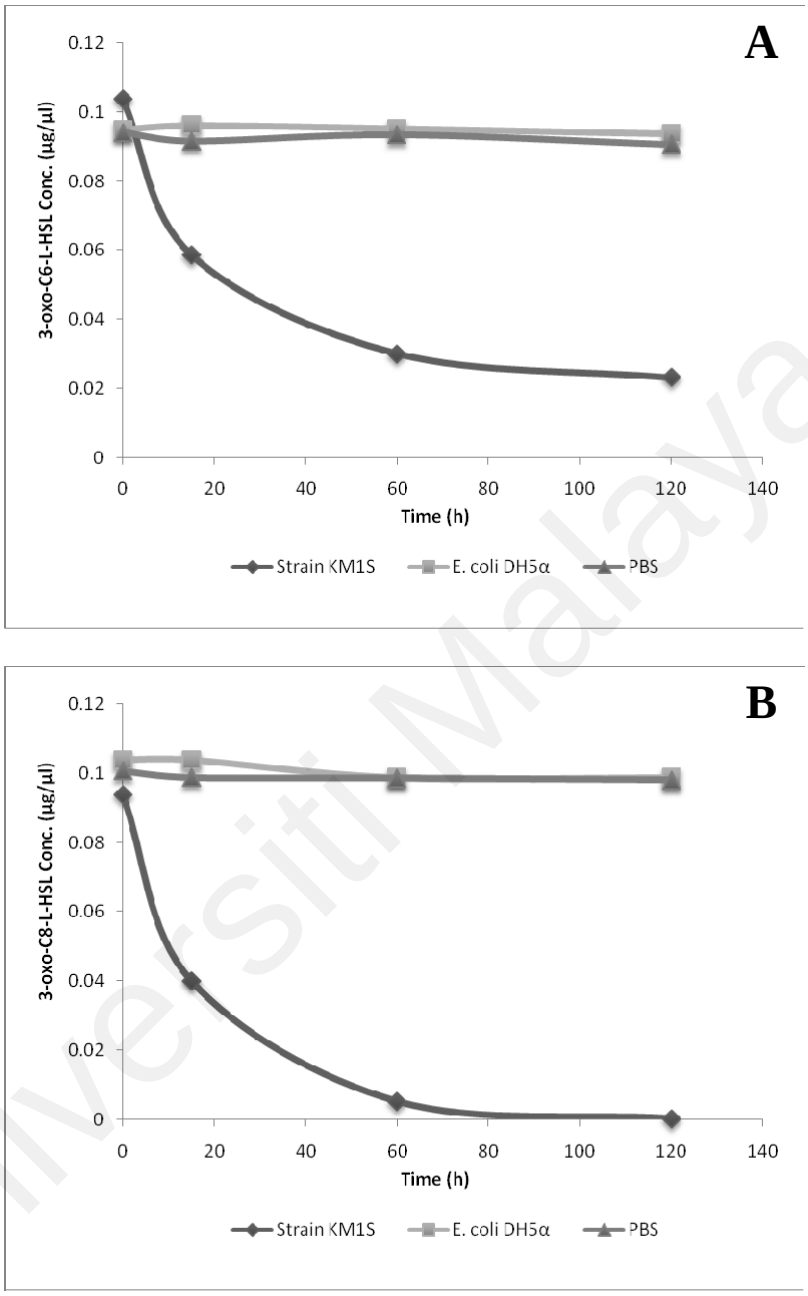
- 3-oxo-C8-HSL incubated with *E. coli* DH5α resting cell for 0-min
- 3-oxo-C8-HSL incubated with *E. coli* DH5α resting cell for 15-min
- 3-oxo-C8-HSL incubated with *E. coli* DH5α resting cell for 60-min
- 3-oxo-C8-HSL incubated with *E. coli* DH5α resting cell for 120-min



Legends:

- 3-oxo-C8-HSL incubated with PBS buffer (100mM, pH 6.5) for 0-min
- 3-oxo-C8-HSL incubated with PBS buffer (100mM, pH 6.5) for 15-min
- 3-oxo-C8-HSL incubated with PBS buffer (100mM, pH 6.5) for 60-min
- 3-oxo-C8-HSL incubated with PBS buffer (100mM, pH 6.5) for 120-min

Figure 4.14 Degradation of (A) 3-oxo-C6-HSL and (B) 3-oxo-C8-HSL by *B. cereus* strain KM1S over a 120-min period.

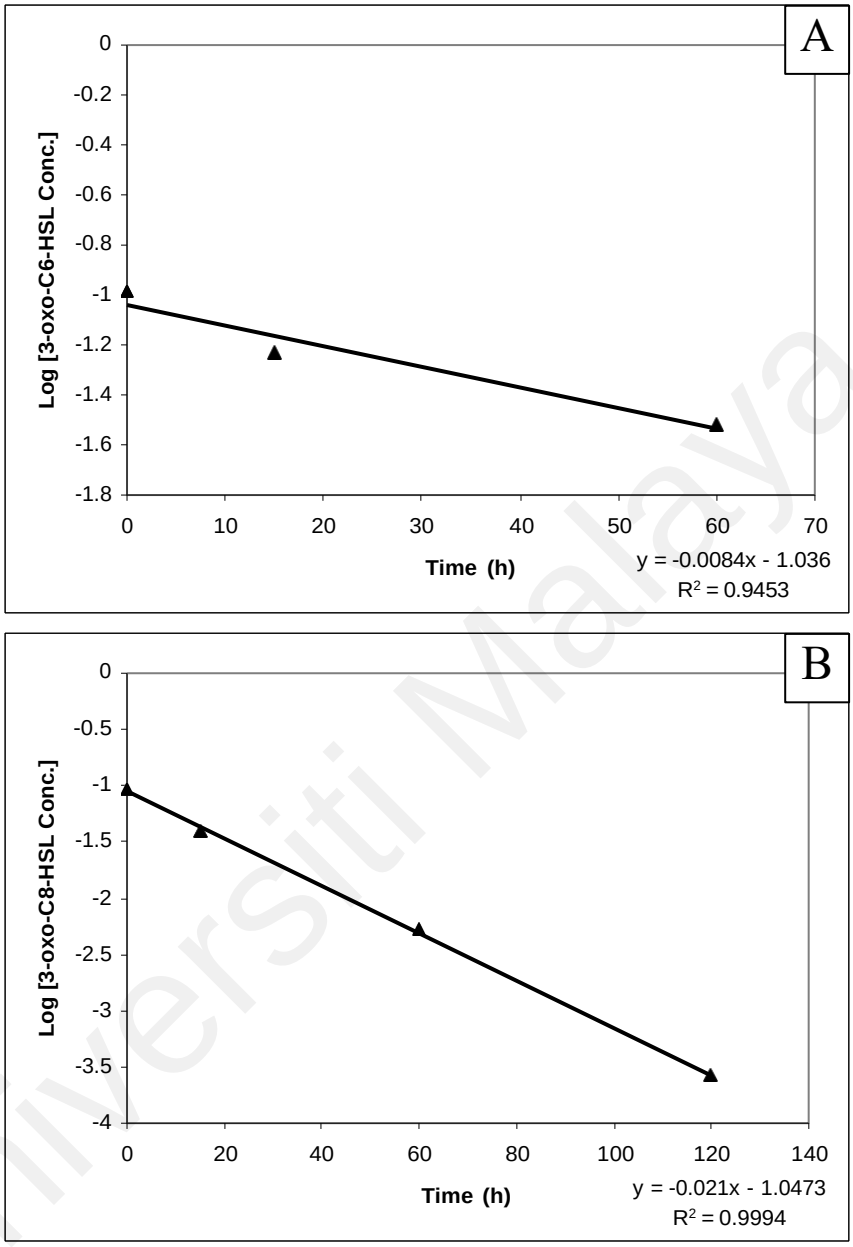


Degradation of (A) 3-oxo-C6-HSL and (B) 3-oxo-C8-HSL by *B. cereus* strain KM1S (◊). Residual AHLs as measured based on calibration curve derived from calibration standards ranging from 0.025 to 0.15 µg/µl plotted as described in Section 2.21. *E. coli* DH5α (□) and extraction buffer (PBS 100 mM, pH 6.5) (Δ) were used as the negative controls.

Legends:

Strain KM1S	:	AHL incubated with *B. cereus* strain KM1S resting cells
E. coli DH5α	:	AHL incubated with *E. coli* DH5α resting cells
PBS	:	AHL incubated with PBS buffer (100mM, pH 6.5)

Figure 4.15 Determination of AHL degradation kinetics from the linear semi-log plot of the residual AHL concentrations against time.



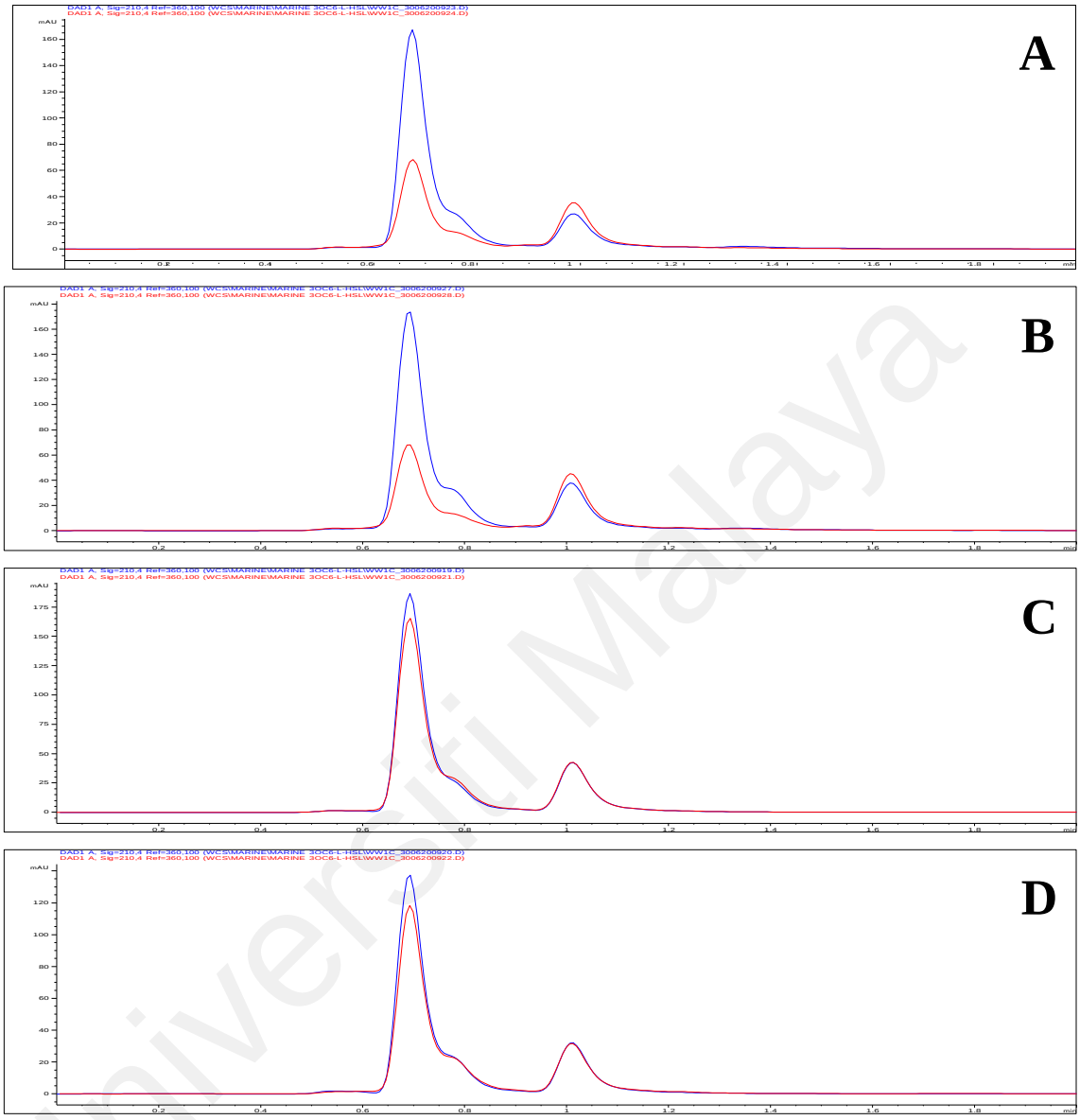
Residual (A) 3-oxo-C6-HSL and (B) 3-oxo-C8-HSL concentrations of resuspension medium incubated with *B. cereus* strain KM1S were linearized by plotting each time point concentration on a semi-log graph. The AHL degradation kinetics were determined from the slope. The equation of the linear trendline and the respective square of correlation coefficient (R^2) was determined by using Microsoft Excel® (shown on the graph).

Strain MW3A and 2WS8

As KG medium was supplemented with 3-oxo-C6-HSL as sole carbon source, the ability of the *Pseudomonas* strains to degrade 3-oxo-C6-HSL was first verified. The isolates were able to degrade 3-oxo-C6-HSL molecules within 24 h (Figure 4.16A and Figure 4.16B). By analyzing the AHL degradation by the isolates using RRLC, it was found that about 40% and 45% of 3-oxo-C6-HSL was degraded after 24 h incubated with *P. aeruginosa* strain MW3A and *P. aeruginosa* strain 2WS8, respectively.

Therefore, further studies were carried out to determine degradation of AHLs other than 3-oxo-C6-HSL in *P. aeruginosa* strain MW3A and *P. aeruginosa* strain 2WS8. RRLC analysis showed that 55 and 65% of the C6-HSL was degraded after 24 h incubated with *P. aeruginosa* strain MW3A and *P. aeruginosa* strain 2WS8, respectively (Figure 4.17A and Figure 4.17B). The degradation activity of C8-HSL was slower as compared to C6-HSL, with only about 40 and 58% degraded after 24 h incubated with *P. aeruginosa* strain MW3A and *P. aeruginosa* strain 2WS8, respectively (Figure 4.18A and Figure 4.18B). No degradation of AHL molecules was obvious when the AHL-inactivation assays were repeated with *E. coli* DH5α cells and PBS buffer (100mM, pH 6.5) (Figure 4.16C and D; Figure 4.17C and D; Figure 4.18C and D).

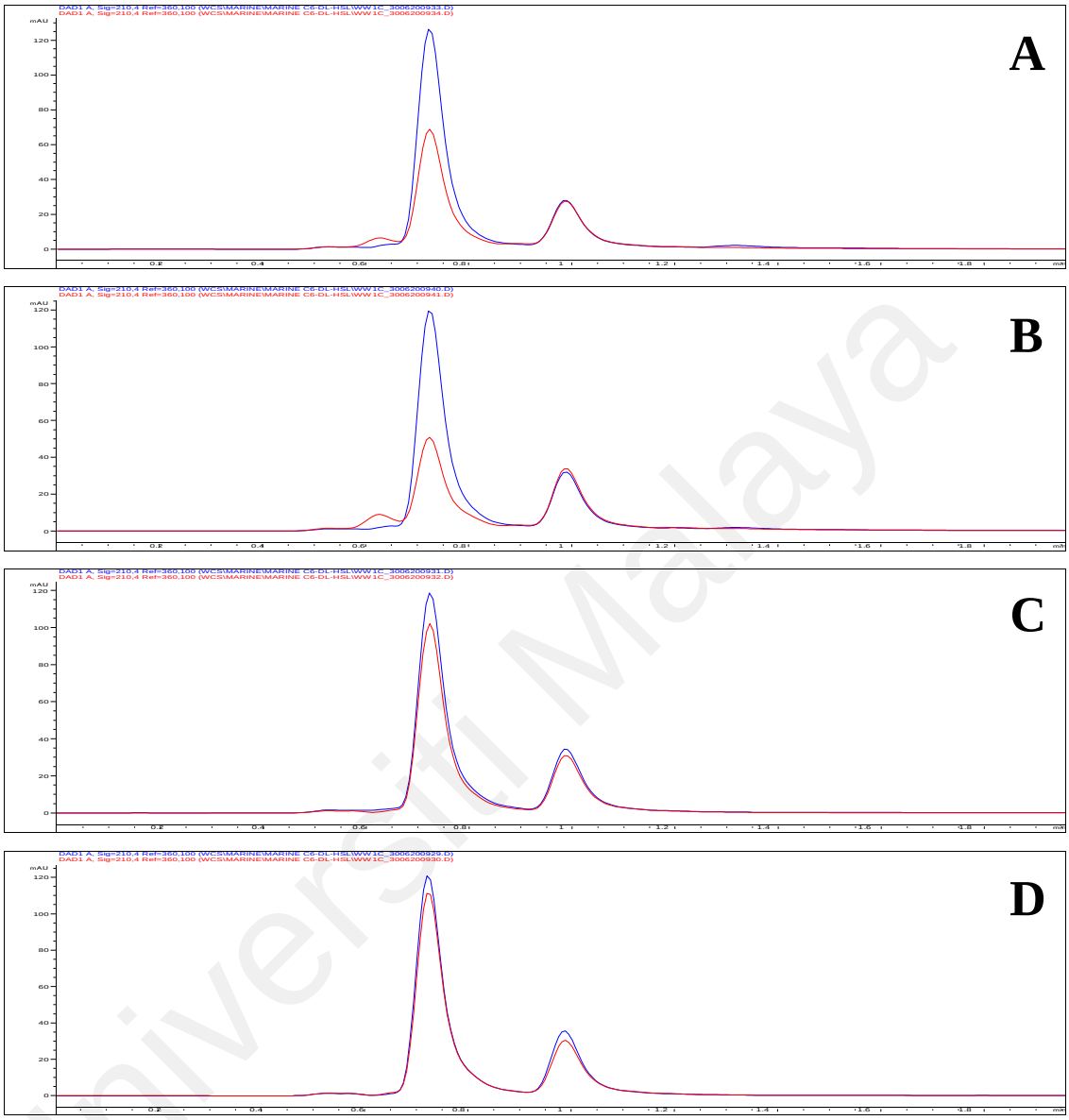
Figure 4.16 RRLC analysis of 3-oxo-C6-HSL after 0- and 24-h incubation with (A) *P. aeruginosa* strain MW3A, (B) *P. aeruginosa* strain 2WS8, (C) *E. coli* DH5α, and (D) PBS buffer (100mM, pH 6.5)



Legends:

- 3-oxo-C6-HSL at 0-h
- 3-oxo-C6-HSL at 24-h

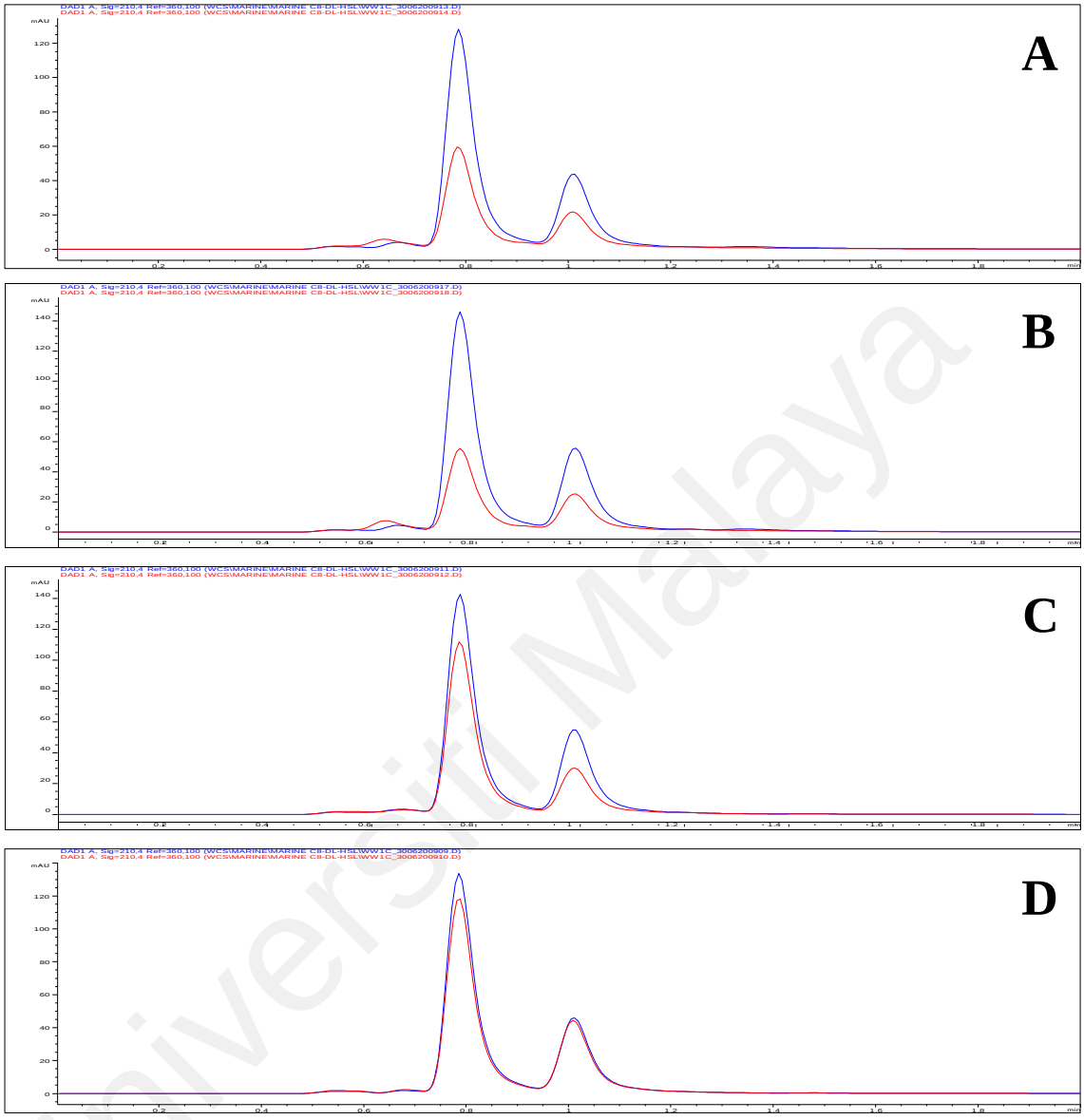
Figure 4.17 RRLC analysis of C6-HSL after 0- and 24-h incubation with (A) *P. aeruginosa* strain MW3A, (B) *P. aeruginosa* strain 2WS8, (C) *E. coli* DH5α, and (D) PBS buffer (100mM, pH 6.5)



Legends:

- C6-HSL at 0-h
- C6-HSL at 24-h

Figure 4.18 RRLC analysis of C8-HSL after 0- and 24-h incubation with (A) *P. aeruginosa* strain MW3A, (B) *P. aeruginosa* strain 2WS8, (C) *E. coli* DH5α, and (D) PBS buffer (100mM, pH 6.5)



Legends:

- C8-HSL at 0-h
- C8-HSL at 24-h

4.8 Characterization of the quorum quenching gene

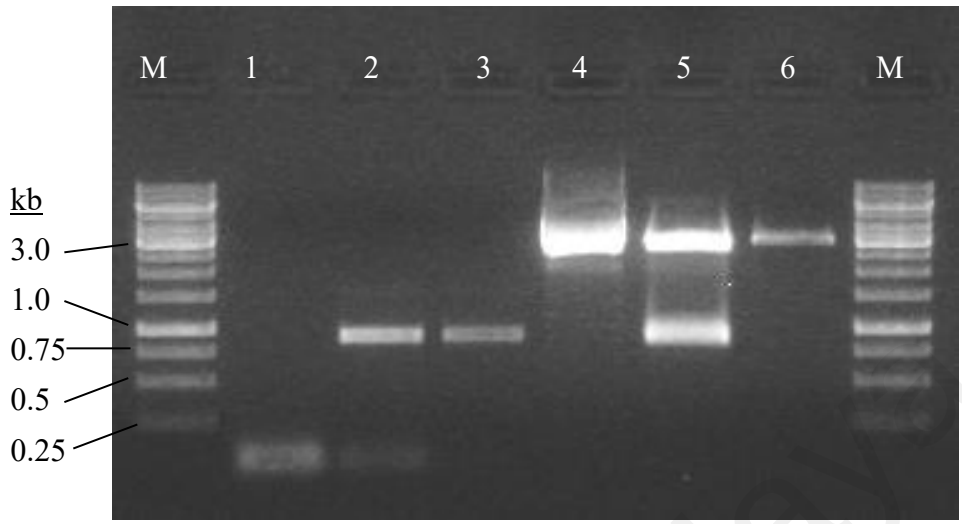
Strain KM1S - Amplification and cloning of the *aiiA* gene

The *aiiA* gene of *B. cereus* strain KM1S was amplified with genomic DNA as the template using *aiiA* gene forward and reverse primers (Table 2.3). The amplicons were subsequently gel-excised, column-purified, and ligated into pGEM®-T Easy cloning vector (Figure 4.19). The clones that conferred AHL-inactivating activity were used for further study (Figure 4.20). The resulting recombinant plasmid was named pGEM-$aiiA_{KM1S}$. Sequencing was performed using vector-specific universal primers T7 and SP6 (Table 2.3). The nucleotides sequences were then aligned and assembled to generate the complete nucleotide sequence. The *aiiA* gene sequence has been deposited at GenBank under the GenBank accession number FJ960449. The sequences obtained are shown in Appendix 1.

Web-based similarity searches against the GenBank using the resulting complete nucleotide sequences of the *aiiA* gene of *B. cereus* strain KM1S (753 nucleotides) suggested that nucleotide sequences were highly similar to a gene encoding hypothetical *N*-acyl homoserine lactone hydrolase in Bacillus thuringiensis clone A, sharing 97.6% sequence identities i.e. (735 out of 753 nucleotides specifically aligned; GenBank accession number DQ440582; last updated on 4th January 2010).

Phylogenetic analysis based on *aiiA* gene indicated that the *B. cereus* strain KM1S *aiiA* homologue was highly conserved as compared with *aiiA* genes from GenBank databases (Figure 4.21). Sequence analysis of the *aiiA* gene cloned indicated that the encoded AiiA contains the motif $_{106}$HXDH-59 amino acids-H_{169}-21 amino acids-D_{191} (Figure 4.22).

Figure 4.19 PCR amplification of *aiiA* gene



Legends:

M: GeneRuler^{TM} 1kb DNA Ladders (Fermentas)

1: Negative control (PCR mixture where DNA was replaced with sdH_2O

2: *aiiA* gene PCR products

3: Purified *aiiA* gene PCR products

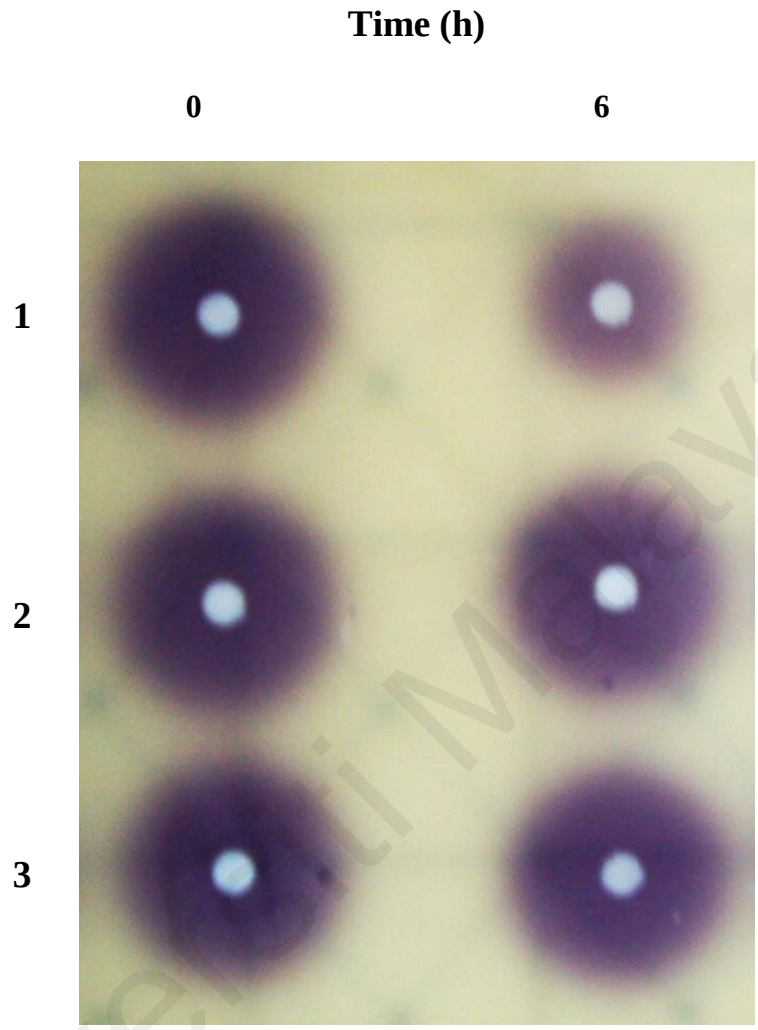
4: pGEM®-T Easy Vector containing *aiiA* gene

5: *EcoR*I digested recombinant plasmid

6: pGEM®-T Easy Vector fragment

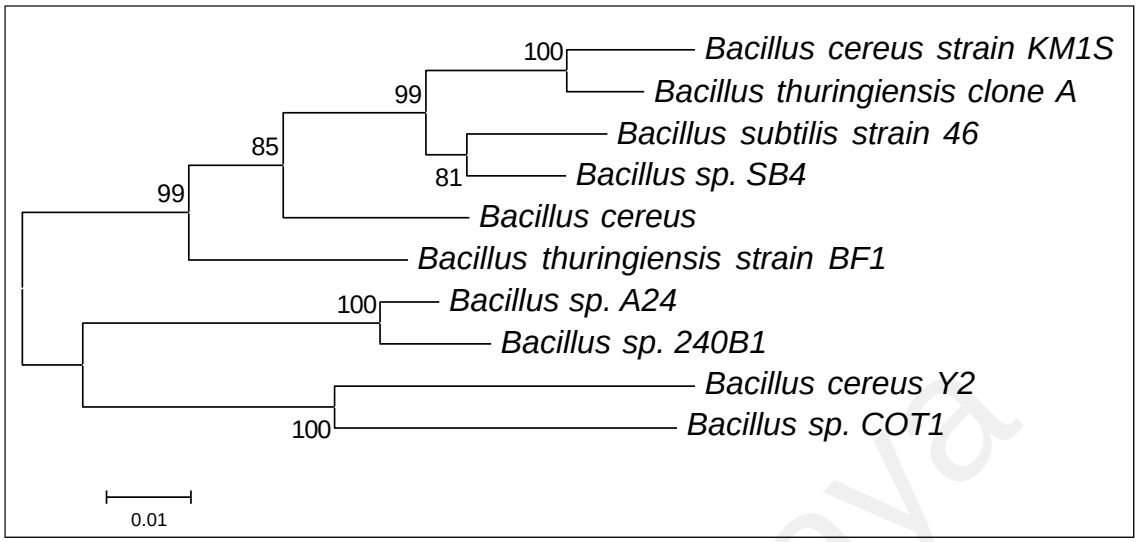
Region containing *aiiA* homologue gene (0.9 kb) was amplified from genomic DNA extracted from strain KM1S. The PCR primers *aiia* gene forward and reverse primers and the PCR conditions were as described in Materials and Methods (Section 2.12). The PCR product was subsequently gel-excised, column-purified and ligated into pGEM®-T Easy cloning vector (Section 2.13). DNA was analyzed by electrophoretic examination in a submerged horizontal agarose gel (0.8 to 1.0% w/v) containing EtBr (0.5 µg/ml), flooded with 1× TBE buffer. DNA was 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).

Figure 4.20 AHL-degrading activity of recombinant *E. coli* DH5*α* carrying the *aiiA* gene from *Bacillus cereus* strain KM1S



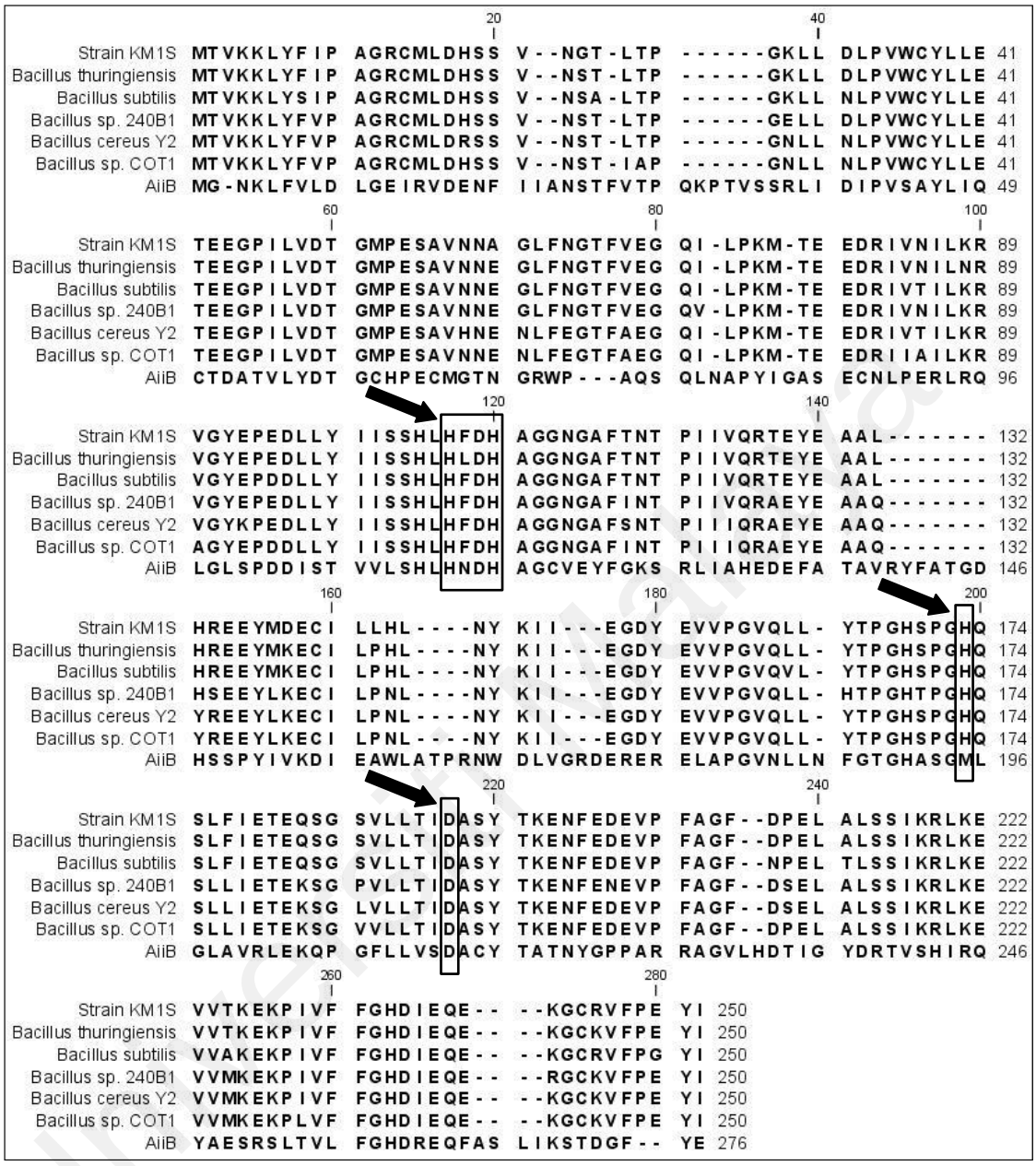
The degradation of 3-oxo-C6-HSL by the recombinant *E. coli* harboring the pGEM-*aiiA*$_{KM1S}$ was monitored using *C. violaceum* CV026 as reporter strains. The *E. coli* DH5*α* carrying pGEM-T vector without any insert was used as negative control, which was named *E. coli* DH5*α*(pGEM). Rows from top to bottom: 1. *E. coli* DH5*α*(pGEM-*aiiA*$_{KM1S}$), 2. *E. coli* DH5*α*(pGEM), 3. PBS buffer (100 mM, pH 6.5)

Figure 4.21 *aiiA* gene-based phylogenetic analysis



aiiA gene-based phylogenetic tree showing the phylogenetic position of *aiiA* gene of *B. cereus* strain KM1S generated using Neighbour-Joining algorithm. The horizontal bar at the bottom represents evolutionary distance as 0.01 changes per nucleotide position, determined by measuring the lengths of the horizontal lines connecting the species. GenBank accession number: *Bacillus thuringiensis* clone A AHL-lactonase gene (DQ440582), *Bacillus subtilis* strain 46 AHL-lactonase (*aiiA*) gene (EF655619), *Bacillus* sp. SB4 AHL-lactonase (AiiA) gene (AY483161), *Bacillus cereus* AiiA gene (AY943831), *Bacillus thuringiensis* strain BF1 AHL-lactonase (*aiiA*) gene (EF219409), *Bacillus* sp. A24 AiiA (*aiiA*) gene (AF397400), *Bacillus* sp. 240B1 putative metallohydrolase (*aiiA*) gene (AF196486), *Bacillus cereus* Y2-*aiiA* gene for Quorum-quenching N-acyl-homoserine lactonase (AM235210), *Bacillus* sp. COT1 AHL-lactonase (*aiiA*-COT1) gene (AF350927).

Figure 4.22 Motif analysis of *aiiA* homologue gene of *B. cereus* strain KM1S



The motif analysis of *aiiA* homologue gene of *B. cereus* strain KM1S indicated that the encoded AiiA contains the motif $_{106}$HXDH-59 amino acids-H$_{169}$-21 amino acids-D$_{191}$ (the highlighted area), which is essential for the AHL degradation activity. GenBank accession number (in parentheses): *Bacillus thuringiensis* strain BF1 *aiiA* (EF219409), *Bacillus subtilis* strain BS1 *aiiA* (DQ000640), *Bacillus* sp. 240B1 *aiiA* (AF196486), *Bacillus* sp. COT1 *aiiA* (AF350927), *B. cereus* Y2 *aiiA* (AM235210), *A. tumefaciens* strain C58 AiiB (NC_003065.3:84344-85174).

Strain MW3A and strain 2WS8

Amplification and cloning of the *pvdQ* and *quiP* gene

The *pvdQ* and *quiP* genes of *P. aeruginosa* strain MW3A and *P. aeruginosa* strain 2WS8 were amplified with genomic DNA as the template using *pvdQ* and *quiP* genes forward and reverse primers (Table 2.3). The amplicons size was approximately 2.5 kb for both genes (Figure 4.23). Sequencing was performed using vector-specific universal primers T7, SP6 and the internal primers PvdQ713F, QuiP664F, and QuiP1410F (Table 2.3). Partial nucleotide sequences were obtained in both of the *pvdQ* and *quiP* genes. The *pvdQ* gene sequences for *P. aeruginosa* strain MW3A and *P. aeruginosa* strain 2WS8 have been deposited at GenBank under the GenBank accession number GQ423680 and GQ423682, respectively. The *quiP* gene sequences for *P. aeruginosa* strain MW3A and *P. aeruginosa* strain 2WS8 have been deposited at GenBank under the GenBank accession number GQ423677 and GQ423679, respectively. The sequences obtained are shown in Appendix 1.

Web-based similarity searches against the GenBank using the partial nucleotide sequences of the *pvdQ* gene of *P. aeruginosa* strain MW3A and *P. aeruginosa* strain 2WS8 (626 nucleotides) suggested that all nucleotide sequences were highly similar to a gene encoding penicillin acylase in *Pseudomonas aeruginosa* UCBPP-PA14, sharing more than 99.4% sequence identities (i.e. more than 622 out of 626 nucleotides specifically aligned; GenBank accession number 115583796:3004988-3007276; last updated on 4th January 2010).

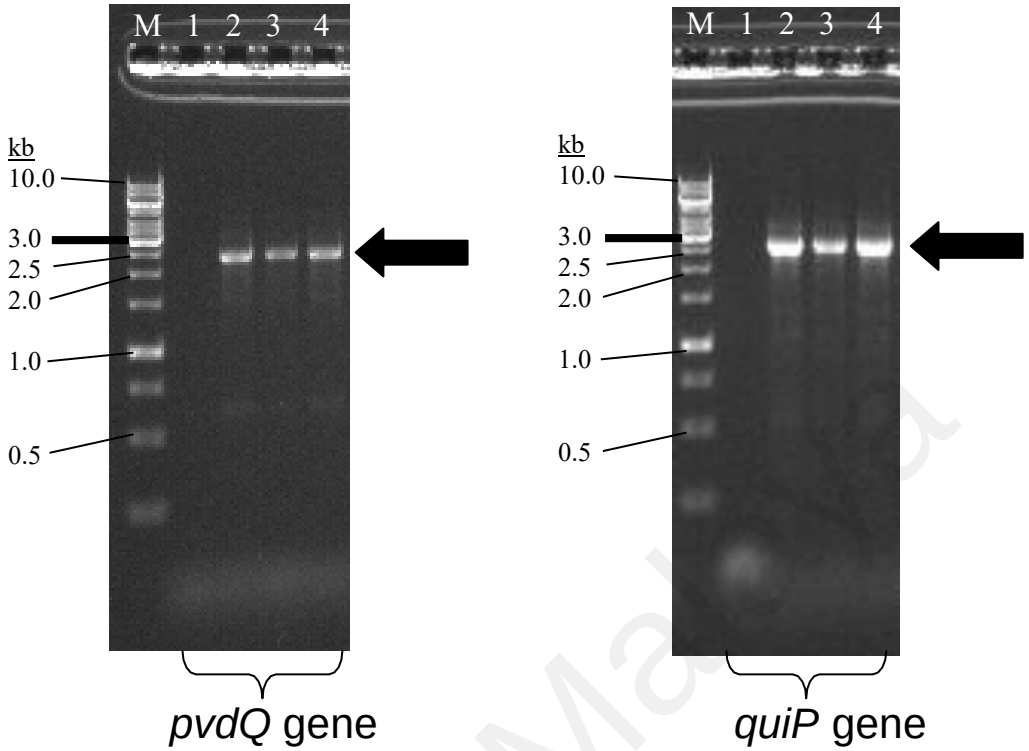
Phylogenetic analysis based on *pvdQ* gene indicated that the *pvdQ* homologue genes cloned from *P. aeruginosa* strain MW3A and *P. aeruginosa* strain 2WS8 were highly conserved as compared with *P. aeruginosa pvdQ* genes (Figure 4.24).

Web-based similarity searches against the GenBank using the partial nucleotide sequences of the *quiP* gene of *P. aeruginosa* strain MW3A and *P. aeruginosa* strain

2WS8 (1459 nucleotides) suggested that all nucleotide sequences were highly similar to a gene encoding QuiP in *Pseudomonas aeruginosa* UCBPP-PA14, sharing more than 99.0% sequence identities (i.e. more than 1444 out of 1459 nucleotides specifically aligned; GenBank accession number 115583796:4527711-4530254; last updated on 4th January 2010).

Phylogenetic analysis based on *quiP* gene indicated that the *quiP* homologue genes cloned from *P. aeruginosa* strain MW3A and *P. aeruginosa* strain 2WS8 were highly conserved as compared with *quiP* genes from GenBank databases (Figure 4.25).

Figure 4.23 *pvdQ* and *quiP* genes PCR amplification



Legends:

M: GeneRuler^{TM} 1kb DNA Ladders (Fermentas)

1: Negative control (PCR mixture where DNA was replaced with sdH_2O)

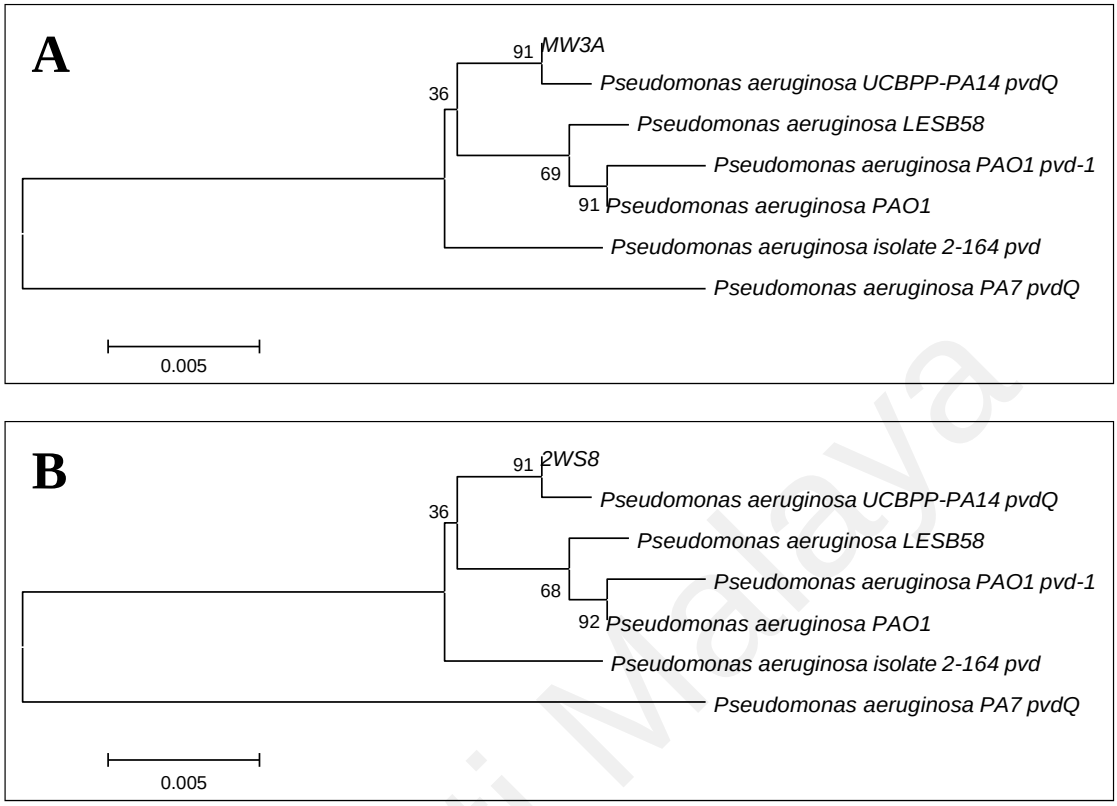
2: Strain MW3A

3: Strain 2WS8

4: Positive control (*Pseudomonas aeruginosa* PAO1)

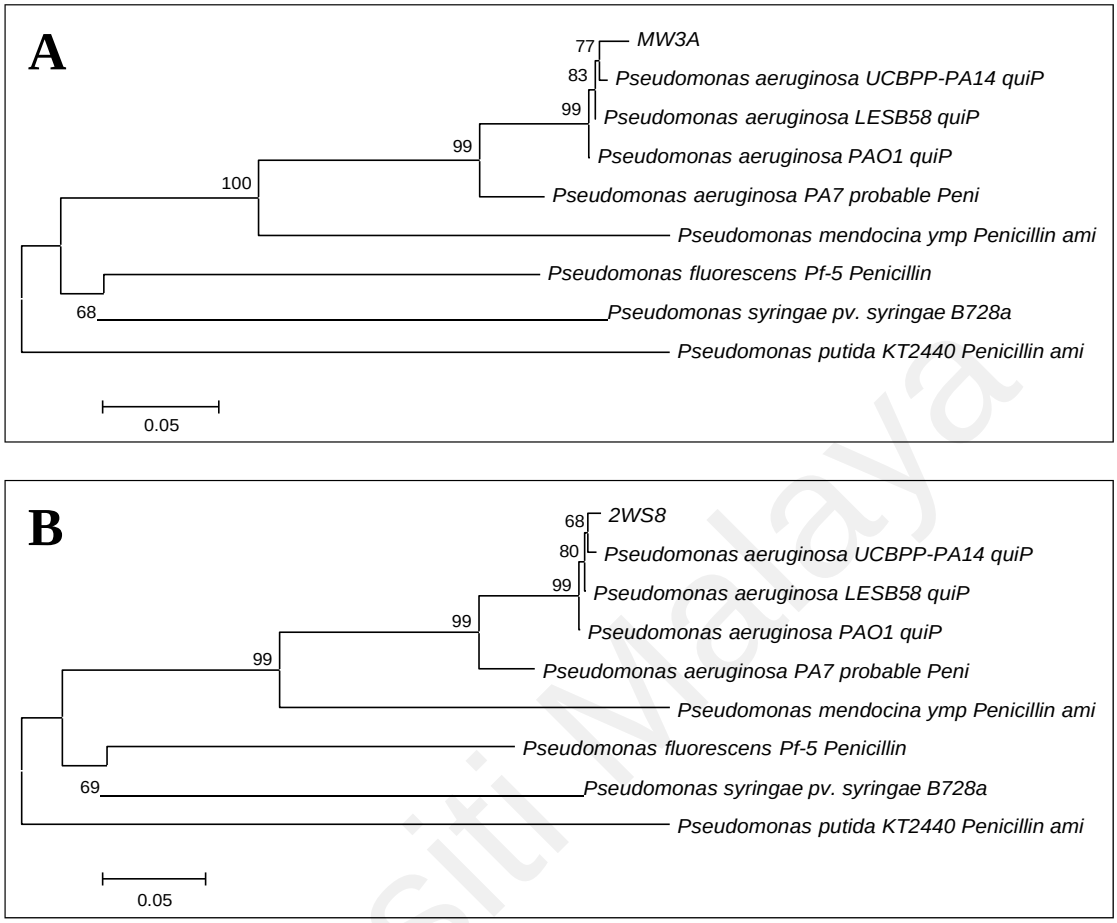
pvdQ and *quiP* homologue genes (approximately 2.5 kb) were amplified from strains MW3A and 2WS8 purified genomic DNA, eletrophoresed and visualized on a 302 nm UV transilluminator (TFM-20V Transilluminator, UVP Inc. USA). The image was digitally photographed with DigiDoc-IT Imaging System (UVP Inc. USA).

Figure 4.24 *pvdQ* gene-based phylogenetic analysis of (A) strain MW3A and (B) strain 2WS8



pvdQ gene-based phylogenetic tree showing the phylogenetic position of *pvdQ* gene of *P. aeruginosa* strain MW3A and *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, determined by measuring the lengths of the horizontal lines connecting the species. GenBank accession number (in parentheses): *Pseudomonas aeruginosa* UCBPP-PA14 *pvdQ* (CP000438: 3004988 to 3007276), *Pseudomonas aeruginosa* LESB58 *pvdQ* (FM209186: 3206318 to 3208606), *Pseudomonas aeruginosa* PAO1 *pvdQ* (AE004091: 2636517 to 2638805), *Pseudomonas aeruginosa* PAO1 *pvd-1* gene (Z25465), *Pseudomonas aeruginosa* isolate 2-164 pyoverdin biosynthetic locus (AF540993), *Pseudomonas aeruginosa* PA7 *pvdQ* (CP000744: 2955521 to 2957803).

Figure 4.25 *quiP* gene-based phylogenetic analysis of (A) strain MW3A and (B) strain 2WS8



quiP gene-based phylogenetic tree showing the phylogenetic position of *quiP* gene of *P. aeruginosa* strain MW3A and *P. aeruginosa* strain 2WS8 generated using Neighbour-Joining algorithm. The horizontal bar at the bottom represents evolutionary distance as 0.05 changes per nucleotide position, determined by measuring the lengths of the horizontal lines connecting the species. GenBank accession number (in parentheses): *Pseudomonas aeruginosa* UCBPP-PA14 *quiP* (CP000438: 4527711 to 4530254), *Pseudomonas aeruginosa* LESB58 *quiP* (FM209186: 4725132 to 4727675), *Pseudomonas aeruginosa* PAO1 *quiP* (AE004091: 1119674 to 1122217), *Pseudomonas aeruginosa* PA7 probable Penicillin amidase (CP000744: 4478165 to 4480708), *Pseudomonas mendocina* ymp Penicillin amidase (CP000680: 1577468 to 1579981),

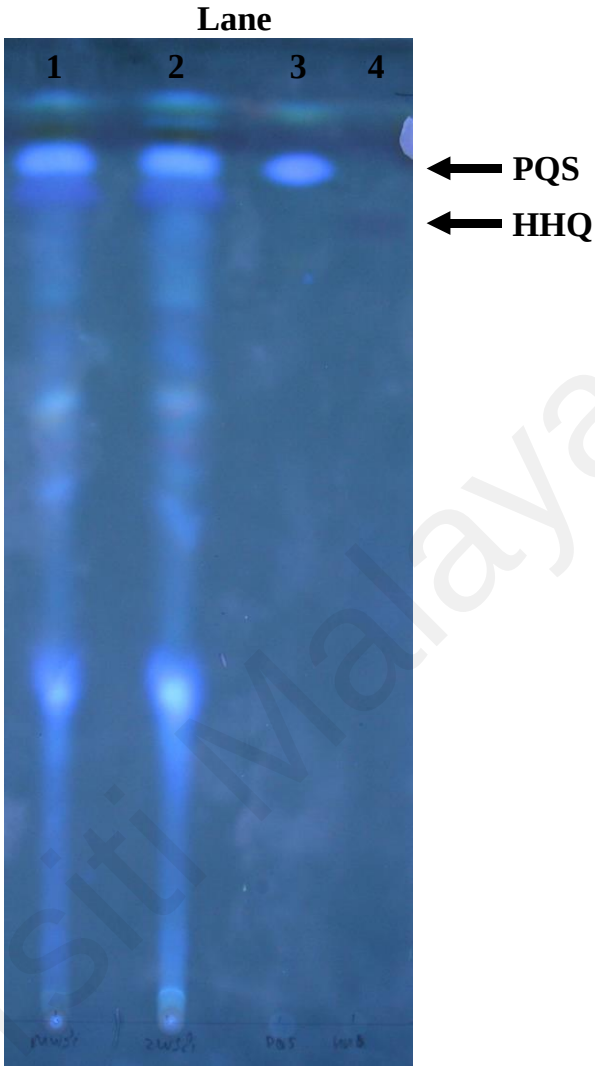
Pseudomonas fluorescens Pf-5 Penicillin amidase family protein (CP000076: 1438348 to 1440777), *Pseudomonas syringae* pv. *syringae* B728a Penicillin amidase (CP000075: 4609176 to 4611650), *Pseudomonas putida* KT2440 Penicillin amidase (AE015451: 1265544 to 1267985).

4.9 Identification of PQS by thin layer chromatography (TLC)

To check for the presence of PQS in cultures of *P. aeruginosa* strain MW3A and *P. aeruginosa* strain 2WS8, organic phase extraction of spent supernatant was performed on culture medium of *P. aeruginosa* strain MW3A and *P. aeruginosa* strain 2WS8 as described in Section 2.19. The resuspended extracts were analysed by TLC and visualized under UV at 302 nm (Figure 4.26).

The PQS synthetic standard migrated with a characteristic relative migration (R_f) value and fluoresced blue under UV light. Based on the comparison of R_f value with synthetic PQS, tentative positive PQS spots were identified on TLC plate in supernatant extracts of the two *Pseudomonas* isolates being tested.

Figure 4.26 Detection of presence of PQS in the bacterial spent supernatants



Legends:

Lane 1: MW3A supernatant extract, 10 µl

Lane 2: 2WS8 supernatant extract, 10 µl

Lane 3: PQS synthetic standard (2.5 µg)

Lane 4: HHQ synthetic standard (2.5 µg)

Spent supernatant extracts were analyzed by TLC by using a mixture of dichloromethane:methanol (95:5) as the mobile phase. The PQS molecules (marked by arrow) 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).

CHAPTER FIVE: DISCUSSION

5.1 Isolation of AHL degrading microorganisms from various environments

The enrichment procedure based on the utilization of 3-oxo-C6-HSL as sole carbon source has yielded four AHL degrading strains from three environmental samples, i.e. rainforest soil, wetland water and marine water. The KG medium has been shown to be able to select and enrich quorum quenching bacteria from a wide range of environments (Chan *et al.*, 2009). In addition to that, the present work demonstrated the isolation of more quorum quenching bacteria from the environmental samples using KG medium.

Malaysian rainforest soil has been selected as one of the source to study because of the potentially diverse flora and fauna that can be harnessed and exploited. Malaysia is known as one of the world's 12 hot spots for biodiversity. The wetland water and marine water were studied using the same method as described in Chan *et al.* (2009), with slight modification on the composition of KG medium. The concentration of all the salts was increased to produce a higher salinity in the modified KG medium, to adjust for the osmotic pressure for halophilic bacteria in these environments. KG medium was buffered to pH 6.5 using MES to prevent lactonolysis of AHLs under alkaline conditions.

A total of three quorum quenching bacteria were isolated from the rainforest soil and marine water. A rod-shaped Gram-positive bacterium, designated strain KM1S, was isolated from rainforest soil. Phylogenetic analysis based on near complete 16S rDNA showed that strain KM1S belonged to *Bacillus cereus* of the Bacillaceae family. On the other hand, two rod-shaped Gram-negative bacteria were isolated from marine water,

designated strain MW3A and strain 2WS8. Phylogenetic analysis based on 16S rDNA gene sequences showed that the two strains isolated from marine water clustered to *Pseudomonas aeruginosa*, belonging to the γ-proteobacteria class, within the Pseudomonaceae family.

5.2 Novel quorum quenching yeast

Interestingly, KG medium was able to enrich and select for quorum quenching fungal strain other than quorum quenching bacteria. A fungal strain WW1C was isolated from wetland water sample. Microscopically, well-developed hyphal structures and barrel-shaped arthroconidia were observed. Phylogenetic analyses based on the 18S rDNA and ITS region showed that this isolate belonged to *Trichosporon loubieri*. Furthermore, strain WW1C could grow at 37°C as well as 42°C in medium containing cycloheximide, these are the characteristics that distinguished *T. loubieri* from other species in the same genus. Thus molecularly and physiologically, it was confirmed that strain WW1C is *T. loubieri.*

Trichosporon sp. had been shown to be readily isolated from various environments such as soil and water, and they are capable of degrading aromatic compounds. For example, it has been reported that *Trichosporon cutaneum* was capable of growing on phenol, resorcinol, α-methylstyrene, acetophenone, and p-cresol as the carbon source (Gaal and Neujahr, 1979; Gerginova *et al.*, 2007). The degradation of AHL by *T. loubieri* strain WW1C expands the list of diverse metabolic traits exhibited by members of this genus.

To our knowledge, this is the first report of AHL degradation activity in basidiomycetous yeast, which is capable of degrading a variety of AHLs and growing on 3-oxo-C6-HSL. Growth curve study showed that the presence or absence of NH_4Cl as the nitrogen source did not affect significantly the growth rate of *T. loubieri* strain

WW1C, suggesting the capability of this isolate to assimilate 3-oxo-C6-HSL as both carbon and nitrogen source. To date, quorum quenching activity by fungi was only reported for *Penicillium* spp. (Rasmussen *et al.*, 2005) and a number of forest root-associated fungi belonging to the *Ascomycota* and *Basidiomycota* lineages (Uroz *et al.*, 2008). Although *T. loubieri* strain WW1C was initially isolated based on its ability to degrade 3-oxo-C6-HSL, the strain was also found to efficiently degrade a variety of AHLs with a carbon chain ranging from C4 to C10, with the highest efficiency in C8-HSL (Table 3.1). Remarkably, *T. loubieri* strain WW1C also degraded the unsubstituted AHLs with odd number *N*-acyl side chain i.e. C7-HSL. Heat-treated resting cells of *T. loubieri* strain WW1C failed to degrade AHL, suggesting that a heat labile factor, for instance enzymes could be involved.

Based on the recyclization of lactone ring by acidification (pH <2) (Yates *et al.*, 2002), *T. loubieri* strain WW1C degraded AHLs via AHL lactonase activity. The presence of putative *aiiA* homologue was investigated in this isolate using PCR method, however, no amplicon was observed. It is likely that *T. loubieri* strain WW1C may possess a novel class of AHL lactonase distinctive of *aiiA*-class of AHL lactonase. Further study is required to determine the gene involved in the metabolic pathway and the degradation of the AHLs.

Recently, it has become apparent that similar to bacteria, fungi also utilize quorum sensing to regulate population-level behavior such as pathogenesis (Hogan, 2006). Various fungal signaling molecules have been identified e.g. farnesol and tyrosol in *Candida albicans*, and trisporic acid produced by zygomycetes (reviewed in Hogan, 2006). However, no production of AHL was reported in fungi hitherto. When tested under a range of incubation temperature (16°C, 23°C, and 28°C) and high salinity (2.5% NaCl), no production of AHL was detected in *T. loubieri* strain WW1C.

The discovery of AHL degrading activity in *T. loubieri* strain WW1C raised the question of the purpose of existence of such catabolic activity in eukaryotic microorganism. In the soil, some bacteria are known to have chitinolytic, antibiosis or antifungal activities regulated by quorum sensing (Chernin *et al.*, 1998). For example, phenazine antibiotics produced by *Pseudomonas aureofaciens* were shown to protect wheat from take-all, a disease caused by the fungus *Gaeumannomyces graminis* var. *tritici* (Pierson *et al.*, 1994; Wood *et al.*, 1997). The degradation of AHL could be a strategy developed by the fungi to disrupt the quorum sensing system and disrupt the deleterious bacterial functions, and thereby giving the fungi survival advantages in the competitive habitats. A number of reports also suggest that higher eukaryotes employ quorum quenching to control colonizing or pathogenic bacteria. In our studies, not only does the *T. loubieri* strain WW1C degrade the AHL signals, it further metabolizes the signaling molecules as energy sources.

Wang and Leadbetter (2005) reported that AHLs were biodegraded and mineralized to CO_2 rapidly in soil samples, thus preventing the signaling molecules from accumulating in the environment. This showed that quorum quenching microorganisms are indeed active in the environment. Other than conferring competitive advantages to the quorum quenching microorganisms, the degradation of AHLs might also serve to either prevent signal cross talk or as a full system reset. Reports have shown that beneficial or deleterious cross talk might occur in species-rich ecosystems such as soil environment (Pierson *et al.*, 1998). So the AHL degradation activity could serve to insulate microbial aggregates that might otherwise occur between spatially separated microbial aggregates (Wang and Leadbetter, 2005). However, whether or not this also confers an advantage specifically through QQ of neighbouring bacteria is not known.

5.3 *Bacillus cereus* strain KM1S and kinetic analysis of AHL turnover

B. cereus strain KM1S was capable of rapid turnover of 3-oxo-C6-HSL and 3-oxo-C8-HSL *in vitro*. Since no reported work has been done to examine the kinetic of AHL turn over using RRLC, we set to study the enzymatic AHL-degradation of *B. cereus* strain KM1S cells using RRLC. From the standard curves constructed using synthetic AHL standards, it was found that the amount of AHL is directly proportional to the areas below the curve of the RRLC chromatograms within the working range of 0.025 to 0.15 µg/µl, showing very high R^2 value (R^2>0.99). Hence, by using linear regression analysis, residual AHL concentration at each time interval can be determined through interpolation. By plotting the residual AHL concentrations against time on a semi-log graph, the AHL degradation kinetic can be determined from the linear portion of the curve.

Interestingly, it was found that this isolate rapidly degraded both 3-oxo-C6-HSL and 3-oxo-C8-HSL within 15 min. About 40% of the 3-oxo-C6-HSL and 60% of the 3-oxo-C8-HSL was degraded within 15 min as determined by RRLC analysis. *B. cereus* strain KM1S degraded 3-oxo-C8-HSL efficiently, with estimated activity of 6.56 µg h^{-1} per 10^9 CFU/ml. In a similar approach, we found that this isolate exhibited a lower activity towards 3-oxo-C6-HSL (4.98 µg h^{-1} per 10^9 CFU/ml) as compared to 3-oxo-C8-HSL, suggesting a preference of AHL degradation with longer acyl chain. Another possible application of this method is the study of environmental variables effect on the enzyme activity. For example, by comparing the kinetics of AHL turnover through a range of pH or temperature, the optimal conditions for the AHLase enzyme activity can be determined.

5.4 Analysis of *aiiA* gene in strain KM1S

Dong *et al.* (2000) reported *Bacillus* sp. 240B1 and the gene encoding autoinducer inactivation enzyme (*aiiA*) that degrades AHLs. Therefore, it is of interest to investigate further the presence of *aiiA* homologue in *Bacillus cereus* strain KM1S. We have PCR-amplified the *aiiA* homologue and the putative zinc-binding motif is conserved in this newly identified AiiA homologue. It has been reported by Dong *et al.* (2000) that this motif played a critical role in the AHL degrading activity. Peptide level analysis revealed that the *aiiA* homologue gene of *B. cereus* strain KM1S indeed contains the motif $_{106}$HXDH-59 amino acids-H$_{169}$-21 amino acids-D$_{191}$. These data suggested that this enzyme belonged to the AHL lactonase family. The presence of lactonase homologue *aiiA* in this isolate was confirmed. However, the ecological role of this AHL lactonase is still not clear.

5.5 *Pseudomonas* strains isolated from marine water

P. aeruginosa is a common bacterium found in diverse environmental conditions due to its metabolic versatility. Consequently, it is ubiquitous in the soil and water ecosystems including the marine ecosystems. *P. aeruginosa* is well known for its ability of metabolize an extensive number of substrated including toxic organic chemicals and aromatic compounds. In this study, the marine *Pseudomonas* strains were able to degrade 3-oxo-C6-HSL, C6-HSL, and C8-HSL, although with differing efficiency. In addition to the reported capability of *P. aeruginosa* PAO1 to degrade AHLs having acyl side chain of C8 or more, the marine *Pseudomonas* strains isolated in this study were able to degrade AHLs with acyl side chain of C6, with or without 3-oxo substitution. The quorum quenching genes, *pvdQ* and *quiP* genes, have been identified and described (Huang *et al.*, 2003; Huang *et al.*, 2006; Sio *et al.*, 2006). By using the reported primers, homologues of *pvdQ* and *quiP* genes were PCR-amplified. Homologues of both *pvdQ*

and *quiP* genes were confirmed present in the marine *Pseudomonas* strains and analysed by phylogenetic approach. It is suggested by Huang and co-workers that these two AHL-acylases play critical role in the AHL degradation and utilization, including the *P. aeruginosa* native 3-oxo-C12-HSL, to control different ratios of their two AHL signaling molecules during different phenotypic stages e.g. biofilm and planktonic states (Huang *et al.*, 2006).

5.6 Production of PQS by strains MW3A and 2WS8

Pesci *et al.* (1999) discovered the presence of a third signaling molecule in *P. aeruginosa* known as *Pseudomonas* quinolone signal (PQS). Therefore it is of interest to investigate the production of PQS in the *Pseudomonas* strains isolated in this study. TLC analysis revealed that these two strains produced PQS molecules under the present culture conditions, by comparing to the R_f values and the fluorescence colour under UV of a synthetic PQS standard. This demonstrates the co-existence of quorum sensing and quorum quenching in these strains. So far, such phenomenon has only been reported in *P. aeruginosa*, as a mean of fine tuning the level of quorum sensing signaling molecules in the population (Sio *et al.*, 2006).

5.7 Future work

A major finding of this study is the identification of a novel quorum quenching yeast strain *T. loubieri* strain WW1C isolated from the wetland water. Many intriguing questions arise from the identification of this novel quorum quencher including: What is the exact chemistry and metabolic pathway of the AHL-degradation? Is the expression of the degradation molecules activated by the presence of AHL, or are they expressed continuously? What is the ecological role of the AHL-degrading mechanism in the

environment and the potential use of the AHL-degradation mechanism to antagonize quorum sensing-regulatory processes?

Little is known regarding eukaryotic quorum quenching, especially by the mechanism of AHL signals inactivation, as compared to quorum quenching in bacteria. Future work may focus on these queries, that extensive study on the novel quorum quenching strain might contribute to quorum sensing research and development of biocontrol agents or therapeutic strategies directed at quorum sensing-dependent infection control in both plants and animals.

Conclusion

The present work demonstrated for the first time the degradation of AHL by basidiomycetes yeast isolated from wetland water. The finding of this quorum queching yeast may implies that the AHL degradation mechanism maybe widespread across the kingdom of fungi and its potential roles in medical, agricultural and industrial applications. As yet, it is by no means to conclude that the AHL-degrading enzymes evolved to degrade AHL signals as their natural substrates, given the diverse catabolic ability of this isolate to degrade various xenobiotic compounds, but their impact on AHL-dependent quorum sensing bacteria cannot be underestimated.

Another important finding in this study is the development of the method to estimate the kinetic of AHL turnover by using RRLC. By comparing the estimated AHL degradation activities, the relative enzyme activity on different AHL derivatives can be determined, whether the length or the substitution at C3 position of acyl side chain has any impact on the enzyme-substrate interaction.

More investigation is needed to gain further insights into the ecological role and the gene level regulation of these AHL degradation mechanisms. These enzymes may hold great promise in infectious disease control through quenching of the microbial quorum sensing signaling.

Bibliography

Andersen, J. B., Heydorn, A., Hentzer, M., Eberl, L., Geisenberger, O., Christensen, B. B., Molin, S. and Givskov, M. (2001). "*gfp*-based *N*-acyl homoserine-lactone sensor systems for detection of bacterial communication." Appl. Environ. Microbiol. **67**: 575-585.

Antonio, R. V. and Creczynski-Pasa, T. B. (2004). "Genetic analysis of violacein biosynthesis by *Chromobacterium violaceum*." Genet. Mol. Res. **3**: 85-91.

Atkinson, S., Chang, C. Y., Patrick, H. L., Buckley, C. M. F., Wang, Y., Sockett, R. E., Cámara, M. and Williams, P. (2008). "Functional interplay between the *Yersinia pseudotuberculosis* YpsRI and YtbRI quorum sensing systems modulates swimming motility by controlling expression of *flhDC* and *fliA*." Mol. Microbiol. **69**: 137-151.

Atkinson, S., Chang, C. Y., Sockett, R. E., Camara, M. and Williams, P. (2006). "Quorum sensing in *Yersinia enterocolitica* controls swimming and swarming motility." J. Bacteriol. **188**: 1451-1461.

Atkinson, S., Throup, J. P., Stewart, G. S. and Williams, P. (1999). "A hierarchical quorum-sensing system in *Yersinia pseudotuberculosis* is involved in the regulation of motility and clumping." Mol. Microbiol. **33**: 1267-1277.

Bainton, N. J., Bycroft, B. W., Chhabra, S. R., Stead, P., Gledhill, L., Hill, P. J., Rees, C. E. D., Winson, M. K., Salmond, G. P., Stewart, G. S. and Williams, P. (1992). "A general role for the *lux* autoinducer in bacterial cell signaling: control of antibiotic biosynthesis in *Erwinia*." Gene **116**: 87-91.

Bainton, N. J., Bycroft, B. W., Chhabra, S. R., Stead, P., Gledhill, L., Hill, P. J., Rees, C. E. D., Winson, M. K., Salmond, G. P. C. and et al. (1992b). "A general role for the *lux* autoinducer in bacterial cell signalling: control of antibiotic synthesis in *Erwinia*." Gene **116**: 87-91.

Bainton, N. J., Stead, P., Chhabra, S. R., Bycroft, B. W., Salmond, G. P. C., Stewart, G. S. A. B. and Williams, P. (1992a). "*N*-(3-oxohexanoyl)-L-homoserine lactone regulates carbapenem antibiotic production in *Erwinia carotovora*." Biochem. J. **288**: 997-1004.

Barnard, A. M. L. and Salmond, G. P. C. (2005). "Quorum sensing: The complexities of chemical communication between bacteria." Complexus **2**: 87-101.

Bertani, I. and Venturi, V. (2004). "Regulation of the *N*-acyl homoserine lactone-dependent quorum-sensing system in rhizosphere *Pseudomonas putida* WCS358 and cross-talk with the stationary-phase RpoS sigma factor and the global regulator GacA." Appl. Environ. Microbiol. **70**: 5493-5502.

Calfee, M. W., Coleman, J. P. and Pesci, E. C. (2001). "Interference with *Pseudomonas* quinolone signal synthesis inhibits virulence factor expression by *Pseudomonas aeruginosa*." Proc. Natl. Acad. Sci. USA **20**: 11633-11637.

Carlier, A., Uroz, S., Smadja, B., Fray, R., Latour, X., Dessaux, Y. and Faure, D. (2003). "The Ti plasmid of *Agrobacterium tumefaciens* harbors an attM-paralogous gene, *aiiB*, also encoding *N*-acyl homoserine lactonase activity." Appl. Environ. Microbiol. **69**: 4989-4993.

Cha, C., Gao, P., Chen, Y. C., Shaw, P. D. and Farrand, S. K. (1998). "Production of acyl-homoserine lactone quorum-sensing signals by Gram-negative plant-associated bacteria." Mol. Plant Microbe Interact. **11**: 1119-1129.

Chan, K. G., Yin, W. F., Sam, C. K. and Koh, C. L. (2009). "A novel medium for the isolation of *N*-acylhomoserine lactone-degrading bacteria." J. Ind. Microbiol. Biotechnol. **36**: 247-251.

Chernin, L. S., Winson, M. K., Thompson, J. M., Haran, S., Bycroft, B. W., Chet, I., Williams, P. and Stewart, G. S. (1998). "Chitinolytic activity in *Chromobacterium violaceum*: substrate analysis and regulation by quorum sensing." J. Bacteriol. **180**: 4435-4441.

Chhabra, S. R., Philipp, B., Eberl, L., Givskov, M., Williams, P. and Camara, M. (2005). Extracellular communication in bacteria. Chemistry of Pheromones and Other Semiochemicals 2. S. Schulz. Berlin/Heidelberg, Springer**:** 279–315.

Chhabra, S. R., Stead, P., Bainton, N. J., Salmond, G. P., Stewart, G. S. A. B., Williams, P. and Bycroft, B. W. (1993). "Autoregulation of carbapenem biosynthesis in *Erwinia carotovora* by analogues of *N*-(3-oxohexanoyl)-L-homoserine lactone." J. Antibiot. (Tokyo) **46**: 441-454.

Chin, A. W. T. F., van den Broek, D., de Voer, G., van der Drift, K. M., Tuinman, S., Thomas-Oates, J. E., Lugtenberg, B. J. and Bloemberg, G. V. (2001). "Phenazine-1-carboxamide production in the biocontrol strain *Pseudomonas chlororaphis* PCL1391 is regulated by multiple factors secreted into the growth medium." Mol. Plant Microbe Interact. **14**: 969-979.

Chin, A. W. T. F., van den Broek, D., Lugtenberg, B. J. and Bloemberg, G. V. (2005). "The *Pseudomonas chlororaphis* PCL1391 sigma regulator *psrA* represses the production of the antifungal metabolite phenazine-1-carboxamide." Mol. Plant Microbe Interact. **18**: 244-253.

Chowdhary, P. K., Keshavan, N., Nguyen, H. Q., Peterson, J. A., Gonzales, J. E. and Haines, D. C. (2007). "*Bacillus megaterium* CYP102A1 oxidation of acyl homoserine lactones and acyl homoserines." Biochemistry **46**: 14429-14437.

Christensen, A. B., Riedel, K., Eberl, L., Flodgaard, L. R., Molin, S., Gram, L. and Givskov, M. (2003). "Quorum-sensing-directed protein expression in *Serratia proteamaculans* B5a." Microbiology **149**: 471-483.

Chun, C. K., Ozer, E. A., Welsh, M. J., Zabner, J. and Greenberg, E. P. (2004). "Inactivation of a *Pseudomonas aeruginosa* quorum-sensing signal by human airway epithelia." Proc. Natl Acad. Sci. USA **101**: 3587–3590.

Cubo, M. T., Economou, A., Murphy, G., Johnston, A. W. and Downie, J. A. (1992). "Molecular characterization and regulation of the rhizosphere-expressed genes *rhiABCR* that can influence nodulation by *Rhizobium leguminosarum* biovar *viciae*." J. Bacteriol. **174**: 4026-4035.

Delalande, L., Faure, D., Raffoux, A., Uroz, S., D'Angelo-Picard, C., Elasri, M., Carlier, A., Berruyer, R., Petit, A., Williams, P. and Dessaux, Y. (2005). "*N*-hexanoyl-L-homoserine lactone, a mediator of bacterial quorum-sensing regulation, exhibits plant-dependent stability and may be inactivated by germinating *Lotus corniculatus* seedlings." FEMS Microbiol. Ecol. **52**: 13-20.

Devine, J. H., Shadel, G. S. and Baldwin, T. O. (1989). "Identification of the operator of the *lux* regulon from *Vibrio fischeri* strain ATCC7744." Proc. Natl. Acad. Sci. USA **86**: 5688-5692.

Dewhirst, F. E., Paster, B. J. and Bright, P. L. (1989). "*Chromobacterium*, *Eikenella*, *Kingella*, *Neisseria*, *Simonsiella*, and *Vitreoscilla* species comprise a major branch of the beta group *Proteobacteria* by 16S ribosomal ribonucleic acid sequence comparison: transfer of *Eikenella* and *Simonsiella* to the family *Neisseriaceae* (emend.)." Int. J. Syst. Bacteriol. **39**: 258-266.

Diggle, S. P., Cornelis, P., Williams, P. and Camara, M. (2006). "4-Quinolone signalling in *Pseudomonas aeruginosa*: Old molecules, new perspectives." Int. J. Medic. Microbiol. **296**: 83-91.

Dong, Y. H., Gusti, A. R., Zhang, Q., Xu, J. L. and Zhang, L. H. (2002). "Identification of quorum quenching *N*-acyl homoserine lactonases from *Bacillus* species." Appl. Environ. Microbiol. **68**: 1754-1759.

Dong, Y. H., Wang, L. H., Xu, J. L., Zhang, H. B., Zhang, X. F. and Zhang, L. H. (2001). "Quenching quorum-sensing-dependent bacterial infection by an *N*-acyl homoserine lactonase." Nature **411**: 813-817.

Dong, Y. H., Xu, J. L., Li, X. Z. and Zhang, L. H. (2000). "AiiA, an enzyme that inactivates the acylhomoserine lactone quorum sensing signal and attenuates the virulence of *Erwinia carotovora*." Proc. Natl. Acad. Sci. USA **97**: 3526-3531.

Dong, Y. H. and Zhang, L. H. (2005). "Quorum sensing and quorum-quenching enzymes." Microbiol. **43**: 101-109.

Dong, Y. H., Zhang, X. F., Xu, J. L. and Zhang, L. H. (2004). "Insecticidal *Bacillus thuringiensis* silences *Erwinia carotovora* virulence by a new form of microbial antagonism, signal interference." Appl. Environ. Microbiol. **70**: 954-960.

Dumenyo, C. K., Mukherjee, A., Chun, W. and Chatterjee, A. K. (1998). "Genetic and physiological evidence for the production of *N*-acyl homoserine lactones by *Pseudomonas syringae* pv. *syringae* and other fluorescent plant pathogenic *Pseudomonas* species." Eur. J. Plant Pathol. **104**: 569-582.

Dunny, G. M. and Winans, S. C., Eds. (1999). Cell-cell signaling in bacteria. Washington, DC, American Society of Microbiology Press.

Dworjanyn, S. A., Nys, R. D. and Steinberg, P. D. (1999). "Localisation and surface quantification of secondary metabolites in the red alga *Delisea pulchra*." Mar. Biol. **133**: 727-736.

Eberhard, A., Burlingame, A. L., Eberhard, C., Kenyon, G. L., Nealson, K. H. and Oppenheimer, N. J. (1981). "Structural identification of autoinducer of *Photobacteriumfischeri* luciferase." Biochemistry **20**: 2444-2449.

Eberl, L., Winson, M. K., Sternberg, C., Stewart, G. S. A. B., Christiansen, G., Chhabra, S. R., Bycroft, B., Williams, P., Molin, S. and Givskov, M. (1996). "Involvement of *N*-acyl-L-homoserine lactone autoinducers in controlling the multicellular behavior of *Serratia liquefaciens*." Mol. Microbiol. **20**: 127-136.

Egland, K. A. and Greenberg, E. P. (1999). "Quorum sensing in *Vibrio fischeri*: elements of the *luxI* promoter." Mol. Microbiol. **31**: 1197-1204.

El-Sayed, A. K., Hothersall, J. and Thomas, C. M. (2001). "Quorum-sensing-dependent regulation of biosynthesis of the polyketide antibiotic mupirocin in *Pseudomonas fluorescens* NCIMB10586." Microbiology **147**: 2127-2139.

Engebrecht, J., Nealson, K. H. and Silverman, M. (1983). "Bacterial bioluminescence: isolation and genetic analysis of the functions from *Vibrio fischeri*." Cell **32**: 773-781.

Engebrecht, J. and Silverman, M. (1984). "Identification of genes and gene products necessary for bacterial bioluminescence." Proc. Natl. Acad. Sci. USA **81**: 4154-4158.

Farah, C., Vera, M., Morin, D., Haras, D., Jerez, C. A. and Guiliani, N. (2005). "Evidence for a functional quorum-sensing type AI-1 system in the extremophilic bacterium *Acidithiobacillus ferrooxidans*." Appl. Environ. Microbiol. **71**: 7033-7040.

Farrand, S. K., Hwang, I. and Cook, D. M. (1996). "The *tra* region of the nopaline-type Ti plasmid is a chimera with elements related to the transfer systems of RSF1010, RP4, and F." J. Bacteriol. **178**: 4233-4247.

Flagan, S., Ching, W. K. and Leadbetter, J. R. (2003). "*Arthrobacter* strain VAI-A utilizes acyl-homoserine lactone inactivation products and stimulates quorum signal biodegradation by *Variovorax paradoxus*." Appl. Environ. Microbiol. **69**: 909-916.

Flavier, A. B., Ganova-Raeva, L. M., Schell, M. A. and Denny, T. P. (1997). "Hierarchical autoinduction in *Ralstonia solanacearum*: control of acylhomoserine lactone production by a novel autoregulatory system responsive to 3-hydroxypalmitic acid methyl ester." J. Bacteriol. **179**: 7089-7097.

Flavier, A. B., Schell, M. A. and Denny, T. P. (1998). "An RpoS (sigmaS) homologue regulates acylhomoserine lactone-dependent autoinduction in *Ralstonia solanacearum*." Mol. Microbiol. **28**: 475-486.

Fletcher, M. P., Diggle, S. P., Camara M., and Williams, P. (2007). "Biosensor-based assays for PQS, HHQ and related 2-alkyl-4-quinolone quorum sensing signal molecules." Nature Protocols **2**:1254-1262.

Fuqua, C. and Greenberg, E. P. (2002). "Listening in on bacteria: acyl-homoserine lactone signalling." Nat. Rev. Microbiol. **3**: 685-695.

Fuqua, C., Parsek, M. R. and Greenberg, E. P. (2001). "Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing." Annu. Rev. Microbiol. **35**: 439-468.

Fuqua, W. C., Winans, S. C. and Greenberg, E. P. (1996). "Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcriptional regulators." Annu. Rev. Microbiol. **50**: 727-751.

Gaal, A. and Neujahr, H. Y. (1979). "Metabolism of phenol and resorcinol in *Trichosporon cutaneum*." J. Bacteriol. **137**: 13-21.

Gallagher, L. A., McKnight, S. L., Kuznetsova, M. S., Pesci, E. C. and Manoil, C. (2002). "Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa*." J. Bacteriol. **184**: 6472-6480.

Gerginova, M., Dimova, N., Ivanova, D. and Alexieva, Z. (2007). Studies on biodegradation of aromatic pollutants by *Trichosporon cutaneum* yeast strain. NATO Science Series. H. J. Heipieper, Springer Netherlands. **76:** 67-74.

Givskov, M., Eberl, L. and Molin, S. (1997). "Control of exoenzyme production, motility and cell differentiation in *Serratia liquefaciens*." FEMS Microbiol. Lett. **148**: 115-122.

Givskov, M., Nys, R. d., Manefield, M., Gram, L., Maximilien, R., Eberl, L., Molin, S., Steinberg, P. D. and Kjelleberg, S. (1996). "Eukaryotic interference with homoserine lactone mediated prokaryotic signaling." J. Bacteriol. **178**: 6618-6622.

Gram, L., Christensen, A. B., Ravn, L., Molin, S. and Givskov, M. (1999). "Production of acylated homoserine lactones by psychrotrophic members of the *Enterobacteriaceae* isolated from foods." Appl. Environ. Microbiol. **65**: 3458-3463.

Gram, L., Grossart, H. P., Schlingliff, A. and Kiorboe, T. (2002). "Posibble quorum sensing in marine snow bacteria: Production of acylated homoserine lactones by Roseobacter strains isolated from marine snow." Appl. Environ. Microbiol. **68**: 4111-4116.

Gray, K. M., Pearson, J. P., Downie, J. A., Boboye, B. E. and Greenberg, E. P. (1996). "Cell-to-cell signaling in the symbiotic nitrogen-fixing bacterium *Rhizobium leguminosarum*: autoinduction of a stationary phase and rhizosphere-expressed genes." J. Bacteriol. **178**: 372-376.

Greenberg, E. P., Chun, C. K., Ozer, E. A., Welsh, M. J. and Zabner, J. (2004). Enzymatic inactivation of a *Pseudomonas aeruginosa* quorum-sensing signal by human airway epithelia. Cell-cell Communication in bacteria (2nd), American Society for Microbiology conferences S5:1.

Gueho, E., Smith, M. T., de Hoog, G. S., Billon-Grand, G., Christen, R. and Batenburg-van der Vegte, W. H. (1992). "Contributions to a revision of the genus *Trichosporon*." Antonie van Leeuwenhoek **61**: 289-316.

Hakenbeck, R. and Stock, J. B. (1996). "Analysis of two-component signal transduction systems involved in transcriptional regulation." Methods Enzymol. **273**: 281-300.

Hanzelka, B. L. and Greenberg, E. P. (1996). "Quorum sensing in *Vibrio fischeri*: evidence that *S*-adenosylmethionine is the amino acid substrate for autoinducer synthesis." J. Bacteriol. **178**: 5291-5294.

Hao, G. and Burr, T. J. (2006). "Regulation of long-chain *N*-acyl-homoserine lactones in *Agrobacterium vitis*." J. Bacteriol. **188**: 2173-2183.

Hao, G., Zhang, H., Zheng, D. and Burr, T. J. (2005). "*luxR* homolog *avhR* in Agrobacterium vitis affects the development of a grape-specific necrosis and a tobacco hypersensitive response." J. Bacteriol. **187**: 185-192.

Hardman, A. M., Stewart, G. S. A. B. and Williams, P. (1998). "Quorum sensing and the cell-cell communication dependent regulation of gene expression in pathogenic and non-pathogenic bacteria." Antonie van Leeuwenhoek **74**: 199-210.

Hentzer, M. and Givskov, M. (2003). "Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections." J. Clin. Invest. **112**: 1300–1307.

Hentzer, M., Riedel, K., Rasmussen, T. B., Heydorn, A., Andersen, J. B., Parsek, M. R., Rice, S. A., Eberl, L., Molin, S., Høiby, N., Kjelleberg, S. and Givskov, M. (2002). "Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound." Microbiology **148**: 87–102.

Hentzer, M., Wu, H., Andersen, J. B., Riedel, K., Rasmussen, T. B., Bagge, N., Kumar, N., Schembri, M. A., Song, Z., Kristoffersen, P., Manefield, M., Costerton, J. W., Molin, S., Eberl, L., Steinberg, P., Kjelleberg, S., Høiby, N. and Givskov, M.

(2003). "Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors." EMBO J. **22**: 3803–3815.

Hoang, T. T. and Schweizer, H. P. (1999). "Characterization of *Pseudomonas aeruginosa* enoyl-acyl carrier protein reducatase (FabI): a target for the antimicrobial triclosan and its role in acylated homoserine lactone synthesis." J. Bacteriol. **181**: 5489–5497.

Hogan, D. A. (2006). "Talking to themselves: autoregulation and quorum sensing in fungi." Eukaryotic Cell **5**: 613-619.

Horng, Y. T., Deng, S. C., Daykin, M., Soo, P. C., Wei, J. R., Luh, K. T., Ho, S. W., Swift, S., Lai, H. C. and Williams, P. (2002). "The LuxR family protein SpnR functions as a negative regulator of *N*-acylhomoserine lactone-dependent quorum sensing in *Serratia marcescens*." Mol. Microbiol. **45**: 1655-1671.

Huang, J. J., Han, J. I., Zhang, L. H. and Leadbetter, J. R. (2003). "Utilization of acyl-homoserine lactone quorum signals for growth by a soil pseudomonad and *Pseudomonas aeruginosa* PAO1." Appl. Environ. Microbiol. **69**: 5941-5949.

Huang, J. J., Petersen, A., Whiteley, M. and Leadbetter, J. R. (2006). "Identification of QuiP, the product of gene PA1032, as the second acyl-homoserine lactone acylase of *Pseudomonas aeruginosa* PAO1." Appl. Environ. Microbiol. **72**: 1190–1197.

Jacobi, C. A., Bach, A., Eberl, L., Steidle, A. and Heesemann, J. (2003). "Detection of *N*-(3-oxohexanoyl)-L-homoserine lactone in mice infected with *Yersinia enterocolitica* serotype O8." Infect. Immun. **71**: 6624-6626.

Jafra, S., Przysowa, J., Czajkowski, R., Michta, A., Garbeva, P. and van der Wolf, J. M. (2006). "Detection and characterization of bacteria from the potato rhizosphere degrading *N*-acyl-homoserine lactone." Can. J. Microbiol. **52**: 1006-1015.

Jones, S., Yu, B., Bainton, N. J., Birdsall, M., Bycroft, B. W., Chhabra, S. R., Cox, A. J., Golby, P., Reeves, P. J., Stephens, S. and et al. (1993). "The *lux* autoinducer regulates the production of exoenzyme virulence determinants in *Erwinia carotovora* and *Pseudomonas aeruginosa*." EMBO J. **12**: 2477-2482.

Juhas, M., Eberl, L. and Tummler, B. (2005). "Quorum sensing: the power of cooperation in the world of *Pseudomonas*." Environ. Microbiol. **7**: 459-471.

Kang, B. R., Lee, J. H., Ko, S. J., Lee, Y. H., Cha, J. S., Cho, B. H. and Kim, Y. C. (2004). "Degradation of acyl-homoserine lactone molecules by *Acinetobacter* sp. strain C1010." Can. J. Microbiol. **50**: 935-941.

Kaplan, H. B. and Greenberg, E. P. (1985). "Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system." J. Bacteriol. **163**: 1210-1214.

Kjelleberg, S., Steinberg, P., Givskov, M., Gram, L., Manefield, M. and Nys, R. d. (1997). "Do marine natural products interfere with prokaryotic AHL regulatory systems?" Aquat. Microb. Ecol. **13**: 85-93.

Labbate, M., Queck, S. Y., Koh, K. S., Rice, S. A., Givskov, M. and Kjelleberg, S. (2004). "Quorum sensing-controlled biofilm development in *Serratia liquefaciens* MG1." J. Bacteriol. **186**: 692-698.

Laue, B. E., Jiang, Y., Chhabra, S. R., Jacob, S., Stewart, G. S., Hardman, A., Downie, J. A., O'Gara, F. and Williams, P. (2000). "The biocontrol strain *Pseudomonas fluorescens* F113 produces the Rhizobium small bacteriocin, *N*-(3-hydroxy-7-cis-tetradecenoyl)homoserine lactone, via HdtS, a putative novel *N*-acylhomoserine lactone synthase." Microbiology **146**: 2469-2480.

Lazazzera, B. A. (2001). "The intracellular function of extracellular signaling peptides." Peptides **22**: 1519-1527.

Lazazzera, B. A. and Grossman, A. D. (1998). "The ins and outs of peptide signaling." Trends Microbiol. **6**: 288-294.

Leadbetter, J. R. and Greenberg, E. P. (2000). "Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*." J. Bacteriol. **182**: 6921-6926.

Lee, S. J., Park, S. Y., Lee, J. J., Yum, D. Y., Koo, B. T. and Lee, J. K. (2002). "Genes encoding the *N*-acyl homoserine lactone-degrading enzyme are widespread in many subspecies of *Bacillus thuringiensis*." Appl. Environ. Microbiol. **68**: 3919-3924.

Lewenza, S., Conway, B., Greenberg, E. P. and Sokol, P. A. (1999). "Quorum sensing in *Burkholderia cepacia*: identification of the LuxRI homologs CepRI." J. Bacteriol. **181**: 748-756.

Lichstein, H. C. and Van de Sand, V. F. (1945). "Violacein, an antibiotic pigment produced by *Chromobacterium violaceum*." J. Infect. Dis. **76**: 47-51.

Lin, Y. H., Xu, J. L., Hu, J., Wang, L. H., Ong, S. L., Leadbetter, J. R. and Zhang, L. H. (2003). "Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes." Mol. Microbiol. **47**: 849–860.

Lithgow, J. K., Wilkinson, A., Hardman, A., Rodelas, B., Wisniewski-Dye, F., Williams, P. and Downie, J. A. (2000). "The regulatory locus *cinRI* in *Rhizobium leguminosarum* controls a network of quorum-sensing loci." Mol. Microbiol. **37**: 81-97.

Luo, Z. Q., Su, S. and Farrand, S. K. (2003). "In situ activation of the quorum-sensing transcription factor TraR by cognate and noncognate acyl-homoserine lactone ligands: Kinetics and consequences." J. Bacteriol. **185**: 5665-5672.

Lynch, M. J., Swift, S., Kirke, D. F., Keevil, C. W., Dodd, C. E. and Williams, P. (2002). "The regulation of biofilm development by quorum sensing in *Aeromonas hydrophila*." Environ. Microbiol. **4**: 18-28.

Malott, R. J., Baldwin, A., Mahenthiralingam, E. and Sokol, P. A. (2005). "Characterization of the *cciIR* quorum-sensing system in *Burkholderia cenocepacia*." Infect. Immun. **73**: 4982-4992.

Manefield, M., Harris, L., Rice, S. A., Nys, R. d. and Kjelleberg, S. (2000). "Inhibition of luminescence and virulence in the black tiger prawn (*Penaeus monodon*) pathogen *Vibrio harveyi* by intercellular signal antagonists." Appl. Environ. Microbiol. **66**: 2079–2084.

Manefield, M., Nys, R. d., Kumar, N., Read, R., Givskov, M., Steinberg, P. and Kjelleberg, S. (1999). "Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein." Microbiology **145**: 283-291.

Manefield, M., Rasmussen, T. B., Henzter, M., Andersen, J. B., Steinberg, P., Kjelleberg, S. and Givskov, M. (2002). "Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover." Microbiol. **148**: 1119 – 1127.

Marketon, M. M. and Gonzalez, J. E. (2002). "Identification of two quorum-sensing systems in *Sinorhizobium meliloti*." J. Bacteriol. **184**: 3466-3475.

Marketon, M. M., Gronquist, M. R., Eberhard, A. and Gonzalez, J. E. (2002). "Characterization of the *Sinorhizobium meliloti sinR/sinI* locus and the production of novel *N*-acyl homoserine lactones." J. Bacteriol. **184**: 5686-5695.

Maximilien, R., Nys, R. d., Holmstrom, C., Gram, L., Givskov, M., Kjelleberg, S. and Steinberg, P. (1998). "Chemical mediation of bacterial surface colonisation by secondary metabolites from the red alga *Delisea pulchra*." Aquat. Microb. Ecol. **15**: 233–246.

McClean, K. H., Winson, M. K., Fish, L., Taylor, A., Chhabra, S. R., Camara, M., Daykin, M., Lamb, J. H., Swift, S., Bycroft, B. W., Stewart, G. S. and Williams, P. (1997). "Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones." Microbiology **143**: 3703-3711.

McFall-Ngai, M. J. (2000). "Negotiations between animals and bacteria: the 'diplomacy' of the squid-*Vibrio* symbiosis." Comp. Biochem. Physiol. A Mol. Integr. Physiol. **126**: 471-480.

McGrath, S., Wade, D. S. and Pesci, E. C. (2004). "Dueling quorum sensing systems in *Pseudomonas aeruginosa* control the production of the *Pseudomonas* quinolone signal (PQS)." FEMS Microbiol. Lett. **230**: 27-34.

McKnight, S. L., Iglewski, B. H. and Pesci, E. C. (2000). "The *Pseudomonas* quinolone signal regulates *rhl* quorum sensing in *Pseudomonas aeruginosa*." J. Bacteriol. **182**: 2702-2708.

Miller, M. B. and Bassler, B. L. (2001). "Quorum sensing in bacteria." Annu. Rev. Microbiol. **55**: 165-199.

Milton, D. L., Chalker, V. J., Kirke, D., Hardman, A., Camara, M. and Williams, P. (2001). "The LuxM homologue VanM from *Vibrio anguillarum* directs the synthesis of *N*-(3-hydroxyhexanoyl)homoserine lactone and *N*-hexanoylhomoserine lactone." J. Bacteriol. **183**: 3537-3547.

Milton, D. L., Hardman, A., Camara, M., Chhabra, S. R., Bycroft, B. W., Stewart, G. S. and Williams, P. (1997). "Quorum sensing in *Vibrio anguillarum*: characterization of the *vanI/vanR* locus and identification of the autoinducer *N*-(3-oxodecanoyl)-L-homoserine lactone." J. Bacteriol. **179**: 3004-3012.

Moré, M. I., Finger, L. D., Stryker, J. L., Fuqua, C., Eberhard, A. and Winans, S. C. (1996). "Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates." Science **272**: 1655–58.

Morohoshi, T., Ebata, A., Nakazawa, S., Kato, N. and Ikeda, T. (2005). "Identification and characterization of *N*-acylhomoserine lactone-acylase from the fish intestinal *Shewanella* sp. strain MIB015." Microb. Environ. **20**: 264-268.

Nasser, W., Bouillant, M. L., Salmond, G. and Reverchon, S. (1998). "Characterization of the *Erwinia chrysanthemi expI-expR* locus directing the synthesis of two *N*-acyl-homoserine lactone signal molecules." Mol. Microbiol. **29**: 1391-1405.

Nealson, K. H. and Hastings, J. W. (1979). "Bacterial bioluminescence: its control and ecological significance." Microbiol. Rev. **43**: 496-518.

Nieto Penalver, C. G., Cantet, F., Morin, D., Haras, D. and Vorholt, J. A. (2006). "A plasmid-borne truncated *luxI* homolog controls quorum-sensing systems and extracellular carbohydrate production in *Methylobacterium extorquens* AM1." J. Bacteriol. **188**: 7321-7324.

Nieto Penalver, C. G., Morin, D., Cantet, F., Saurel, O., Milon, A. and Vorholt, J. A. (2006). "*Methylobacterium extorquens* AM1 produces a novel type of acyl-homoserine lactone with a double unsaturated side chain under methylotrophic growth conditions." FEBS Lett. **580**: 561-567.

Niu, C., Clemmer, K. M., Bonomo, R. A. and Rather, P. N. (2008). "Isolation and characterization of an autoinducer synthase from *Acinetobacter baumannii*." J. Bacteriol. **190**: 3386-3392.

Padhye, A. A., Verghese, S., Ravichandran, P., Balamurugan, G., Hall, L., Padmaja, P. and Fernandez, M. C. (2003). "*Trichosporon loubieri* infection in a patient with adult polycystic kidney disease." J. Clin. Microbiol. **41**: 479-482.

Park, S. Y., Hwang, B. J., Shin, M. H., Kim, J. A., Kim, H. K. and Lee, J. K. (2006). "*N*-acylhomoserine lactonase producing *Rhodococcus* spp. with different AHL-degrading activities." FEMS Microbiol. Lett. **261**: 102 - 108.

Park, S. Y., Kang, H. O., Jang, H. S., Lee, J. K., Koo, B. T. and Yum, D. Y. (2005). "Identification of extracellular *N*-acylhomoserine lactone acylase from a *Streptomyces* sp. and its application to quorum quenching." Appl. Environ. Microbiol. **71**: 2632-2641.

Park, S. Y., Lee, S. J., Oh, T. K., Oh, J. W., Koo, B. T., Yum, D. Y. and Lee, J. K. (2003). "AhlD, an *N*-acylhomoserine lactonase in *Arthrobacter* sp., and predicted homologues in other bacteria." Microbiol. **149**: 1541–1550.

Pearson, J. P., Gray, K. M., Passador, L., Tucker, K. D., Eberhard, A., Iglewski, B. H. and Greenberg, E. P. (1994). "Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes." Proc. Natl. Acad. Sci. USA **91**: 197-201.

Pearson, J. P., Passador, L., Iglewski, B. H. and Greenberg, E. P. (1995). "A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*." Proc. Natl. Acad. Sci. USA **92**: 1490-1494.

Pearson, J. P., Pesci, E. C. and Iglewski, B. H. (1997). "Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes." J. Bacteriol. **179**: 5756-5767.

Persson, T., Hansen, T. H., Rasmussen, T. B., Skinderso, M. E., Givskov, M. and Nielsen, J. (2005). "Rational design and synthesis of new quorum-sensing inhibitors derived from acylated homoserine lactones and natural products from garlic." Org. Biomol. Chem. **3**: 253–262.

Pesci, E. C., Milbank, J. B. J., Pearson, J. P., McKnight, S., Kende, A. S., Greenberg, E. P. and Iglewski, B. H. (1999). "Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*." Proc. Natl. Acad. Sci. USA **96**: 11229-11234.

Pesci, E. C., Pearson, J. P., Seed, P. C. and Iglewski, B. H. (1997). "Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*." J. Bacteriol. **179**: 3127-3132.

Peters, L., König, G. M., Wright, A. D., Pukall, R., Stackebrandt, E., Eberl, L. and Riedel, K. (2003). "Secondary metabolites of *Flustra foliacea* and their influence on bacteria." Appl. Environ. Microbiol. **69**: 3469-3475.

Pierson, E. A., Wood, D. W., Cannon, J. A., Blachere, F. M. and Pierson, L. S. (1998). "Interpopulation signaling via *N*-acyl-homoserine lactones among bacteria in the wheat rhizosphere." Mol. Plant-Microbe Interact **11**: 1078-1084.

Pierson, L. S., III, Keppenne, V. D. and Wood, D. W. (1994). "Phenazine antibiotic biosynthesis in *Pseudomonas aureofaciens* 30-84 is regulated by PhzR in response to cell density." J. Bacteriol. **176**: 3966-3974.

Piper, K. R., von Bodman, S. B. and Farrand, S. K. (1993). "Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction." Nature **362**: 448-450.

Pirhonen, M., Flego, D., Heikinheimo, R. and Palva, E. T. (1993). "A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*." EMBO J. **12**: 2467-2476.

Puskas, A., Greenberg, E. P., Kaplan, S. and Schaefer, A. L. (1997). "A quorum-sensing system in the free-living photosynthetic bacterium *Rhodobacter sphaeroides*." J. Bacteriol. **179**: 7530-7537.

Quinones, B., Pujol, C. J. and Lindow, S. E. (2004). "Regulation of AHL production and its contribution to epiphytic fitness in *Pseudomonas syringae*." Mol. Plant Microbe Interact. **17**: 521-531.

Rasmussen, T. B., Manefield, M., Andersen, J. B., Eberl, L., Anthoni, U., Christophersen, C., Steinberg, P., Kjelleberg, S. and Givskov, M. (2000). "How *Delisea pulchra* furanones affect quorum sensing and swarming motility in *Serratia liquefaciens* MG1." Microbiology **146**: 3237–3244.

Rasmussen, T. B., Skindersoe, M. E., Bjarnsholt, T., Phipps, R. K., Christensen, K. B., Jensen, P. O., Andersen, J. B., Koch, B., Larsen, T. O., Hentzer, M., Eberl, L., Høiby, N. and Givskov, M. (2005). "Identity and effects of quorum-sensing inhibitors produced by *Penicillium* species." Microbiology **151**: 1325-1340.

Reverchon, S., Bouillant, M. L., Salmond, G. and Nasser, W. (1998). "Integration of the quorum-sensing system in the regulatory networks controlling virulence factor synthesis in *Erwinia chrysanthemi*." Mol. Microbiol. **29**: 1407-1418.

Rice, S. A., Koh, K. S., Queck, S. Y., Labbate, M., Lam, K. W. and Kjelleberg, S. (2005). "Biofilm formation and sloughing in *Serratia marcescens* are controlled by quorum sensing and nutrient cues." J. Bacteriol. **187**: 3477-3485.

Rivas, M., Seeger, M., Holmes, D. S. and Jedlicki, E. (2005). "A Lux-like quorum sensing system in the extreme acidophile *Acidithiobacillus ferrooxidans*." Biol. Res. **38**: 283-297.

Rivas, M., Seeger, M., Jedlicki, E. and Holmes, D. S. (2007). "Second acyl homoserine lactone production system in the extreme acidophile *Acidithiobacillus ferrooxidans*." Appl. Environ. Microbiol. **73**: 3225-3231.

Rodelas, B., Lithgow, J. K., Wisniewski-Dye, F., Hardman, A., Wilkinson, A. and et al. (1999). "Analysis of quorum-sensing-dependent control of rhizosphere-

expressed (*rhi*) genes in *Rhizobium leguminosarum* bv. *viciae*." J. Bacteriol. **181**: 3816-3823.

Romero, M., Diggle, S. P., Heeb, S., Cámara, M. and Otero, A. (2008). "Quorum quenching activity in *Anabaena* sp. PCC 7120: identification of AiiC, a novel AHL-acylase." FEMS Microbiol. Lett. **280**: 73-80.

Rosemeyer, V., Michiels, J., Verreth, C. and Vanderleyden, J. (1998). "*luxI*- and *luxR*-homologous genes of *Rhizobium etli* CNPAF512 contribute to synthesis of autoinducer molecules and nodulation of *Phaseolus vulgaris*." J. Bacteriol. **180**: 815-821.

Ruby, E. G. (1996). "Lessons from a cooperative, bacterial-animal association: the *Vibrio fischeri-Euprymna scolopes* light organ symbiosis." Annu. Rev. Microbiol. **50**: 591-624.

Ruby, E. G. and McFall-Ngai, M. J. (1992). "A squid that glows in the night: development of an animal-bacterial mutualism." J. Bacteriol. **174**: 4865-4870.

Ruiz, L. M., Valenzuela, S., Castro, M., Gonzalez, A., Frezza, M., Soulere, L., Rohwerder, T., Queneau, Y., Doutheau, A., Sand, W., Jerez, C. A. and Guiliani, N. (2008). "AHL communication is a widespread phenomenon in biomining bacteria and seems to be involved in mineral-adhesion efficiency." Hydrometallurgy **94**: 133-137.

Salmond, G. P. C., Bycroft, B. W., Stewart, G. S. A. B. and Williams, P. (1995). "The bacterial enigma: cracking the code of cell-cell communication." Mol. Microbiol. **16**: 615-624.

Sambrook, J., E. F. Fritsch, and T. Maniatis (1989). "Molecular cloning: a laboratory manual." 2 ed. Cold Spring Harbor Laboratory.

Schaefer, A. L., Greenberg, E. P., Oliver, C. M., Oda, Y., Huang, J. J., Bittan-Banin, G., Peres, C. M., Schmidt, S., Juhaszova, K., Sufrin, J. R. and Harwood, C. S. (2008). "A new class of homoserine lactone quorum-sensing signals." Nature **454**: 595-599.

Schaefer, A. L., Val, D. L., Hanzelka, B. L., J E Cronan, J. and Greenberg, E. P. (1996). "Generation of cell-to-cell signals in quorum sensing: acyl homoserine lactone synthase activity of a purified *Vibrio fischeri* LuxI protein." Proc. Natl. Acad. Sci. USA **93**: 9505–9509.

Schauder, S. and Bassler, B. L. (2001). "The languages of bacteria." Genes dev. **15**: 1468-1480.

Schripsema, J., de Rudder, K. E. E., van Vliet, T. B., Lankhorst, P. P., de Vroom, E., Kijne, J. W. and van Brussel, A. A. N. (1996). "Bacteriocin *small* of *Rhizobium leguminosarum* belongs to the class of *N*-acyl-L-homoserine lactone molecules

known as autoinducers and as quorum sensing co-transcription factors." J. Bacteriol. **178**: 366-371.

Schuster, M., Lostroh, C. P., Ogi, T. and Greenberg, E. P. (2003). "Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis." J. Bacteriol. **185**: 2066-2079.

Shaw, P. D., Gao, P., Daly, S. L., Chung, C., Cronan, J. E., Jr., Rinehart, K. L. and Farrand, S. K. (1997). "Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography." Proc. Natl. Acad. Sci. USA **94**: 6036-6041.

Sio, C. F., Otten, L. G., Cool, R. H., Diggle, S. P., Braun, P. G., Bos, R., Daykin, M., Càmara, M., Williams, P. and Quax, W. J. (2006). "Quorum quenching by an *N*-acyl-homoserine lactone acylase from *Pseudomonas aeruginosa* PAO1." Infect. Immun. **74**: 1673-1682.

Smith, R. S. and Iglewski, B. H. (2003). "*P. aeruginosa* quorum-sensing systems and virulence." Curr. Opin. Microbiol. **6**: 56-60.

Sokol, P. A., Sajjan, U., Visser, M. B., Gingues, S., Forstner, J. and Kooi, C. (2003). "The CepIR quorum-sensing system contributes to the virulence of *Burkholderia cenocepacia* respiratory infections." Microbiology **149**: 3649-3658.

Song, Y., Xie, C., Ong, Y. M., Gan, Y. H. and Chua, K. L. (2005). "The BpsIR quorum-sensing system of *Burkholderia pseudomallei*." J. Bacteriol. **187**: 785-790.

Steidle, A., Allesen-Holm, M., Riedel, K., Berg, G., Givskov, M., Molin, S. and Eberl, L. (2002). "Identification and characterization of an *N*-acylhomoserine lactone-dependent quorum-sensing system in *Pseudomonas putida* strain IsoF." Appl. Environ. Microbiol. **68**: 6371-6382.

Steinberg, P. D., Schneider, R. and Kjelleberg, S. (1997). "Chemical defences of seaweeds against microbial colonization." Biodegradation **8**: 211–220.

Swift, S., Karlyshev, A. V., Fish, L., Durant, E. L., Winson, M. K., Chhabra, S. R., Williams, P., Macintyre, S. and Stewart, G. S. (1997). "Quorum sensing in *Aeromonas hydrophila* and *Aeromonas salmonicida*: identification of the LuxRI homologs AhyRI and AsaRI and their cognate *N*-acylhomoserine lactone signal molecules." J. Bacteriol. **179**: 5271-5281.

Swift, S., Lynch, M. J., Fish, L., Kirke, D. F., Tomas, J. M., Stewart, G. S. and Williams, P. (1999). "Quorum sensing-dependent regulation and blockade of exoprotease production in *Aeromonas hydrophila*." Infect. Immun. **67**: 5192-5199.

Swift, S., Winson, M. K., Chan, P. F., Bainton, N. J., Birdsall, M., Reeves, P. J., Rees, C. E., Chhabra, S. R., Hill, P. J., Throup, J. P. and et al. (1993). "A novel strategy for the isolation of *luxI* homologues: evidence for the widespread

distribution of a LuxR:LuxI superfamily in enteric bacteria." Mol. Microbiol. **10**: 511-520.

Tait, K., Williamson, H., Atkinson, S., Williams, P., Cámara, M. and Joint, I. (2009). "Turnover of quorum sensing signal molecules modulates cross-kingdom signalling." Environ. Microbiol. **11**: 1792-1802.

Teplitski, M., Chen, H., Rajamani, S., Gao, M., Merighi, M., Sayre, R. T., Robinson, J. B., Rolfe, B. G. and Bauer, W. D. (2004). "*Chlamydomonas reinhardtii* secretes compounds that mimic bacterial signals and interfere with quorum sensing regulation in bacteria." Plant Physiol. **134**: 137-146.

Teplitski, M., Robinson, J. B. and Bauer, W. D. (2000). "Plants secrete substances that mimic bacterial *N*-acyl homoserine lactone signal activities and affect population density-dependent behaviors in associated bacteria." Mol. Plant-Microbe Interact. **13**: 637–648.

Throup, J. P., Camara, M., Briggs, G. S., Winson, M. K., Chhabra, S. R., Bycroft, B. W., Williams, P. and Stewart, G. S. (1995). "Characterisation of the *yenI/yenR* locus from *Yersinia enterocolitica* mediating the synthesis of two *N*-acylhomoserine lactone signal molecules." Mol. Microbiol. **17**: 345-356.

Ulrich, R. L. (2004). "Quorum quenching: Enzymatic disruption of *N*-acylhomoserine lactone-mediated bacterial communication in *Burkholderia thailandensis*." Appl. Environ. Microbiol. **70**: 6173–6180.

Ulrich, R. L., DeShazer, D., Brueggemann, E. E., Hines, H. B., Oyston, P. C. and Jeddeloh, J. A. (2004a). "Role of quorum sensing in the pathogenicity of *Burkholderia pseudomallei*." J. Med. Microbiol. **53**: 1053-1064.

Ulrich, R. L., DeShazer, D., Hines, H. B. and Jeddeloh, J. A. (2004b). "Quorum sensing: a transcriptional regulatory system involved in the pathogenicity of *Burkholderia mallei*." Infect. Immun. **72**: 6589-6596.

Uroz, S., Chhabra, S. R., Càmara, M., Wiliams, P., Oger, P. M. and Dessaux, Y. (2005). "*N*-Acylhomoserine lactone quorum-sensing molecules are modified and degraded by *Rhodococcus erythropolis* W2 by both amidolytic and novel oxidoreductase activities." Microbiol. **151**: 3313-3322.

Uroz, S. and Heinonsalo, J. (2008). "Degradation of *N*-acyl homoserine lactone quorum sensing signal molecules by forest root-associated fungi." FEMS Microbiol. Ecol. **65**: 271-278.

Uroz, S., Oger, P. M., Chapelle, E., Adeline, M. T., Faure, D. and Dessaux, Y. (2008). "A *Rhodococcus qsdA*-encoded enzyme defines a novel class of large-spectrum quorum-quenching lactonases." Appl. Environ. Microbiol. **74**: 1357–1366.

Uroz, S., Oger, P. M., Chhabra, S. R., Cámara, M., Williams, P. and Dessaux, Y. (2007). "*N*-acyl homoserine lactones are degraded via an amidolytic activity in *Comamonas* sp. strain D1." Arch. Microbiol. **187**: 249–256.

Val, D. L. and Cronan, J. E., Jr. (1998). "In vivo evidence that *S*-adenosylmethionine and fatty acid synthesis intermediates are the substrates for the LuxI family of autoinducer synthases." J. Bacteriol. **180**: 2644-2651.

Valade, E., Thibault, F. M., Gauthier, Y. P., Palencia, M., Popoff, M. Y. and Vidal, D. R. (2004). "The PmlI–PmlR quorum-sensing system in *Burkholderia pseudomallei* plays a key role in virulence and modulates production of the MprA protease." J. Bacteriol. **186**: 2288-2294.

Van Houdt, R., Moons, P., Buj, M. H. and Michiels, C. W. (2006). "*N*-acyl-L-homoserine lactone quorum sensing controls butanediol fermentation in *Serratia plymuthica* RVH1 and *Serratia marcescens* MG1." J. Bacteriol. **188**: 4570-4572.

Venturi, V. (2006). "Regulation of quorum sensing in *Pseudomonas*." FEMS Microbiol. Rev. **30**: 274-291.

Visick, K. L. and McFall-Ngai, M. J. (2000). "An exclusive contract: specificity in the *Vibrio fischeri-Euprymna scolopes* partnership." J. Bacteriol. **182**: 1779-1787.

Visick, K. L. and Ruby, E. G. (2006). "*Vibrio fischeri* and its host: it takes two to tango." Curr. Opin. Microbiol. **9**: 1-7.

von Bodman, S. B., Ball, J. K., Faini, M. A., Herrera, C. M., Minogue, T. D., Urbanowski, M. L. and Stevens, A. M. (2003). "The quorum sensing negative regulators EsaR and ExpR Ecc, homologues within the LuxR family, retain the ability to function as activators of transcription." J. Bacteriol. **185**: 7001-7007.

von Bodman, S. B. and Farrand, S. K. (1995). "Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an *N*-acylhomoserine lactone autoinducer." J. Bacteriol. **177**: 5000-5008.

Wagner, V. E., Bushnell, D., Passador, L., Brooks, A. I. and Iglewski, B. H. (2003). "Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment." J. Bacteriol. **185**: 2080-2095.

Wang, Y. H., Zhang, L. Q., Li, J. Y., Wang, J. H. and Wang, H. M. (2008). "The quorum-sensing system AvsR-AvsI regulates both long-chain and short-chain acyl-homoserine lactones in *Agrobacterium vitis* E26." Antonie van Leeuwenhoek **93**: 267-273.

Waters, C. M. and Bassler, B. L. (2005). "Quorum sensing: Cell-to-cell communication in bacteria." Annu. Rev. Cell Dev. Biol. **21**: 319-346.

Wei, J. R., Tsai, Y. H., Horng, Y. T., Soo, P. C., Hsieh, S. C., Hsueh, P. R., Horng, J. T., Williams, P. and Lai, H. C. (2006). "A mobile quorum-sensing system in *Serratia marcescens*." J. Bacteriol. **188**: 1518-1525.

Williams, P., Winzer, K., Chan, W. and Camara, M. (2007). "Look who's talking: communication and quorum sensing in the bacterial world." Philos. Trans. R. Soc. Lond. B. Biol. Sci. **362**: 1119-1134.

Winson, M. K., Swift, S., Fish, L., Throup, J. P., Jorgensen, F., Chhabra, S. R., Bycroft, B. W., Williams, P. and Stewart, G. S. A. B. (1998). "Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl homoserine lactone-mediated quorum sensing." FEMS Microbiol. Lett. **163**: 185-192.

Winzer, K. and Williams, P. (2001). "Quorum sensing and the regulation of virulence gene expression in pathogenic bacteria." Int. J. Medic. Microbiol. **291**: 131-143.

Wisniewski-Dyé, F. and Downie, J. A. (2002). "Quorum-sensing in *Rhizobium*." Antonie van Leeuwenhoek **81**: 397-407.

Wood, D. W., Gong, F., Daykin, M. M., Williams, P. and Pierson, L. S., III (1997). "*N*-acyl-homoserine lactone-mediated regulation of phenazine gene expression by *Pseudomonas aureofaciens* 30-84 in the wheat rhizosphere." J. Bacteriol. **179**: 7663-7670.

Xu, F., Byun, T., Dussen, H. J. and Duke, K. (2003). "Degradation of *N*-acylhomoserine lactones, the bacterial quorum sensing molecules, by acylase." J. Biotechnol. **101**: 89–96.

Yang, F., Wang, L. H., Wang, J., Dong, Y. H., Hu, J. Y. and Zhang, L. H. (2005). "Quorum quenching enzyme activity is widely conserved in the sera of mammalian species." FEBS Lett. **579**: 3713–3717.

Yates, E. A., Philipp, B., Buckley, C., Atkinson, S., Chhabra, S. R., Sockett, R. E., Goldner, M., Dessaux, Y., Camara, M., Smith, H. and Williams, P. (2002). "*N*-acylhomoserine lactones undergo lactonolysis in a pH-, temperature-, and acyl chain length-dependent manner during growth of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*." Infect. Immun. **70**: 5635-5646.

Zhang, H. B., Wang, L. H. and Zhang, L. H. (2002). "Genetic control of quorum-sensing signal turnover in *Agrobacterium tumefaciens*." Proc. Natl. Acad. Sci. USA **99**: 4638-4643.

Zhang, L., Murphy, P. J., Kerr, A. and Tate, M. E. (1993). "*Agrobacterium* conjugation and gene regulation by *N*-acyl-L-homoserine lactones." Nature **362**: 446-448.

Zhang, Z. and Pierson, L. S., III (2001). "A second quorum-sensing system regulates cell surface properties but not phenazine antibiotic production in *Pseudomonas aureofaciens*." Appl. Environ. Microbiol. **67**: 4305-4315.

Zheng, D., Zhang, H., Carle, S., Hao, G., Holden, M. R. and Burr, T. J. (2003). "A *luxR* homolog, *aviR*, in *Agrobacterium vitis* is associated with induction of necrosis on grape and a hypersensitive response on tobacco." Mol. Plant Microbe Interact. **16**: 650-658.