GENOME AND TRANSCRIPTOME ANALYSIS OF QUORUM SENSING PROPERTIES IN *Vibrio variabilis* STRAIN T01

NUR IZZATI BINTI MOHAMAD

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

GENOME AND TRANSCRIPTOME ANALYSIS OF QUORUM SENSING PROPERTIES IN *Vibrio variabilis* STRAIN T01

NUR IZZATI BINTI MOHAMAD

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

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Nur Izzati binti Mohamad

Matric No: SGR130116

Name of Degree: Master of Science (MSc)

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):
GENOME AND TRANSCRIPTOME ANALYSIS OF QUORUM SENSING PROPERTIES IN *Vibrio variabilis* STRAIN T01

Field of Study: Genetics and Molecular Biology

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this Work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this Work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this Work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this Work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature Date:

Subscribed and solemnly declared before,

Witness's Signature Date:

Name:

Designation:

ABSTRACT

Quorum sensing (QS) is bacterial communication that allows bacteria to respond to stimuli in population density-dependent manner. In this work, seawater sampling site was Morib Beach, Malaysia. This work has successfully isolated 60 bacterial strains. Five of them namely T01, T08, T14, T33 and T47 showed *N*-acyl homoserine lactones (AHLs) production screened by QS biosensor. These QS bacteria were identified using MALDI-TOF mass spectrometry and analyses of 16S rRNA gene sequence which showed that all of them are members of *Vibrio* genus. Profiling of the AHLs produced by these isolates revealed production of both short-chain and long-chain AHL production by all of the strains. The AHLs profiles are as follows; T01 produced *N*-hexanoyl-L-homoserine lactone (C6-HSL), *N*-(3-oxo-decanoyl)-L-homoserine lactone (OC10 HSL) and *N*-dodecanoyl homoserine-L-lactone (C12-HSL), T08 produced *N*-butanoyl-L-homoserine lactone (C4-HSL) and C6-HSL, T14 produced C6-HSL, T33 produced C6-HSL and OC10-HSL and T47 produced C4-HSL. Next, draft genomes of all five strains were obtained using MiSeq platform followed by bioinformatics analyses. Of these, *V. variabilis* strain T01 showed the presence of a QS synthase gene *vanM* (contig 16, 1203 bp) in *V. variabilis* strain T01 vanM was heterologously expressed in *Escherichia coli* BL21 (DE3) pLysS. From the overexpression analyses, *vanM* is responsible for the production of three AHL which are C6-HSL, OC10 HSL and C12-HSL. Using a known anti-QS compound, gallic acid, biofilm assay was performed on *V. variabilis* strain T01 and treatment of gallic acid significantly reduced its biofilm production.

To further study the mode of action of gallic acid on *V. variabilis* strain T01, transcriptomic analyses were carried out. From the RNA-sequencing data, most of the *V. variabilis* strain T01 genes were affected upon gallic acid treatment involved in transport and metabolism (inorganic ions, amino acids, carbohydrates and lipids). Genes related to QS such as cell motility (*fliS*, *fliT* and *fliL*) were down-regulated and cell wall/membrane/envelope biogenesis (*tonB*) were notably up-regulated

ABSTRAK

Penderiaan kuorum (PK) ialah komunikasi antara bakteria yang membolehkan bakteria untuk bertindak ke atas rangsangan bergantung kepada populasi. Pengambilan sampel air laut yang dilakukan di Pantai Morib, Malaysia telah berjaya mengisolasi 60 strain bakteria. Lima daripadanya iaitu, T01, T08, T14, T33 dan T47 telah mendapat keputusan positif dalam ujian awal pengesanan AHL. Identiti lima bakteria tersebut yang dikenalpasti dengan menggunakan spektometri jisim MALDI-TOF dan analysis gen 16S rRNA telah menunjukkan kesemua bakteria tersebut tergolong dalam genera Vibrio daripada spesis Vibrio yang baru dikenalpasti. Analisis profil AHL menunjukkan lima bakteria ini menghasilkan kombinasi AHL rantaian panjang dan pendek. Seterusnya, draf genom bagi lima bakteria ini telah dihasilkan melalui jujukan personal MiSeq dan analisis hiliran telah dilakukan. Satu gen KP iaitu *vanM*, telah dikenal pasti dalam *V. variabilis* strain T01 dan pengesahan fungsi *vanM* dijalankan melalui klon heterologous ke dalam *Escherichia coli* BL21 (DE3) pLysS. Daripada analisis tersebut, *vanM* terbukti menghasilkan tiga jenis AHL iaitu N-hexanoyl-L-homoserin lakton (C6-HSL), *N*-(3-oxo-decanoyl)-L-homoserin lakton (OC10 HSL) dan *N*-dodecanoyl homoserin-L-lakton (C12-HSL). Seterusnya, dengan meggunakan kompaun anti-QS yang diketahui iaitu asid gallic, ujian penghasilan biofilm telah dilakukan ke atas *V. variabilis* T01 and pengurangan penghasilan biofilm yang signifikan telah diperhatikan. Bagi menyelidik cara tindakan asid gallic ke atas *V. variabilis* strain T01, analisis transkriptomik telah dilakukan. Daripada data jujukan RNA, gen-gen yang terjejas daripada rawatan asid gallic terlibat dalam metabolism dan transport (ion tidak organic, asid amino, karbohidrat dan lipid). Gen yang berkaitan dengan KP seperti motilitas sel (*fliS*, *fliT* dan *fliL*) telah turun-dikawal selia dan biogenesis dinding sel/membrane/sampul (*tonB*) telah meningkat-dikawal selia.

ACKNOWLEDGEMENTS

First and foremost, I would like to offer this endeavor to Allah S. W. T, GOD Almighty for the opportunity, the wisdom He has bestowed upon me, the peace of mind and good health He has given me to finish my MSc study. He has given me spiritual strength in times of need and guidance during difficult times. This thesis becomes a reality with the kind support and help of many individuals. I would like to extend my sincere thanks to all of them.

I would like to express my gratitude towards my family for the encouragement which helped me in completion of my MSc studies. To my beloved and supportive parents, Kholijah binti Mahamud and Mohamad bin Zainal, who is always by my side, as they are my inspiration to pursue this undertaking. I would also like to thank my closest friend, Ms. Tan Wen Si who has been a great help in making this study a success and for her constant support and cherished friendship.

Next, I would like to thank my supervisor, Assoc. Prof. Dr. Chan Kok Gan for his tremendous support during my studies and for imparting his knowledge and expertise in this study.

I am highly indebted to High Impact Research, University Malaya for their guidance and constant supervision as well as providing the funds for my research.

Many thanks and appreciations also go to my colleague and people who have willingly helped me during my studies and completion of this thesis

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percent
×	:	Times
× *g*	:	Gravity
°C	:	Degree Celsius
µg	:	Microgram
µL	:	Microlitre
µm	:	Micron
µM	:	Micromolar
HC4-HSL	:	*N*-(3-hydroxybutanoyl)-L-homoserine lactone
HC6-HSL	:	*N*-(3-hydroxyhexanoyl)-L-homoserine lactone
OC4-HSL	:	*N*-(3-oxo-butyryl)-L-homoserine lactone
OC6-HSL	:	*N*-(3-oxo-hexanoyl)-L-homoserine lactone
OC8-HSL	:	*N*-(3-oxo-octanoyl)-L-homoserine lactone
OC10-HSL	:	*N*-(3-oxo-decanoyl)-L-homoserine lactone
OC12-HSL	:	*N*-(3-oxo-dodecanoyl)-L-homoserine lactone
ACN	:	Acetonitrile
AGE	:	Agarose gel electrophoresis
AHL	:	*N*-acyl homoserine lactone
AI-2	:	Autoinducer-2
AIP	:	Autoinducer peptide
bp	:	Basepair
C4-HSL	:	*N*-butanoyl-L-homoserine lactone
C6-HSL	:	*N*-hexanoyl-L-homoserine lactone
C8-HSL	:	*N*-octanoyl-L-homoserine lactone
C12-HSL	:	*N*-dodecanoyl-L-homoserine lactone
COG	:	Clusters of Orthologous Groups
dH_2O	:	Distilled water
dsDNA	:	Double-stranded deoxyribonucleic acid
DNA	:	Deoxyribonucleic acid
dNTP	:	Deoxyribonucleotide triphosphate
EDTA	:	Ethylenediaminetetraacetic acid
g	:	Gram
HMDS	:	Hexamethydisilazane
Hrs	:	Hour
HS	:	High sensitivity
IPTG	:	Isopropyl-β-D-1-thiogalactopyranoside
Kb	:	Kilobase pair
L	:	Litre
LB	:	Luria-Bertani
LC-MS/MS	:	Triple quadrupole liquid chromatography mass spectrometry
M	:	Molarity
m/z	:	Mass to charge ration
MEGA	:	Molecular Evolutionary Genetic Analysis

MIGS	:	Minimum Information about the Genome Sequence
min	:	Minute
mg	:	Milligram
mL	:	Mililitre
mm	:	Milimetre
mM	:	Milimolar
MOPS	:	3-(*N*-morpholino) propanesulfonic acid
MS	:	Mass spectrometry
MTA	:	Methylthioadenosine
N	:	Normal acid or base
NA	:	Not applicable
NCBI	:	National Center for Biotechnology Information
n	:	Number
ng	:	Nanogram
nM	:	Nanomolar
PBS	:	Phosphate buffer saline
PCR	:	Polymerase chain reaction
psi	:	Pounds per square rich
qRT-PCR	:	Quantitative real-time polymerase chain reaction
QQ	:	Quorum quenching
QS	:	Quorum sensing
QSI	:	Quorum sensing inhibitors
RAST	:	Rapid Annotation using Subsystem Technology
RNA	:	Ribonucleic acid
TLC	:	Thin-layer chromatography
s	:	Second
SAM	:	*S*-adenosyl-L-methionine
SEM	:	Scanning electron microscope
SI	:	Super Integrons
TBE	:	Tris-boric acid ethylenediaminetetraacetic acid
TOF	:	Time of flight
V	:	Voltage
v/v	:	Volume per volume
w/v	:	Weight per volume
X-gal	:	5-bromo-4-chloro-indoyl-galactopyranoside

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

The ability to coordinate cellular behavior was once thought to be a privilege shared only amongst eukaryotic organisms such as plants and animals while bacterial way of communication was assumed to happen indirectly such as detecting differences in nutrient availability. However, in the last two decades, the intense study on bacterial communication shows that other than carrying out simple nutrient sensing, bacteria are also able to carry out advanced communication and coordination to perform complex life activities such as the exhibition of virulence factors and the production of biofilms (Rutherford & Bassler, 2012). As the saying 'many hands make light work' goes, bacteria are unable to carry out these complex processes on their own and need to achieve a certain concentration of population in order for it to happen. Advanced communication is a cell density dependent response which relies on the production of small pheromone-like biomolecule produced by the bacteria themselves. In their own social community, bacteria exhibit certain behavior based on the regulation of specific genes in response to the intracellular communication (Sifri, 2008). Hence, quorum sensing (QS) is a termed coined to define the need for sufficient population/concentration to activate the system thus regulating differential gene expression.

QS involves four main stages which begin with the production of small biomolecule called autoinducers by the bacteria followed by the second step which is the release of these autoinducer into the environment which occurs in both passive and active manner. The third step is the recognition of these molecules by a specialized signal receptor and once these signal achieve a threshold level required, the fourth step will be carried out which is the differential expression in genes regulation. Simultaneously with the induction on gene expression, the synthesis of the proteins involved in autoinducer production will also be regulated (Fuqua, Parsek, & Greenberg, 2001). A positive feedback loop exists to prevent from overproduction of the signaling molecules which explains the term autoinducer for the quorum sensing signals.

There are a lot of cell functions such as sporulation, antibiotic production and fruiting body development depend on QS in both Gram-positive and Gram-negative bacteria (Diggle, Crusz, & Cámara, 2007). One of the most important functions regulated by QS is the production of virulence factors in several bacterial species such as *Burkholderia pseudomallei*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Vibrio cholerae* (Rutherford & Bassler, 2012). For example, *P. aeruginosa* is an opportunistic pathogen which causes cystic fibrosis in patients with compromised immune system (Davies, 2002). Various types of virulence factors such as lectins, elastase and pyocyanin are regulated by QS in *P. aeruginosa* (Winzer et al., 2000).

Although often associated with the exhibition of virulence factors, QS-regulated genes also encode for other type of proteins such as proteins involved in metabolic processes. About 4-10 % of the bacterial genome and 20 % of the proteome is influenced by QS (Schuster et al., 2003). These findings indicate that QS is a mechanism not only to exhibit virulence but also to enable them to adapt to the metabolic demands living in communities.

Understanding and studying QS system, their signals and their effects will provide a window of opportunity represents a novel target for the production of compounds/ agents to counter bacterial infections. However, despite the positive impression made by the production of anti-QS compounds/agents to combat bacterial infections, new discovery shows that QS is far more complex that what was first estimated and interference in the QS signaling pathway may produce unexpected and adverse effects. Furthermore, the breakthroughs in QS studies lately revealed that bacteria are also capable of conducting interspecies communication which adds to the complexity of established QS systems so far. Thus, more studies should be conducted in order to understand more about QS in bacteria.

1.1 Research objective

This research aims to provide insights to the community of QS marine bacteria and cellular processes affected by QS mechanisms.

1.2 Detailed objectives of the research

The detailed objectives of this thesis are as follow:

1. To culture and identify QS bacteria in the marine environment using polyphasic approach
2. To study the QS properties of bacterial isolates and identify their signaling molecules
3. To sequence the genome of QS isolates
4. To carry out functionality study of the LuxM and its homologue found in the sequenced genome
5. To conduct transcriptome analyses via RNA-sequencing of selected strain treated with a known anti-QS compound

CHAPTER 2: LITERATURE REVIEW

2.1 Initial Study of QS in Gram-negative Bacteria

The first discovery on QS in bacteria was made in *Vibrio fischeri* and since then it has become the archetype of QS system in Gram-negative bacteria (Nealson & Hastings, 1979). The prominent blue-green bioluminescence is portrayed by *V. fischeri* when their population density reaches a threshold level while during low cell density, *V. fischeri* does not produce luminescence. Symbiosis relationship exists between *V. fischeri* and multiple species of fish and squids and the symbiosis between *V. fischeri* and *Euprymna scolopes*, the nocturnal Hawaiian bobtail squiid is one of the most extensively studied so far (Ruby, 1999). Exploitation of the bioluminescence generated by *V. fischeri* by *E. scolopes* enables the squid to avoid predation and masks itself by manipulating the downward projection of the light intensity thus removing the moonlight casted shadow in the waters, a phenomenon known as counterillumination. Approximately $10^{10} – 10^{11}$ cfu per ml of *V. fischeri* cells populate the light organ of the squid and in turn benefits from the nutrients supplied by the squid(Graf & Ruby, 1998).

2.1.1 Regulation of Bioluminescence Mediated by QS

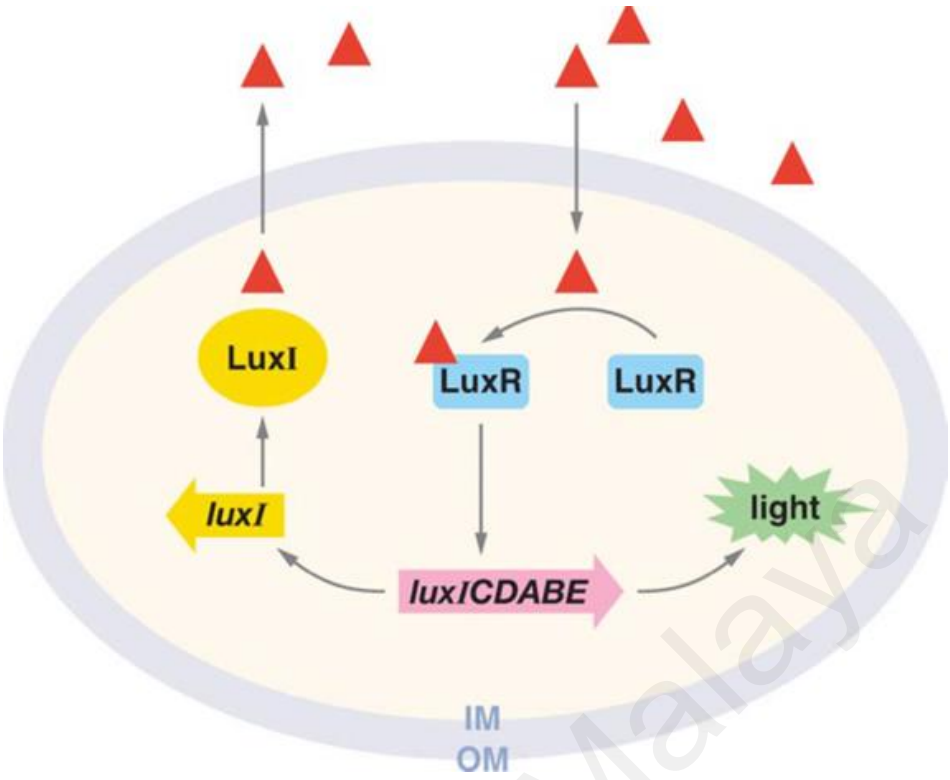
Eight *lux* genes which are *luxA-E*, *luxG*, *luxI* and *luxR* positioned in two bi-directional transcribed operons are responsible for bioluminescence in *V. fischeri* (Engebrecht, Nealson, & Silverman, 1983), (Swartzman, Kapoor, Graham, & Meighen, 1990). The main entity for bioluminescence in *V. fischeri* are the products derived from the *luxI* and *luxR* genes (Engebrecht & Silverman, 1984) while the subunits required for the production of heterodimeric luciferase enzyme are produced from the *luxA* and *luxB* genes. Bioluminescence are generated from the surplus discharge of free energy from the reduction of flavin and oxidation of aldehyde; an enzymatic reaction catalyzed by the heterodimeric luciferase enzyme (Dunn, Michaliszyn, Bogacki, & Meighen, 1973), (Hastings, 1978). On the other hand, *luxC-E* are translated to produce components necessary for the complex structure of multiple enzymes to assemble the aldehyde substrate used by the luciferase (Boylan, Graham, & Meighen, 1985), (Boylan, Miyamoto, Wall, Graham, & Meighen, 1989). The final component in the bioluminescence regulation in *V. fischeri* is *luxG* which translates into flavin reductase (Zenno & Saigo, 1994) ensued by terminal region (Swartzman et al., 1990).

Early lag phase was observed in liquid culture of *V. fischeri* (Nealson, 1999) mainly because there is a need to achieve a threshold level of signaling molecules before bioluminescence could commence (Nealson et al., 1970), (Eberhard, 1972). In the growing medium, each component is differentially metabolized by the bacterial population. The presence of signaling molecules in a population of bacteria affect the acclimatization of bacteria in the growing medium thus triggering the induction of certain genes, a process known as autoinduction (Nealson et al., 1970).

Later researches discovered a chemical signal, coined as autoinducer which is *N*-(3-oxo-hexanoyl)-L-homoserine lactone (OC6-HSL) (Eberhard et al., 1981). The signal, produced by *luxI* gene diffuses in and out of the cell membrane and able to induce bioluminescence even at low concentrations (10 nM) (Kaplan & Greenberg, 1985). The induction of bioluminescence in *V.fischeri* begins with the interception of OC6-HSL with the transcriptional regulator protein, LuxR (Engebrecht et al., 1983), (Engebrecht & Silverman, 1984). OC6-HSL are produced by the LuxI protein and the cell density directly affects the concentration of the OC6-HSL molecules. In the light organ of *E. scolopes*, as the cell density of *V. fishceri* increases, the OC6-HSL molecules also increases and diffuses in and out of the cell membrane.

Once the OC6-HSL molecule reaches a threshold level, it will bind to the LuxR receptor protein creating the LuxR-OC6-HSL complex. This complex then binds to the *lux* box (20-bp DNA) (Devine, Countryman, & Baldwin, 1988) and the transcription of *luxICDABEG* (*lux* box) begins (Figure 2.1). The mRNA transcripts for OC6-HSL synthesis and bioluminescence functions increased with the increment of transcription of the *lux* box thus increasing the output of OC6-HSL molecules and light. Since more OC6-HSL molecules are being produced, it can continuously bind with the LuxR complex and constantly produce luminescence in the light organ.

However, autoinduction does not occur in singular cell of *V. fischeri* as it requires substantial amount of energy to produce bioluminescence if not assisted by the host(Boettcher & Ruby, 1995). Positive loop back arrangement exists in the mechanisms of bioluminescence whereby the transcription of *luxR* by LuxR is repressed when OC6-HSL molecules concentration is high (high cell density) while in low OC6-HSL molecules conditions, transcription of *luxR* is induced (Shadel & Baldwin, 1991), (Dunlap & Ray, 1989) besides that, another receptor protein, cAMP (CRP) ensure the steady transcription of *luxR* (Dunlap & Grennberg, 1988). On the other hand, a different acyl HSL molecule known as *N*-octanoyl-L-homoserine lactone (OHL) produced by *V. fischeri* helps to avoid early induction of bioluminescence by competing with OC6-HSL to bind with LuxR (Callahan & Dunlap, 2000). The OHL molecule is generated from a protein, AinS transcribe from *ainS* gene. Although it is an autoinducer synthase protein, AinS shares no homology with LuxI (Hanzelka et al., 1999).



▲	*N*-(3-oxo-hexanoyl)-L-homoserine lactone (OC6-HSL)
IM	Inner membrane
OM	Outer membrane
luxICDABE	*lux* box

Figure 2.1: QS in *V. fischeri*. During low cell density, transcription of the *lux* box is weak and unable to produce bioluminescence. Threshold level of OC6-HSL molecules achieved during high cell density allows continuous transcription of the *lux* box producing bioluminescence and continuous production of OC6-HSL molecules.

2.2 QS in Gram-negative Bacteria

2.2.1 Receptor Protein LuxR

Previous studies used *E. coli* to study the genetic aspects of LuxR and have provided insights to the mechanisms involved in QS by LuxR and its homologues. In order to be activated, LuxR, a 250-amino acid polypeptide requires the presence of GroESL protein chaperones for protein folding and to form a stable structure (Adar & Ulitzur, 1993), (Dolan & Greenberg, 1992). Furthermore, membrane spamming domain is absent in LuxR, however LuxR is located on the cytoplasmic surface of the inner membrane (Kolibachuk & Greenberg, 1993). Interestingly, homology among LuxR and its homologues are very low as they share only five conserved residues (Whitehead, Barnard, Slater, Simpson, & Salmond, 2001).

Direct interaction between LuxR and acyl HSL molecules was observed in TraR, a LuxR homologue from *Agrobacterium tumefaciens* and in CarR, a LuxR homologue from *Erwinia carotovora* whereby the binding occurs in a stoichiometric fashion; one TraR/CarR binds with one molecule of acyl HSL (Qin et al., 2000), (Welch et al., 2000). Multimerisation is one of the significant outcomes from the binding of LuxR and acyl HSL molecules. When exposed to acyl HSL, purified TraR forms a dimeric structure and dissociates when acyl HSL molecules was removed (Zhu & Winans, 2001). The main cause of this event is the presence of mapping domain located in the center of LuxR (Qin et al., 2000).

The complex formed by the LuxR and acyl HSL molecules allows DNA binding to trigger the differential regulation of genes associated with QS. DNA binding capacity of LuxR depends on specific residues located in a region in the third carboxy-terminal containing a helix shaped motif (Choi & Greenberg, 1992). The specific region of DNA binding to LuxR is known as the *lux* box. Majority of Gram-negative bacteria *lux* box are located upstream of the QS regulated genes (Whitehead et al., 2001). The presence of RNA polymerase with efficient α-subunit C-terminal domain (αCTD) is crucial to enable transcription of the *lux* operon (Stevens, Fujita, Ishihama, & Greenberg, 1999).

2.2.2 Acyl HSL Synthase Protein

Initial studies show that AHL was generated by LuxI and its homologues through sequences of enzymatic activity when it is provided with suitable substrates (Moré et al., 1996). LuxI biologically synthesize AHL utilizing two different types of compounds which are amino acid and coenzyme A (CoA) or acyl carrier protein (ACP). Early research done by Eberhard et al. (Eberhard, Longin, Widrig, & Stranik, 1991) supplied *S*-adenosylmethionine (SAM) together with a derivative of fatty acid to *V. fischeri* extract and successful OC6-HSL production was observed. This observation is supported by many researches after, indicating SAM is crucial amino acid substrate for production of AHL molecules by homologues of LuxI (Jiang et al., 1998), (Hoang, Yufang, Stem, McNeil, & Shweizer, 1999).

For the production of the fatty acid side chain of the OC6-HSL molecules, early study (Eberhard et al., 1991) hypothesized that both CoA and ACP are required as substrates. However, further research states that the production of the fatty acid moiety of OC6-HSL used acylated ACP from cellular pools instead of the acylated CoA (Val & Cronan, 1998), (Hoang & Schweizer, 1999). Sequentially, the production of OC6-HSL by LuxI begins with the attachment of SAM to the enzymes' active site followed by the transfer of suitable acyl group from a charged ACP to the SAM-enzyme complex. Next, lactonisation of the complex occurs hence catalyzed the synthesis of OC6-HSL by its product, 5'-methylthioadenosine. Figure 2.2 illustrates the production of OC6-HSL.

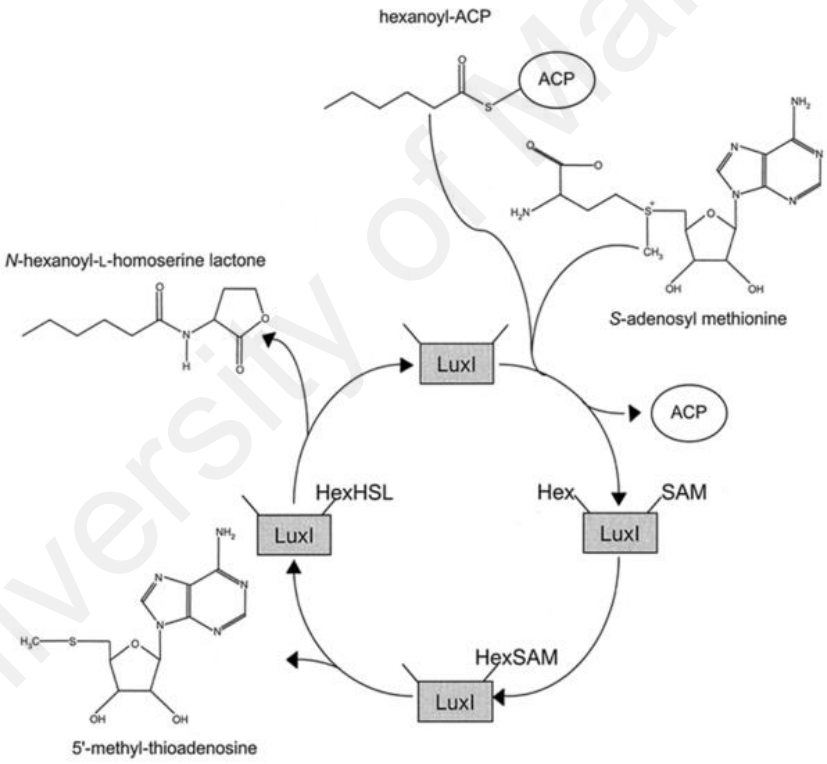


Figure 2.2: Schematic diagram showing the OC6-HSL synthesis by LuxI adapted from Whitehead and colleagues (2001). First, the binding of SAM onto the active site of LuxI, coupled with the transfer of hexanoyl groups of charged ACP triggers the formation of amide bond. From the reaction, 5'-Methylthioadenosine is produced as a by-product while the lactonisation of the complex result in formation of OC6-HSL (Whitehead et al., 2001)

LuxI is not the only protein which possesses the ability to produce AHL signals and generally the LuxI homologues is made up of 200 amino acids. Another AHL synthase, LuxM found in *V. harveyi* is classified into the novel non-LuxI synthase. LuxM produces 3-OH-C4-HSL in *V. harveyi* and was initially wrongly annotated as two proteins, LuxM and LuxL (Bassler et al., 1993). Now, the gene has been corrected and renamed as LuxM. In the *Vibrio* family, both AinS and VanM are the homologues of LuxM and shares 34 % homology and biochemical similarities (Milton, Chalker, Kirke, Hardman, Cámara, et al., 2001). Similar like LuxI, LuxM, VanM and AinS also uses the octanoyl-ACP and SAM to produce AHL signal molecules. Despite sharing some of biochemical specificity with LuxI, LuxM and its homologues are able to effectively use charged acylated CoA as a substrate (Hanzelka et al., 1999). Distinctly different from the LuxI and LuxM family of synthases, another protein known as HdtS, a member of the lysophosphatidic acid acyltransferase family was shown to direct the production of AHL molecules in *Pseudomonas fluorescens* F113 (Laue et al., 2000). It uses both acylated ACP and acylated CoA as substrates for the acylation of lysophosphatidic acid and AHL synthesis (Cullinane, Baysse, Morrissey, & O'Gara, 2005).

2.2.3 AHL Signaling Molecules

The signaling molecules produced by bacteria can be detected by using many methods such as employing biosensor as preliminary screening or employing quantification method via mass spectrometry (McClean, Winson, Fish, et al., 1997), (Charlton et al., 2000), (Ortori et al., 2011). From the studies done, in Gram-negative bacteria, the structure of AHL is divided into two main components; a homoserine lactone ring coupled with an acyl side chain (Churchill & Chen, 2011). The side chain in AHL molecule consists of four to 14 carbon atoms and often has a double bond and a hydroxyl or oxo group on the third carbon.

Usually the side chain in AHL produced by Gram-negative bacteria has an even number of carbons and most of them are unique to their own bacterial genera. Due to the substrate specificity of LuxI homologue, AHL production correlates with differently charged ACPs used in the AHL synthesis. Therefore, most of the bacteria can produce more than one type of AHL (Parsek, Val, Hanzelka, Cronan, & Greenberg, 1999), (Eberhard et al., 1991). Differential gene expression by bacteria relies on the production of different types of AHL intercepted by their cognate R- proteins during virulence factor expression or biofilm formation per se. However, factors such as the physiological functions related to QS and the signal receptors are still unknown thus add to the obscurity of the complex QS signaling mechanism.

Production of AHL depends on the quantity and concentration of both enzymes and substrates and the effective specific enzymes binding. On the other hand, although *E. coli* possesses no AHL synthase gene, it is able to interact with exogenous signaling molecules including AHLs using its receptor SdiA (Michael, Smith, Swift, Heffron, & Ahmer, 2001). Furthermore, the AHL produces by the bacteria also acts as a control mechanism whereby in *A. tumefaciens*, positive feedback mechanism takes place as the continuous expression of AHL synthase TraI is up regulated by continuous *N*-3-oxo-octanoyl-L-homoserine lactone (OC8-HSL)-TraR complex formation which triggers increase the production of TraI and OC8-HSL. The presence of an anti-activator ; TraM which binds to TraR forming the TraR-TraM anti-activation complex is responsible for positive control of TraR in avoiding premature portrayal of energy exhaustive QS during low cell population (Piper & Farrand, 2000).

Degradation of AHLs can be achieved via enzymatic and chemical reactions by both active and passive methods (Eberhard et al., 1981). When infected by *Erwinia* sp., plants produce alkaline substances leading to hydrolysis of the AHL produced (Byers, Lucas, Salmond, & Welch, 2002; Nachin & Barras, 2000). Temperature also plays a role in the shelf life of AHLs (Kirwan et al., 2006) while enzymatic hydrolysis and deacylation of the AHLs occurs in a bacterial cell by lactonases and acylases as it recycles the material as other useful products (Huang et al., 2003; Roche et al., 2004).

2.3 *Vibrio* spp.

Currently there are approximately more than 100 species of *Vibrio* identified and they are classified into 14 clades. *Vibrio* can be found in almost all aquatic landscape such as in coastal waters, estuarine and sediments (Reen, Almagro-Moreno, Ussery, & Boyd, 2006). They are also commonly associated with aquatic life forms such as in fishes, shrimps, molluscs and crustaceans either pathogenically, commensality or symbiotically. With regards to advance taxonomical tools, there are more than 50 species of *Vibrio* identified and characterized in the last decade while studies in the environmental field have greatly improved the existing information on *Vibrionaceae* family.

A bacteria described as *Vibrions* was isolated by Filippo Pacini about 150 years ago, which was later coined as type strain *Vibrio pacini* (*Vibrio cholerae Pacini*) (Nardi, 1954). *Vibrios* are curved, Gram-negative, comma-shaped rods bacteria when visualized under the electron microscope (Baumann, Furniss, & Lee, 1984). Flagella are present in *Vibrios* to enable motility with multiple lateral flagella observed when cultured on solid media while in liquid media *Vibrios* are equipped with polar flagella enclosed in a sheathe motility (Garrity et al., 2006). *Vibrios* are facultative anaerobes with ability to ferment D-glucose producing acid and thrive in growing media containing sodium ions (0.03 – 4.1%) or seawater. They are oxidase positive in nature and capable of reducing nitrate to nitrite, *Vibrio* grows well in temperature ranging from 20 – 30 °C with round entire margin (Baumann et al., 1984). The spread of *Vibrio* in the aquatic environment has increase rapidly due to the increase in global temperature due to global warming and have cause significant implications to the aquatic life and aquaculture industry (Romalde, Dieguez, Lasa, & Balboa, 2014).

Some of the species in *Vibrios* portray bioluminescent properties such as in *V. fischeri* and in *V. harveyi*. Some of them exhibit pathogenicity to both aquatic animals and humans. Several *Vibrio* species such as *V. anguillarum*, *V. harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *V. splendidu*s have a major threat to the shrimp farming industry by causing severe vibriosis infection (Chatterjee & Haldar, 2012). The alarming global loss sustained by the aquatic farming industry accumulated to USD 3 Billion/year in 1997 indicating that the outbreak of diseases caused by *Vibrio* is a critical issue (Subasinghe et al., 2001). In humans, *Vibrio* infections can be classified into two categories which are *Vibrio cholera* infection and noncholera *Vibrio* infections. In the non-cholera *Vibrio* infections, there are two subcategories; halophilic and non-halophilic (Blake, Merson, Weaver, Hollis, & Heublein, 1979).

To date, there are at least 12 species of *Vibrio* associated with human infections (J. Janda, Powers, Bryant, & Abbott, 1988) usually contracted through the ingestion of contaminated seafood and open cuts or wound exposed to contaminated waters. Early detection and treatment are the key factors to cure the infections as untreated infections may cause severe effect such as vomiting and acute diarrhea that could lead to death (Scallan et al., 2011; Vollberg & Herrera, 1997; Zide, Davis, & Ehrenkranz, 1974).

Although some of the *Vibrio* species are considered pathogenic and poses a threat to humans and aquatic life, other species in *Vibrio* do not possess the pathogenicity trait. For example, *V. fischeri* shares symbiotic relationship with the Hawaiian bobtail squid (Ruby & Lee, 1998). Although *Vibrio* species such as *V. vulnificus* and *V. parahaemolyticus* are known as the pathogenic, the encounter and isolation of a pathogenic strain is extremely rare. Scientists inferred the situation as a completely random event, whereby environmental cues may trigger certain species of *Vibrio* to contain small population of pathogenic strain (Julie et al., 2010).

2.3.1 QS in *Vibrio*

The QS systems in *Vibrio* are quite complex often involving more than one QS circuits. The LuxI/R system is the main QS system in *V. fischeri*; the paradigm of QS regulatory systems in Gram-negative bacteria. Generally, almost all QS systems involve cascades of reaction from one QS circuit to another depending on the cell density and environmental condition. The LuxI/R system is positioned at the bottom of the cascade reaction with two QS systems on top of the hierarchy which are the LuxS/PQ and AinS/R systems. All of these systems work in unification to successfully exhibit luminescence (Lupp & Ruby, 2004).

On the other hand, LuxS/PQ circuit can also be found in *V. harveyi* which is well known for its pathogenicity effect on aquaculture (Surette, Miller, & Bassler, 1999). Unlike the LuxI/R system found in *V. fischeri*, *V. harveyi* employs a slightly different QS circuit which is LuxM/N which relies on *N*-(3-hydroxybutanoyl)-L-homoserine lactone (HC4-HSL), synthesized by LuxM (Bassler, Wright, Showalter, & Silverman, 1993). There are three QS circuits in *V. harveyi* which are the LuxM/N aforementioned, LuxS/PQ which uses AI-2 signaling molecules and CqsA/S system which depends on the CAI-1 signal molecules. These three signal molecules works synergistically in gene regulations to induce bioluminescence, metalloprotease, siderophore and exopolysaccharide production and negatively regulate type III secretion which majorly relies on the cell population (Bassler, Wright, & Silverman, 1994).

Unlike in *V. fischeri*, in low cell density, three of the response regulators; LuxN, LuxQ and CqsS work in parallel getting phosphorylated and delivering the phosphate to a shared phosphotransferase; LuxU. Another response regulator, LuxO is responsible for the gene induction by the master regulator which is LuxR (Miller & Bassler, 2001). Meanwhile, in higher cell density, the signal molecules bind to their respective regulators converting the complex into phosphatase and dephosphorylation of LuxO and activation of LuxR inducing the specific set of genes (Milton, 2006). However, as the population increases, the signal molecules produced increases and diffuse in and out of the cell membrane (Figure 2.3). When the signaling molecules reach a threshold level, it will bind to its respective receptor and triggers multiple gene expression (Milton, 2006)

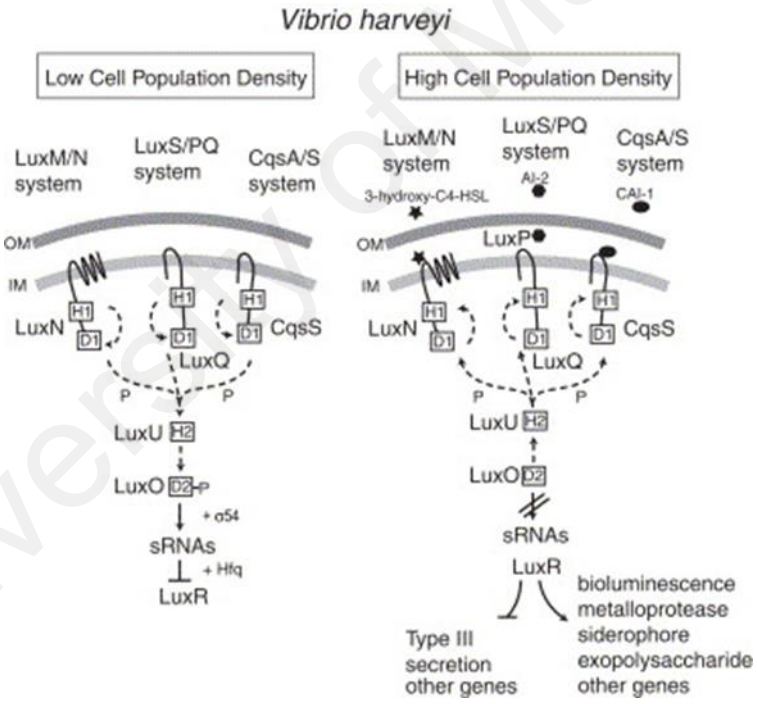


Figure 2.3: Outline of QS systems in *V. harveyi* adapted from Milton (2006) during low and high cell density. Signaling molecules from LuxM, LuxS and CqsA were absent during low cell density.

In the human pathogen; *V. cholerae* possess two similar QS systems as in *V. harveyi*; CqsA/S and LuxS/PQ system and a third system which are not fully identified and documented yet (Miller, Skorupski, Lenz, Taylor, & Bassler, 2002). The LuxM/N system is not found in *V. cholerae*. CqsA/S and LuxS/PQ systems sensors work in parallel identical to the one described in *V. harveyi*; the sensory information is passed along from LuxU to LuxO. System 3 is believed to channel the sensory information through LuxO. The master regulatory in *V. cholerae* is HapR instead of LuxR in *V. Harveyi* (Jobling & Holmes, 1997).

More complex QS system can be observed in *Vibrio anguillarum* which uses four QS circuits. Some of the QS systems in *V. anguillarum* are homologues to the QS systems in *V. harveyi* (VanM/N and VanS/Q) (Milton, Chalker, Kirke, Hardman, Camara, et al., 2001). Interestingly, the third QS system in *V. anguillarum* is similar to the *V. fischeri* which is VanI/R and works in a hierarchy manner with VanM/N and VanS/PQ. The fourth system in *V. anguillarum* is the CqsA/S also found in *V. cholerae* that enhance the level of complexity of QS system in *V. anguillarum* (Henke & Bassler, 2004).

2.4 Physiological Traits Affected by QS

QS allows certain type of bacteria to form a group and acts as a multicellular organism. By being a multicellular organism, bacteria are able to carry out complex life processes such as swarming, biofilm formation and expression of virulence factors (Sifri, 2008). In search of suitable environmental niches with nutrients, motility is crucial to ensure the survival of certain bacteria (Harshey, 2003). On a surface, bacteria employ swarming, gliding, twitching and sliding to acquire nutrients, move to suitable environments and find new host to colonize (Verstraeten et al., 2008). Swarming is a multicellular behavior and is triggered by the flagella rotation and secretion of mucoid layer by the bacteria to facilitate motility. The mucoid layer serves to extract water from the environs or as surfactants to reduce surface tension. Exopolysaccharide (EPS) is produced in the cell and exported to outside of the cell and is postulated to be involved in swarming by being the water reaping agent and surfactant (Liu et al., 2016). The response regulator LitR and OpaR found in *V. fisheri* and *V. parahaemolyticus* respectively, triggers the expression of EPS production to promote swarming ability by binding to their respective AHL signal (Yildiz & Visick, 2009). Some evidence also suggests that long chained AHLs play a role as the surfactant to ease the swarming movement of the bacteria (Daniels et al., 2006)

On the other hand, biofilm formation is a density dependent process involving populations of bacteria attached on a surface and encapsulated in a extracellular matrix or biofilm (Costerton et al., 1987). Biofilm production begins with strong surface adhesion, followed by multiplication of bacterial population. Next, the bacterial population produces the extracellular matrix by up-regulating the production of EPS and lastly the bacteria will disperse itself from the biofilm (O'Toole, Kaplan, & Kolter, 2000). The extracellular matrix also known as biofilm provides protection from the environmental stresses, immune response, antimicrobials and protozoa. QS plays a role in the latter part of the biofilm development; during the multiplication of the bacterial colonies. During this time, the QS signals can reach a threshold level and coordinate a stream of gene expression and trigger the dispersal of bacteria from its biofilm (Solano, Echeverz, & Lasa, 2014).

The QS pathway plays extremely important role in the physiological behavior of bacteria. As production of EPS, swarming and biofilm are interconnected, QS reacts to environmental cues to portray these activities to their gain. For example, *V. parahaemolyticus*, are able to carry out reversible phase change, whereby there are two distinct colony morphologies: translucent (TR) and opaque (OP). The TR strains are able to swarm, produce less capsular polysaccharide (CPS) and forms translucent colonies. On the other hand, OP strains are the exact opposite: unable to swarm; produce thick CPS and forms opaque colonies (Enos-Berlage, Guvener, Keenan, & McCarter, 2005).

The master regulator for this reversible behavior is OpaR QS regulator. OpaR is a homologue of LuxR and the QS pathway upstream of OpaR is similar to the pathway described in *V. harveyi* (Gode-Potratz & McCarter, 2011). During low cell density, low concentration of autoinducers cause the phosphorylation of LuxU which eventually leads to phosphorylation of LuxO and halt the translation of *luxR*/*opaR* (Pompeani et al., 2008). The absence of OpaR causes the strain to become translucent, exhibit swarming and expressing virulence factors such as the type III secretion system. However, during high cell density, high concentration of autoinducer de-phosphorylation of LuxO occurs and continuous translation of OpaR begins. During constitutive expression of OpaR, *V. parahemolyticus* forms opaque colonies with reduced swarming ability and virulence factor expression (Burke et al., 2015).

2.5 AHL Biosensors

AHL biosensors play a great role in aiding the identification and elucidation of QS systems. Unable to produce its own AHL signals, these biosensors respond to the exogenous AHL via their functional LuxR receptor cloned with specific promoter namely *luxI* synthase promoter. It then would be able to coordinate the transcription of certain reporter genes which exhibit visible indicators such as violacein pigment production, β-galactosidase, bioluminescence and green-fluorescence. Since each biosensor harbors a specific set of LuxR protein and will only bind to its cognate AHL/s, the use of several biosensors is crucial when testing a certain bacterium for AHL/s production. There are several ways to deploy the biosensor; one of them is by growing the biosensor and the tester strain on solid media close together by forming a 'T'.

The phenotypic changes that occur to the biosensor can be observed when it reacts with the exogenous AHL produced from the tester strain. Another method is partial characterization of the AHL whereby the AHL is extracted from the tester strain during the late exponential phase and subjected to thin liquid chromatography (TLC) on a reversed-phase C_{18} plates. Next, the TLC plates dried and overlaid with agar suspension containing the biosensor strain (Schaefer et al., 2000).

Some biosensors are only able to detect short and medium acyl chain AHLs which range from C4 to C8 chain length. A Gram-negative bacteria; *Chromobacterium violaceum* produces *N*-hexanoyl-L-homoserine lactone (C6-HSL) via *cviI* synthase. C6-HSL then binds to its receptor, CviR instigating gene regulations which lead to purple pigmentation. Exploiting this visible phenotypic change, an AHL-negative and violacein double mini Tn*5* mutant of *C. violaceum* CV026 was created and purple pigmentation was observed when supplemented with exogenous AHLs (McClean, Winson, Fish, et al., 1997). Potent reaction was observed when tested using C6-HSL while six-fold reduction in purple pigment production was observed when tested against OC6-HSL and *N*-octanoyl-homoserine lactone (C8-HSL). CV026 was also able to induce weak purple pigmentation in the presence of exogenous OC8-HSL and *N*-butanoyl-L-homoserine lactone (C4-HSL). Poor response was observed when using exogenous *N*-(3-oxo-butyryl)-L-homoserine lactone (OC4-HSL) and AHLs with acyl side chain ≥. Furthermore, AHL which contains the 3-hydroxy bonds are undetectable by CV026 (Steindler & Venturi, 2007). Usually, CV026 biosensor is applied by conducting the close 'T' streak on solid agar and TLC-soft agar overlay method.

Another biosensor commonly used for detection of long chain AHL is constructed based on QS system of *P. aeruginosa*. A plasmid pSB1075 which contains the promoter of both LasI and LasR, manipulates the expression of *luxCDABE* (Winson et al., 1998). pSB1075 which can only function in *E. coli* strain is used to detect the presence of *N*-(3-oxo-decanoyl)-L-homoserine lactone (C10-HSL),*N*-dodecanoyl-L-homoserine lactone (C12-HSL) and their 3-oxo derivatives whereby it produces luminescence when supplied with exogenous AHLs when viewed under a luminometer. Another biosensor, *E. coli* pKDT17 which harbors the *lac* promoter and *lasB*::*lacZ* translational fusion responds to the same long chain AHLs as in *E. coli* pSB1075 can be detected via TLC analysis and quantified by conducting the standard β-galactosidase determination protocol (Pearson et al., 1994). Both of these *P. aeruginosa* based biosensors do not respond to short acyl chain AHL. A *P. aeruginosa lasI* knock-out mutant, PAO1 M71LZ with *rsaL*::*lacZ* transcriptional fusion is also a biosensor used to detect long chain AHLs via the standard β-galactosidase detection protocol (Dong et al., 2005).

Multiple AHLs containing acyl chain ranging from C4 to C12 can be detected by a biosensor with broad range detection, *A. tumefaciens*. *A. tumefaciens* produces its own AHL (OC8-HSL) and the QS system of *A. tumefaciens* TraI/R regulates the conjugal transfer of plasmids needed for their life processes (Farrand, Qin, & Oger, 2002). The biosensor strain of *A. tumafaciens* NT1 (pZLR4) does not contain the Ti plasmid rendered them unable to produce their own AHL. However, it contains plasmid pZLR4 containing the *traR* gene and its operon together with reporter fusion *traG*::*lacZ* which enable it to respond to exogenous AHLs and easy detection by conducting the β-galactosidase essay (Umesha & Shivakumar, 2013).

In some cases, negative result may be obtained in detecting AHL production by using the biosensor. Nevertheless, it does not mean that the tester strain is incapable of producing AHL/s. The reason behind this occurrence is because the tester strain might be producing novel AHLs that are undetectable by the biosensor strain or the concentration of the AHL/s produced by the tester strain is below the threshold level needed to activate the biosensor (Venturi, 2006). A false-negative result can also occur due to the bacteriostatic effect of the compounds produced by either the tester strain or the biosensor. To combat this problem, the plasmid containing the AHL sensor system could be transformed or conjugated into the target bacteria. The growing media condition should contain the suitable pH so that the AHL/s would not be hydrolyzed. In addition, long chain AHL/s usually has low cell membrane permeability thus making it undetectable by the biosensor. By growing the tester strain using whole culture or using its spent supernatant, the problem may be averted (Llamas, Keshavan, & Gonzalez, 2004).

2.6 Interference with QS

The use of antibiotic to control and treat bacterial infections is becoming more irrelevant as bacteria have come up with resistance traits to ensure their survival by rapid evolution. Antimicrobial resistance may be subjected to the use of similar antibiotic for the last century and the rise of multiple drug resistant (MDR) bacterial strains (Kalia, Rani, Lal, Cheema, & Raut, 2007). Apart from infecting humans, bacterial infections also affect the business industry such as the aqua farming of shrimps, fishes and shellfishes, aquatic based tourism and food supply (Skandamis & Nychas, 2012). Virulence and pathogenicity factors such as biofilm formation are directly related to QS and studies have shown that bacteria encapsulated inside a biofilm is 1000 times more resistant to the use of antibiotic compared to bacteria in the planktonic state (Olson, Ceri, Morck, Buret, & Read, 2002).

Hence, the intensive study on the biofilm production and its QS system has led to discovery of molecules produced by both prokaryotes and eukaryotes that are able to manipulate the QS system known as quorum quenching (QQ) (Kalia & Purohit, 2011). Apart from QQ, the production of synthetic chemical compounds and the quorum sensing inhibitors (QSIs) compounds are also able to attenuate QS (Janssens et al., 2008; Thomas Bovbjerg Rasmussen et al., 2005). Attenuating the QS process can be accomplished by several methods such as decreasing the AHL synthase or its respective receptor, inhibiting the assembly of signaling molecules, hydrolyzing the AHL molecules, and using synthetic molecules identical to AHLs to bind to the receptor (Kalia, 2013). The concept of biosensor strain such as CV026 and *A. tumefaciens* NT1 has given insight to researchers to search for analogues molecules of AHLs. The ability of the LuxR receptor and its homologue to accept certain sets of AHL molecules

without undergoing the gene expression except for the intended identifiable phenotypes has led to screening of natural QSI molecules with no toxic properties (McLean, Pierson, & Fuqua, 2004). There are many types of natural QSI molecules that can be found in nature. One of them originates from the prokaryotic cells itself by producing QQ enzymes that can degrade the AHLs.

There are four types of enzymes that can degrade AHL which are: decarboxylases, lactonases, acylase and deaminase. Both decarboxylases and lactonases degrade AHLs by hydrolyzing the lactone ring present in the AHL molecule structure and acylase and deaminase acts by removing the acyl side chain. Lactonases are commonly produced by the *Bacillus* spp. sharing 90 % homology among them (Dong & Zhang, 2005). Lactonase produced by *B. megaterium* has a wide range of substrate specificity ranging from short chain AHL (C4-HSL) to long chain AHL such as *N*-(3-oxo-dodecanoyl)-L-homoserine lactone (OC12-HSL) (Chowdhary et al., 2007). There are many acylase producing bacteria and interestingly, the similarities shared between this acylases are quite low about 32 – 39 % only. Some of the acylases produced only cleave a certain length of AHL chain. For example, the AHL-acylase produced by *P. aeruginosa* specifically cleaves OC12-HSL but could not cleave the side chain of C4-HSL (Sio et al., 2006). On the other hand, the acylase-AHL produced by *Streptomyces* sp. is capable to cleave acyl chains with 6 or more carbons (Park et al., 2005). Another QQ enzyme; oxidoreductase acts by replacing the 3C keto group of the AHL side chain with a hydroxyl group thus rendering the AHL inactive (Uroz et al., 2005). Oxidoreductase are produced by *Burkholderia* sp. GG4 was shown to modify OC6-HSL and reduce *E carotovora* virulence (Chan et al., 2011).

Since bacteria and other eukaryotic organisms such as animals, humans and plants have co-existed together, they have adapted several mechanisms in order to exist harmonically together. It has been discovered that some animals also produce QQ enzymes in order to hinder or disrupt the QS between bacteria. Paraoxonases (PON) are enzymes produced by both animals and humans act by hydrolyzing the esters and lactones (Camps, Pujol, Ballester, Joven, & Simó, 2011). The difference between PON-lactonases produced by mammalian and prokaryotic cells is the absence of "HCDH ~ H ~ D" motif in prokaryotic cells. PON-lactonases require calcium for their activity and evidence has shown that the epithelial cells' lactonase in humans are able in inactivate OC12HSL produced by *P. aeruginosa* (Stoltz et al., 2007).

Passive in nature, plants and fungi have adapted to producing multiple QSI molecules as their first line of defense against pathogenic bacteria. Extracts of multiple plants have shown QSI potential by sharing structure similarities to the AHL molecules thus creating competition for the AHL signals to bind to their receptor. Another factor is the ability of the extracts to degrade the signal receptors; LuxR and its homologues (Teplitski, Mathesius, & Rumbaugh, 2010). An example of interference in LuxR is the penicilic acid and patulin produced by *Penicillium* fungus species which disrupts the RhlR and LasR function in *P. aeruginosa* (Rasmussen & Givskov, 2006).

AHL degradation in *A. tumafaciens* by lactonase was aided by the GABA (γ-aminobutyric acid) produced by majority of plants (Chevrot et al., 2006). In fact, traditional medicine often uses plants extracts as a therapy to cure or prevent bacterial infection. Studies made on the effectiveness of the plants extracts used eventually revealed the connection between molecules produced by the plants which can attenuate QS. For example, cinnamaldehyde and its derivative from cinnamon (*Cinnamomum verum*) successfully inhibit QS related behavior in both *Vibrio* spp. and *P. aeruginosa* such as biofilm formation (Brackman et al., 2008; Niu, Afre, & Gilbert, 2006).

Other well-known plants extracts used in traditional medicine are carrot, bean, chamomile, propolis, habanero, water lily and garlic were also reported capable of attenuating QS (Ivanova, Fernandes, & Tzanov, 2013). Among all these reported plant extracts, garlic was proven to be the most effective by asserting at least three different strategies to inhibit QS pathway and the combined effect of garlic and tobramycin reduced biofilm formation effectively (Thomas Bovbjerg Rasmussen et al., 2005). In addition, some of the polyphenol produced by plants have the potential to disrupt QS and the formation of biofilm. The polyphenol produced by *Camellia sinensis* (Green tea), epigallocatechin gallate (EGCG) demonstrated alteration in virulence factors expression in *P. aeruginosa* PAO1 (Mihalik, Chung, Crixell, McLean, & Vattem, 2008).

Furocoumarins is an organic compound present naturally in almost all plants. The furocoumarins produced by grapefruit were shown to have anti-QS properties by successfully inhibiting the AI-1 and AI-2 actions in several bacteria such as *Salmonella typhimurium* and *E. coli* O157:H7. Concentrated purified furocoumarins compound which are berggamottin and dihydroxybergamottins successfully inhibits the autoinducer at rate of 94.6 – 97.7 %.

Seeds of sour orange contains compound known as limonoids. In *V. harveyi* limonoids shown more than 90 % inhibition on AI-2 mechanisms (Amit Vikram, Jesudhasan, Jayaprakasha, Pillai, & Patil, 2011). Interestingly, both furocoumarins and limonoids share similarity with another compound; halogenated furanones (fimbrolides). Naturally produced by red microalgae *D. pulchra*, there are more than 30 different furanones produced as secondary metabolites with QS inhibition capacities demonstrated (Kociolek, 2009).

Subsequently, synthetic derivatives of halogenated furanones were produced and it was proven to be more effective than the natural furanones when administered to rats infected with fatal *P. aeruginosa* (Wu et al., 2004). The mode of action of furanones is still unclear and even though the results obtained using synthetic furanones were promising, synthetic furanones were too reactive and unstable thus rendering it unsuitable to be use on humans due to its toxic effects (Defoirdt, Boon, Sorgeloos, Verstraete, & Bossier, 2008).

2.7 Omics Technology

It was until 1975, Sanger and Coulson have successfully sequenced the DNA of a bacteriophage by using the 'plus and minus' sequencing method (Sanger & Coulson, 1975). With the breakthrough in DNA sequencing multiple sequencing strategies have emerged such as the chain-terminator and dye terminator Sanger sequencing together with automated sequencing. Sanger sequencing technology is limited to only a few samples at one time and larger sequences output would have to be separated and purified using gels and polymers. In 2005, new method of sequencing termed the next generation sequencing (NGS) allowing massive data generated from a single experiment drastically reducing the time taken for a sequencing project from years to weeks with increased accuracy (Hutchison, 2007).

At low cost and faster generation of data, NGS allows better understanding of a genome by annotation of genes via certain platforms with annotation tools and reference to existing database. Since bacteria genomes are much smaller than higher organisms such as humans, animals and plants, more samples can be sequenced at a time using platform such as Illumina MiSeq personal sequencer. The availability of whole genome sequences provides us with better understanding of the processes and mechanisms throughout an organism as well as allowing us to map out the positions of genes and insert hence determine their respective functions. As such, with more bacteria being sequenced from time to time, we are able to conduct comparison studies between the bacteria and understand the micro events that take place and study the evolutionary pathway of a certain organism. The revolutionary advancement in genomics study which involves DNA recombinants, sequencing of the DNA and bioinformatics to

annotate data obtained have help many research areas such as functional genomics, structural genomics, epigenomics and metagenomics.

Advancing from genomic approach, in order to know more about the micro events that occur during a specific growing stage or physiological condition, the quantity of transcripts and the presence of complete set of transcripts is studied. Transcriptomic study is to differentiate and classify all types of transcripts including mRNAs, small RNAs and and non-coding RNAs, to uncover the gene transcriptional structure; and to quantify the fold change of expression levels observed during growth and varied conditions (Wang, Gerstein, & Snyder, 2009). A method to analyze the eukaryotic transcriptomes known as RNA-sequencing (RNA-seq) basically uses a population of RNA extracted from the organism of interest and converts them to cDNA fragments attached with adapter on both or one end. The cDNA were then prepped and underwent high throughput sequencing and the obtained reads are mapped to a reference genome or assembled *de novo*. RNA-seq technology can be used to compare two different growing conditions on an organism such as bacteria by introducing treatment with certain compound or exposure to certain stress to quantify the level of expressed transcripts thus knowing which genes are up or down regulated. By using this technology, a more precise quantification of transcripts and isoforms can be obtained as well as increase the knowledge in more depth and complexity of eukaryotic and prokaryotic organism.

CHAPTER 3: METHODOLOGY

3.1 Chemicals and Solvents

The research grade chemicals used in this study were purchased from Merck, Germany; Sigma, USA; Promega Ltd, USA; Sigma-Aldrich, USA; Thermo Fisher Scientific, USA; BDH Ltd, UK; BD Difco™ Laboratories, USA; Cayman Chemicals, USA. Solvents utilized during the experiment were purchased from Fisher Scientific, UK.

3.2 Equipment and Instruments

The equipment and instruments used in this study are as follows; thermal cycler (Bio-Rad; ABI), shaking incubator (N-biotek), Libra S4 spectrophotometer (Biochrom, UK), Tecan microplate reader (Infinite M200®, Mannerdorf, Switzerland), incubators and ovens (Memmert, Germany), thermomixer (Eppendorf), weighing balance (Sartorius), centrifuge machine (Eppendorf),UV transilluminator (UV Products, Inc.), NanoDrop spectrophotometer (Thermo Scientific, USA), Qubit 2.0 fluorometer (Life Tech., USA), Microflex MALDI-TOF MS (Bruker Daltonik Gmbh, Leipzig, Germany), high resolution tandem liquid chromatography quadrupole mass spectrometry (LC-MS/MS) (Agilent Tech., USA), Illumina MiSeq personal sequencer (Illumina Inc., CA) available in HIR Central facility, CFX96™ Real-Time Polymerase Chain Reaction (RT-PCR) Detection System (Bio-Rad Laboratories Ltd., USA), Milli-Q® water purification system (Merck Millipore, Germany), Analytical Table-top Microscope SEM TM3030 (Hitachi), Bioanalyzer (Agilent Technologies Inc., USA), and pipettes (Eppendorf).

Other equipment include; petri dishes, tips, tubes, Schott's bottle, conical flask, dryer and cuvettes.

3.3 Growth Media

Growing media used throughout the experiment are Luria-Bertani (LB) medium (Scharlab, Barcelona, Spain) supplemented with NaCl concentration from 1 - 3 % (w/v) when needed and were prepared and autoclaved at 121 °C, 15 psi for 15 mins. For pH adjustment, 1N of HCl and 1N of NaOH were used. Growing media were left to cool before adding antibiotics and the heat labile items were syringe filtered using sterile filter of 0.22 µm pore size (SartoriusMinisart).

3.4 Stock Solutions

3.4.1 Synthetic *N*-acyl Homoserine Lactone (AHL)

The synthetic *N*-acyl-homoserine lactone (AHL) stock solutions (Sigma-Aldrich and Cayman Chemicals, USA) stock solutions were dissolved in HPLC grade acetonitrile (ACN) (Merck, Germany).

3.4.2 Antibiotics Stock Solutions

Stock solutions for antibiotic were prepared and filtered using sterile syringe filter (pores size of 0.22 µM) and stored in -20 °C until used (Table 3.1)

Table 3.1: Stock solutions of antibiotics used in this study

Antibiotics	Stock Concentration (mg/mL)	Solvent
Ampicillin	100	dH_2O
Chloramphenicol	34	100% ethanol
Kanamycin	50	dH_2O

3.4.3 1× Phosphate Buffered Saline (PBS) Solution

In 100 mL dH_2O, three chemical components which are, 900 mg of NaCl, 115 mg of Na_2HPO_4 and 23 mg of NaH_2PO_4 were mixed thoroughly. The pH was adjusted to 6.5 followed by sterilization of the solution by autoclaving.

3.5 Gallic Acid Compound

The pure gallic acid (3,4,5-trihydroxybenzoic acid) was purchased from Sigma-Alrich. It was dissolved in dH_2O and the stock concentration used in this study was 10 mg/mL and was filter sterilized using syringe filter (pores size of 0.22 µm). The solution was kept in room temperature until used or needed.

3.6 Agarose Gel Electrophoresis (AGE)

3.6.1 10× TBE (Tris Borate EDTA) Buffer

TBE was prepared by adding 10.8 g Tris base, 7.44 g $Na_2EDTA●2H_2O$ and 55 g boric acid was dissolved in 100 mL of dH2O. The pH was adjusted to 8.0 and subsequently autoclaved at 121 °C for 20 mins

3.6.2 1 % (w/v) Agarose Gel

In a conical flask, 50 mL of 1× TBE was poured and added with 500 mg of agarose powder. The mixture was heated until a clear solution is obtained and left to cool. Once cooled, 1 µL of gel dye, GelStar™ Nucleic Acid Gel Stain (Lonza, Basel) was added and mixed thoroughly before casting into 50 mL gel.

3.6.3 DNA Ladder

Both 100 bp and 1 kb DNA ladder from GeneRuler™ purchased from Fermentas International Inc., Canada, was used throughout this study.

3.7 Commercial Kits

Table 3.2: Commercial kits used in this study

Kit	Application	Manufacturer
***i-Taq*™ DNA Polymerase Kit**	Amplification of PCR	iNtRON Biotechnology, Korea
MasterPure™ DNA Purification Kit	Extraction of genomic DNA	Epicentre Biotechnologies, USA
QIAquick Gel Extraction Kit	Agarose gel DNA purification	Qiagen Pty. Ltd., Germany
QIAquick PCR Purification Kit	Purification of PCR product	Qiagen Pty. Ltd., Germany
QIAquick Spin Miniprep Kit	Extraction of plasmid DNA	Qiagen Pty. Ltd., Germany
NucleoSpin® RNA Kit	Extraction of RNA	Macherey-Nagel GmbH & Co. KG, Duren, Germany
QuantiTect® Reverse Transcription Kit	Conversion of RNA to cDNA	Qiagen Pty. Ltd., Germany
SolGent™ Real-Time PCR kit	Real-time PCR amplification	SolGent Co., Ltd., Korea
pGEM®-T Easy Vector Systems	PCR products cloning	Promega, USA
Qubit dsDNA High Sensitivity (HS) Assay Kit	DNA qualities assessment	Life Technologies, USA

Kit	Application	Manufacturer
Nextera™ DNA Sample Preparation Kit; Nextera Index Kit	Whole genome sequencing DNA sample preparation	Illumina, Inc., CA
Illumina Library Quantification Kit	Libraries authentication prepared for Illumina platform	KAPA Biosystems, Woburn MA
Agilent High Sensitivity DNA Kit	Low concentration dsDNA segregation, sizing and quantification (50-7000 bp)	Agilent Technologies Inc., USA
Agilent RNA 6000 Nano Kit	For evaluation and calculation of total mRNA samples with concentration of 25-500 ng/µL	Agilent Technologies Inc., USA
Agilent RNA 6000 Pico Kit	For evaluation and calculation of total mRNA samples with 500-5000 pg/µL concentration	Agilent Technologies Inc., USA
Ribo-Zero rRNA Removal Kit	Single pass removal of ribosomal RNA	Epicentre Biotechnologies, USA
Scriptseq v2 RNA-Seq Library Prep Kit	Construction of diverse sequencing libraries which requires a very little RNA: 500 pg+ of rRNA-depleted RNA or poly(A)+ RNA	Illumina, Inc., CA

3.8 Primers /Oligonucleotide

Table 3.3: List of oligonucleotide used together with their sequence and length

Primer	Sequence	Length (-mer), Reference
16S rRNA gene amplification		
16S rDNA forward primer 27F	AGA GTT TGA TCM TGG CTC AG	20, (Ott, Musfeldt, Ullmann, Hampe, & Schreiber, 2004)
16S rDNA reverse primer 1525F	AAG GAG GTG WTC CAR CC	17, (Dewhirst et al., 1999)
	Gene Cloning of QS-related genes	
T7	TAA TAC GAC TCA CTA TAG GG	20, Universal Primer
SP6	TTC TAT AGT GTC ACC TAA AT	19, Universal Primer
VanM_F	CCA TGG AAT GGC TAG TTA CGA TCA TAC AA	29, This study
VanM_ R	CTC GAG ACT AAG CAT GGT TGA GCT CA	26, This study
	Colony PCR Verification for Gene Cloning	
VanM_CP_F	TCG GTT TGT CTC TCA GCG TCT	21, This study
VanM_CP_R	CGC CGA TAA TCT GAG GAT GT	20, This study
	Housekeeping Genes for Normalization of RT-PCR Expression Data	
GyrB_F	TGA TTA CGG CTC TTG GTT GTG	21, This study
GyrB_R	TTG GTT CAT CGC ATC TTC ATC	21, This study
Rec A_F	CCG CGC AAT GGA TGT AGA AA	20, This study
RecA_R	AAG TGC TTG CTC ACC AGT GT	20, This study
TnaA_F	TGC ATT ACC GTA TCA GCC AAG	21, This study
TnaA_R	**CCC AGC TTC CAA ATA AAG TGC**	21, This study

Primer	Sequence	Length (-mer), Reference
	RT-PCR for RNA-Seq with Anti-QS Compound	
HmuU_F	TTC CTC TTT CCG CTA GTC TGC	21, This study
HmuU_R	AGC CCC GAC TAA CAT ACA GAG	21, This study
SufD_F	AAG AAG AGG GGT GGA AGT ACA	21, This study
SufD_R	GCG CAC TTT AGG AAT CCA ATC	21, This study
SODMn_F	GCA TCA CCG CAC TTA CTT TGA	21, This study
SODMn_R	AAA GTA TTA ATC GCC GCC TCA	21, This study
Fis_F	TCA AAA GCC TTT ACG TGA CTC TG	23, This study
Fis_R	TTG GTT ACC GCG AGT GTA CTG	21, This study
YhjX_F	CGG GCT TTG TTA ACT CTC CAC	21, This study
YhjX_R	CAA GAA TAC CCA CAC CAA GCA	21, This study
PilP_F	GTG GAC TTA AAG CCC GTG TTC	21, This study
PilP_R	GTT CAA GCT GTC CGG TTT TTC	21, This study

3.9 Plasmids

There are two plasmids used in the study, pGEM®-T Easy Vector and pET-28a for cloning and overexpression purposes respectively (Table 3.4).

Table 3.4: Plasmid used together with their description and source

Plasmid	Description	Source/Reference
pGEM®-T Easy Vector	F1 ori, Amp^R, used as cloning vector	Promega, USA
pET-28a	F1 ori, Kan^R, used as expression vector	Novagen, Inc., Gemany

3.10 Bacterial Strains and Growth Conditions

The bacterial strains used in this study are listed in Table 3.5. The strains listed are routinely cultured on LB medium (1 % w/v casein peptone; 1 % w/v sodium chloride; 0.5 % w/v yeast extract). *C. violaceum* CV026, *Erwinia carotovora* (GS101) and *Erwinia carotovora* (PNP22) were cultured at the optimum growth temperature; 28°C. *E. coli* DH5α and BL21 (DE3) pLysS were incubated at 37°C as its optimum growth temperature.

Table 3.5: List of bacterial strains used

Strains	Description	Source
***Chromobacterium violaceum* CV026**	Derived from *C. violaceum* ATCC 31532. This mini-Tn*5* mutant acts as a biosensor with the formation of purple pigment in the presence of exogenous short chain AHL/s signaling molecules	(McClean, Winson, Fish, et al., 1997)
***Erwinia carotovora* GS101**	Restrictionless, modificationless derivatives that produces OC6-HSL for the regulation of its production of carbapenem	(McGowan et al., 1995)
***Erwinia carotovora* PNP22**	Mutants derive from GS101 with defective *carI* gene which code for the production of AHL synthase. It serve as the negative control for the AHL preliminary test due to its inability to synthesize OC6-HSL	(McGowan et al., 1995)
***Escherichia coli* DH5α**	Host that carries no plasmid that yield high quality and concentration of inserted plasmid	(Taylor, Walker, & McInnes, 1993)
***Escherichia coli* BL21 (DE3) pLysS**	Under the control of T7 promoter enables high-efficiency of targeted protein expression coupled with ribosome binding site. Lac UV5 promoter controlled the expression of T7 RNA polymerase and the presence of pLysS with T7 lysozyme to decrease background noise governed by the T7 promoter with no restriction to level of expression when induced with IPTG	Novagen Inc., Germany

3.11 Seawater Sampling and Isolation of Bacteria

The location of sampling was at a commercial recreation spot in Morib Beach, Hulu Selangor. Samping was done once and sterile 15 ml falcon tubes were used to collect the seawater samples by holding the tubes against the currents. The sample was transferred on ice immediately and kept cool until further processing at the lab. At the lab, ten-fold serial dilution of the seawater samples were conducted with saline buffer (0.9 % NaCl) and spread on Luria-Bertani agar (LBA) with different NaCl concentration ranging from 1% to 3% (w/v). From this stage, the routine culturing of isolated colonies of bacteria will be done on the medium that showed the most bacterial growth. All the isolated colonies from the processing stage were further cultured to obtain the pure culture.

3.12 Preliminary Test of AHLs Production

After obtaining the pure culture, the isolated bacterial strains were tested against a biosensor; *C. violaceum* CV026 which will produce purple pigment in the presence of exogenous short chain AHL/s (McClean, Winson, Leigh, et al., 1997). *E. carotovora* GS101 and *E. carotovora* PNP22 were used as the positive and negative control respectively. The preliminary AHL/s detection test was done on the LB agar and incubated at 28°C for 24 hrs. Purple pigment production in the CV026 indicates the production of short chain AHL/s from the isolated strains.

3.13 Identification of Bacteria

3.13.1 Matrix-assisted Laser Desorption Ionization Time-of-flight Mass Spectrometry (MALDI-TOF MS)

Rapid identification of the QS positive strains were conducted by using Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). The sample preparation for MALDI-TOF MS analysis was carried out as reported previously by(Mellmann et al., 2008). Firstly, fresh culture of the QS positive strains cultured on LB agar were smeared on a MSP 96 target polished steel BC plate and overlaid with 1 µL of MALDI matrix (Robson, Yin, & Chan, 2013) and the sample was air-dried before further analysis using the Microflex MALDI-TOF bench-top MS (equipped with UV laser at wavelength of 337 nm). The method for analysis of the sample was conducted as previously described (Robson et al., 2013) and the identity of the sample was evaluated based on a dedicated scoring system where the spectra information of the sample was compared to the best match in the Bruker database. The scoring value used was according to those described previously(Robson et al., 2013).

3.13.2 Molecular Identification of Strains Positive for AHLs Production

3.13.2.1 Extraction of Genomic DNA

The isolated strains which showed positive result in the preliminary AHL/s detection test, were subjected to genomic DNA extraction. Using overnight culture of isolates grown in LB broth was pelleted (13, 000 rpm) for 10 min. The genomic DNA was extracted using MasterPure^{TM} DNA Purification Kit (Epicentre) following the guidelines illustrated in the instructions manual. The extracted genomic DNA was eluted with 50 µl of elution buffer and kept in -20 °C.

3.13.2.2 16S rRNA Gene Amplification by Polymerase Chain Reaction (PCR)

Amplification of the 16S rDNA was conducted to identify the genus and species of the strain/s of interest precisely. The extracted DNA was amplified with the 27F, and 1525R primers (Chong et al, 2012). The polymerase chain reaction (PCR) parameters was set as 32 cycles; 1 cycle of denaturation step at 94 °C for 5 min, subsequently followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 63 °C for 30 s and extension at 72 °C for 90 s and lastly 1 cycle of final extension step at 72 °C for 7 min. The PCR component was obtained from *iTaq*^{TM} DNA polymerase kit (iNtRON) and details of the components used are listed in table 3.7. Ultrapure water was set as negative control substituting the genomic DNA in the PCR run.

Table 3.6: PCR components utilized in 16S rRNA amplification

Items	**Volume/sample (µL)**
Ultrapure H_2O	9.9
10× buffer with 2mM $MgCl_2$	1.5
dNTP (200 µM)	1.2
***Taq* polymerase (5 U/µL)**	0.2
27F forward primer (10 µM)	0.6
1525 R reverse primer (10 µM)	0.6
Genomic DNA	1.0
Total reaction volume/sample	15.0

3.13.2.3 Agarose Gel Electrophoresis (AGE)

1.0 % (w/v) of agarose gel pre-stained with GelStar™ was submerged in the 1× TBE buffer. The 16S rRNA PCR products were mix with 6× loading dye at 5:1 ratio and loaded in the wells in the agarose gel. The parameters of the gel electrophoresis were set at 80 V and 400 mA and ran for 40 mins. After electrophoresis, the stained gels containing the products were viewed under UV illuminator (UVP, USA). The size of PCR products as well as DNA sample were estimated by using both 100 bp and 1 kb DNA ladder (GeneRuler™) as reference respectively.

3.13.2.4 Purification of PCR Products

Viewed using UV transilluminator (UV Product), the band with the target DNA size was excised using a clean and sharp scalpel and placed in a sterile1.5 mL microcentrifuge tube. The excised gel was weighed and purified using QIAquick® Gel Extraction Kit (Qiagen).

3.13.2.5 16S rDNA Sequencing Analysis

Sanger sequencing analysis of the purified PCR products were conducted by 1st base (Malaysia) and Chromas software (Technelysium) were used to view and interpret the results. The reads were trimmed, aligned and compared with other 16S DNA sequences from the GenBank database via the BLASTN application available on the National Center for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). The identities of the isolates were determined with regard to the closest species match.

3.13.2.6 Phylogenetic Analysis

The phylogenetic analysis using the 16S DNA in FASTA file format sequences were carried out via Molecular Evolutionary Genetic Analysis (MEGA) 6.0 software (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The phylogenetic analysis employ the maximum likelihood statistical method with bootstrap value of 1000 replicates. For each analysis, the in-group taxa data was obtained from closest match using the BLASTN application and included together with an out-group from distantly related taxon to produce a rooted phylogenetic tree.

3.14 Extraction of AHLs

Fresh cultures of the strains that are positive for AHL/s production were subsequently cultured into 5.0 ml of LB broth supplemented with 50 mM of 3-[*N*-morpholino] propanesulfonic acid (MOPS) to avoid the alkaline hydrolysis of the AHL by adjusting the pH to 5.5. The cultures were incubated overnight (28 °C) on the shaking incubator. From the inoculated broth, 1.0 ml of each culture were sub-cultured into 200 ml of fresh LB-MOPS and kept at 28 °C for 18 hrs. Next, using equal amounts of acidified (0.1 % (v/v) glacial acetic acid) ethyl acetate, the spent supernatant of each culture were extracted twice by vigorously mixing both the cultured broth and acidified ethyl acetate. The mixtures were left to separate into two distinct layers and the top clear layer was extracted into new sterile tubes. Each separated clear extracts were then left to dry completely in the fume hood and later kept in -20 °C.

3.15 AHLs Profiling via High Resolution Tandem Liquid Chromatography Quadrupole Mass Spectrometry (LC-MS/MS)

The dried AHLs extract was re-suspended by using 2 ml of acetonitrile (ACN). The re-suspended extracts were then further diluted by dissolving it in 150 µl of ACN. 100 µl of the dissolved extract were then inserted into individual vials and sent for LCMS analysis. The mobile phases used in the LCMS analysis were LCMS grade water and ACN at 80:20 ratios respectively. Both the mobile phases were added with 0.1 % v/v formic acid. The column (solid phase) used was the ZORBAX Rapid Resolution High Definition SB-C18 Threaded column (Agilent, USA) with 1.8 µm particle size, 2 µl injection volume with 0.21 cm × 0.50 cm. Additional operating parameters such as the capillary voltage, sheath gas, ion source, desolvation temperature and nebulizer pressure were set as reported before. To detect the AHLs, precursor ion scan mode was activated with *m*/*z* value ranging from 150 to 400; scanning the $[M+H]^+$ ion in the lactone ring moiety with *m*/*z* value of 102. The flow rate was set at 0.4 ml/min at room temperature.

3.16 Whole-Genome Sequencing (WGS)

3.16.1 Genome Sequencing via MiSeq Platform

The genomic DNA of the isolated strains was extracted as previously mentioned in section 3.13.2.1. The quality of the DNA extracted was then checked using agarose gel electrophoresis and measured using NanoDrop spectrophotometer (Thermo Scientific) and Qubit dsDNA High Sensitivity (HS) Assay Kit (Life Technologies). The sequencing library was then prepped using Nextera™ DNA Sample Preaparation Kit and Nextera Index Kit (llumina) following the guidelines provided by the manufacturer. After the sample has been prepped, it was sequenced using the Illumina MiSeq personal sequencer (Illumina) with paired-end read length configuration of 2 × 75. Paired read length of 2 × 75 was used in this sequencing project due to the amount of samples sequenced in this study (five strains) together with other bacterial strains non-related to this study (other individual study) rounding up to a total of 12 bacterial strains in one run.

3.16.2 Bioinformatics Data Analysis

The sequenced genomes were then assembled using the CLC Genomics Workbench 5.1 (CLC Bio, Denmark). The assembled genome was then deposited into GenBank to obtain their respective accession numbers. Gene predictions of the sequenced reads were done using Prodigal 2.60 while annotation of the genomes were done using an online server database; Rapid Annotation using Subsystem Technology (RAST)(R K Aziz et al., 2008). To identify the non-coding sequences and the miscellaneous genes, RNAmmer (Lagesen et al., 2007) and tRNA scan SE online servers (Lowe & Eddy, 1997) were used respectively. From the annotated genome, LuxI and its homologues from strain T01 were fished out and sequence similarity searched was conducted using

the BLASTP application on NCBI GenBank database. The sequences were aligned and phylogenetic analysis was conducted using MEGA 6.0 as mentioned in section 3.13.2.6.

3.17 Morphology Study using Scanning Electron Microscope (SEM) and Classification of Bacteria

The established protocol by Fischer et. al., (2012) with slight modifications was adapted in sample preparation to be viewed under the SEM (Fischer, Hansen, Nair, Hoyt, & Dorward, 2012). First of all, fresh bacterial culture were immersed in 5 % glutaraldehyde containing 0.1 M of PBS with pH adjusted to 7.2 and left to incubate for 24 hrs to fix them. Next, the fixed cells were then washed twice with 0.1 M PBS (pH 7.2) followed by post-fixing of the cells by immersing them in 1 % osmium (OsO_4) for an hour. The osmium covering the cells were then washed off with dH_2O twice and subsequently subjected to gradient dehydration using ethanol starting with 50 %, 75 %, 95 % and 100 % ethanol. Hexamethyldisilazane (HMDS) was then used to overlay the cells for 10 min before drying of the cells in the dessicator for 24 hrs. Prior to viewing under Analytical TableTop Microscope SEM TM 3030 (Hitachi), the dried fixed cells were mounted on aluminum stubs and coated with gold particles using SC7620 mini sputter coater (Quorum Technologies). The selected bacteria was then sorted according to the Minimum Information about a Genome Sequences (MIGS) (Field et al., 2008) which will provide a better understanding of the source of genome with relation to its geographic location and sampling date.

3.18 Gene Functionality Study via Cloning

3.18.1 Recombinant VanM Expression Plasmids

One LuxI homologue namely VanM was found in strain T01 from the annotation platform. The autoinducer synthase gene was fished out and phylogenetic analysis was carried out using MEGA 6.0 (Tamura et al., 2013) based on the Maximum Likelihood method based on the JTT matrix model (Jones, Taylor, & Thornton, 1992). Besides phylogeny analysis, comparison analyses were also carried out on RAST and BLASTP application in available on NCBI database. In gene cloning; the *vanM* gene was amplified from the genomic DNA using PCR using a set of primer, VanM_F and VanM_R. The forward primer was equipped with two non-specific bases and an NcoI restriction site (underlined) while one non-specific base together with an XhoI restriction site was added on the reverse primer.

PCR parameters for amplification of *vanM* gene were set as follows: initial denaturation at 95 °C (5 min); 30 cycles of denaturation at 95 °C (30 s), annealing at 57 °C (30 s) and extension at 72 °C (1 min); final extension at 72 °C (5 min). For negative control, dH_2O was used substituting the template DNA. The PCR products were subjected to AGE for size verification and purification of PCR products were conducted using QIAquick Gel Extraction Kit (Qiagen).

Subsequently, pGEM-T Easy cloning vector supplied by Promega was utilized in ligating the purified PCR product according to the manufacturer's instructions. Heat shock transformation of the recombinant plasmid, pGEM-T-*vanM* generated into *E. coli* DH5α was conducted based on the protocol established by Sambrook and Russel (2001) with slight modifications (Sambrook & Russell, 2001). The transformants were left to grow for 16 hrs on LB agar supplemented with ampicillin for selection. Transformants with the recombinant plasmid, pGEM-T-*vanM* will form a colony and the agar and inoculated into a fresh LB broth with ampicillin to maintain the recombinant plasmid and grown for 16 hrs. The overnight culture was then pelleted and the recombinant plasmid was extracted using QIAquick Spin Miniprep Kit (Qiagen).

Next, the *vanM* gene was excised using NcoI and XhoI restriction enzymes (Promega) and later verified using AGE and extracted from the gel using using QIAquick Gel Extraction Kit (Qiagen). Next, purified *vanM* gene was ligated into linearized pET-28a obtained from Novagen and the recombinant plasmid produced, pET-28a-*vanM* was transformed into *E. coli* Bl21 (DE3) pLysS and grown on media containing both kanamycin and chloramphenicol for selection of successful transformants.

3.18.2 Verification of Transformants

Colony PCR were conducted to screen transformed cells with PCR parameters set as in section 3.13.2.2 and later sent for sequencing as mentioned in section 3.13.2.5. The cloned *vanM* gene was characterized by streaking the *E. coli* BL21 (DE3) pLysS employing the pET-28a-*vanM* against the biosensor, *C. violaceum* CV026 (refer to section 3.12). The positive control used was *E. carotovora* GS101 and two negative controls were used namely,- *E. carotovora* PNP22 and *E. coli* BL21 (DE3) pLysS harboring pET-28a only.

AHL extraction and profiling were conducted in order to further verify the production of AHL by the transformants. *E. coli* BL21 (DE3) pLysS harboring pET28a-*vanM* was cultured in 50 ml of LB broth supplemented with 50 mM of 3MOPS to avoid AHL degradation, along with kanamycin and chloramphenicol at 37°C until it reaches OD600 of 0.4 – 0.5. Induction of vanM gene expression in *E. coli* BL21 was achieved by adding isopropyl-D-thiogalactopyranoside (IPTG, Sigma) to a final concentration of 1.0 mM. *E. coli* BL21 (DE3) pLysS harboring only pET-28a was used as the negative control. The induced culture was allowed to grow for 4 h and 8 h at 25°C and after incubation time achieved, the culture was prepared to undergo AHL extraction. The AHL extraction was conducted as in section 3.14 and profiling of AHL was conducted as in section 3.15.

3.19 Biofilm Assay

The biofilm assay procedures were adapted from work done by Vandeputte et al., (2010) with slight modifications. Biofilm assay was performed using cullture treated with gallic acid and un-treated culture. Firstly, the bacterial culture was inoculated into fresh LB broth supplemented with 3 % NaCl (w/v) and incubated overnight at 28 °C. Next, the overnight culture was further diluted with LB medium and adjusted to OD_{600} of 0.1. In a 96-well microtitre plate, approximately 50 µl of the diluted culture was added into 930 ml of LB broth supplemented with 3 % NaCl (w/v) with two conditions, one of them contains1 mg/mL of gallic acid; a known anti-QS compound and the other without added gallic acid. Both types of cultures were incubated statically at 28 °C for 72 hrs. The planktonic bacteria were removed by washing three times with sterile distilled water (Lade, Paul, & Kweon, 2014) and the plate was air-dried for 15 min and was stained with 0.1% (w/v) crystal violet (200 µL per well) for 30 min. Excess crystal violet was removed and the wells were washed with sterile distilled water twice followed by addition of 95% (v/v) ethanol (200 µL) and 100 µL of the resulting solution was transferred to a new, sterile microtitre plate. The absorbance of the solution was read at OD_{590} with Tecan microplate reader (Infinite M200®). All experiments were repeated thrice.

3.20 Transcriptomics Study Using Known Anti-QS Compound, Gallic Acid

3.20.1 Extraction of RNA and Synthesis of cDNA

Strain T01 was cultured in two different conditions; without gallic acid and with gallic acid. Prior to culture, the minimal inhibitory concentration (MIC) assay for gallic acid was conducted and 1 mg/mL was selected as the gallic acid concentration used throughout this study. The biological triplicates for each condition containing strain T01 was cultured in LB medium supplemented with 3 % (w/v) NaCl until the OD_{600nm} reached 0.3 – 0.5. For extraction of total RNA, Macherey-Nagel Mucleospin RNA Kit (Macherey-Nagel) was used according to the manufacturer's instructions. The extracted RNA quality was quantified using the NanoDrop 1000 spectrophotometer (Thermo Fischer) at 260-nm/280-nm ratio. RNA reliability was measured using Agilent 2100 Bioanalyzer via Agilent RNA 6000 Nano Kit (Agilent) and the integrity value should be within 9.0 – 10.0. After all the necessary qualities meet, the half of the amount of purified RNA were subjected to reverse transcription assay for cDNA conversion using QuantiTect® Reverse Transcription Kit (Qiagen) by following the manufacturer's instruction.

3.20.2 RNA-sequencing

Other half of the extracted RNA underwent rRNA depletion using Ribo-zero rRNA Removal Kit (Epicentre), whereby the total rRNA content in the sample was depleted and subsequently followed by quality assessment via Agilent RNA 6000 Pico kit (Agilent). Next, the cDNA sequencing libraries were built using ScriptSeq v2 RNA-Seq Library Preparation Kit (Agilent) according to the manufacturer's instructions. Agilent High Sensitivity (HS) DNA Kit (Agilent) and Library Quantification Kits (KAPA Biosystems) were used to validate the sequencing libraries built. Once the libraries are validated, the templates were diluted to 2 nM prior to denaturation of the cDNA. Next, the denatured cDNA were then further diluted to a concentration of 10 pM before being sequenced by the MiSeq personal sequencer (Illumina). In this sequencing project, 2 × 250 bp paired-end read length configuration was used.

3.20.3 Transcriptomic Data Analyses

The data obtained from the sequencer, in FASTQ file format were aligned to the reference genome of strain T01 obtained from the WGS in section 3.16 in CLC Genomic Workbench 7.0 (CLC Bio) software. The paired reads obtained were subjected to trimming and further transcriptomic analyses with specific parameters. The RNA-reads were then exported in BAM file format for normalization of data and statistical analyses in Partek Genomics Suite (Saint Louis, MO). In Partek Genomics Suite (Saint Louis, MO), calculates the reads /kb of exon model/million mapped (RPKM)-normalized reads and estimates and determine differential expression level of genes using ANOVA. From the lists of differential expression level of genes, the false discovery rate (FDR)-corrected cut off P-value (P) ≤ 0.05 with fold change ≤ -2.0 and ≥ 2.0 were set as the deciding factor for subsequent validation procedure.

3.20.4 Quantitative Real-time PCR Assay

In order to validate the differential gene expression triggered by the anti-QS compound, gallic acid, several genes related to QS were selected to undergo qualitative RT_PCR (qRT-PCR). The template for this assessment is the cDNA generated in section 3.20.1. Primers used in amplification of specific genes are listed in Table 3.3 and the qRT-PCR components used in this experiment and their cycling conditions are listed in Table 3.8 obtained from SolGentTM qPCR assay (SolGent). The amplification of genes will be conducted using the Bio-Rad CFX-96 real-time detection system (Bio-Rad). Triplicates were applied for each reaction and evaluation of primer efficiency for each gene was done by performing standard curves using cDNA serially diluted into10-fold range. Next, three housekeeping genes namely; *recA*, *gyrB* and *tnaA* were used as internal control gene and as normalization of variability in level of expression for both conditions mentioned in section 3.20.1.

Table 3.7: qRT-PCR Components and cycle conditions

qRT-PCR Mixture (reaction volume : 15 µL)		**qRT-PCR Cycle Conditions**		
2× SolGent RT-PCR Master Mix	7.5 µL	95 °C	15 min	×1
Forward Primer (10 µM)	0.75 µL	95 °C	20 sec	
Reverse Primer (10 µM)	0.75 µL	55 °C	40sec	×40
cDNA Template (> 300 ng)	1 µL	72 °C	1 min/kb	
Ultrapure H_2O	5 µL	72 °C	5 min	×1

At the end of the cycle, a melt curve step whereby the temperature increases from 75 °C to 95 °C with 0.5 °C increase per second. The melt curve data was taken every 5 s. The melt curve data obtained was used to generate melting peaks by plotting the negative derivative versus temperature. The efficiency, correlation coefficient and slope for each qRT-PCR run was determine using the CFX Manager Software (Bio-Rad).

CHAPTER 4: RESULTS

4.1 Seawater Sampling

The sampling parameters are listed in Table 4.1 and routine streaking of the isolated bacteria were conducted on LB agar supplemented with NaCl concentration of 3 % (w/v) this agar allowed more colonies formed as compared to LB media supplemented with 1 % or 2 % NaCl (w/v).

Table 4.1: Sampling parameters

Parameters	**Respective value**
Date of sampling	18th April 2014
Location	Morib Beach, Banting, Hulu Selangor
GPS coordinate	N02° 45.023' E101° 26.623'
Time of sampling	10:00 A.M
Temperature of seawater	27 °C
Depth of sampled seawater	5 cm from seawater surface
pH	8.00

Serial dilution was performed to isolate pure colonies of 60 isolates on 3 % NaCl (w/v) LB agar. The isolates were labeled as T01 to T60 respectively.

4.2 Preliminary Test for AHL Production

The ability of the 60 isolates to produce AHL molecules was determined by testing them against a AHL biosensor namely, *C. violaceum* CV026. Out of the 60 isolates, only 5 of them, namely, T01, T08, T14, T33 and T47 showed positive result as indicated by the purple pigmentation produced by *C. violaceum* CV026 biosensor (Figure 4.1).

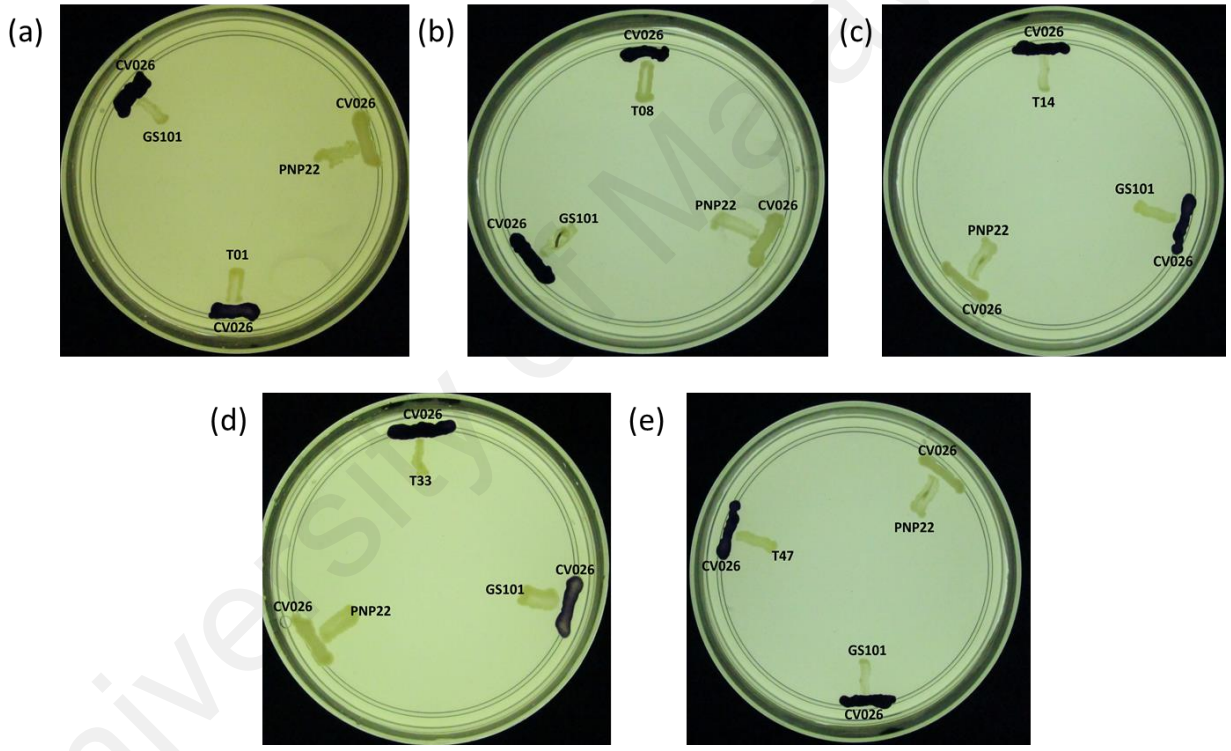


Figure 4.1: Detection of QS activity by 5 bacterial strains; (a) T01, (b) T08, (c) T14, (d) T33 and (e) T47. The biosensor used was C. violaceum CV026 with E. carotovora GS101 and E. carotovora PNP22 set as the positive and negative control respectively.

4.3 Identification of Bacteria

4.3.1 MALDI-TOF Identification

General observations of the five strains isolated indicated that they share similar characteristics such as having round entire margin, beige in colour with smooth surface after incubation at 28 °C for 24 hrs grown on LB medium supplemented with 3% NaCl (w/v) concentration. MALDI-TOF serves is a reliable method that provides high throughput for the classification and identification of microorganisms in a shorter turnover time (Dworzanski & Snyder, 2005) and Table 4. 2 summarizes the result from MALDI-TOF identification.

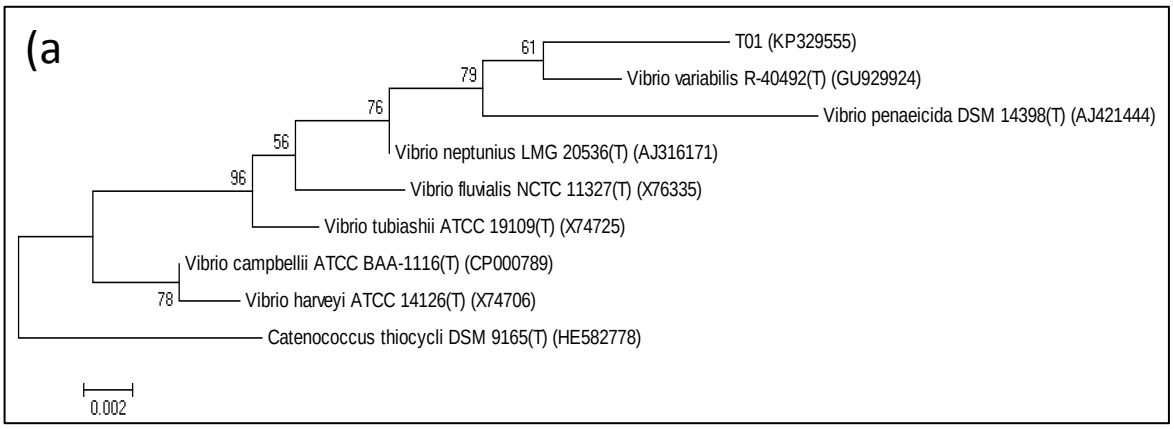
Table 4.2: MALDI-TOF Identification of QS Positive Strains

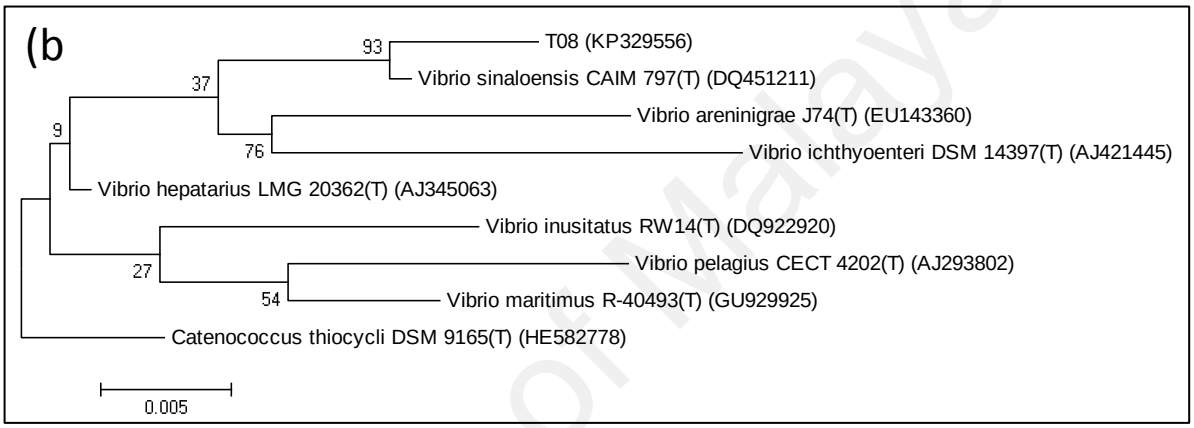
Strain Name	Strain Identification	Score Value
T01	*Vibrio orientalis*	1.652
T08	*Vibrio brasiliensis*	1.630
T14	*Vibrio nereis*	1.459
T33	*Vibrio orientalis*	1.679
T47	*Pseudomonas cedrina*	1.431

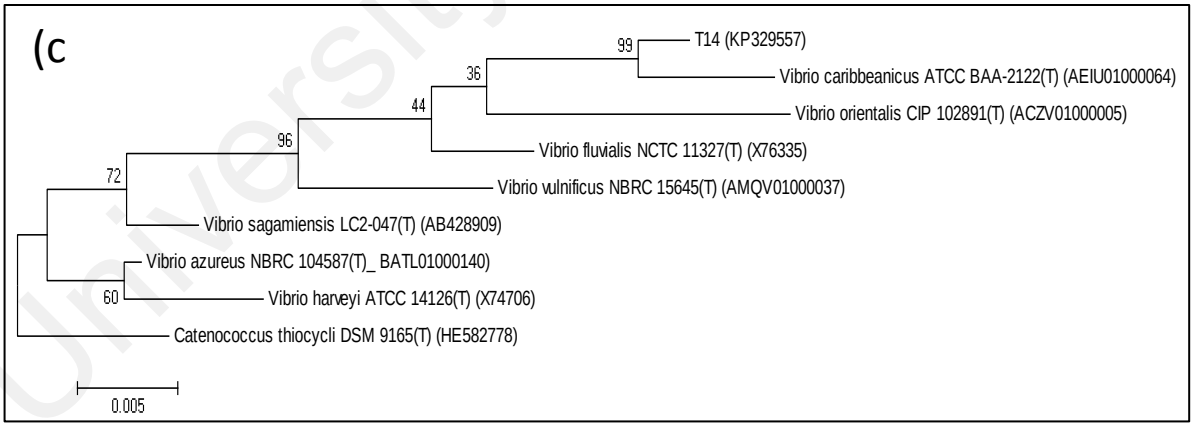
As judged by the score values obtained (Table 4.2), all of the isolates obtained score values less than 1.7 which indicates unreliable identification thus the identity of the five QS positive strains were determined by amplification of the 16S rRNA gene.

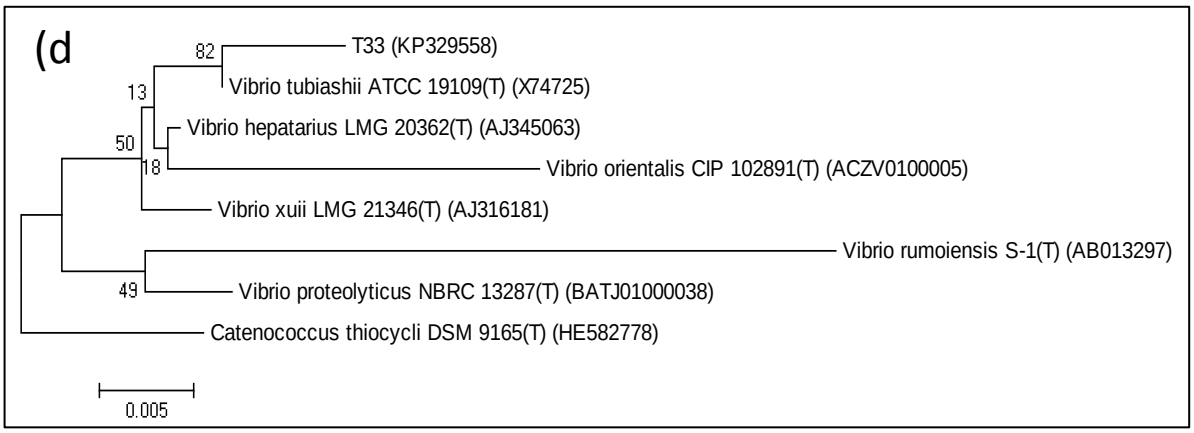
4.3.2 Molecular Identification of QS Positive Strains

The analysis of the amplified 16S rDNA nucleotides of the five QS positive strains enabled the molecular identification of these strains. The bacterial 16S rDNA nucleotides were compared to the highest matching percentage of the Ez-Taxon database (Ramy K Aziz et al., 2008) to construct the phylogeny analysis using the MEGA6.0 software (Tamura et al., 2013). The phylogenetic analysis shows that all five strains belongs to the vibrio clade with T01 sharing strain T01 shares 98.77% similarity with that of *V. variabilis* R–40492^{T} isolated from soft corals (zoanthids) in São Paolo, Brazil (Chimetto et al., 2011). By using the MEGA 6.0 software, the 16S rRNA gene sequences of strain T01 was aligned against *V. variabilis* R–40492^{T}. As for the identification of T08, the amplified 16S rDNA nucleotide sequence shows high matching percentage with *V. sinaloensis* CAIM 797^{T} which is 99.20% similarities between them. As for T14 16S rDNA nucleotide sequence analysis, the identity was a 99.15% matching similarity to *V. caribbeanicus* ATCC BAA-2122^{T} isolated by Hoffman and friends (Hoffmann et al., 2012). *V. tubiashii* ATCC 19109^{T} shares 99.36% of 16S rDNA nucleotide sequence similarities with strain T33 and T47 was identified as *V. sinaloensis* as it shares 98.92% 16S rDNA sequence as *V. sinaloensis* CAIM 797^{T}.









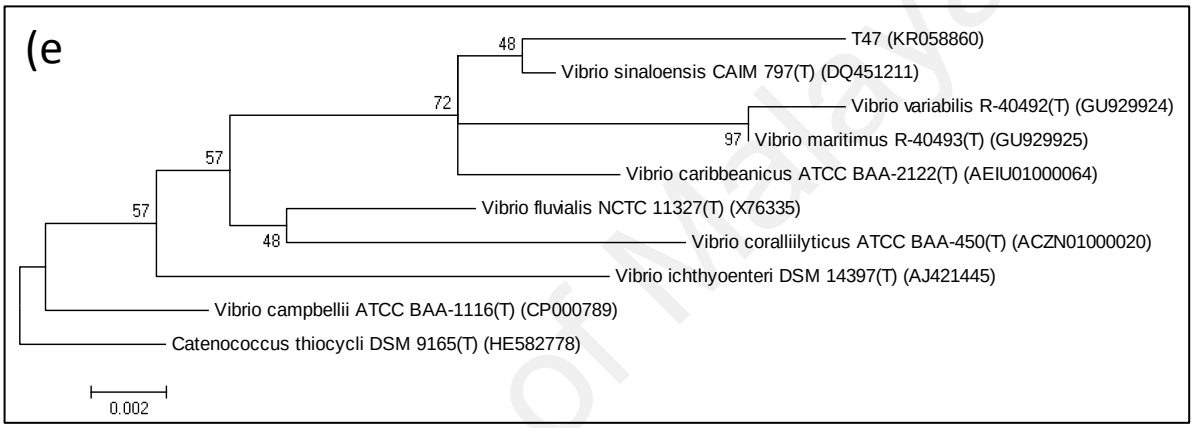


Figure 4.2: Phylogenetic trees of T01 (a), T08 (b), T14 (c), T33 (d) and T47 (e) highlighting the position of the strains relative to the type strains of other species within the genus *Vibrio*. The strains and their corresponding GenBank accession numbers of 16S rRNA genes are indicated in the parentheses. The sequences were aligned using MEGA 6.0 and all the phylogenetic tree inferences were obtained using the Maximum Likelihood method(Tamura et al., 2013) based on the Tamura-Nei model (Tamura & Nei, 1993). The number at nodes are the percentage of bootstrap values obtained by 500 replicates and the number of total positions for each phylogenetic tree are; T01: 1209, T08: 1235, T14: 1294, T33: 1388 and T47: 1170. *Catenococcus thiocycli* DSM 9165^{T} (HE82772) was used as outgroup for all phylogenetic tree.

4.4 Identification of AHLs Profiles Using High Resolution Tandem Liquid Chromatography Quadrupole Mass Spectrometry (LC-MS/MS)

Agilent 6490 Triple Quadrupole LC/MS system was used to detect the homoserine lactone ring moiety fragmented in the collision cell thus, identifying the AHL profiles of the five QS positive strains. All of the strains produced short-chain AHLs. *V. variabilis* strain T01 and *V. tubiashii* strain T33 produced long-chain AHLs namely *N*-(3-oxo-decanoyl)-L-homoserine lactone (OC10-HSL) and C12-HSL. All of the strains except for *V. sinaloensis* T47 produced C6-HSL.The summarize data of AHL profiles are listed in Table 4.3 below.

Table 4.3: Summary of AHL molecules produced by the QS Positive Strains

Strains	Identity Based on 16S rRNA Gene Analysis	QS Signaling Molecules Detected by LCMS (*m/z* ration)
T01	*V. variabilis*	C6-HSL (*m/z*: 200.50)
		OC10-HSL (*m/z*: 270.30)
		C12-HSL (*m/z*: 284.50)
T08	*V. sinaloensis*	C4-HSL (*m/z*: 172.20)
		C6-HSL (*m/z*: 200.30)
T14	*V. caribbeanicus*	C6-HSL (*m/z*: 200.30)
T33	*V. tubiashii*	C6-HSL (*m/z*: 200.30)
		OC10-HSL (*m/z*: 270.40)
T47	*V. sinaloensis*	C4-HSL (*m/z*: 172.20)

The mass spectra for all the AHL profiles are in Appendix A.

4.5 Whole Genome Sequencing Analyses of QS Positive Strains

The information of the sequencing project are tabulated in Table 4.4 and all of the draft sequences were deposited in NCBI GenBank database and have obtained their respective accession number. The general genome features of all 5 strains are summarised in Table 4.5. Since they are all from the *Vibrio* genus, some of the features are quite similar among each other.

Table 4.4: Information on genome sequencing project

Property	**Term**				
Finishing quality	Draft				
Libraries used	One paired-end Illumina library				
Sequencing platforms	Illumina MiSeq				
Assemblers	CLCBio CLC Genomics Workbench, 6.5.1				
Gene Calling	RAST and IMG-ER				
Sequencing coverage	***V. variabilis* T01**	***V. sinaloensis* T08**	***V. caribbeanicus* T14**	***V. tubiashii* T33**	***V. sinaloensis* T47**
	61.95 ×	66.56 ×	75.13 ×	60.05 ×	43.80 ×
GenBank ID	JRWM00000000	JRWP00000000	JRWR00000000	JRWQ00000000	JXBJ00000000
Release date	2014/11/14	2014/11/13	2014/11/19	2014/11/13	2015/01/08
NCBI project ID	PRJNA263994	PRJNA264124	PRJNA264129	PRJNA264132	PRJNA271552
Project relevance	Environmental				

Table 4.5: Genome features of 5 QS strains

Attributes	*V. variabilis* T01		*V. sinaloensis* T08		*V. caribbeanicus* T14		*V. tubiashii* T33		*V. sinaloensis* T47	
	Value	% of Total	Value	% of Total	Value	% of Total	Value	% of Total	Value	% of Total
Length of sequence (bp)	4, 529, 728	100	4, 565, 090	100	4, 568, 683	100	4, 138, 417	100	4,599, 504	100
Coding region (bp)	4, 003, 944	88.39	4, 031, 551	88.31	4, 041, 295	88.46	3, 677, 566	88.86	4, 053, 747	88.13
G + C content (bp)	2, 093, 692	46.22	2, 109, 479	46.21	2, 108, 490	46.15	1, 864, 840	45.06	2, 121, 494	46.12
Total genes	4, 147	100	4146	100	4, 135	100	3, 866	100	4, 232	100
Protein-coding genes	4, 053	97.73	4065	98.05	4,048	97.9	3, 764	97.36	4, 105	97.00
RNA genes	94	2.27	81	1.95	87	2.10	102	2.64	127	3.00
rRNA genes	9	0.22	6	0.14	10	0.24	5	0.13	19	0.45
tRNA genes	83	2.00	74	1.78	76	1.84	69	1.78	106	2.50
Pseudo genes	62	1.50	64	1.54	76	1.84	N/A	N/A	65	1.54
Genes with function prediction(protein)	3, 620	87.29	3, 423	82.56	3, 609	87.28	3, 144	81.32	3, 428	81.00
Genes assigned to COGs	3, 047	73.47	3, 070	74.05	3, 061	74.03	2,926	75.69	3, 085	72.90
Genes with Pfam domains	3. 566	85.99	3, 579	86.32	3,562	86.14	3, 382	87.48	3, 595	84.95

Attributes	*V. variabilis* T01		*V. sinaloensis* T08		*V. caribbeanicus* T14		*V. tubiashii* T33		*V. sinaloensis* T47	
	Value	**% of Total**	Value	% of Total	Value	% of Total	Value	% of Total	Value	% of Total
Genes in paralog clusters	2, 997	72.27	2, 389	66.75	2, 957	71.51	2, 311	68.33	2, 394	66.59
Genes with signal peptides	445	10.73	430	10.37	431	10.42	351	9.08	445	10.52
Genes with transmembranes helices	1, 038	25.03	1, 040	25.08	1, 037	25.08	942	24.37	1, 034	24.43

4.6 Cell Morphology via Scanning Electron Microscope (SEM) and MIGS Classification

Out of all five strains that display QS ability, *V. variabilis* strain T01 was chosen for further analysis due to it is a newly discovered species and produces both short and long-chained AHLs. Its morphology was observed using tabletop SEM (Hitachi, Japan). Figure 4.3 shows strainT01 are curved-rods shaped bacteria with 0.9 µm width and 1.35 µm long. Table 4.6 provides Minimum Information about the Genome Sequences (MIGS)

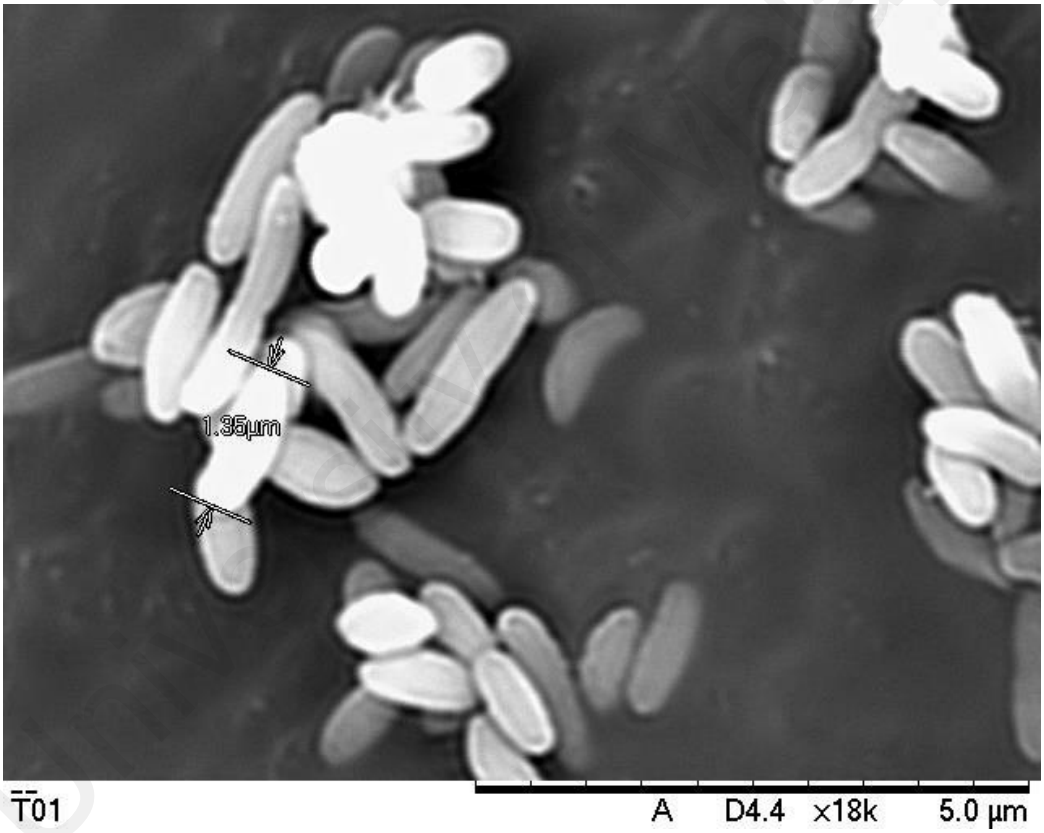


Figure 4.3: Electromicrograph of *V. variabilis* strain T01

Table 4.6: Classification and general features of *V. variabilis* strain T01 according to the MIGS recommendations

MIGS ID	Property	Term	Evidence code
	Current classification	Domain *Bacteria*	TAS (Woese, Kandler, & Wheelis, 1990)
		Phylum *Proteobacteria*	TAS (G. M. Garrity & J. G. Holt, 2001)
		Class *Gammaproteobacteria*	TAS (Williams et al., 2010)
		Order *Vibrionales*	TAS (G. Garrity & J. Holt, 2001)
		Family *Vibrionaceae*	TAS (SKERMAN, McGowan, & Sneath, 1980)
		Genus *Vibrio*	TAS (Shewan & Veron, 1974)
		Species *Vibrio variablis*	TAS (Chimetto et al., 2011)
		Type strain T01	
	Gram stain	Negative	IDA
	Cell shape	Curved rods (vibroids)	IDA
	Motility	Motile via single polar flagellum	TAS (Chimetto et al., 2011)
	Sporulation	Non-sporulating	IDA
	Temperature range	4–37°C	IDA
	Optimum temperature	28°C	TAS (Chimetto et al., 2011)
	Salinity	Considerably hydrophilic; 1–3% NaCl (optimum)	IDA
MIGS-22	Oxygen requirement	Aerobic	IDA
	Carbon source	Highly diverse	IDA
	Energy metabolism	Highly diverse	IDA
MIGS-6	Habitat	Marine environment	TAS (Chimetto et al., 2011), IDA
MIGS-15	Biotic relationship	Free-living	NAS
MIGS-14	Pathogenicity	Non-pathogenic	NAS
	Biosafety level	1	NAS
MIGS-23.1	Isolation	Coastal marine waters	IDA

MIGS ID	Property	Term	Evidence Code
MIGS-4	Geographic location	Morib Beach, Hulu Selangor, Malaysia	IDA
MIGS-5	Sample collection time	10 a.m	IDA
MIGS-4.1	Latitude	2° 45' 2.7" N	
MIGS-4.2	Longitude	101° 26' 34.7" E	
MIGS-4.3	Depth	5 cm from water surface	IDA

Evidence codes - IDA: Inferred from Direct Assay; TAS: Traceable Author Statement (i.e., a direct report exists in the literature); NAS: Non-traceable Author Statement (i.e., not directly observed for the living, isolated sample, but based on a generally accepted property for the species, or anecdotal evidence). These evidence codes are from the Gene Ontology project (Ashburner et al., 2000). If the evidence is IDA, the properties was directly observed by the authors.

4.7 *vanM* Functionality Study

From the RAST annotation platform, *V. variabilis* strain T01 genome showed a total of 519 subsystems which represents 55 % of the subsystem coverage (Figure 4.4). The most abundant genes reported in *V.variabilis* strain T01 genome could be related to the metabolic pathways and basic fundamental functions which are subsystems related to amino acids and derivative (417 genes), carbohydrates (448 genes), cofactors, vitamins, prosthetic groups, pigments (294 genes), protein metabolism (284 genes), RNA metabolism (221 genes), and membrane transport (210 genes).

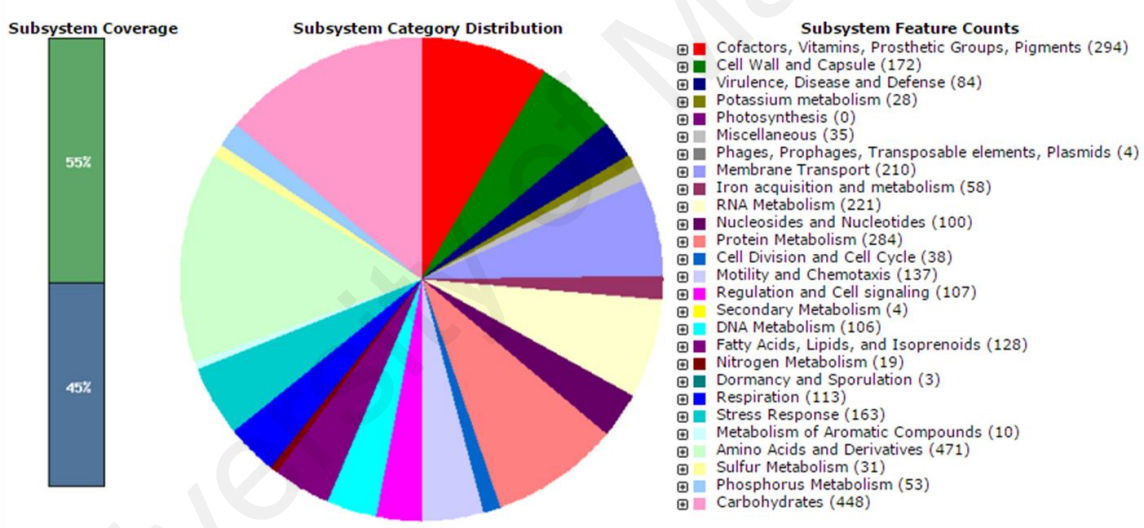


Figure 4.4: Distribution of subsystem of *V. variabilis* strain T01 via the RAST server. The green region indicated the proteins covered by the subsystem while the blue region indicates the regions not covered by the subsystem. The RAST subsystem cover 519 (55%) subsystems from the 3, 321 CDS predicted.

From the subsystem features of strain *V. variabilis* strain T01, 107 CDS are responsible for regulation and cell signaling while 84 CDS are linked with virulence disease and defense. A study by Deep at al. has shown that both of these distinctive features are associated with quorum sensing (Deep, Chaudhary, & Gupta, 2011). From the subsystem, the location of the autoinducer synthase gene was located and fished out from the genome. The LuxM homologue of *V. variabilis* strain T01 was located on contig 16 with 1203 bp in size. The nucleotide sequence was translated to approximately 400 amino acids and used to construct the phylogenetic tree visualized in Figure 4.5. From the figure, autoinducer synthase protein in strain T01 shares 99.0 % similarity with VanM in *Vibrio* sp. 16 (WP_005476971.1).

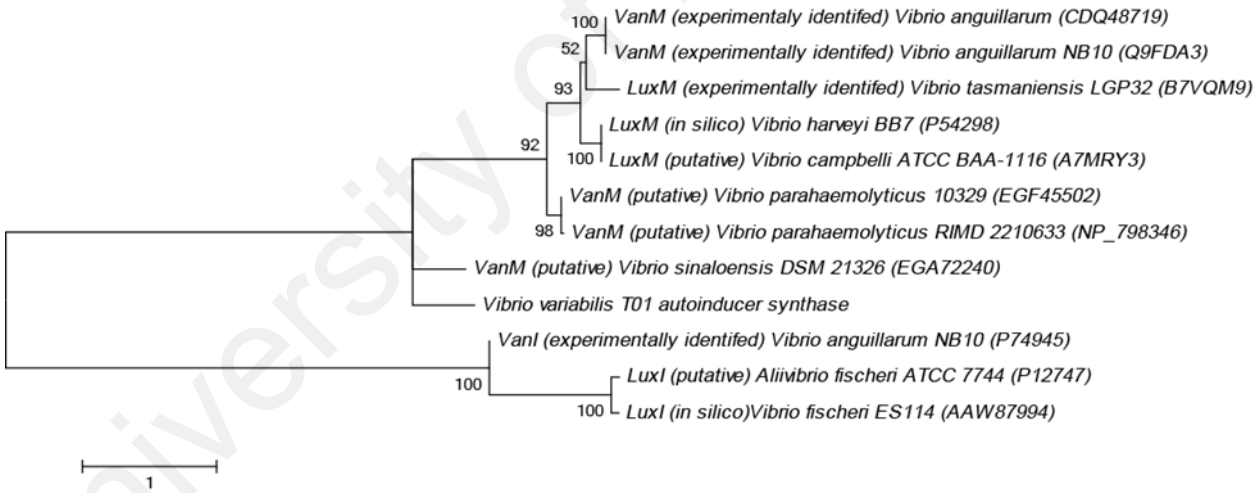


Figure 4.5: Phylogenetic analyses of LuxM homologue in strain T01

The l*uxI* homologue in *V. variabilis* strain T01 was further compared with other genes of closely related bacteria species via the RAST annotation database and Figure 4.6 illustrates the arrangements of autoinducer synthase gene paired with respective autoinducer receptor.

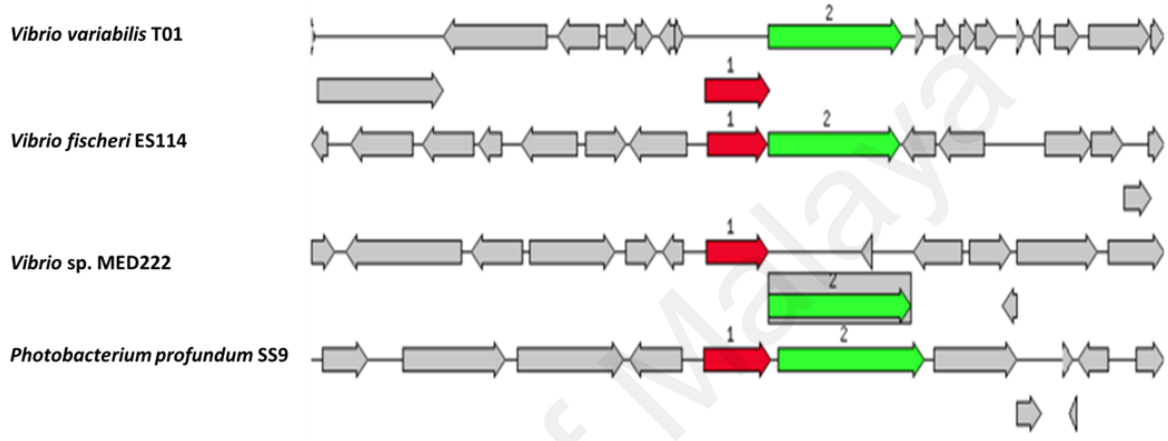


Figure 4.6: Position of autoinducer synthase gene (*luxI* homologue*)* (red arrow) paired with respective autoinducer receptor (*luxR* homologue) (green arrow).

For more confirmation, the amino acid sequence of autoinducer synthase protein in *V. vraiabilis* strain T01 was compared and aligned with five highly similar amino acid sequences obtained from the NCBI database. Figure 4.7 shows similarity of amino acid sequences indicated by the black background signifying exact similarity while grey background represents similar residues shared between the sequences. The autoinducer synthase sequence in *V. variabilis* strain T01 denoted as *vanM* , was deposited in the NCBI database with accession number of KT258634.

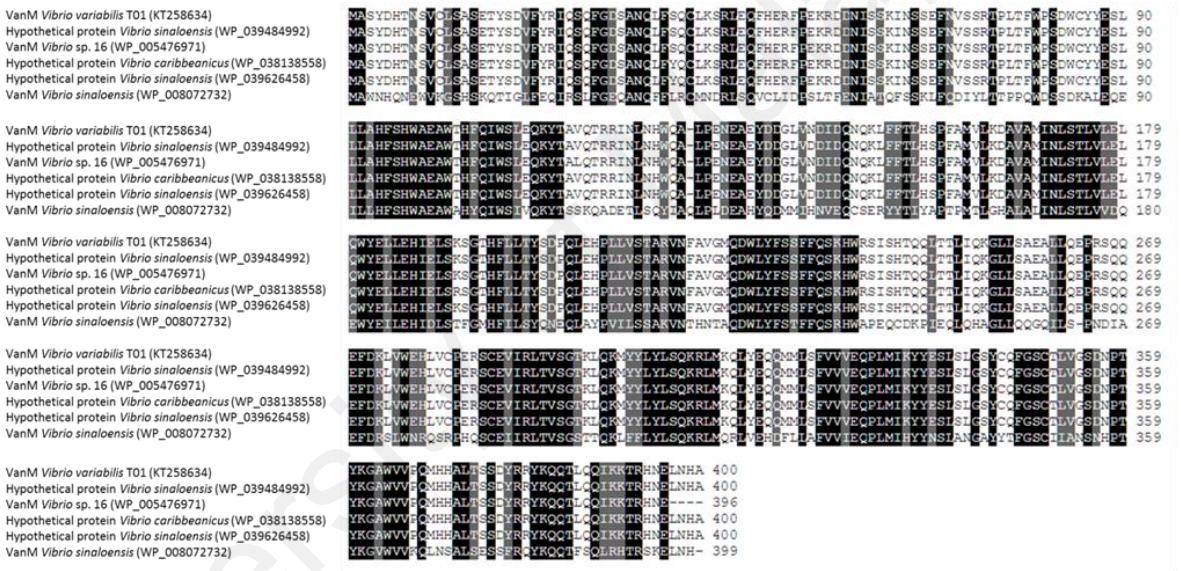


Figure 4.7: Amino acid alignment of LuxI homologue in *V. variabilis* strain T01 with other highly similar proteins

Cloning of the *vanM* gene was conducted using both cloning and overexpression plasmids and preliminary AHL detection assay using biosensor *C. violaceum* CV026 was conducted using *E. coli* BL21 (DE3) pLysS harboring the fusion plasmid pET-28a-*vanM*. Purple pigmentation produced by the biosensor indicated production of short chain AHL by cloned *vanM* gene. Negative control used in the assay namely, *E. coli* BL21 (DE3) pLysS harboring only pET-28a did not induce purple pigmentation (Figure 4.8)

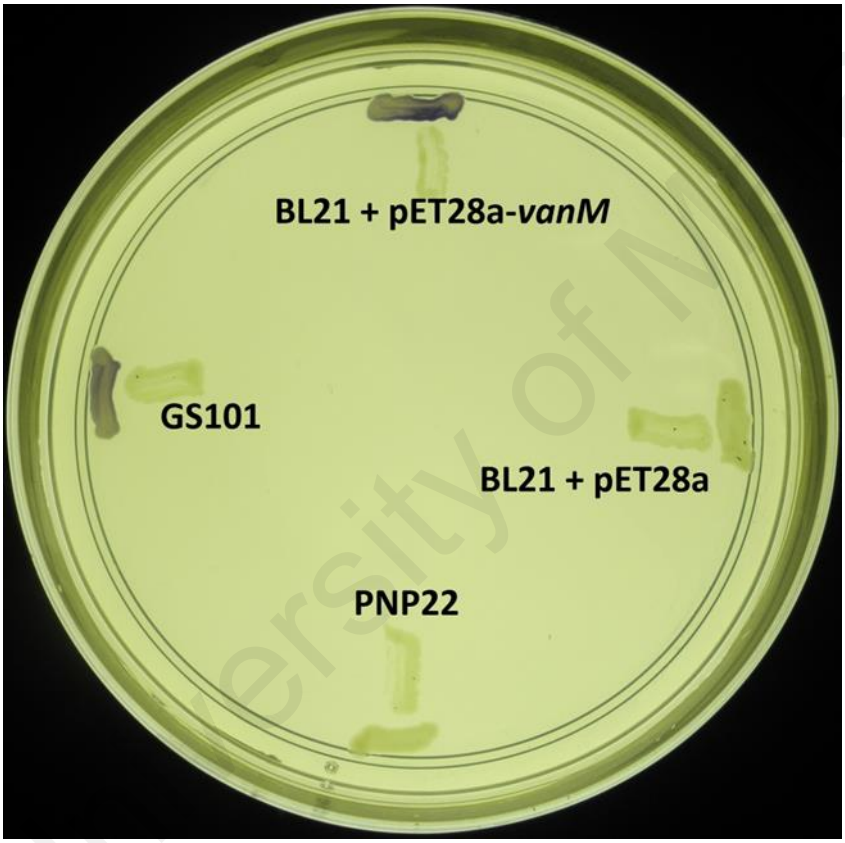
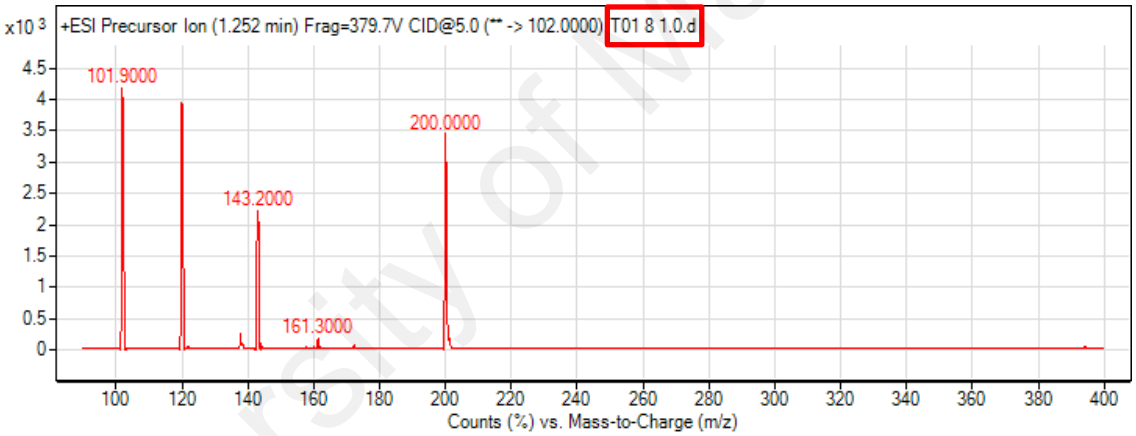


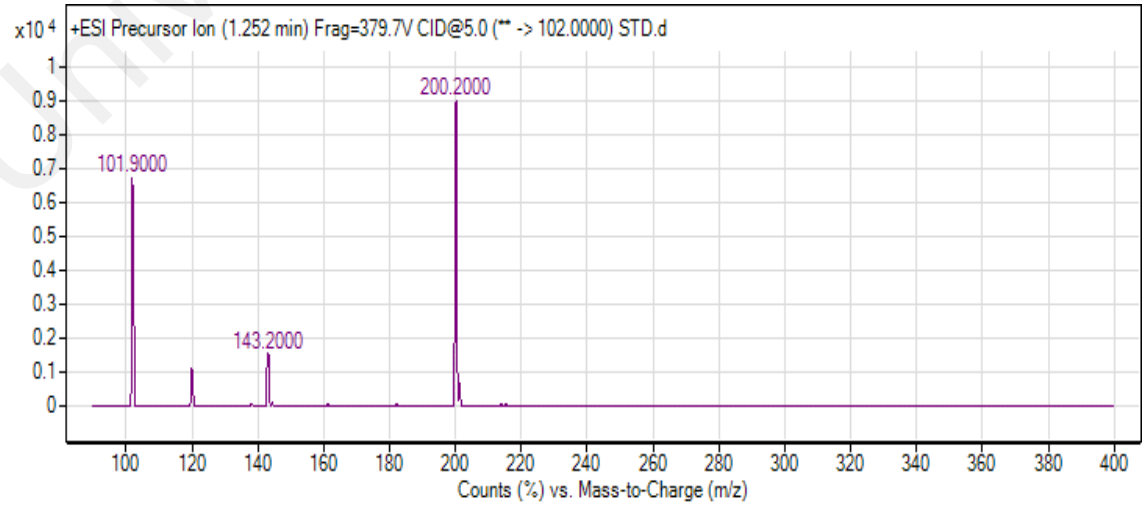
Figure 4.8: Preliminary AHL detection assay on *E coli* BL21 harboring pET-28a-*vanM* insert using biosensor *C. violaceum* CV026. The positive control was set as *E. carotovora* (GS101) and negative control used in the assay are and *E. carotovora* (PNP22) and *E. coli* BL21 harboring only pET-28a

E. coli BL21 containing the overexpressed *vanM* fused in pET-28a, was subjected to AHL extraction and the AHL extract was analysed using the LC-MS/MS system. The mass spectrometry analyses in Figure 4.9 revealed 3 AHL profiles namely, *N*-hexanoyl-L-homoserine lactone (C6-HSL), *N*-(3-oxo-decanoyl)-L-homoserine lactone (OC10 HSL) and *N*-dodecanoyl homoserine-L-lactone (C12-HSL) produced by *E. coli* BL21 with pET-28a-*vanM*. C6-HSL and OC10-HSL were detected after 4 h induction while C12-HSL was only present after 8 h induction with IPTG (highlighted in red box). The AHL profiles produced were identical with the corresponding standard at their respective retention time.

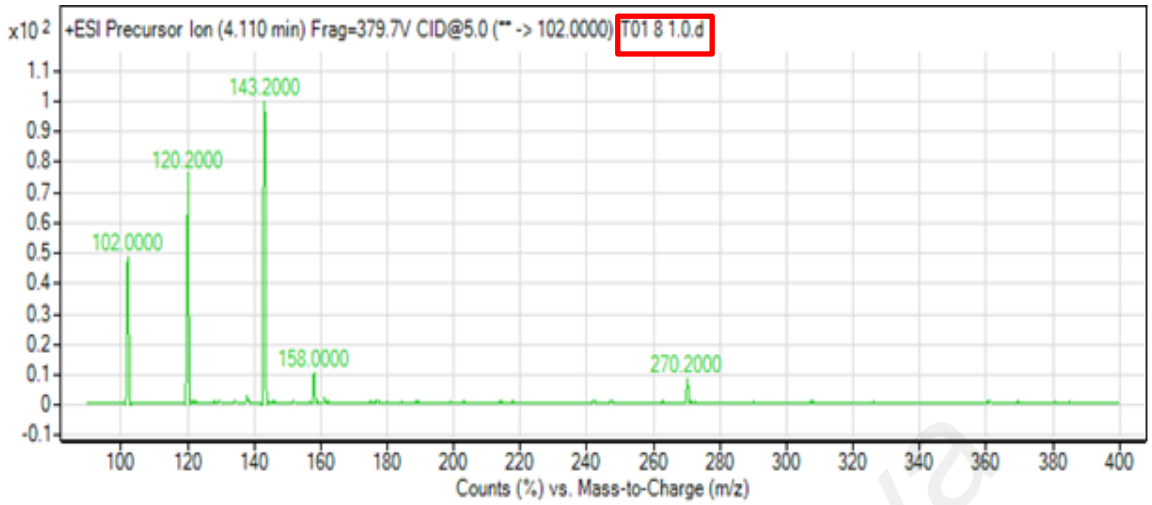
1 (a)



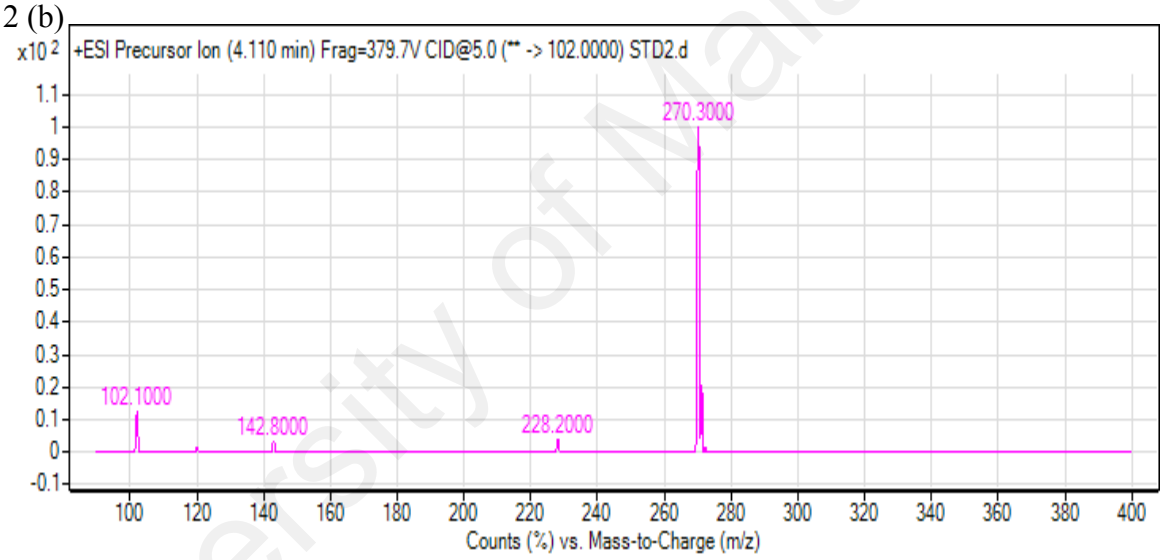
1 (b)



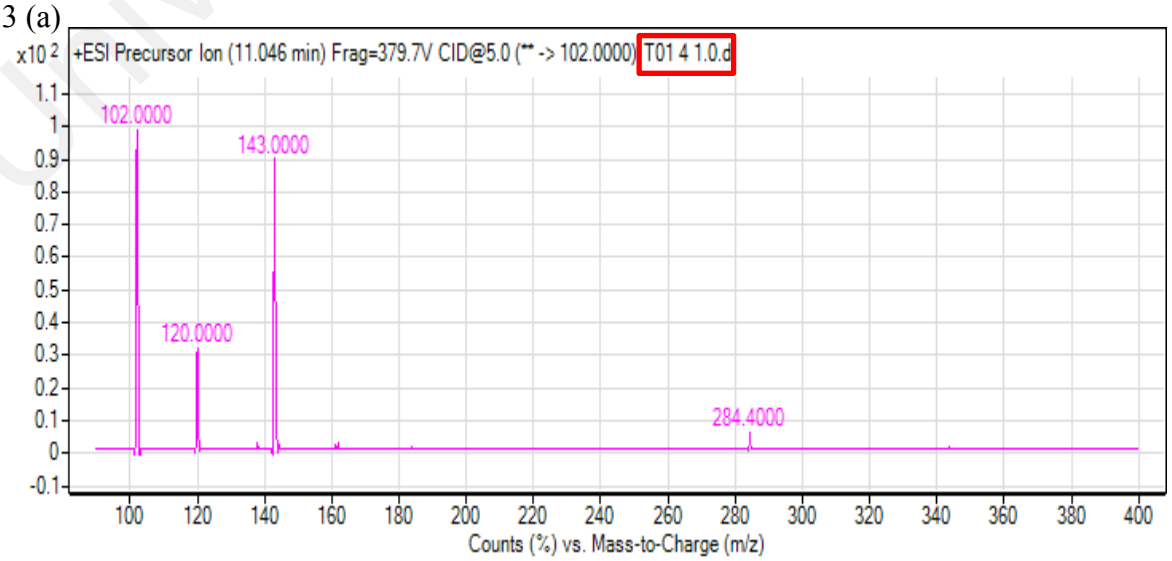
2 (a)



2 (b)



3 (a)



3 (b)

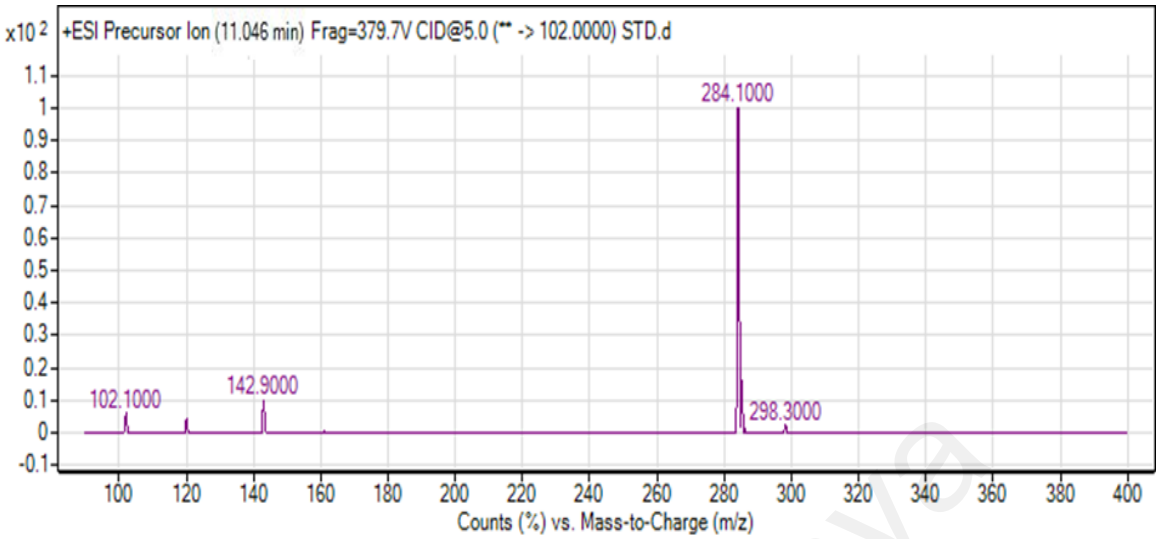


Figure 4.9: AHL profiles produced by *E. coli* BL21 pET-28a-*vanM*. Mass spectra of the AHL profiles obtained in the induced cultures and their respective standards are placed in an interval manner. 1 (a) shows C6-HSL (*m*/*z* = 200.00) production after 8 h of incubation with its standard; 1 (b), 2 (a) shows OC10-HSL production after 8 h incubation (*m*/*z* = 270.20) with its standard; 2 (b) and 3(a) shows C12-HSL production after 4 h incubation (*m*/*z* = 284.40) with its standard; 3 (b).

4.8 Biofilm Assay Using Gallic Acid, a Known Anti-QS Compound

The concentration of gallic acid used in this biofilm assay was adapted from a study by Borges et al. (2012) where 1 mg/mL of gallic acid was shown to reduce 59% violacein production in *C. violaceum* CV12472. Biofilm assay on *V. variabilis* strain T01 was conducted using 1 mg/mL gallic acid and incubated for 72 h. The production of biofilm by the planktonic bacteria can clearly be seen after 24 h of incubation. In Figure 4.9, the bar chart indicates a significant difference ($p < 0.0016$) in biofilm productin between culture containing strain T01 and culture containing both strain T01 and 1 mg/mL gallic acid.

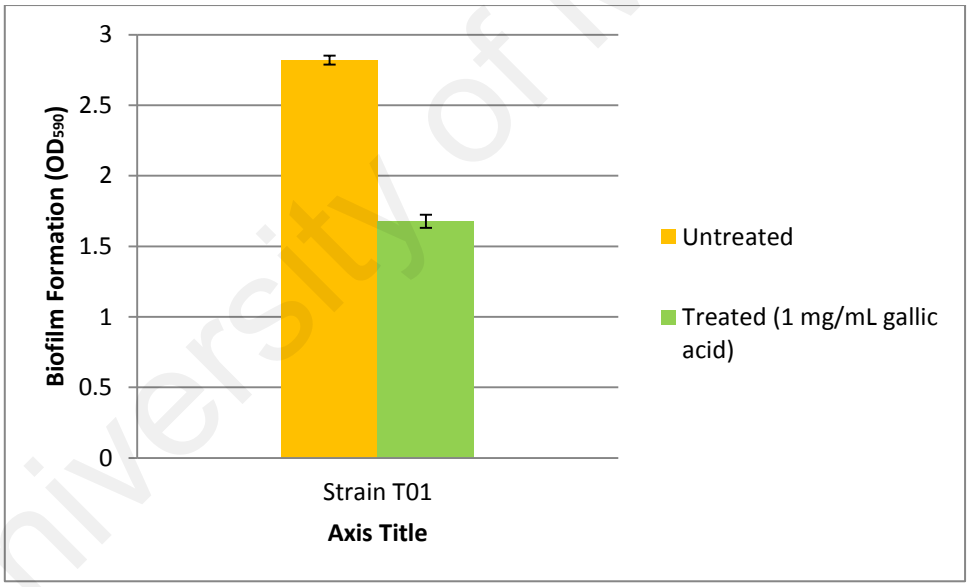


Figure 4.10: Biofilm assay using gallic acid on *V variabilis* strain T01. Using a paired T-test, the two-tailed p-value obtained for the data is less than 0.0016 which denotes significant result.

4.9 Transcriptomic Study Using Gallic Acid, a Known Anti-QS Compound, Against *V. variabilis* Strain T01

From the RNA-sequencing data analyses, there were a total of 674 with fold change value ≥2 and ≤-2 genes were affected by the gallic acid treatment. From the total of 674 genes, 298 genes were upregulated while remaining 376 genes were downregulated in *V. variabilis* strain T01. The 675 affected genes and together with their $\log_2$ values are given in Appendix D.

Table 4.7: Upregulated and downregulated genes of *V. variabilis* strain T01 affected by gallic acid treatment

Protein Name	p-value(treated vs. untreated)	Log^2 value
Upregulated		
hypothetical protein	0.018838	6.935141
Pyridoxamine 5'-phosphate oxidase	0.004772	6.420216
Carbonic anhydrase precursor	0.011732	6.406929
colicin uptake protein TolR	0.002883	6.344678
ChuX-like family protein	0.000551	6.330413
Hemin-binding periplasmic protein HmuT precursor	0.000139	6.090781
biopolymer transport protein ExbB	0.0002	6.011207
Iron-binding protein IscA	0.006046	5.96901
transport protein TonB	0.002392	5.904759
Hemoglobin and hemoglobin-haptoglobin-binding protein A precursor	0.000878	5.808992
Oxygen-independent coproporphyrinogen-III oxidase 1	0.000605	5.791165
hypothetical protein	0.039698	5.646883
putative ABC transporter solute-binding protein YclQ precursor	0.001299	5.516103
Glutaredoxin-like protein NrdH	0.002494	5.37113
Hemin import ATP-binding protein HmuV	0.001674	5.326145
Superoxide dismutase [Mn]	0.002433	5.258217
Fumarate hydratase class II	0.001488	5.185874
N(6)-hydroxylysine O-acetyltransferase	0.000479	5.009213
Putative NrdI-like protein	0.004137	4.851074
Ferric aerobactin receptor precursor	0.000943	4.786602
FeS cluster assembly protein SufD	0.001568	4.772287
6-phospho-beta-glucosidase BglB	0.000111	4.769401

Protein Name	p-value (treated vs. untreated)	Log_2 value
Multidrug resistance protein MdtL	0.001698	4.764856
Protein YceI	0.014173	4.694652
hypothetical protein	0.018394	4.645915
hypothetical protein	0.004444	4.592648
hypothetical protein	0.000884	4.572277
N(2)-citryl-N(6)-acetyl-N(6)-hydroxylysine synthase	0.01329	4.547943
Hemin transport system permease protein HmuU	0.00611	4.52095
Succinate-semialdehyde dehydrogenase [NADP(+)] GabD	0.001121	4.505269
Cysteine desulfurase	0.000455	4.38102
hypothetical protein	0.006204	4.335104
Ferrous iron transport protein B	0.003268	4.272777
L-lysine N6-monooxygenase	0.002639	4.26126
Glyoxylate/hydroxypyruvate reductase B	0.003677	4.131186
hypothetical protein	0.008139	4.026632
Ribonucleoside-diphosphate reductase subunit alpha 2	0.003144	3.792939
Aerobactin synthase	0.000354	3.749352
Iron(3+)-hydroxamate import system permease protein FhuB	0.000593	3.743633
Iron(3+)-hydroxamate import ATP-binding protein FhuC	0.000693	3.738022
hypothetical protein	0.006385	3.732519
Bacterial regulatory proteins, tetR family	0.012534	3.700462
EamA-like transporter family protein	0.047809	3.66487
Ribonucleoside-diphosphate reductase subunit beta nrdF2	0.002497	3.635975
Iron(3+)-hydroxamate import ATP-binding protein FhuC	0.002175	3.584205
HTH-type transcriptional regulator CysL	0.005037	3.55508
hypothetical protein	0.001539	3.475837

Protein Name	p-value (treated vs. untreated)	Log_2 value
Downregulated		
Protein Name	p-value(treated vs. untreated)	Log2 value
Flp/Fap pilin component	0.00095	4.436428
putative MFS-type transporter YhjX	0.000642	4.434174
hypothetical protein	0.001485	3.60959
Nucleoside permease NupX	0.000884	3.121107
hypothetical protein	0.000632	2.980769
SnoaL-like domain protein	0.033899	2.779718
Periplasmic nitrate reductase, electron transfer subunit precursor	0.000189	2.761006
Cytochrome c-type protein NapC	0.000764	2.662021
putative amino-acid import ATP-binding protein YxeO	0.000247	2.476978
Hypoxanthine phosphoribosyltransferase	0.00011	2.462144
hypothetical protein	0.020624	2.459028
Efflux pump periplasmic linker BepF	0.015216	2.426399
Bicarbonate transporter BicA	0.007881	2.412516
Metallothiol transferase FosB	0.000109	2.412153
hypothetical protein	0.011567	2.408633
hypothetical protein	0.001024	2.344953
HTH-type transcriptional regulator BetI	0.001305	2.341217
hypothetical protein	0.017686	2.262755
flagellar protein FlaG	0.001187	2.199547
tRNA (guanosine(18)-2'-O)-methyltransferase	0.004006	2.186916
hypothetical protein	0.000232	2.186184
putative antibiotic transporter	0.004882	2.151752
Serine/threonine-protein kinase PrkC	0.000144	2.142103

Protein Name	p-value (treated vs. untreated)	Log_2 value
hypothetical protein	0.046676	2.12619
hypothetical protein	0.0013	2.113297
putative amino-acid permease protein YxeN	0.004234	2.113204
hypothetical protein	0.000128	2.107062
Siroheme synthase	0.000505	2.093557
Pilus assembly protein, PilP	0.028126	2.051024
Sulfite reductase [NADPH] flavoprotein alpha-component	0.000697	2.043134
Lichenan permease IIC component	0.033603	2.017619
Outer membrane protein A precursor	0.000244	2.010304
Threonine efflux protein	0.006135	1.968345
HTH-type transcriptional activator AllS	0.000386	1.962134
O-acetyltransferase OatA	0.000114	1.95471
Glutamine synthetase	0.00106	1.952505
DNA-binding protein Fis	0.001048	1.94102
Inositol-1-monophosphatase	0.00129	1.93864
hypothetical protein	0.005615	1.937472
putative amino-acid-binding protein YxeM precursor	0.000815	1.936131
tRNA modification GTPase MnmE	0.000138	1.9268
ATP-binding region	0.000354	1.918863
Transketolase 1	0.000102	1.880713
L-2,4-diaminobutyric acid acetyltransferase	0.001298	1.860184
Sulfate adenylyltransferase subunit 2	0.000698	1.85289
Sulfate adenylyltransferase subunit 1	0.000965	1.851215
MltA-interacting protein MipA	0.000646	1.838847
hypothetical protein	0.002213	1.807656

Table 4.7 shows three of the upregulated genes highlighted in blue and three of the downregulated genes highlighted in red were randomly chosen for quantitative RT-PCR (qPCR) analysis to validate the RNA-seq results(Figure 4.11). Parallel expression patterns between the QS-regulated genes from RNA-seq analysis and expression levels in qPCR were observed. The fold-change obtained in qPCR analysis are slightly different due to sensitivity of each technique used. Three housekeeping genes are used for normalization purposes namely, DNA gyrase subunit B (*gyrB*), Tryptophanase (*tnaA*) and RecA protein (*recA*).

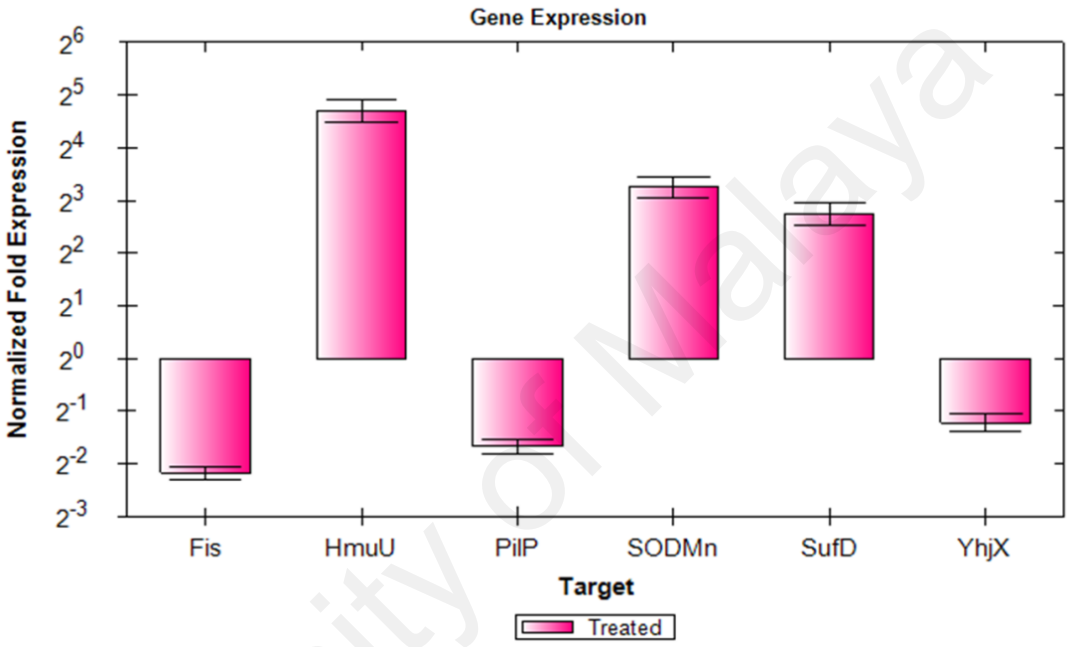


Figure 4.11: Validation of RNA-seq analysis using qPCR

The genes that were chosen at random underwent validation using qPCR and the from Figure 4.11 it can be seen that the normalised fold expression reflects the result obtained in the transcriptomic analyses done using the sample. the downregulated genes were Fis, Pilp and YhjX while HmuU, SODMn and SufD were found upregulated which is paralllel with the result obtained in RNA-seq (Table 4.7).

The differentially expressed genes were then mapped to the cluster of orthologous groups (COGs) database containing protein sequences from completely sequenced bacterial genomes. From this analysis, the distribution of the differentially expressed genes can be visualised in respective COG categories indicating their function.

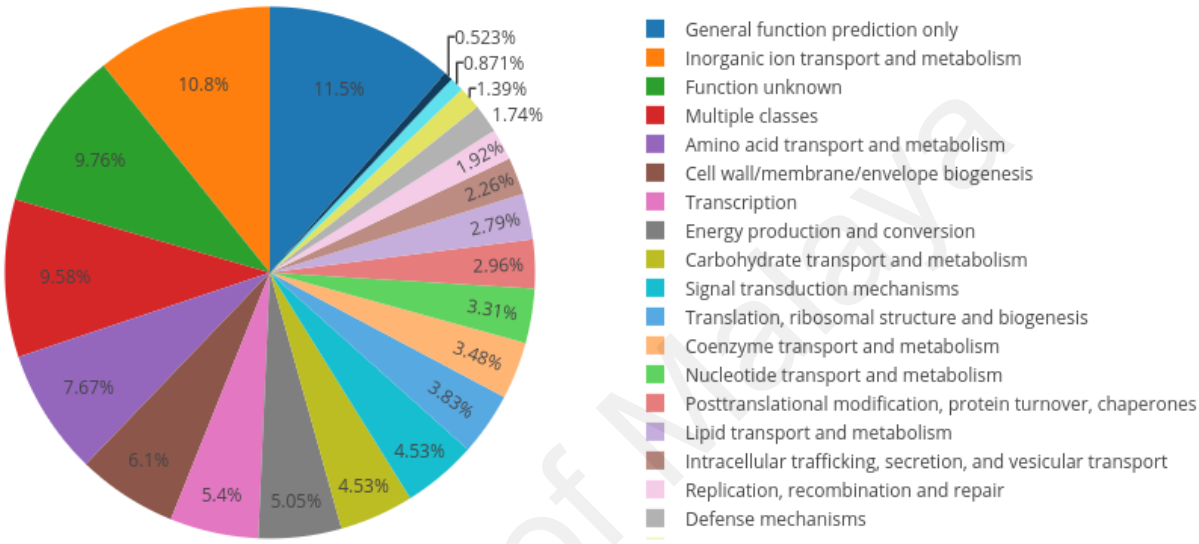


Figure 4.12: Classification of *V. variabilis* strain T01 differentially expressed genes into COG categories

From the pie chart generated (Figure 4.12), most of the differentially expressed genes are involved in transport and metabolism of inorganic ion (10.8%), amino acid (7.67%), carbohydrate (4.53%), coenzyme (3.48%), nucleotide (3.31%) and lipid (2.79%). There are a total of 574 differentially expressed genes which are successfully classified into their COG categories.

CHAPTER 5: DISCUSSION

5.1 Isolation and Identification of Bacteria

The sampling site was chosen due to its location as a known tourist spot with moderate human activities. For the isolation of bacteria, LB medium was chosen as the growing medium as it is suitable for isolating environmental isolates based on previous study (Corry, Curtis, & Baird, 2011; Ringeisen, Rincon, Fitzgerald, Fulmer, & Wu, 2015). The growth medium, LB was added with additional NaCl and in this study, LB agar supplemented with 3 % NaCl (w/v) was chosen as the optimal growth media since it supported more colonies growth. This observation was in accordance with a study states that marine bacteria need more than 0.5% NaCl (w/v) in order to thrive in the media, and 3% NaCl (w/v) concentration in the growth media was optimum for growth while 8% NaCl (w/v) content was detrimental to the bacteria (Farmer Iii & Hickman-Brenner, 2006). Due to the osmotic tolerance of marine bacteria, diluted medium are unfavorable for growth as the bacteria have the tendency to lyse (Harvey, 1915; Kocasoy, 1989).

The use of *C. violaceum* CV026 biosensor as a preliminary assay to detect the production of AHL enables rapid and accurate detection of QS isolates. Identification of QS positive isolates were first conducted using MALDI-TOF method which provides rapid identification. A score of ³2.0 indicates acceptable species identification (assuming that there is at least a 10% score difference between the top match and different genera or species with closely related spectra). According to score cutoffs specified by Bruker Daltonics, a score of 1.7 to 1.999 indicates acceptable genus identification. If the score is <1.7, the system does not reliably identify the organism (El-Bouri et al., 2012). However, In this study, allof the isolates obtain score lower than 1.7 indicating unreliable identification maybe due to limited information of environmental strains in the Bruker database since it is usually used to identify clinical isolates (Panda, Kurapati, Samantaray, Srinivasan, & Khalil, 2014) .

Further confirmation of bacteria isolates identification was done by performing 16S rRNA gene sequence analysis on the QS positive strains. To date, the 16S rRNA gene sequences have been extensively used as a universal housekeeping genetic marker. Several reasons such as the presence of it in almost all bacteria and the adequate gene size (1, 500 bp) contributes to the use of 16S rRNA sequences for identification purposes. Another main reason is the stability of the gene over time with minor sequence changes thus serves as a measuring ruler for evolution of species (J. M. Janda & Abbott, 2007). In this study, all of the QS positive bacteria identified belongs to the *Vibrio* genus and some of them is very closely related species such as *V. sinaloensis* strain T08, *V. variabilis* strain T01 and *V. carribbeanicus* strain T14.

5.2 AHL Profiling

LC-MS/MS was used to validate the AHL profiles of the QS positive strains. This method enables accurate detection of AHL signal based on the chemical and physical characteristics of the AHL molecules such as molecular ions *m*/*z* ratio, product ions produced in the collision cell and retention time coupled with chromatogram profiles (Cataldi, Bianco, Fonseca, & Schmitt-Kopplin, 2013; Gould, Herman, Krank, Murphy, & Churchill, 2006). From the LC-MS/MS analysis, all of the QS positive strains produced short chain AHLs while only *V. variabilis* strain T01 and *V. tubiashii* T33 produced long chain AHL namely, OC10-HSL and C12-HSL. Among the five strains, *V. caribbeanicus* strain T14 and *V. sinaloensis* strain T47 produced only one type of AHL while *V. variabilis* strain T01, *V. sinaloensis* strain T08 and *V. tubiashii* strain T33 produced multiple AHL molecules.

Four of the QS positive strains produce C6-HSL except for *V. sinaloensis* strain T47. To date, the only published work reporting on the production of C6-HSL by *Vibrio* is by *V. anguillarum* (Milton, Chalker, Kirke, Hardman, Cámara, et al., 2001). Milton et al., reported there are 2 QS systems present in *V anguillarum* which are VanI/R and VanM/N. There are 3 AHL molecules produced by *V. anguillarum*, C6-HSL, *N*-(3-hydroxyhexanoyl)-L-homoserine lactone (HC6-HSL) and OC10 HSL. Both C6-HSL and HC6-HSL are produced by *vanI.* Interestingly, both of these AHL molecules were needed for the production of another AHL molecule, (OC10-HSL) produced by *vanM*. In this study, both *V. variabilis* strain T01 and *V. tubiashii* strain T33 produced both C6-HSL and OC10-HSL. This leads to the speculation that similar QS system might be shared between these species.

On the other hand, C4-HSL production was observed in *V. sinaloensis* strains T08 and T47. So far, there are no report on the production of this C4-HSL molecule by the *Vibrio* family. In *P. aeruginosa* PAO1, C4-HSL is needed for the production of biofilm as the *rhlI* mutant (defective in production of C4-HSL) produces 70 % less biofilm compared to the wildtype (Favre-Bonté, Köhler, & Van Delden, 2003). In the LCMS analysis, C12-HSL was also detected in *V. variabilis* strain T01. So far, there is no report of the production of C12-HSL in the *Vibrio* species. According to recent research, long-chain AHLs are assumed to influence gene transfer thus enhancing the genetic exchange (Chang et al., 2012; Amy L Schaefer, Taylor, Beatty, & Greenberg, 2002).

Vibrio spp. are known to employ many QS circuits which often depends on one another to carry out certain phenotypic changes. For instance, in *V. harveyi* employs four different QS systems, involving LuxM/LuxN, LuxS/LuxPQ, CqsA/CqsS and H-NOX/HqsK pathway (Henares, Xu, & Boon, 2013). Similar QS systems could also be seen in *V. cholera,* a notable pathogenic bacterium responsible for cholera. *V. cholerae* QS systems are made of LuxS/LuxPQ and CqsA/CqsS (Donnell, 2015). A study reports on the QS system of *V. tubiashii* NCIMB 1337 involving LuxM/N, LuxS/P,Q and CqsA/S, however, there are no reports on the AHL or other signaling molecules used in these systems (Temperton et al., 2011). The QS system/s in T01 *V. variabilis* strain T01, *V. sinaleoensis* strain T08 and T47 and *V. caribbeanicus* strain T14 are still unshed and many studies need to be done since they are all newly discovered strains dated as early as 2011.

5.3 Genome Analysis of *Vibrio* Isolates

The genome attributes of the five *Vibrio* strains are similar to each other and the fact that they are sampled from the same site triggered questions about their phylogenetic relationship with each other. Acording to Hunt and colleagues (2008), the temporal and spatial separation of resources among *Vibrionaceae* also known as ecological population boundaries may trigger speciation occuring at deep phylogenetic levels. These variation maybe due to acquisition of genetic materials from the environment or other external source. Several mechanisms are employed by microbes to enables the transfer of genetic materials such as the use of phages, phage elements, conjugative plasmids and transposable elements (Chen et al., 2003). Horizontal transfers of genetic materials from other species, contributes to the evolutionary events that occurred within the *Vibrio* species (Antonova & Hammer, 2011). One of the reasons for genetic variability in *Vibrio* species is the presence of a consensus sequence known as super-integron (SI) cassette (Rowe-Magnus & Mazel, 2001) which are present in a number of *Vibrio* species such as *V.anguillarum*, *V. cholerae*, *V. fischeri*, and *V. parahaemolyticus*. In *Vibrio* the SI systems contents varies among species and it is present in almost all proteobacteria as it allows the insertion of open reading frames (ORFs) together with conversion of exogenous sequences into functional genes in the bacteria (Rowe-Magnus, Guérout, & Mazel, 1999).

On the other hand, all of the sequenced strains shares similar subsystems generated by the RAST platform. All of the QS positive strains shares the same class of subsystem in their data from the RAST server which is the cell signaling subsystem. Cell signaling in bacteria begins with the sensing of population density by the autoinducer molecules. Once the population reaches a threshold level, modulation of gene expression is initiated by the transduction cascade reaction. As the autoinducer signaling molecules diffuse into the cell membranes, secondary messenger systems is triggered thus inserting multiple sensory inputs and inducing appropriate response (Camilli & Bassler, 2006).

In bacteria, there are several common second messengers of cell signaling such as the adenosine 3' ,5' monophosphate (cAMP) and guanosine-3 which is used to activate the catabolite regulation protein (CRP) involved in carbon sources usage (Harman, 2001). In addition, guanosine-3, 5-bis (pyrophosphate) (ppGpp) derived from guanosine 5' –triphosphate (GTP) manipulates the RNA polymerase in low levels of tRNAs (Reiness, Yang, Zubay, & Cashel, 1975). These observations provide a clear link between the extracellular signaling information involving the autoinducers and the intra-cellular secondary messengers that translates the signaling cascades into altered gene expression.

5.4 VanM Functionality Study in *V. variabilis* Strain T01

According to previous study (Milton, Chalker, Kirke, Hardman, Cámara, et al., 2001), the autoinducer synthase VanM produces one AHL which is OC10-HSL which depends on the production of C6-HSL and HC6-HSL by VanI. In this study, the VanM gene of *V. variabilis* strain T01 was cloned and transformed into *E. coli* BL21 and the AHL profiles obtained from the transformed *E. coli* BL21 are identical to the wildtype. This finding indicates that VanM in *V. variabilis* strain T01 is capable of producing three types of AHLs which contradicts with previous findings mentioned above. Since specific AHLs are needed to trigger specific phenotypic response, the new findings could be used to discover the roles of the AHLs produced. For example, the *luxM*/*N* and *luxS*/*P*,*Q* influence bioluminescence, polysaccharide, siderophore and metalloprotease production as well as type III secretion.

In addition to this, there is no other autoinducer synthase gene that can be found in the annotated genome sequence of *V. variabilis* strain T01. This finding adds more information to the existing QS systems discovered so far and signals produced from each system in the *Vibrio* family. Species which are very closely related to *V. variabilis* strain T01 could share the same QS profile for example, the VanM homologue, LuxM activity are restricted between very closely related *V. harveyi* and *V. parahaemolyticus* as the signal molecules produced by both of them are used specifically between them (Henke & Bassler, 2004).

V. variabilis strain T01 also produced one long chain AHL namely, C12-HSL. To my best knowledge, this is the first report of long chain AHL production in the *Vibrio* family. In this study, C12-HSL was produced after 8 h of incubation compared with C6-HSL and OC10-HSL that were produced after 4 h of incubation. A study done on the presence of AHL in biofilms in subtidal bacterial population revealed that the production of biofilm was dominated by the *Vibrio* species and shorter AHLs such as C6-HSL was detected in young biofilm while long chain AHLs were detected in mature biofilm (Huang, Ki, Lee, & Qian, 2009). However, a clear reason behind this occurrence still remains enigmatic and more research should be done in order to gain a better understanding in this.

Generally, AHLs produced by the bacteria diffuse freely in and out of the cell, however, accumulation of long chain AHLs in the cell membrane occurred as they are unable to diffuse freely in or out of the cell membrane. Thus, efflux pumps are needed to transport these signaling molecules (Pearson, Van Delden, & Iglewski, 1999). These mechanisms of transport are important as long chain AHLs facilitates in gene transfer among species by activating the production of bacteriophage-like particle which acts as a gene transfer agent (Schaefer et al., 2002). In this study, the long chain AHLs namely, OC10-HSL and C12-HSL produced by V. variabilis strain T01 could be involved in gene transfer needed for the perseverence in the environment as well as evolution.

5.5 Biofilm Formation

QS is the key for regulation biofilm formation (Yildiz & Visick, 2009). A known anti-QS compound, 3,4,5-trihydroxybenzoic acid also known as gallic acid was reported to be a potential QSI compound reported in multiple studies. In strain T01, the biofilm production has reduced significantly with gallic acid treatment. To date, there is no report on the effect of gallic acid on *Vibrio* species. On the other hand, gallic acid has proven to inhibit biofilm production in multiple bacterial species including *E. coli*, *P. aeruginosa*, *S. aureus* and *Listeria monocytogenes* by interfering with bacterial adhesion (Borges et al., 2012). Gallic acid contains polyphenols which are secondary metabolites of plants with multiple biological effects such as anti-fungal, anti-bacterial and anti-viral (Borges et al., 2013). An example of polyphenols effect on biofilm production is in *V. harveyi* treated with naringenin and quercetin found in citrus and a synthetic polyphenol, pyrogallol treatment also on *V. harveyi* (de Lima Pimenta et al., 2013; Vikram et al., 2010). From these observations, it could be inferred that gallic acid inhibits the biofilm production in *V. variabilis* strain T01 via polyphenol compound present in it.

5.6 Differential Gene Expression in *V. variabilis* Strain T01 with Gallic Acid

Plants and bacteria have co-existed with one another leading to the establishment of synthrophic and symbiotic relationship with each other. However, some of the bacteria in the enviroment are pathogenic to plants and one of the plant's defense mechanisms against the bacteria is by producing secondary metabolites. Phenolic compounds are one of the most abundant secondary metabolites produced by plants and they are also proven as anti-QS compound. Many studies conducted using phenolic compounds such as methanolic extracts from *Phyllanthus amarus* (Priya, Yin, & Chan, 2013) and malabaricone C from *Myrictica cinnnamomea* (Chong et al., 2011) has proven to intefere with bacterial QS hence making them a potential natural alternatives compared to the use of antibiotics.

Plyuta et al., (2013) reported that the biofilm produced by *P. aeruginosa* PAO1 has reduced by 30% upon treatment with 200 µg/mL of gallic acid (Plyuta et al., 2013). Apart from that, biofilms produced by *Eikenella corrodens* was dramatically reduced by 80% with treatment of 1 mM of gallic acid (Matsunaga et al., 2010). Known as anti-bacterial, anti-fungal and anti-viral compund, the use of gallic acid however, does not affect the growth of the bacteria as reported by Campos et al., (2003) where high concentration of gallic acid did not affect the bacterial growth and viability. Another study reports inhibition and prevention of biofilm by gallic acid affecting the pathogenicity of several bacteria (Borges et al., 2013). Initial adherence of biofilms as well as filamentous growth which supports the formatuon of biofilms in *Candida albicans* were significantly inhibted by gallic acid displaying potent activity against the formation of biofilms in *Candida albicans* (Wang et al., 2009).

In this study, approximately 40.5 % biofilm production of *V. variabilis* strain T01 was reduced upon treatment using 1 mg/mL of gallic acid concentration adapted from Borges et al., (2012). The use of 1 mg/mL of gallic acid showed no inhibition of growth on *V. variabilis* strain T01.

Differential expression of genes in *V. variabilis* strain T01 was observed upon treatment with 1 mg/mL of gallic acid. The differential expressed genes were then categorised into their respective funtions according to the COG categories and from the pie chart generated (Figure 4.12), all of the matched COG categories corresponds to all of the processes involved in cell's biology (Alberts et al., 2013). In the data generated by mapping the differentially expressed genes with the COGs database, ATP-binding cassette (ABC) transporter family was present in all COG categories. For example, ABC-type proline/glycine betaine transport systems, periplasmic components (COG: amino acid transport and metabolism), ABC-type sugar transport system, periplasmic component (COG: carbohydrate transport and metabolism), ABC-type tungstate transport system, permease component (COG: coenzyme transport and metabolism), ABC-type multidrug transport system, ATPase component (COG: Defense mechanisms), and ABC-type phosphate/phosphonate transport system, periplasmic component (COG: inorganic ion transport and metabolism) indicates that gallic acid treatment on *V. variabilis* strain T01 affects most of its cellular function by affecting the function of ABC transporter. ABC transporters are a part of a large proteins superfamily which carry very important role in physiological functions in all organisms. ABC transporters are located mostly between membranes and transports a multiple types of substrates through lipid membranes (Broehan, Kroeger, Lorenzen, & Merzendorfer, 2013). In a study done on *Saccharomyces cerevisae*, gallic acid was proven to inhibit its ABC transporter by inhibiting the efflux of substrates and affecting the ATPase activity

(Rangel et al., 2010). Based on this observation, the gallic acid treatment on *V. variabilis* strain T01 might act in a similar way thus affecting multiple cellular processes. The RNA-seq data obtained also showed a decrease in fold change value as compared with the untreated sample which indicates a down regulation of genes. some of the genes affected are ABC transporter ATP-binding protein uup, Inner membrane amino-acid ABC transporter permease protein YhdY and putative ABC transporter ATP-binding protein YheS.

Apart from that, *cgtA* gene in *V. variabilis* strain T01 was downregulated upon treatment with gallic acid. CgtA belongs to a small group within GTP-binding proteins which is involved in repairing damage DNA by stimulating expression of *recA* and the subsequently activating the DNA repair pathways in *V. harveyi* (Zielke, Sikora, Dutkiewicz, Wegrzyn, & Czyż, 2003). DNA repair is one of the main components for evolution as it maintains genetic stability from DNA damage factors which would evidently change the DNA sequences and alter their functions and the majority a cells energy is invested in DNA repair enzymes involved in repair pathways (Alberts et al., 2002). Gallic acid possess bactericidal effect on bacterial cells due to polyphenols compound present in it (Nakamura et al., 2015). Downregulation of DNA repair gene *ctgA*, upon treatment of gallic acid on *V. variabilis* strain T01 indicates the inference of DNA repair mechanisms which affect the capability of the bacterial cells to perform certain functions related to QS.

From the RNA-seq data, transport protein TonB is highly up-regulated with fold chain of 59.9114 upon treatment with gallic acid. *tonB* are classified as group M (cell wall/membrane/envelope biogenesis) in COG groups.TonB is a bacterial outer membrane protein responsible for binding and transporting siderophores, nickel complexes, carbohydrates and vitamin B (12) (Noinaj, Guillier, Barnard, & Buchanan, 2010). In *Vibrio,* iron is an important component for growth and multiple mechanisms is employed to obtain iron from its surrounding using siderophore produced (Griffiths, Sigel, Payne, & Neilands, 1984). In pathogenic *Vibrios* such as *V. anguillarum* and *V. vulnificus,* iron uptake plays a key role in regulating virulence (Wolf & Crosa, 1986). Since virulence is mediated by QS, the treatment of gallic acid, a known anti QS compound which induce a change in membrane permeability might be responsible in increased uptake of iron into the cell (Borges et al., 2013).

On another note, a gene classified in group G of COGs (carbohydrate transport and metabolism), PTS system glucose-specific EIICB component (*ptsG*), is downregulated. In *Vibrio, ptsG* functions as phosphorylator of sugar substrates and subsequently distributes them across the membranes of the cell. In *P. aeruginosa,* QS was proven to regulate carbohydrate and lipid metabolism based on the environmental stresses (Davenport, Griffin, & Welch, 2015). When population density is high, energy in the forms of sugars and carbohydrates are utilised for exhibiting various phenotypes associated with QS (Schuster et al., 2003). Gallic acid is a compound known to inhibit or interfering with QS systems, so a decreased in QS associated processes would likely to have negative impact on the regulation of carbohydrates *V. variabilis* strain T01.

5.7 Future Work

Future research could be directed at constructing a knock out mutant of LuxI of *V. variabilis* strain T01 to further understand and confirm the genes affected by QS. The λ red recombineering protocol could be adapted to perform this mutation project as it provides a straightforward and efficient procedure using plasmids and cassettes to induce mutation (Sawitzke et al., 2013). Once *luxI* mutant is obtained, transcriptomic profiling of the mutant coupled with phenotypic microarray can be carried out to study the relationship between differentially expressed genes and the phenotypic changes by the mutant. This approach would give a clearer view of the processes affected in the mutant and a direct link between genotypes and phenotypes.

CHAPTER 6: CONCLUSION

The isolation of bacteria from Morib beach led to the isolation of 60 bacterial strains of which five of them exhibited QS activity. From LC-MS/MS analysis, both short and long chained AHL namely, C4-HSL, C6-HSL, OC10-HSL and C12-HSL were produced by the QS positive strains and this study reports the first findings of C4-HSL production in *Vibrio* spp. which are produced by *V. sinaloensis* strains T08 and T47. Whole genome sequencing, and functionality studies on selected strain, the QS gene was identified and validated. Treatment with anti -QS compound (gallic acid) provides some useful insights to the mechanism of QS in newly identified *V. variabilis* strain T01. The RNA-seq transcriptomic profiling on *V. variabilis* strain T01 treated with gallic acid showed the differential regulations of genes in relation to QS such as genes involved in cell wall/envelope biogenesis (*tonB*) and cell motility (*fliL*, *fliT* and *fliS*). Gallic acid treatment on *V. variabilis* strain T01 affected the over all transport and metabolism process (inorganic ion, amino acid, carbohydrates and lipids.

REFERENCES

Adar, Y. Y., & Ulitzur, S. (1993). GroESL proteins facilitate binding of externally added inducer by LuxR protein-containing *E. coli* cells. *J Biolumin Chemilumin, 8*, 261-266.

Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., . . . Walter, P. (2013). *Essential cell biology*: Garland Science.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). DNA replication, repair, and recombination.

Antonova, E. S., & Hammer, B. K. (2011). Quorum-sensing autoinducer molecules produced by members of a multispecies biofilm promote horizontal gene transfer to Vibrio cholerae. *FEMS microbiology letters, 322*(1), 68-76.

Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., . . . Kubal, M. (2008). The RAST Server: rapid annotations using subsystems technology. *BMC Genomics, 9*(1), 75.

Bassler, B. L., Wright, M., Showalter, R. E., & Silverman, M. R. (1993). Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence. *Mol Microbiol, 9*.

Bassler, B. L., Wright, M., & Silverman, M. R. (1994). Multiple signaling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a secong sensory pathway. *Mol Microbiol, 13*, 273-286.

Baumann, P., Furniss, A., & Lee, J. V. (1984). Genus I. *Vibrio*. *Bergey's manual of systematic bacteriology, 1*, 518-538.

Blake, P. A., Merson, M. H., Weaver, R. E., Hollis, D. G., & Heublein, P. C. (1979). Disease caused by a marine *vibrio*: clinical characteristics and epidemiology. *N Engl J Med, 300*(1), 1-5.

Boettcher, K. J., & Ruby, E. G. (1995). Detection and quantification of *Vibrio fischeri* autoinducer from symbiotic squid organs. *J Bacteriol, 177*, 1053-1058.

Borges, A., Ferreira, C., Saavedra, M. J., & Simoes, M. (2013). Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. *Microbial Drug Resistance, 19*(4), 256-265.

Borges, A., Saavedra, M. J., & Simões, M. (2012). The activity of ferulic and gallic acids in biofilm prevention and control of pathogenic bacteria. *Biofouling, 28*(7), 755-767.

Boylan, M., Graham, A. F., & Meighen, E. A. (1985). Functional identification of the fatty acid reductase components encoded in the luminescence operon of *Vibrio fischeri*. *J Bacteriol, 163*, 1186-1190.

Boylan, M., Miyamoto, C., Wall, L., Graham, A., & Meighen, E. A. (1989). Lux C, D and E genes of the *Vibrio ficheri* luminescence operon code for the reductase, transferase and synthetase enzymes involved in aldehyde biosynthesis. *Photochem Photobiol, 49*, 681-688.

Brackman, G., Defoirdt, T., Miyamoto, C., Bossier, P., Van Calenbergh, S., Nelis, H., & Coenye, T. (2008). Cinnamaldehyde and cinnamaldehyde derivatives reduce virulence in *Vibrio* spp. by decreasing the DNA-binding activity of the quorum sensing response regulator LuxR. *BMC Microbiol, 8*(1), 149.

Broehan, G., Kroeger, T., Lorenzen, M., & Merzendorfer, H. (2013). Functional analysis of the ATP-binding cassette (ABC) transporter gene family of *Tribolium castaneum*. *BMC Genomics, 14*(1), 1.

Burke, A., Guthrie, L. T. C., Modise, T., Cormier, G., Jensen, R. V., McCarter, L. L., & Stevens, A. M. (2015). OpaR controls a network of downstream transcription factors in *Vibrio parahaemolyticus* BB22OP. *PLoS ONE, 10*(4), e0121863. doi: 10.1371/journal.pone.0121863

Byers, J. T., Lucas, C., Salmond, G. P., & Welch, M. (2002). Nonenzymatic turnover of *an Erwinia carotovora* quorum-sensing signaling molecule. *J Bacteriol, 184*(4), 1163-1171.

Callahan, S. M., & Dunlap, P. V. (2000). LuxR- and acyl-homoserine-lactone-controlled non-lux genes define a quorum-sensing regulon in *Vibrio fischeri*. *J Bacteriol, 182*, 2811-2822.

Camilli, A., & Bassler, B. L. (2006). Bacterial small-molecule signaling pathways. *Science, 311*(5764), 1113-1116.

Campos, F., Couto, J., & Hogg, T. (2003). Influence of phenolic acids on growth and inactivation of *Oenococcus oeni* and *Lactobacillus hilgardii*. *J Appl Microbiol, 94*(2), 167-174.

Camps, J., Pujol, I., Ballester, F., Joven, J., & Simó, J. M. (2011). Paraoxonases as potential antibiofilm agents: their relationship with quorum-sensing signals in gram-negative bacteria. *Antimicrob Agents Chemother, 55*(4), 1325-1331.

Cataldi, T. R., Bianco, G., Fonseca, J., & Schmitt-Kopplin, P. (2013). Perceiving the chemical language of Gram-negative bacteria: listening by high-resolution mass spectrometry. *Anal Bioanal Chem, 405*(2-3), 493-507.

Chan, K.-G., Atkinson, S., Mathee, K., Sam, C.-K., Chhabra, S. R., Cámara, M., . . . Williams, P. (2011). Characterization of *N*-acylhomoserine lactone-degrading bacteria associated with the *Zingiber officinale* (ginger) rhizosphere: Co-existence of quorum quenching and quorum sensing in *Acinetobacter* and *Burkholderia*. *BMC Microbiol, 11*(1), 51.

Chang, C.-Y., Koh, C.-L., Sam, C.-K., Chan, X.-Y., Yin, W. F., & Chan, K. G. (2012). Unusual long-chain *N*-acyl homoserine lactone production by and presence of quorum quenching activity in bacterial isolates from diseased tilapia fish. *PLoS ONE, 7*(8), e44034.

Charlton, T. S., de Nys, R., Netting, A., Kumar, N., Hentzer, M., Givskov, M., & Kjelleberg, S. (2000). A novel and sensitive method for the quantification of *N*-3-oxoacyl homoserine lactones using gas chromatography-mass spectrometry: application to a model bacterial biofilm. *Environ Microbiol, 2*, 530=541.

Chatterjee, S., & Haldar, S. (2012). *Vibrio* related diseases in aquaculture and development of rapid and accurate identification methods. *J Mar Sci: Res & Dev, 2012*.

Chen, C.-Y., Wu, K.-M., Chang, Y.-C., Chang, C.-H., Tsai, H.-C., Liao, T.-L., . . . Li, J.-C. (2003). Comparative genome analysis of *Vibrio vulnificus*, a marine pathogen. *Genome Res, 13*(12), 2577-2587.

Chevrot, R., Rosen, R., Haudecoeur, E., Cirou, A., Shelp, B. J., Ron, E., & Faure, D. (2006). GABA controls the level of quorum-sensing signal in *Agrobacterium tumefaciens*. *Proc Natl Acad Sci, 103*(19), 7460-7464.

Chimetto, L. A., Cleenwerck, I., Moreira, A. P. B., Brocchi, M., Willems, A., De Vos, P., & Thompson, F. L. (2011). *Vibrio variabilis* sp. nov. and *Vibrio maritimus* sp. nov., isolated from *Palythoa caribaeorum*. *Int J Syst Evol Microbiol, 61*(12), 3009-3015.

Choi, S. H., & Greenberg, E. P. (1992). Genetic dissection of DNA binding and luminescence gene activation by the *Vibrio fischeri* LuxR protein. *J Bacteriol, 1774*, 4064-4069.

Chong, Y. M., Yin, W. F., Ho, C. Y., Mustafa, M. R., Hadi, A. H. A., Awang, K., . . . Chan, K.-G. (2011). Malabaricone C from *Myristica cinnamomea* exhibits anti-quorum sensing activity. *J Nat Prod, 74*(10), 2261-2264.

Chowdhary, P. K., Keshavan, N., Nguyen, H. Q., Peterson, J. A., González, J. E., & Haines, D. C. (2007). *Bacillus megaterium* CYP102A1 oxidation of acyl homoserine lactones and acyl homoserines. *Biochemistry, 46*(50), 14429-14437.

Churchill, M. E. A., & Chen, L. (2011). Structural Basis of Acyl-homoserine Lactone-Dependent Signaling. *Chem Rev, 111*(1), 68-85.

Corry, J. E., Curtis, G. D., & Baird, R. M. (2011). *Handbook of culture media for food and water microbiology*: Royal Society of Chemistry.

Costerton, J. W., Cheng, K. J., Geesey, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M., & Marrie, T. J. (1987). Bacterial biofilms in nature and disease. *Annu Rev Microbiol, 41*, 435-464. doi: 10.1146/annurev.mi.41.100187.002251

Cullinane, M., Baysse, C., Morrissey, J. P., & O'Gara, F. (2005). identification of two lysophosphatidic acid acyltransferase genes with overlapping function in *Pseudomonas fluorescens*. *Microbiology, 151*(9), 3071-3080.

Daniels, R., Reynaert, S., Hoekstra, H., Verreth, C., Janssens, J., Braeken, K., . . . Lambrichts, I. (2006). Quorum signal molecules as biosurfactants affecting swarming in *Rhizobium etli*. *Proc Natl Acad Sci, 103*(40), 14965-14970.

Davenport, P. W., Griffin, J. L., & Welch, M. (2015). Quorum sensing is accompanied by global metabolic changes in the opportunistic human pathogen *Pseudomonas aeruginosa*. *J Bacteriol, 197*(12), 2072-2082.

Davies, J. C. (2002). *Pseudomonas aeruginosa* in cystic fibrosis: pathogenesis and persistence. *Paediatric respiratory reviews, 3*(2), 128-134.

de Lima Pimenta, A., Chiaradia-Delatorre, L. D., Mascarello, A., de Oliveira, K. A., Leal, P. C., Yunes, R. A., . . . Smânia, A. (2013). Synthetic organic compounds with potential for bacterial biofilm inhibition, a path for the identification of compounds interfering with quorum sensing. *Int J Antimicrob Agents, 42*(6), 519-523.

Deep, A., Chaudhary, U., & Gupta, V. (2011). Quorum sensing and bacterial pathogenicity: from molecules to disease. *Journal of laboratory physicians, 3*(1), 4.

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., & Bossier, P. (2008). Quorum sensing and quorum quenching in *Vibrio harveyi*: lessons learned from in vivo work. *ISME J, 2*(1), 19-26.

Devine, J. H., Countryman, C., & Baldwin, T. O. (1988). Nucleotide sequence of the *luxR* and *luxI* genes and structure of the primary regulatory region of the *lux* regulon of *Vibrio fischeri* ATCC 7744. *Biochemistry, 27*, 837-842.

Dewhirst, F. E., Chien, C.-C., Paster, B. J., Ericson, R. L., Orcutt, R. P., Schauer, D. B., & Fox, J. G. (1999). Phylogeny of the defined marine microbiota: altered Schaedler flora. *Appl Environ Microbiol, 65*(8), 3287-3292.

Diggle, S. P., Crusz, S. A., & Cámara, M. (2007). Quorum sensing. *Curr Biol, 17*(21), R907-R910.

Dolan, K. M., & Greenberg, E. P. (1992). Evidence that GroEL, not o32, is involved in transcriptional regulation of the *Vibrio fischeri* luminescence genes in *Escherichia coli*. *J Bacteriol, 174*, 5132-5135.

Dong, Y. H., Zhang, X. F., Soo, H. M. L., Greenberg, E. P., & Zhang, L. H. (2005). The two-component response regulator PprB modulates quorum-sensing signal production and global gene expression in *Pseudomonas aeruginosa*. *Mol Microbiol, 56*(5), 1287-1301.

Donnell, Z. N. (2015). *Regulation of CqsA and CqsS in Vibrio cholerae.* PRINCETON UNIVERSITY.

Dunlap, P. V., & Grennberg, E. P. (1988). Control of *Vibrio fischeri* lux gene transcription by a cyclic AMP receptor protein-LuxR protein regulatory circuit. *J Bacteriol, 170*, 4040-4046.

Dunlap, P. V., & Ray, J. M. (1989). Requirement for autoinducer in transcriptional negative autoregulation of the *Vibrio fischeri luxR* gene in *Escherichia coli*. *J Bacteriol, 171*, 3549-3552.

Dunn, G. K., Michaliszyn, G. A., Bogacki, I. G., & Meighen, E. A. (1973). Conversion of aldehyde to acid in the bacterial bioluminescent reaction. *Biochemistry, 12*, 4911-4918.

Dworzanski, J. P., & Snyder, A. P. (2005). Classification and identification of bacteria using mass spectrometry-based proteomics. *Expert review of proteomics, 2*(6), 863-878.

Eberhard, A. (1972). Inhibition and activation of bacterial luciferase synthesis. *J Bacteriol, 109*, 1101-1105.

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

Eberhard, A., Longin, T., Widrig, C. A., & Stranik, S. J. (1991). Synthesis of the lux gene autoinducer in *Vibrio fischeri* is positively autoregulated. *Arch Microbiol, 155*, 294-297.

El-Bouri, K., Johnston, S., Rees, E., Thomas, I., Bome-Mannathoko, N., Jones, C., . . . Harris, L. (2012). Comparison of bacterial identification by MALDI-TOF mass spectrometry and conventional diagnostic microbiology methods: agreement, speed and cost implications. *Brit J Biomed Sci, 69*(2), 47.

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

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

Enos-Berlage, J. L., Guvener, Z. T., Keenan, C. E., & McCarter, L. L. (2005). Genetic determinants of biofilm development of opaque and translucent *Vibrio parahaemolyticus*. *Mol Microbiol, 55*(4), 1160-1182.

Farmer Iii, J., & Hickman-Brenner, F. (2006). The genera *Vibrio* and *Photobacterium. The prokaryotes* (pp. 508-563): Springer.

Farrand, S. K., Qin, Y., & Oger, P. (2002). Quorum-sensing system of *Agrobacterium* plasmids: analysis and utility. *Methods Enzymol, 358*, 452-484.

Favre-Bonté, S., Köhler, T., & Van Delden, C. (2003). Biofilm formation by *Pseudomonas aeruginosa*: role of the C4-HSL cell-to-cell signal and inhibition by azithromycin. *J Antimicrob Chemo, 52*(4), 598-604.

Field, D., Garrity, G., Gray, T., Morrison, N., Selengut, J., Sterk, P., . . . Wipat, A. (2008). The minimum information about a genome sequence (MIGS) specification. *Nat Biotechnol, 26*(5), 541-547. doi: 10.1038/nbt1360

Fischer, E. R., Hansen, B. T., Nair, V., Hoyt, F. H., & Dorward, D. W. (2012). Scanning electron microscopy. *Curr Protoc Microbiol, Chapter 2*, Unit 2B.2. doi: 10.1002/9780471729259.mc02b02s25

Fuqua, C., Parsek, M. R., & Greenberg, E. P. (2001). Regulation of gene expression by cell-to-cell communication: Acyl-homoserine lactone quorum sensing. *Annu Rev Genet, 35*(1), 439-468.

Garrity, G., & Holt, J. (2001). Taxonomic outline of the Archaea and Bacteria: Bergey's Manual of Systematic Bacteriology.

Garrity, G., Staley, J. T., Boone, D. R., De Vos, P., Goodfellow, M., Rainey, F. A., . . . Krieg, N. R. (2006). *Bergey's Manual® of Systematic Bacteriology: Volume Two: The Proteobacteria*: Springer Science & Business Media.

Garrity, G. M., & Holt, J. G. (2001). The road map to the manual *Bergey's Manual® of Systematic Bacteriology* (pp. 119-166): Springer.

Gode-Potratz, C. J., & McCarter, L. L. (2011). Quorum sensing and silencing in *Vibrio parahaemolyticus*. *J Bacteriol, 193*(16), 4224-4237. doi: 10.1128/jb.00432-11

Gould, T. A., Herman, J., Krank, J., Murphy, R. C., & Churchill, M. E. (2006). Specificity of acyl-homoserine lactone synthases examined by mass spectrometry. *J Bacteriol, 188*(2), 773-783.

Graf, J., & Ruby, E. G. (1998). Host-derived amino acids support the proliferation of symbiotic bacteria. *Proc Natl Acad Sci USA, 95*, 1818-1822.

Griffiths, G., Sigel, S., Payne, S., & Neilands, J. (1984). Vibriobactin, a siderophore from *Vibrio cholerae*. *J Bio Chem, 259*(1), 383-385.

Hanzelka, B. L., Parsek, M. R., Val, D. L., Dunlap, P. V., Cronan, J. E. J., & Greenberg, E. P. (1999). Acylhomoserine lactone synthase activity of the *Vibrio fischeri* AinS protein. *J Bacteriol, 181*, 5766-5770.

Harman, J. G. (2001). Allosteric regulation of the cAMP receptor protein. *Biochim Biophys Acta, 1547*(1), 1-17.

Harshey, R. M. (2003). Bacterial motility on a surface: many ways to a common goal. *Annu Rev Microbiol, 57*, 249-273. doi: 10.1146/annurev.micro.57.030502.091014

Harvey, E. N. (1915). The effect of certain organic and inorganic substances upon light production by luminous bacteria. *The Bio Bull, 29*(5), 308-311.

Hastings, J. W. (1978). Bacterial bioluminescence: an overview. *Methods Enzymol, 57*, 125-135.

Henares, B. M., Xu, Y., & Boon, E. M. (2013). A nitric oxide-responsive quorum sensing circuit in *Vibrio harveyi* regulates flagella production and biofilm formation. *Int J Mol Sci, 14*(8), 16473-16484.

Henke, J. M., & Bassler, B. L. (2004). Three parallel quorum sensing systems regulate gene expression in *Vibrio harveyi*. *J Bacteriol, 186*, 6902-6914.

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

Hoang, T. T., Yufang, M., Stem, R. J., McNeil, m. R., & Shweizer, H. P. (1999). Construction and use of low-copy number T7 expression vectors for purification of problem proteins: purification of *Mycobacterium tuberculosis* RmID and *Pseudomonas aeruginosa* LasI and RhlI proteins, and functional analysis of purified RhlI. *Gene, 237*, 361-371.

Hoffmann, M., Monday, S. R., Allard, M. W., Strain, E. A., Whittaker, P., Naum, M., . . . Brown, E. W. (2012). *Vibrio caribbeanicus* sp. nov., isolated from the marine sponge *Scleritoderma cyanea*. *Int J Syst Evol Microbiol, 62*(8), 1736-1743.

Huang, J. J., Han, J. I., Zhang, L. H., & 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*(10), 5941-5949.

Huang, Y.-L., Ki, J.-S., Lee, O. O., & Qian, P.-Y. (2009). Evidence for the dynamics of Acyl homoserine lactone and AHL-producing bacteria during subtidal biofilm formation. *ISME J, 3*(3), 296-304.

Hunt, D. E., David, L. A., Gevers, D., Preheim, S. P., Alm, E. J., & Polz, M. F. (2008). Resource partitioning and sympatric differentiation among closely related bacterioplankton. *Science, 320*(5879), 1081-1085.

Hutchison, C. A. (2007). DNA sequencing: bench to bedside and beyond. *Nucleic Acids Res, 35*(18), 6227-6237.

Ivanova, K., Fernandes, M., & Tzanov, T. (2013). Current advances on bacterial pathogenesis inhibition and treatment strategies. *Microbial pathogens and strategies for combating them: science, technology and education. Mendez-Vilas A (ed), 1*, 322-336.

Janda, J., Powers, C., Bryant, R., & Abbott, S. (1988). Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clin Microbiol Rev, 1*(3), 245-267.

Janda, J. M., & Abbott, S. L. (2007). 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. *J Clin Microbiol, 45*(9), 2761-2764.

Janssens, J. C., Steenackers, H., Robijns, S., Gellens, E., Levin, J., Zhao, H., . . . Marchal, K. (2008). Brominated furanones inhibit biofilm formation by *Salmonella enterica* serovar Typhimurium. *Appl Environ Microbiol, 74*(21), 6639-6648.

Jiang, Y., Camara, M., Chhabra, S. R., hardie, k. R., Bycroft, b. W., Lazdunski, A., . . . Williams, P. (1998). In vitro biosynthesis of the *Pseudomonas aeruginosa* quorum-sensing signal molecule *N*-butanoyl-l-homoserine lactone. *Mol Microbiol, 28*, 193-203.

Jobling, M. G., & Holmes, R. K. (1997). Characterization of *hapR*, a positive regulator of the *Vibrio cholerae* HA/protease gene hap, and its identification as a functional homologue of the *Vibrio harveyi luxR* gene. *Mol Microbiol, 26*, 1023-1034.

Jones, D. T., Taylor, W. R., & Thornton, J. M. (1992). The rapid generation of mutation data matrices from protein sequences. *Computer applications in the biosciences: CABIOS, 8*(3), 275-282.

Julie, D., Solen, L., Antoine, V., Jaufrey, C., Annick, D., & Dominique, H. H. (2010). Ecology of pathogenic and non-pathogenic *Vibrio parahaemolyticus* on the French Atlantic coast. Effects of temperature, salinity, turbidity and chlorophyll a. *Environ Microbiol, 12*(4), 929-937.

Kalia, V. C. (2013). Quorum sensing inhibitors: an overview. *Biotechnol Adv, 31*(2), 224-245.

Kalia, V. C., & Purohit, H. J. (2011). Quenching the quorum sensing system: potential antibacterial drug targets. *Critical reviews in microbiology, 37*(2), 121-140.

Kalia, V. C., Rani, A., Lal, S., Cheema, S., & Raut, C. (2007). Combing databases reveals potential antibiotic producers. *Expert Opin Drug Discovery, 2*(2), 211-224.

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

Kirwan, J. P., Gould, T. A., Schweizer, H. P., Bearden, S. W., Murphy, R. C., & Churchill, M. E. (2006). Quorum-sensing signal synthesis by the *Yersinia pestis* acyl-homoserine lactone synthase YspI. *J Bacteriol, 188*(2), 784-788. doi: 10.1128/jb.188.2.784-788.2006

Kocasoy, G. (1989). The relationship between coastal tourism, sea pollution and public health: a case study from Turkey. *Environmentalist, 9*(4), 245-251.

Kociolek, M. G. (2009). Quorum-sensing inhibitors and biofilms. *Anti-Infective Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Infective Agents), 8*(4), 315-326.

Kolibachuk, D., & Greenberg, E. P. (1993). The *Vibrio fischeri* gene activator LuxR is a membrane-associated protein. *J Bacteriol, 175*, 7307-7312.

Lade, H., Paul, D., & Kweon, J. H. (2014). Isolation and molecular characterization of biofouling bacteria and profiling of quorum sensing signal molecules from membrane bioreactor activated sludge. *Int J Mol Sci, 15*, 2255-2273.

Lagesen, K., Hallin, P., Rodland, E. A., Staerfeldt, H. H., Rognes, T., & Ussery, D. W. (2007). RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res., 35*, 3100-3108.

Laue, B. E., Jiang, y., Chhabra, S. R., Jacob, S., Stewart, G. S. A. B., Hardman, A., . . . 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.

Liu, A., Mi, Z. H., Zheng, X. Y., Yu, Y., Su, H. N., Chen, X. L., . . . Qin, Q. L. (2016). Exopolysaccharides play a role in the swarming of the benthic bacterium *Pseudoalteromonas* sp. SM9913. *Front Microbiol, 7*, 473. doi: 10.3389/fmicb.2016.00473

Llamas, I., Keshavan, N., & Gonzalez, J. E. (2004). Use of *Sinorhizobium meliloti* as an indicator for specific detection of long-chain *N*-acyl homoserine lactones. *Appl Environ Microbiol, 70*(6), 3715-3723. doi: 10.1128/aem.70.6.3715-3723.2004

Lowe, T. M., & Eddy, S. R. (1997). tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. *Nucleic Acids Res., 25*, 955-964.

Lupp, C., & Ruby, E. G. (2004). *Vibrio fischeri* LuxS and AinS: comparative study for two signal synthases. *J Bacteriol, 186*, 3873-3881.

Matsunaga, T., Nakahara, A., Minnatul, K. M., Noiri, Y., Ebisu, S., Kato, A., & Azakami, H. (2010). The inhibitory effects of catechins on biofilm formation by the periodontopathogenic bacterium, *Eikenella corrodens*. *Bioscience, Biotechnol Biochem, 74*(12), 2445-2450.

McClean, K. H., Winson, M. K., Fish, L., Taylor, A., Chhabra, S. R., Camara, M., . . . 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.

McClean, K. H., Winson, M. K., Leigh, F., Taylor, A., Chhabra, S. R., Camara, M., . . . Williams, P. (1997). Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactone. *Microbiology, 143*, 3703-3711.

McGowan, S., Sebaihia, M., Jones, S., Yu, B., Bainton, N., Chan, P. F., . . . Salmond, G. P. C. (1995). Carbapenem antibiotic production in *Erwinia carotovora* is regulated by *carR*, a homologue of the LuxR transcriptional activator. *Microbiology, 141*(3), 541-550.

McLean, R. J., Pierson, L. S., & Fuqua, C. (2004). A simple screening protocol for the identification of quorum signal antagonists. *J Microbiol Methods, 58*(3), 351-360.

Mellmann, A., Cloud, J., Maier, T., Keckevoet, U., Ramminger, I., Iwen, P., . . . Lasala, P. (2008). Evaluation of matrix-assisted laser desorption ionization-time-of-flight mass spectrometry in comparison to 16S rRNA gene sequencing for species identification of nonfermenting bacteria. *J Clin Microbiol, 46*, 1946-1954.

Michael, B., Smith, J. N., Swift, S., Heffron, F., & Ahmer, B. M. (2001). SdiA of *Salmonella enterica* is a LuxR homolog that detects mixed microbial communities. *J Bacteriol, 183*(19), 5733-5742. doi: 10.1128/jb.183.19.5733-5742.2001

Mihalik, K., Chung, D., Crixell, S., McLean, R., & Vattem, D. (2008). Quorum sensing modulators of *Pseudomonas aeruginosa* characterized in *Camellia sinensis*. *Asian J Trad Med, 3*(12), e23.

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

Miller, M. B., Skorupski, K., Lenz, D. H., Taylor, R. K., & Bassler, B. L. (2002). Parallel quorum sensing systems converge to regulate virulence in *Vibrio cholerae*. *Cell, 110*, 303-314.

Milton, D. L. (2006). Quorum sensing in *vibrios*: Complexity for diversification. *Int J Med Microbiol, 296*(2–3), 61-71. doi: http://dx.doi.org/10.1016/j.ijmm.2006.01.044

Milton, D. L., Chalker, V. J., Kirke, D., Hardman, A., Cámara, M., & Williams, P. (2001). The LuxM homologue VanM from *Vibrio anguillarum* directs the synthesis of *N*-(3-hydroxyhexanoyl) homoserine lactone and *N*-hexanoyl homoserine lactone. *J Bacteriol, 183*(12), 3537-3547.

Moré, M. I., Finger, D., Stryker, J. L., Fuqua, C., Eberhard, A., & Winans, S. C. (1996). Enzymatic synthesis of a quorum-sensing autoinducer through use of defined substrates. *Science*, 1655-1658.

Nachin, L., & Barras, F. (2000). External pH: an environmental signal that helps to rationalize pel gene duplication in *Erwinia chrysanthemi*. *Mol Plant Microbe Interact, 13*(8), 882-886. doi: 10.1094/mpmi.2000.13.8.882

Nakamura, K., Ishiyama, K., Sheng, H., Ikai, H., Kanno, T., & Niwano, Y. (2015). Bactericidal activity and mechanism of photoirradiated polyphenols against gram-positive and-negative bacteria. *J Agric Food Chem, 63*(35), 7707-7713.

Nardi, M. G. (1954). [Discovery of *Vibrio cholerae* by Filippo Pacini, of Pistoia, established in the initial phases of microbiological thought and judged after a century]. *Minerva Med, 45*(102), Varia, 1024-1029.

Nealson, K. H. (1999). Early observations defining quorum-dependent gene expression. In G. M. Dunny & S. C. Winans (Eds.), *Cell-Cell Signaling in Bacteria* (pp. 277-289). Washington, DC: ASM Press.

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

Nealson, K. H., Platt, T., & Hastings, J. W. (1970). Cellular control of the synthesis and activity of the bacterial luminescent system. *J Bacteriol, 104*, 313-322.

Niu, C., Afre, S., & Gilbert, E. (2006). Subinhibitory concentrations of cinnamaldehyde interfere with quorum sensing. *Lett Appl Microbiol, 43*(5), 489-494.

Noinaj, N., Guillier, M., Barnard, T. J., & Buchanan, S. K. (2010). TonB-dependent transporters: regulation, structure, and function. *Annu Rev Microbiol, 64*, 43.

O'Toole, G., Kaplan, H. B., & Kolter, R. (2000). Biofilm formation as microbial development. *Annu Rev Microbiol, 54*(1), 49-79.

Olson, M. E., Ceri, H., Morck, D. W., Buret, A. G., & Read, R. R. (2002). Biofilm bacteria: formation and comparative susceptibility to antibiotics. *Canadian J Vet Res, 66*(2), 86.

Ortori, C. A., Dubern, J. F., Chhabra, S. R., Cámara, M., Hardie, k., Williams, P., & Barrett, D. A. (2011). Simultaneous quantitative profiling of *N*-acyl-L-homoserine lactone and 2-alkyl-4(1H)-quinolone families of quorum-sensing signaling molecules using LC-MS/MS. *Anal Bioanal Chem, 399*(2), 839-850.

Ott, S. J., Musfeldt, M., Ullmann, U., Hampe, J., & Schreiber, S. (2004). Quantification of intestinal bacterial populations by real-time PCR with a universal primer set and minor groove binder probes: a global approach to the enteric flora. *J Clin Microbiol, 42*(6), 2566-2572.

Panda, A., Kurapati, S., Samantaray, J. C., Srinivasan, A., & Khalil, S. (2014). MALDI-TOF mass spectrometry proteomic based identification of clinical bacterial isolates. *Ind J Med Res, 140*(6), 770.

Park, S.-Y., Kang, H.-O., Jang, H.-S., Lee, J.-K., Koo, B.-T., & 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*(5), 2632-2641.

Parsek, M. R., Val, D. L., Hanzelka, B. L., Cronan, J. E. J., & Greenberg, E. P. (1999). Acyl homoserine lactone quorum-sensing signal generation. *Proc Natl Acad Sci USA, 96*, 4360-4365.

Pearson, J. P., Gray, K. M., Passador, L., Tucker, K. D., Eberhard, A., Iglewski, B. H., & Greenberg, E. (1994). Structure of the autoinducer required for expression of *Pseudomonas aeruginosa* virulence genes. *Proc Natl Acad Sci, 91*(1), 197-201.

Pearson, J. P., Van Delden, C., & Iglewski, B. H. (1999). Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J Bacteriol, 181*(4), 1203-1210.

Piper, K. R., & Farrand, S. K. (2000). Quorum sensing but not autoinduction of Ti plasmid conjugal transfer requires control by the opine regulon and the antiactivator TraM. *J Bacteriol, 182*(4), 1080-1088.

Plyuta, V., Zaitseva, J., Lobakova, E., Zagoskina, N., Kuznetsov, A., & Khmel, I. (2013). Effect of plant phenolic compounds on biofilm formation by *Pseudomonas aeruginosa*. *Apmis, 121*(11), 1073-1081.

Pompeani, A. J., Irgon, J. J., Berger, M. F., Bulyk, M. L., Wingreen, N. S., & Bassler, B. L. (2008). The *Vibrio harveyi* master quorum-sensing regulator, LuxR, a TetR-type protein is both an activator and a repressor: DNA recognition and binding specificity at target promoters. *Mol Microbiol, 70*(1), 76-88. doi: 10.1111/j.1365-2958.2008.06389.x

Priya, K., Yin, W.-F., & Chan, K.-G. (2013). Anti-quorum sensing activity of the traditional Chinese herb, *Phyllanthus amarus*. *Sensors, 13*(11), 14558-14569.

Qin, Y., Luo, Z. Q., Smyth, A. J., Gao, P., Beck von Bodman, S., & Farrand, S. K. (2000). Quorum-sensing signal binding results in dimerisation of TraR and its release from membranes into the cytoplasm. *EMBO J, 19*, 5212-5221.

Rangel, L. P., Fritzen, M., Yunes, R. A., Leal, P. C., Creczynski-Pasa, T. B., & Ferreira-Pereira, A. (2010). Inhibitory effects of gallic acid ester derivatives on *Saccharomyces cerevisiae* multidrug resistance protein Pdr5p. *FEMS yeast research, 10*(3), 244-251.

Rasmussen, T. B., Bjarnsholt, T., Skindersoe, M. E., Hentzer, M., Kristoffersen, P., Köte, M., . . . Givskov, M. (2005). Screening for quorum-sensing inhibitors (QSI) by use of a novel genetic system, the QSI selector. *J Bacteriol, 187*(5), 1799-1814.

Rasmussen, T. B., & Givskov, M. (2006). Quorum sensing inhibitors: a bargain of effects. *Microbiology, 152*(4), 895-904.

Reen, F. J., Almagro-Moreno, S., Ussery, D., & Boyd, E. F. (2006). The genomic code: inferring *Vibrionaceae* niche specialization. *Nat Rev Microbiol, 4*(9), 697-704. doi: 10.1038/nrmicro1476

Reiness, G., Yang, H.-L., Zubay, G., & Cashel, M. (1975). Effects of guanosine tetraphosphate on cell-free synthesis of *Escherichia coli* ribosomal RNA and other gene products. *Proc Natl Acad Sci, 72*(8), 2881-2885.

Ringeisen, B. R., Rincon, K., Fitzgerald, L. A., Fulmer, P. A., & Wu, P. K. (2015). Printing soil: a single-step, high-throughput method to isolate micro-organisms and near-neighbour microbial consortia from a complex environmental sample. *Methods Ecol Evol, 6*(2), 209-217.

Robson, E. H. J., Yin, W. F., & Chan, K. G. (2013). *Pandoraea* sp., RB-44, a novel quorum sensing soil bacterium. *Sensors, 13*, 14121-14132.

Roche, D. M., Byers, J. T., Smith, D. S., Glansdorp, F. G., Spring, D. R., & Welch, M. (2004). Communications blackout? Do *N*-acylhomoserine-lactone-degrading enzymes have any role in quorum sensing? *Microbiology, 150*(Pt 7), 2023-2028. doi: 10.1099/mic.0.26977-0

Romalde, J. L., Dieguez, A. L., Lasa, A., & Balboa, S. (2014). New *Vibrio* species associated to molluscan microbiota: a review. *Front Microbiol, 4*, 413. doi: 10.3389/fmicb.2013.00413

Rowe-Magnus, D. A., Guérout, A.-M., & Mazel, D. (1999). Super-integrons. *Res Microbiol, 150*(9), 641-651.

Rowe-Magnus, D. A., & Mazel, D. (2001). Integrons: natural tools for bacterial genome evolution. *Curr Opin Microbiol, 4*(5), 565-569.

Ruby, E. G., & Lee, K. H. (1998). The Vibrio fischeri-Euprymna scolopes light organ association: current ecological paradigms. *Applied and environmental microbiology, 64*(3), 805-812

Ruby, E. G. (1999). The *Euprymna scolopes-Vibrio fischeri* symbiosis: a biomedical model for the study of bacterial colonization of animal tissue. *J Mol Microbiol Biotechnol, 1*, 13-21.

Rutherford, S. T., & Bassler, B. L. (2012). Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold Spring Harbor Perspectives in Medicine, 2*(11), a012427.

Sambrook, J., & Russell, D. (2001). Molecular Cloning: A Laboratory Manual . 2001: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanger, F., & Coulson, A. R. (1975). A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *J Mol Bio, 94*(3), 441-448.

Sawitzke, J. A., Thomason, L. C., Bubunenko, M., Li, X.-t., Costantino, N., & Court, D. L. (2013). Recombineering: highly efficient in vivo genetic engineering using single-strand oligos. *Methods Enzymol, 533*, 157-177.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., ... & Griffin, P. M. (2011). Foodborne illness acquired in the United States—major pathogens. *Emerg Infect Dis*, *17*(1)

Schaefer, A. L., Hanzelka, B. L., Parsek, M. R., & Greenberg, E. P. (2000). Detection, purification, and structural elucidation of the acylhomoserine lactone inducer of Vibrio fischeri luminescence and other related molecules. *Methods Enzymol, 305*, 288-301.

Schaefer, A. L., Taylor, T. A., Beatty, J. T., & Greenberg, E. (2002). Long-chain acyl-homoserine lactone quorum-sensing regulation of *Rhodobacter capsulatus* gene transfer agent production. *J Bacteriol, 184*(23), 6515-6521.

Schuster, M., Lostroh, C. P., Ogi, T., & Greenberg, E. (2003). Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol*, *185*(7), 2066-2079.

Schuster, M., Lostroh, C. P., Ogi, T., & Greenberg, E. P. (2003). Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol, 5*, 1350-1369.

Shadel, G. S., & Baldwin, T. O. (1991). The *Vibrio fischeri* LuxR protein is capable of bidirectional stimulation of transcription and both positive and negative regulation of the *luxR* gene. *J Bacteriol, 173*, 568-574.

Shewan, J., & Veron, M. (1974). Genus I. Vibrio Pacini 1854, 411. *Bergey's Manual of Determinative Bacteriology, Eighth Edition, The Williams and Wilkins Co., Baltimore*, 340-345.

Sifri, C. D. (2008). Quorum sensing: bacteria talk sense. *Clin Infect Dis, 47*(8), 1070-1076.

Sio, C. F., Otten, L. G., Cool, R. H., Diggle, S. P., Braun, P. G., Bos, R., . . . Quax, W. J. (2006). Quorum quenching by an *N*-acyl-homoserine lactone acylase from *Pseudomonas aeruginosa* PAO1. *Infecti Immun*, *74*(3), 1673-1682.

Skandamis, P. N., & Nychas, G.-J. E. (2012). Quorum sensing in the context of food microbiology. *Appl Environ Microbiol, 78*(16), 5473-5482.

SKERMAN, V. B. D., McGowan, V., & Sneath, P. H. A. (1980). Approved lists of bacterial names. *Int J Syst Evo Microbiol, 30*(1), 225-420.

Solano, C., Echeverz, M., & Lasa, I. (2014). Biofilm dispersion and quorum sensing. *Curr Opin Microbiol, 18*, 96-104.

Steindler, L., & Venturi, V. (2007). Detection of quorum-sensing *N*-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiol Lett, 266*(1), 1-9.

Stevens, A. M., Fujita, N., Ishihama, A., & Greenberg, E. P. (1999). Involvement of the RNA polymerase α-subunit C-terminal domain in LuxR-dependent activation of the *Vibrio fischeri* luminescence genes. *J Bacteriol, 181*, 4704-4707.

Stoltz, D. A., Ozer, E. A., Ng, C. J., Janet, M. Y., Reddy, S. T., Lusis, A. J., . . . Shih, D. M. (2007). Paraoxonase-2 deficiency enhances *Pseudomonas aeruginosa* quorum sensing in murine tracheal epithelia. *American J Physiology-Lung Cell Mol Physiology, 292*(4), L852-L860.

Subasinghe, R., Bueno, P., Phillips, M., Hough, C., McGladdery, S., & Arthur, J. (2001). *Aquaculture in the Third Millennium. Technical proceedings.* Paper presented at the Conference on Aquaculture in the Third Millennium, Bangkok (Thailand), 20-25 Feb 2000.

Surette, M. G., Miller, M. B., & Bassler, B. L. (1999). Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsibe for autoinducer production. *Proc Natl Acad Sci USA, 96*, 1639-1644.

Swartzman, E., Kapoor, S., Graham, A. F., & Meighen, E. A. (1990). A new *Vibrio fischeri* lux gene precedes a bidirectional termination site for the lux operon. *J Bacteriol, 172*, 6797-6802.

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol Biol Evol, 10*(3), 512-526.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol*, mst197.

Taylor, R. G., Walker, D. C., & McInnes, R. R. (1993). *E. coli* host strains significantly affect the quality of small scale plasmid DNA preparations used for sequencing. *Nucleic Acids Res, 21*(7), 1677.

Temperton, B., Thomas, S., Tait, K., Parry, H., Emery, M., Allen, M., . . . Gilbert, J. (2011). Permanent draft genome sequence of *Vibrio tubiashii* strain NCIMB 1337 (ATCC19106). *Standards in genomic sciences, 4*(2), 183.

Teplitski, M., Mathesius, U., & Rumbaugh, K. P. (2010). Perception and degradation of *N*-acyl homoserine lactone quorum sensing signals by mammalian and plant cells. *Chem Rev, 111*(1), 100-116.

Umesha, S., & Shivakumar, J. (2013). Bcterial quorum sensing and its application in biotechnology. *Int J Pharma Bio Sci*, *4*, 850-861.

Uroz, S., Chhabra, S. R., Camara, M., Williams, P., Oger, P., & Dessaux, Y. (2005). *N*-Acylhomoserine lactone quorum-sensing molecules are modified and degraded by *Rhodococcus erythropolis* W2 by both amidolytic and novel oxidoreductase activities. *Microbiology, 151*(10), 3313-3322.

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

Vandeputte, O. M., Kiendrebeogo, M., Rajaonson, S., Diallo, B., Mol, A., El Jaziri, M., & Baucher, M. (2010). Identification of catechin as one of the flavonoids from *Combretum albiflorum* bark extract that reduces the production of quorum-sensing-controlled virulence factors in *Pseudomonas aeruginosa* PAO1. *Appl Environ Microbiol, 76*(1), 243-253.

Venturi, V. (2006). Regulation of quorum sensing in *Pseudomonas. FEMS Microbiol Rev, 30*(2), 274-291.

Verstraeten, N., Braeken, K., Debkumari, B., Fauvart, M., Fransaer, J., Vermant, J., & Michiels, J. (2008). Living on a surface: swarming and biofilm formation. *Trends Microbiol, 16*(10), 496-506. doi: 10.1016/j.tim.2008.07.004

Vikram, A., Jayaprakasha, G., Jesudhasan, P., Pillai, S., & Patil, B. (2010). Suppression of bacterial cell–cell signalling, biofilm formation and type III secretion system by citrus flavonoids. *J Appl Microbiol, 109*(2), 515-527.

Vikram, A., Jesudhasan, P. R., Jayaprakasha, G., Pillai, S. D., & Patil, B. S. (2011). Citrus limonoids interfere with *Vibrio harveyi* cell–cell signalling and biofilm formation by modulating the response regulator LuxO. *Microbiology, 157*(1), 99-110.

Vollberg, C. M., & Herrera, J. L. (1997). *Vibrio vulnificus* infection: an important cause of septicemia in patients with cirrhosis. *Southern Med J, 90*(10), 1040-1042.

Wang, C., Cheng, H., Guan, Y., Wang, Y., & Yun, Y. (2009). In vitro activity of gallic acid against Candida albicans biofilms. *Zhongguo Zhong yao za zhi= Zhongguo zhongyao zazhi= China journal of Chinese materia medica*, *34*(9), 1137-1140.

Wang, Z., Gerstein, M., & Snyder, M. (2009). RNA-Seq: a revolutionary tool for transcriptomics. *Nat Rev Gen, 10*(1), 57-63.

Welch, M., Todd, D. E., Whitehead, N. A., McGowan, S. J., Bycroft, B. W., & Salmond, G. P. C. (2000). *N*-Acyl homoserine lactone binding to the CarR receptor determines quorum-sensing specificity in Erwinia. *EMBO J, 19*, 631-641.

Whitehead, N. A., Barnard, A. M. L., Slater, H., Simpson, N. J. L., & Salmond, G. P. C. (2001). Quorum sensing in Gram-negative bacteria. *FEMS Microbiol Rev, 25*, 365-404.

Williams, K. P., Gillespie, J. J., Sobral, B. W., Nordberg, E. K., Snyder, E. E., Shallom, J. M., & Dickerman, A. W. (2010). Phylogeny of gammaproteobacteria. *J Bacteriol, 192*(9), 2305-2314.

Winson, M. K., Swift, S., Fish, L., Throup, J. P., Jørgensen, F., Chhabra, S. R., . . . Stewart, G. S. (1998). Construction and analysis of luxCDABE-based plasmid sensors for investigating *N*-acyl homoserine lactone-mediated quorum sensing. *FEMS Microbiol Lett, 163*(2), 185-192.

Winzer, K., Falconer, C., Nachman, G. C., Stephen, D. P., Camara, M., & Williams, P. (2000). The *Pseudomonas aeruginosa* lectins PA-IL and PA-IIL controlled by quorum sensing and by RpoS. *J Bacteriol, 182*(22), 6401-6411.

Woese, C. R., Kandler, O., & Wheelis, M. L. (1990). Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. *Proc Natl Acad Sci, 87*(12), 4576-4579.

Wolf, M. K., & Crosa, J. H. (1986). Evidence for the role of a siderophore in promoting *Vibrio anguillarum* infections. *Microbiology, 132*(10), 2949-2952.

Wu, H., Song, Z., Hentzer, M., Andersen, J. B., Molin, S., Givskov, M., & Høiby, N. (2004). Synthetic furanones inhibit quorum-sensing and enhance bacterial clearance in *Pseudomonas aeruginosa* lung infection in mice. *J Antimicrob Chemo, 53*(6), 1054-1061.

Yildiz, F. H., & Visick, K. L. (2009). *Vibrio* biofilms: so much the same yet so different. *Trends Microbiol, 17*(3), 109-118.

Zenno, S., & Saigo, K. (1994). Identification of the genes encoding the NAD(P)H-flavin oxidoreductase that are siimilar in sequence to *Escherichia coli* fre in four species of luminous bacteria: *Photorhabdus luminescens, Vibrio fischeri, Vibrio harveyi*, and *Vibrio orientalis*. *J Bacteriol, 176*, 3544-3551.

Zhu, J., & Winans, S. C. (2001). The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance, and dimerisation. *Proc Natl Acad Sci USA, 98*, 1507-1512.

Zide, N., Davis, J., & Ehrenkranz, N. J. (1974). Fulminating *Vibrio parahemolyticus* septicemia: A syndrome of erythemia multiforme, hemolytic anemia, and hypotension. *Arch Internal Med, 133*(3), 479-481.

Zielke, R., Sikora, A., Dutkiewicz, R., Wegrzyn, G., & Czyż, A. (2003). Involvement of the *cgtA* gene function in stimulation of DNA repair in *Escherichia coli* and *Vibrio harveyi*. *Microbiology, 149*(7), 1763-1770.

SUPPLEMENTARY

LIST OF PUBLICATIONS AND PAPER PRESENTED

A : List of Publications

Mohamad, N. I., Adrian, T.-G.-S., Tan, W.-S., Muhamad Yunos, N. Y., Tan, P.-W., Yin, W.-F., & Chan, K.-G. (2016). Vibrio variabilis T01: A tropical marine bacterium exhibiting unique N-acyl homoserine lactone production. *Frontiers in Life Science, 9*(1), 17-23.

Mohamad, N. I., Yin, W.-F., & Chan, K.-G. (2015). Whole-genome sequence of quorum-sensing Vibrio tubiashii strain T33. *Genome announcements, 3*(1), e01362-01314.

Tan, P.-W., Tan, W.-S., Yunos, N. Y. M., **Mohamad, N. I.**, Adrian, T.-G.-S., Yin, W.-F., & Chan, K.-G. (2014). Short chain N-acyl homoserine lactone production in tropical marine Vibrio sinaloensis strain T47. *Sensors, 14*(7), 12958-12967.

Tan, W.-S., Yunos, N. Y. M., Tan, P.-W., **Mohamad, N. I.**, Adrian, T.-G.-S., Yin, W.-F., & Chan, K.-G. (2014). Characterisation of a marine bacterium Vibrio brasiliensis T33 producing N-acyl homoserine lactone quorum sensing molecules. *Sensors, 14*(7), 12104-12113.

B : Paper Presentation

Mohamad, N. I., Tan, W. S., Yunos, N. Y. M., Tan, P. W., Adrian, T. G. S., Yin, W. F., & Chan, K. G. (2014, June). Bacterial dialogue: Deeper approach, Poster presented at Monash Science Symposium – Monash University (International)

Frontiers in Life Science

ISSN: 2155-3769 (Print) 2155-3777 (Online) Journal homepage: http://www.tandfonline.com/loi/tfls20

Vibrio variabilis T01: A tropical marine bacterium exhibiting unique N-acyl homoserine lactone production

Nur Izzati Mohamad, Tan-Guan-Sheng Adrian, Wen-Si Tan, Nina Yusrina Muhamad Yunos, Pui-Wan Tan, Wai-Fong Yin & Kok-Gan Chan

To cite this article: Nur Izzati Mohamad, Tan-Guan-Sheng Adrian, Wen-Si Tan, Nina Yusrina Muhamad Yunos, Pui-Wan Tan, Wai-Fong Yin & Kok-Gan Chan (2016) Vibrio variabilis T01: A tropical marine bacterium exhibiting unique N-acyl homoserine lactone production, Frontiers in Life Science, 9:1, 17-23, DOI: 10.1080/21553769.2015.1066716

To link to this article: http://dx.doi.org/10.1080/21553769.2015.1066716

Frontiers in Life Science, 2016
Vol. 9, No. 1, 17–23, http://dx.doi.org/10.1080/21553769.2015.1066716

Taylor & Francis
Taylor & Francis Group

Vibrio variabilis T01: A tropical marine bacterium exhibiting unique *N*-acyl homoserine lactone production

Nur Izzati Mohamad, Tan-Guan-Sheng Adrian, Wen-Si Tan, Nina Yusrina Muhamad Yunos, Pui-Wan Tan, Wai-Fong Yin and Kok-Gan Chan*

Division of Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

(*Received 17 September 2014; accepted 24 June 2015*)

Vibrio spp. have been widely studied for their unique properties such as pathogenicity and quorum sensing (QS) abilities. This article presents the identification of *Vibrio variabilis* strain T01 isolated from Malaysian coastal waters. Strain T01 of *V. variabilis* was identified to produce QS molecules as tested using a biosensor. High-resolution tandem mass spectrometry was used to identify *N*-acyl homoserine lactone (AHL) profiles of strain T01. Three AHLs, *viz. N*-hexanoyl-L-homoserine lactone (C6-HSL), *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL) and *N*-dodecanoyl-L-homoserine lactone (C12-HSL), were confirmed. To the authors' knowledge, this is the first documentation of AHL profiles from *V. variabilis* strain T01, which expands the number of QS members in *Vibrio* spp.

Keywords: mass spectrometry; *Vibrio variabilis*; quorum sensing; *N*-hexanoyl-L-homoserine lactone (C6-HSL); *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL); *N*-dodecanoyl-L-homoserine lactone (C12-HSL)

Introduction

In the marine environment, many types of microorganisms inhabit a complex environment, creating a unique microbial ecology. Members of the Vibrionaceae family such as *Vibrio*, *Grimontia* and *Photobacterium* thrive in the marine environment. *Vibrio*, a curved Gram-negative bacterium, can be found in almost all aquatic environments such as the sea, freshwater and estuaries, which include both shallow and deep water ecological environments (Reen et al. 2006). Although *Vibrio* spp. are often associated with pathogenic effect in fishes, shrimps and humans, such cases are rare (Gomez-Gil et al. 2008). The pathogenic strains of *Vibrio* studied so far are mostly opportunistic in character, causing disease only when the health of the host is compromised (Lightner & Redman 1998). According to Weil et al. (2006), *Vibrio* spp. occupy a significant portion of both healthy and ailing coral varieties. In corals, *Vibrio* spp. assist in nitrogen fixation (Shashar et al. 1994), chitin decomposition (Ducklow & Mitchell 1979), food production (Chimetto et al. 2008) and antimicrobial production (Ritchie 2006). Another example is *Vibrio fischeri*, which shares a commensal relationship with squids and jellyfish by providing luminescence when its cell density reaches a threshold level, thus regulating sets of autoinducers which activate the transcription of *lux* genes (Kaplan & Greenberg 1985).

On the other hand, the negative impact of elevated *Vibrio* spp. in the aquatic environment has been widely debated. For example, increased numbers of *Vibrio* in coral mucus indicate an unhealthy environment (Dinsdale et al. 2008). As another example, *Vibrio anguillarum*, found in the kidney, liver and muscle of rainbow trout in the Danish sea, causes fatal vibriosis (Buch et al. 2003). Furthermore, a recent study found that *Vibrio* spp. also have devastating effects on molluscan species (Romalde et al. 2013).

The focus of the present study is on *Vibrio variabilis*, which was first identified by Chimetto et al. (2011). Isolated from zoanthids, *V. variabilis* was discovered by Chimetto and colleagues as a new novel species by applying DNA–DNA hybridization (DDH) and multilocus sequence analysis (MLSA). In accordance with the species name, *variabilis*, the bacterium changes colour from light beige to black if cultured under limited light conditions. Since this particular bacterium was isolated from a healthy zoanthid (*Palythoa caribaeorum*), it is assumed to be non-pathogenic (Chimetto et al. 2011).

Quorum sensing (QS) refers to bacterial cell-to-cell communication, which uses signalling molecules termed autoinducers to interact by sensing bacterial cell density to regulate the target genes (González & Marketon 2003). Gram-negative bacteria mostly use *N*-acyl homoserine lactone (AHL) as the autoinducer; this diffuses and binds

*Corresponding author. Email: kokgan@um.edu.my

to its cognate receptor proteins and triggers a cascade or reaction that induces the expression of particular sets of genes (Fuqua et al. 2001). Even though the AHL signals produced by bacteria are unique to each species, the mode of AHL biosynthesis and gene regulation appears to be evolutionarily conserved and typically involves *luxI* and *luxR* (Dong et al. 2002). For instance, *V. harveyi* uses three parallel QS systems that regulate biofilm and protease production, type III secretion (TTS) and bioluminescence expression (Waters & Bassler 2006). The autoinducer synthase/regulator system can be seen in all three QS systems in *V. harveyi*. The first system involves LuxM/LuxN (HAI-1), the second system consists of LuxS/LuxPQ (AI-2) and the third system CqsA/CqsS (CAI-1) (Henke & Bassler 2004). In this study, the QS properties of *V. variabilis,* a marine isolate, are confirmed and its putative AHL synthase is identified.

Experimental section

Seawater sampling and isolation of bacteria

The seawater sample was taken at Morib, Banting, Selangor, Malaysia (GPS coordinates of the sample site: 101° 26′ 34.7″ E, 2° 45′ 2.7″ N) in 2014. A water sample was taken using a sterile plastic tube at a depth of 15 cm below the water surface. The sample was transferred on ice and immediately processed at the laboratory. The sample underwent serial dilution and was then spread on Luria–Bertani agar (LBA) with NaCl concentration ranging from 1% to 3%. The culture medium that optimally supported bacterial growth was selected as the main culture medium and the cultures were isolated.

Bioassay of N-acyl homoserine lactones

Isolate T01 was cultured at 37°C on LBA (3% w/v NaCl). For detection of bacteria that exhibited AHL production, *Chromobacterium violaceum* CV026, which shows purple pigmentation in the presence of short-chain AHLs, was used (McClean et al. 1997). *Erwinia carotovora* GS101 (positive control for AHL detection) and *E. carotovora* PNP22 (negative control) were included as controls for AHL bioassay. These bacteria were cultured on LBA and incubated at 28°C.

Extraction of N-acyl homoserine lactones

For AHL extraction, Luria–Bertani (LB) broth was used, supplemented with 50 mM of 3-(*N*-morpholino)propanesulfonic acid (MOPS) to avoid the alkaline hydrolysis of the AHL by adjusting the pH to 5.5. Pure colonies of isolate T01 were inoculated into the MOPS-LB broth and incubated with shaking using a shaking incubator at 28°C for 18 h. Using an equal amount of acidified (0.1% v/v glacial acetic acid) ethyl acetate, the spent supernatant of the cultures was extracted twice, as described previously (Chong et al. 2012). The supernatant was dried completely in the fume hood and resuspended with 200 µl acetonitrile (ACN) followed by centrifugation at 24,000 × *g* for 5 min. Next, supernatant (75 µl) was inserted into a vial together with 25 µl of ACN ready for liquid chromatography–mass spectrometry (LC/MS) analysis.

Molecular identification of bacterial strain

Using the MasterPure™ DNA Purification Kit (Epicentre, Madison, WI, USA), the genomic DNA of T01 was extracted and used as a polymerase chain reaction (PCR) template. PCR mix (Promega Kit, Madison, WI, USA) was used to amplify the 16S rRNA gene of isolate T01 utilizing the 1525R reverse primer (5′-AAGGAGG TGWTCCARCC-3′), 515F forward primer (5′-GTGCCAG CMGCCGCGGTAA-3′) and 27F forward primer (5′-AGAGTTTGATCMTGGCTCAG-3′). The parameters of the PCR amplification were as reported previously (Chan et al. 2009) and 16S rRNA gene nucleotides were compared to the highest percentage of similarities using the Ez-taxon database (Kim et al. 2012). Phylogenetic analysis was conducted using MEGA 6.0 software utilizing the maximum likelihood method (Tamura et al. 2013) based on the Tamura–Nei model (Tamura & Nei 1993).

N-Acyl homoserine lactone profiling via high-resolution tandem liquid chromatography–quadrupole mass spectrometry

The extracted AHLs dissolved in ACN were injected into the LC delivery system. The mobile phases were water and ACN at an 80:20 ratio, all solvents used were of LC/MS grade , and 0.1% v/v formic acid was added to each mobile phase. A C18 column (1.8 µm particle size, 2 µl injection volume, 0.21 cm × 0.50 cm) was used for the chromatography. Other operating parameters, such as the ion source, capillary voltage, sheath gas, desolvation temperature and nebulizer pressure, were set as reported before (Wong et al. 2012; Chen et al. 2013; Robson et al. 2013). The precursor ion scan mode was activated (*m/z* 150–400), scanning the acyl chain moiety $[M+H]^+$ and the lactone ring moiety with an *m/z* value of 102.

DNA extraction, library arrangement, sequencing and assembly

Vibrio variabilis T01 genomic DNA was extracted using the MasterPure DNA Purification Kit. After prepping the sample, the Illumina MiSeq personal sequencer (Illumina, CA, USA) was used to perform whole-genome shotgun sequencing. The sequenced genome was assembled using CLC Genomics Workbench 5.1 (CLC Bio, Aarhus, Denmark).

Gene prediction and annotation

Prodigal 2.60 was used for gene prediction and an online server database, Rapid Annotation using Subsystem Technology (RAST) (Aziz et al. 2008), was used for annotation.

Results and discussion

Sampling and screening bacteria with N-acyl homoserine lactone production

The seawater samplewas taken at a beach in Morib, Selangor, which is a commercial recreation site. The pH of the seawater was 8.0 and the temperature was 27°C. Among the isolates tested, T01 was selected as it activated the AHL biosensor *C. violaceum* CV026. The purple pigmentation of *C. violaceum* CV026 showed that short-chain AHLs were produced by strain T01 (McClean et al. 1997) (Figure 1). Hence, isolate T01 was selected for further analysis.

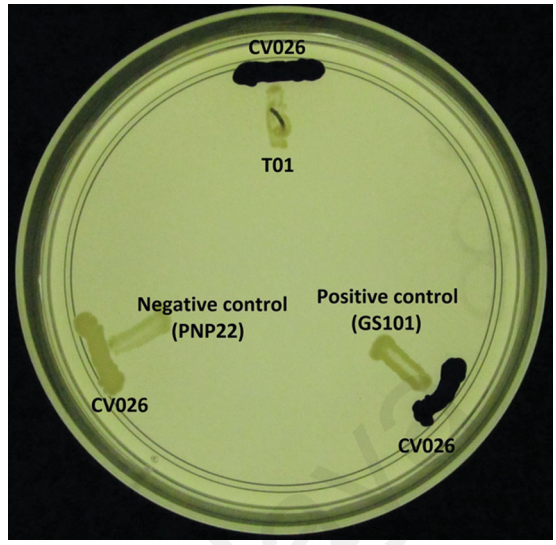


Figure 1. Bioassay of *N*-acyl homoserine lactone (AHL) molecules screening by isolate T01. The bioassay was conducted using the AHL biosensor *Chromobacteriumviolaceum* CV026. Positive control = *Erwinia carotovora* GS101; negative control = *E. carotovora* PNP22; T01 = isolate T01. The dark purple pigmentation shows production of quorum sensing molecules.

Molecular identification of bacterial strain

To identify isolate T01, its 16S rRNA gene was amplified using PCR and the resulting sequence was aligned against the database from Ez-Taxon (Kim et al. 2012). The 16S rRNA gene sequence of isolate T01 shares 98.77% similarity with that of *V. variabilis* R-40492^T isolated from soft corals (zoanthids) in São Paolo, Brazil (Chimetto et al. 2011). Using the MEGA 6.0 software (Tamura et al. 2013), the 16S rRNA gene sequence of strain T01 was aligned against *V. variabilis* R-40492^T (Figure 2). A phylogenetic tree was constructed and it was perceived that strain T01 clustered within the *Vibrio* genus (Figure 2) by comparing the ingroup of closely related (95–98%) *Vibrio* species with *Cycloclasticus* sp., set as the outgroup. The phylogenetic tree was built using the maximum likelihood method (Tamura et al. 2013) with the Tamura–Nei model (Tamura & Nei 1993). The 16S rRNA gene sequence of strain T01 V. *variabilis* was deposited into GenBank with an accession number KP329555. The version described in this article is the first version of KP329555.

High-resolution tandem liquid chromatography–quadrupole mass spectrometry to identify N-acyl homoserine lactone profile of Vibrio variabilis T01

The Agilent 6490 triple quadrupole LC/MS system was used to detect the homoserine lactone ring moiety fragmented in the collision cell, thus identifying the AHL profiles of strain T01. Three different types of AHL molecule were detected in the supernatant of T01. As shown in Figure 3(a–c), the LC/MS data showed the detection of *m/z* values for *N*-hexanoyl-L-homoserine lactone (C6-HSL) ($m/z = 200.500$), *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL) ($m/z = 270.300$) and *N*-dodecanoyl-L-homoserine lactone (C12-HSL) ($m/z = 284.500$). Based on the chromatograms

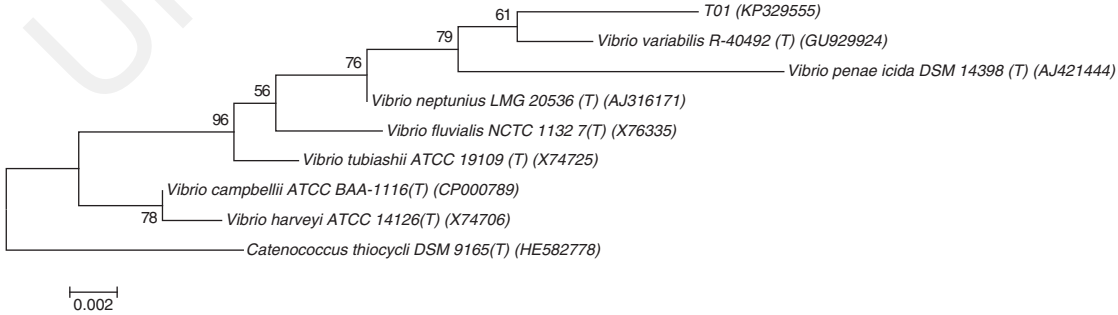


Figure 2. Phylogenetic tree highlighting the position of *Vibrio variabilis* T01 relative to the type strains of other species within the genus *Vibrio*. The strains and their corresponding GenBank accession numbers of 16S rRNA genes are indicated in parentheses. The sequences were aligned using MEGA 6.0 and the phylogenetic inferences were obtained using the maximum likelihood method based on the Tamura–Nei model (Tamura & Nei 1993). The numbers at nodes are the percentage of bootstrap values obtained by 500 replicates and there was a total of 1209 positions in the final dataset. *Catenococcus thiocycli* (HE582778) was used as the outgroup.

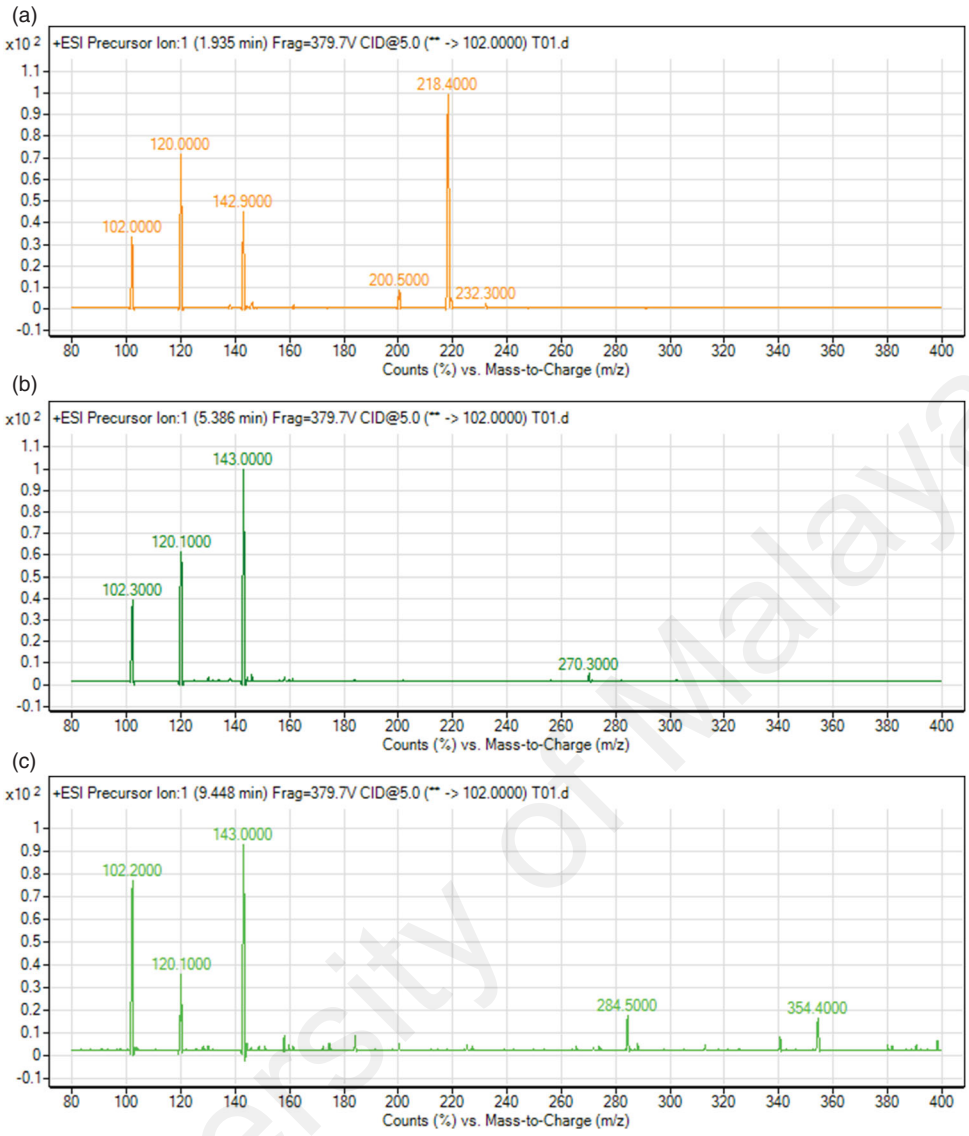


Figure 3. Detection of T01 *N*-acyl homoserine lactone (AHL) profiles: (a) *N*-hexanoyl-L-homoserine lactone (C6-HSL) ($m/z = 200.500$), (b) *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL) ($m/z = 270.300$) and (c) *N*-dodecanoyl-L-homoserine lactone (C12-HSL) ($m/z = 284.500$). Respective *m/z* values (boxed) and the molecular structure of respective AHLs are shown.

obtained, *V. variabilis* strain T01 produced 3-oxo-C10-HSL most abundantly, followed by C6-HSL and then C12-HSL. Both short- and long-chain AHLs are produced by isolate T01. To the authors' knowledge, this is the first report of *V. variabilis* producing three different AHLs.

Since three different AHLs are produced by *V. variabilis* T01, many assumptions can be made. The QS system was first discovered in the Gram-negative bacterium *V. fischeri* (Fuqua et al. 1994), which consists of two AHL-dependent QS systems, LuxI/LuxR and AinS/AinR. The AinS/AinR system activates the expression of luminescence genes at lower cell density compared to the LuxI/LuxR system (Lupp et al. 2003; Lupp & Ruby 2005). Another example of a complex QS system can be observed in *V. harveyi*, which employs four different systems involving LuxM/LuxN, LuxS/LuxPQ, CqsA/CqsS and H-NOX/HqsK pathways (Ng & Bassler 2009; Henares et al. 2013). A similar QS system to *V. harveyi* can also be seen in *V. cholerae*, a notable pathogenic bacterium responsible for cholera. The *V. cholerae* QS system comprises LuxS/LuxPQ and CqsA/CqsS (Ng & Bassler 2009). The complex QS systems in the different bacteria synthesize different autoinducers: HAI-1, AI-2 and CAI-1 are synthesized by LuxM, LuxS and CqsA, respectively (Ng & Bassler 2009). Although LuxM is not a homologue of LuxI described in the *V. fischeri* QS system, it is analogous to another AHL synthase, AinS (Gilson et al. 1995). The QS system in *V. variabilis* is still unclear and more studies need to be carried out.

In the LC/MS analysis, *N*-dodecanoyl-L-homoserine lactone (C12-HSL) was also detected. There has been no previous report of the production of C12-HSL in *Vibrio*

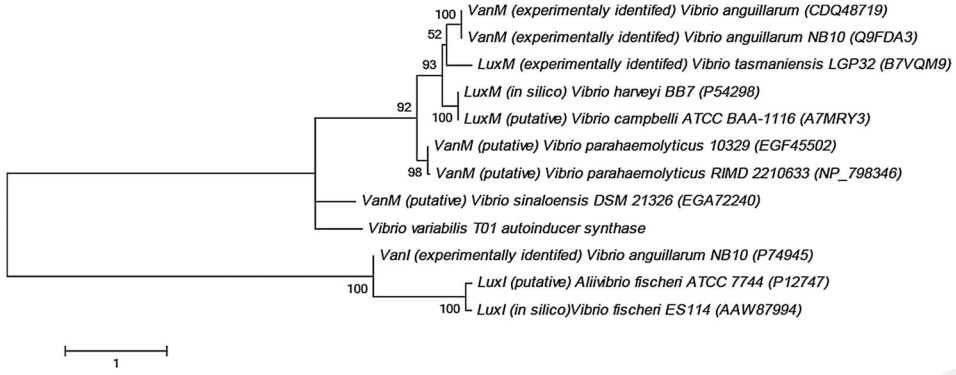


Figure 4. Evolutionary history phylogenetic tree inferred using the maximum likelihood method based on the JTT matrix-based model (Jones et al. 1992). The sequences and their corresponding reference sequence are indicated in parentheses. The reference sequences used to construct the phylogenetic tree were from putative, *in silico* and experimentally identified homoserine lactone synthase sequences. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved four amino acid sequences. All positions containing gaps and missing data were eliminated. There was a total of 187 positions in the final dataset. Evolutionary analyses were conducted in MEGA 6.0.

species. According to recent research, long-chain AHLs are assumed to influence gene transfer, thus enhancing the genetic exchange (Schaefer et al. 2002; Chang et al. 2012). These new findings will act as a platform to understand the communication of *V. variabilis* with its surrounding organisms, and more research should be conducted at the molecular level.

Genomic annotation and gene of interest in Vibrio variabilis T01

There is a total of 83 contigs in the 4,529,728 bp of the sequenced genome of T01. There is 46.22% G + C content in the bases. Protein coding genes make up 97.73% of the genome, with 87.29% being functional protein coding genes.

After the annotation process, the gene responsible for the QS properties were fished out from the contigs and located in contig 16 (1203 bp). The nucleotide sequence of the QS gene in isolate T01 was translated into amino acid sequences using MEGA 6.0 software (Tamura et al. 2013) and compared to the BLASTx database. The translated nucleotide of the QS gene in strain T01 made up about 400 amino acid length, which is the average length for most QS genes reported so far. The translated amino acid sequence shares 99.0% similarity with the amino acid sequence of putative synthase VanM of *Vibrio* sp. 16 (NCBI reference sequence WP_005476971.1). A phylogenetic tree was constructed using MEGA 6.0 (Tamura et al. 2013) with the maximum likelihood method based on the JTT matrix model (Jones et al. 1992) (Figure 4).

The phylogenetic tree built using the fished out autoinducer synthase-encoding gene indicates that its product is closely related to the synthase VanM, which is a homologue of the synthase LuxM in *V. harveyi*. The autoinducer *N*-(3-hydroxybutanoyl)homoserine lactone is produced by LuxM, while its homologue VanM synthesizes *N*-hexanoylhomoserine lactone (C6-HSL) and *N*-(3-hydroxyhexanoyl)homoserine lactone (3-hydroxy-C6-HSL) (Milton 2006). These autoinducer molecules then bind to the receptor known as VanN (Milton et al. 2001). The tree was built using both putative and experimentally identified homoserine lactone synthase. Mutant studies on both *V. anguillarum* (NB10) (Milton et al. 1997, 2001) and *V. tasmaniensis* (LGP32) (Le Roux et al. 2007) provided the experimentally identified amino acid sequences of LuxM, VanM and VanI. Other amino acid sequences of homoserine lactone synthase were identified using *in silico* studies (Bassler et al. 1993; Ruby et al. 2005), while *V. sinaloensis* (DSM 21326) VanM, *V. parahaemolyticus* (10329, RIMD 2210633) VanM and *V. campbelli* (ATCC BAA-1116) LuxM are putative sequences.

From the phylogenetic tree, it can be observed that the autoinducer synthase in T01 is grouped into the VanM/LuxM categories. An example of the VanM/VanN QS system is found in *V. anguillarum*, which produces 3-hydroxy-C6-HSL and C6-HSL and 3-oxo-C6-HSL (Milton et al. 2001). *Vibrio anguillarum* also produces another autoinducer, *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10-HSL), synthesized by another QS system which is the VanI, a LuxI homologue found in *V. fischeri* (Milton et al. 1997). VanM null mutant in *V. anguillarum* failed to produce all of the autoinducers, indicating that the QS system may work in hierarchical cascades (Milton et al. 2001). Since both C6-HSL and 3-oxo-C10-HSL were also found in *V. variabilis* T01, it is possible that there is more than one QS system regulated in T01.

Conclusions and outlook

In this study, the bacterium *V. variabilis* T01 was isolated from tropical marine water and shown to possess a unique

AHL profile, as confirmed by high-resolution mass spectrometry. Three AHLs, namely 3-oxo-C10 HSL, C6-HSL and C12-HSL, in descending abundance, were detected in the spent supernatant of *V. variabilis* T01. The putative AHL synthase of this isolate was identified by analysing its genome sequence. Hence, further studies could be conducted to identify the role of the AHLs in this isolate. To the authors' knowledge, this is the first report on the QS activities of *V. variabilis* T01.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the University of Malaya through high impact research grants [UM-MOHE HIR grant UM.C/625/1/HIR/MOHE/CHAN/14/1, no. H-50001-A000027; UM-MOHE HIR grant UM.C/625/1/HIR/MOHE/CHAN/01, no. A000001-50001] awarded to Kok-Gan Chan.

References

Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O. 2008. The RAST server: rapid annotations using subsystems technology. BMC Genomics. 9:1–15.

Bassler BL, Wright M, Showalter RE, Silverman MR. 1993. Intercellular signaling in *Vibrio harveyi*: a sequence function of genes regulating expression of luminescence. Mol Microbiol. 9:773–786.

Buch C, Sigh J, Nielsen J, Larsen JL, Gram L. 2003. Production of acylated homoserine lactones by different serotypes of *Vibrio anguillarum* both in culture and during infection of rainbow trout. Syst Appl Microbiol. 26:338–349.

Chan KG, Yin WF, Sam CK, Koh CL. 2009. A novel medium for the isolation of *N*-acylhomoserine-lactone-degrading bacteria. J Ind Microbiol Biotechnol. 36:247–251.

Chang CY, Koh CL, Sam CK, Chan XY, Yin WF, Chan KG. 2012. Unusual long-chain *N*-acyl homoserine lactone production by and presence of quorum quenching activity in bacterial isolates from diseased Tilapia fish. PLoS One. 7:1–8.

Chen JW, Koh CL, Sam CK, Yin WF, Chan KG. 2013. Short chain *N*-acyl homoserine lactone production by soil isolate *Burkholderia* sp. strain A9. Sensors. 13:13217–13227.

Chimetto LA, Brocchi M, Thompson CC, Martins RCR, Ramos HR, Thompson FL. 2008. *Vibrios* dominate as culturable nitrogen-fixing bacteria of the Brazilian coral *Mussismilia hispida*. Syst Appl Microbiol. 31:312–319.

Chimetto LA, Cleenwerck I, Moreira APB, Brocchi M, Willems A, De Vos P, Thompson FL. 2011. *Vibrio variabilis* sp. nov. and *Vibrio maritimus* sp. nov., isolated from *Palythoa caribaeorum*. Int J Syst Evol Microbiol. 61:3009–3015.

Chong TM, Koh CL, Sam CK, Choo YM, Yin WF, Chan KG. 2012. Characterization of quorum sensing and quorum quenching soil bacteria isolated from Malaysian tropical montane forest. Sensors. 12:4846–4859.

Dinsdale EA, Pantos O, Smriga S, Edwards RA, Angly F, Wegley L, Hatay M, Hall D. 2008. Microbial ecology of four coral atolls in the northern line islands. PLoS One. 3:e1584–0.

Dong YH, Gusti AR, Zhang Q, Xu JL, Zhang LH. 2002. Identification of quorum quenching *N*-acyl homoserine lactonases from *Bacillus* species. Appl Environ Microbiol. 68:1754–1759.

Ducklow HW, Mitchell R. 1979. Composition of mucus released by coral reef coelenterates. Limnol Oceanogr. 24:706–714.

Fuqua C, Parsek MR, Greenberg EP. 2001. Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. Annu Rev Genet. 35:439–468.

Fuqua WC, Winans SC, Greenberg EP. 1994. Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. J Bacteriol. 176:269–275.

Gilson L, Kuo A, Dunlap PV. 1995. AinS and a new family of autoinducer synthesis proteins. J Bacteriol. 177:6946–6951.

Gomez-Gil B, Fajer-Avilla E, Pascual J, Macián MC, Pujalte MJ, Garay E, Roque A. 2008. *Vibrio sinaloensis* sp. nov., isolated from the spotted rose snapper, *Lutjanus guttatus* Steindachner, 1869. Int J Syst Evol Microbiol. 58: 1621–1624.

González JE, Marketon MM. 2003. Quorum sensing in nitrogen fixing rhizobia. Microbiol Mol Biol Rev. 67:574–592.

Henares BM, Xu Y, Boon EM. 2013. A nitric oxide-responsive quorum sensing circuit in *Vibrio harveyi* regulates flagella production and biofilm production. Int J Mol Sci. 14: 16473–16484.

Henke JM, Bassler BL. 2004. Three parallel quorum-sensing systems regulate gene expression in *Vibrio harveyi*. J Bacteriol. 186:6902–6914.

Jones DT, Taylor WR, Thornton JM. 1992. The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci. 8:275–282.

Kaplan HB, Greenberg EP. 1985. Diffusion of autoinducer is involved in regulation of the *Vibrio fischeri* luminescence system. J Bacteriol. 163:1210–1214.

Kim OS, Cho YJ, Lee K, Yoon SH, Kim M, Na H, Park SC, Jeon YS, Lee JH, Yi H, Won S, Chun J. 2012. Introducing EzTaxon-e: a prokaryotic 16S rRNA gene sequence database with phylotypes that represent uncultured species. Int J Syst Evol Microbiol. 62:716–721.

Le Roux F, Binesse J, Saulnier D, Mazel D. 2007. Construction of a *Vibrio splendidus* mutant lacking the metalloprotease gene vsm by use of a novel counterselectable suicide vector. Appl Environ Microbiol. 73:777–784.

Lightner DV, Redman RM. 1998. Shrimp diseases and current diagnostic methods. Aquaculture. 164:201–220.

Lupp C, Ruby EG. 2005. *Vibrio fischeri* uses two quorum-sensing for the regulation of early and late colonization factors. J Bacteriol. 187:3620–3629.

Lupp C, Urbanowski M, Greenberg EP, Ruby EG. 2003. The *Vibrio fischeri* quorum-sensing systems ain and lux sequentially induce luminescence gene expression and are important for persistence in the squid host. Mol Microbiol. 50:319–331.

McClean KH, Winson MK, Fish L, Taylor A, Chhabra SR, Cámara M, Daykin M, Lamb JH, Swift S, Brcroft BW, Stewart GS, 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.

Milton DL. 2006. Quorum sensing in vibrios: complexity for diversification. Int J Med Microbiol. 296:61–71.

Milton DL, Chalker VJ, Kirke D, Hardman A, Cámara M, 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. 183:3237–3247.

Milton DL, Hardman A, Cámara M, Chhabra SR, Bycroft BW, Stewart GSAB, 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.

Ng WL, Bassler BL. 2009. Bacterial quorum-sensing network architectures. Annu Rev Genet. 43:197–222.

Reen FJ, Almagro-Moreno S, Ussery D, Boyd EF. 2006. The genomic code inferring Vibrionaceae niche specialization. Nat Rev Microbiol. 4:697–704.

Ritchie KB. 2006. Regulation of microbial populations by coral surface mucus and mucus-associated bacteria. Mar Ecol Prog Ser. 322:1–14.

Robson EHJ, Yin WF, Chan KG. 2013. *Pandoraea* sp. RB-44, A novel quorum sensing soil bacterium. Sensors. 13: 14121–14132.

Romalde JL, Diéguez AL, Lasa A, Balboa S. 2013. New *Vibrio* species associated to molluscan microbiota: a review. Front Microbiol. 4:1–11.

Ruby EG, Urbanowski M, Campbell J, Dunn A, Faini M, Gunsalus R, Lostroh P, McCann J, Millikan D, Schaefer A, Stabb E, Stevens A, Viscik K, Whistler C, Greenberg EP. 2005. Complete genome sequence of *Vibrio fischeri*: a symbiotic bacterium with pathogenic congeners. Proc Natl Acad Sci USA. 102:3004–3009.

Schaefer AL, Taylor TA, Beatty JT, Greenberg EP. 2002. Long-chain homoserine lactone quorum-sensing regulation of *Rhodobacter capsulatus* gene transfer agent production. J Bacteriol. 184:6515–6521.

Shashar N, Cohen Y, Loya Y, Sar N. 1994. Nitrogen fixation (acetylene reduction) in stony corals: evidence for coral-bacteria interactions. Mar Ecol Prog Ser. 111:259–264.

Tamura K, Nei M. 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol. 10:512–526.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 30:2725–2729.

Waters CM, Bassler BL. 2006. The *Vibrio harveyi* quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. Genes Dev. 20:2754–2767.

Weil E, Smith G, Gil-Agudelo DL. 2006. Status and progress in coral reef disease research. Dis Aquat Organ. 69:1–7.

Wong CS, Yin WF, Choo YM, Sam CK, Koh CL, Chan KG. 2012. Coexistence of quorum-quenching and quorum-sensing in tropical marine *Pseudomonas aeruginosa* strain MW3A. World J Microbiol Biotechnol. 28:453–461.

genomeA
Journals.ASM.org

Whole-Genome Sequence of Quorum-Sensing *Vibrio tubiashii* Strain T33

Nur Izzati Mohamad, Wai-Fong Yin, Kok-Gan Chan

Division of Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia

***Vibrio tubiashii* strain T33 was isolated from the coastal waters of Morib, Malaysia, and was shown to possess quorum-sensing activity similar to that of its famous relative *Vibrio fischeri*. Here, the assembly and annotation of its genome are presented.**

Received 18 November 2014 **Accepted** 20 November 2014 **Published** 2 January 2015

Citation Izzati Mohamad N, Yin W-F, Chan K-G. 2015. Whole-genome sequence of quorum-sensing *Vibrio tubiashii* strain T33. Genome Announc. 3(1):e01362-14. doi:10.1128/genomeA.01362-14.

Address correspondence to Kok-Gan Chan, kokgan@um.edu.my.

In 1965, Tubiash, Chanley, and Leifson (1) first discovered pathogenic bacteria that infect the mollusk, and these were classified as *Vibrio anguillarum*. However, due to the advancement of molecular taxonomical approaches, further work done in 1984 by Hada and colleagues (2) confirmed that the bacterium isolated in 1965 by Tubiash, Chanley, and Leifson is a new species, which was named *Vibrio tubiashii*. Isolated in 2014, *V. tubiashii* strain T33 forms a round yellow colony on Luria-Bertani agar (LBA) with 3% (wt/vol) NaCl concentration. Here, we sequenced the whole genome of *V. tubiashii* strain T33, as this will contribute to the understanding of its pathogenic properties and pave the way to solutions to combat severe vibriosis in mollusks, such as clams, oysters, and shellfish.

By using the QIAamp DNA minikit (Qiagen, Germany), the genomic DNA of *V. tubiashii* strain T33 was isolated according to the manufacturer's recommendations. DNA quality was checked via a NanoDrop spectrophotometer (Thermo Scientific) and Qubit 2.0 fluorometer (Life Technologies). Using the platform Illumina MiSeq personal sequencer (Illumina, Inc., CA), the whole genome of *V. tubiashii* strain T33 was sequenced. The number of calculated filtered reads was 1,308,193, with approximately 60.05-fold coverage. Assembly of the filtered reads was done using CLC Genomics Workbench version 5.1 (CLC bio, Denmark) (3) and resulted in 46 contig numbers, with an N_{50} value of 410,629. A total of 4,144,653 bp makes up the draft genome of *V. tubiashii* strain T33, with a G+C content of 45%. Annotation was done using the Rapid Annotations using Subsystems Technology (RAST) server (4). The number of open reading frames (ORFs) of strain T33 is 3,815, and the number of tRNAs predicted using tRNAscan-SE (version 1.21) (5) is 76. There are 5 main housekeeping genes in strain T33, which consist of three copies of 5S rRNA genes, one copy of 23S rRNA gene, and one copy of 16S rRNA gene, which was characterized using RNAmmer (6).

From the RAST server annotation, we discovered a protein that was responsible for the *N*-acyl homoserine lactone (AHL), LuxM, in contig number 8. AHL is a quorum-sensing (QS) signaling molecule that has been reported in many proteobacteria, including marine vibrios (7, 8). LuxM is a homologue to LuxI, which is a protein responsible for the production of signal molecules, namely, AHLs (9, 10). The QS signaling system, which consists of genes and regulators, may play a key role in the pathogenic properties of *V. tubiashii*. Therefore, this study may provide insight to better understand the virulence factors of *V. tubiashii*, hence leading to an effective solution for severe vibriosis.

Nucleotide sequence accession number. This whole-genome shotgun project has been deposited at DDBJ/EMBL/GenBank under the accession no. JRWQ00000000. The version described in this paper is the first version.

ACKNOWLEDGMENT

This work was supported by a High Impact Research Grant (A000001-50001; awarded to K.-G. Chan) from the University of Malaya.

REFERENCES

1. **Tubiash HS, Chanley PE, Leifson E.** 1965. Bacillary necrosis, a disease or larval and juvenile bivalve mollusks. I. Etiology and epizootiology. J Bacteriol **90:**1036–1044.
2. **Hada HS, West PA, Lee JV, Stemmler J, Colwell RR.** 1984. *Vibrio tubiashii* sp. nov., a pathogen of bivalve mollusks. Int J Syst Bacteriol **34:**1–4. http://dx.doi.org/10.1099/00207713-34-1-1.
3. **Chan XY, Chua KH, Puthucheary SD, Yin WF, Chan KG.** 2012. Draft genome sequence of an *Aeromonas* sp. strain 159 clinical isolate that shows quorum-sensing activity. J Bacteriol **194:**6350. http://dx.doi.org/10.1128/JB.01642-12.
4. **Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O.** 2008. The RAST server: Rapid Annotations using Subsystems Technology. BMC Genomics **9:**75. http://dx.doi.org/10.1186/1471-2164-9-75.
5. **Lowe TM, Eddy SR.** 1997. tRNAscan-SE: a program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res **25:**955–964. http://dx.doi.org/10.1093/nar/25.5.0955.
6. **Lagesen K, Hallin P, Rødland EA, Staerfeldt HH, Rognes T, Ussery DW.** 2007. RNAmmer: consistent and rapid annotation of ribosomal RNA genes. Nucleic Acids Res **35:**3100–3108. http://dx.doi.org/10.1093/nar/gkm160.
7. **Tan WS, Yunos NYM, Tan PW, Mohamad NI, Adrian TGS, Yin WF, Chan KG.** 2014. Characterisation of a marine bacterium *Vibrio brasilien-*

sis T33 producing *N*-acyl homoserine lactone quorum sensing molecules. Sensors **14:**12104–12113. http://dx.doi.org/10.3390/s140712104.
8. **Cámara M, Hardman A, Williams P, Milton D.** 2002. Quorum sensing in *Vibrio cholerae*. Nat Genet **32:**217–218. http://dx.doi.org/10.1038/ng1002-217.
9. **Engebrecht J, Nealson K, Silverman M.** 1983. Bacterial bioluminescence: isolation and genetic analysis of functions from *Vibrio fischeri*. Cell **32:**773–781. http://dx.doi.org/10.1016/0092-8674(83)90063-6.
10. **Hong KW, Koh CL, Sam CK, Yin WF, Chan KG.** 2012. Whole-genome sequence of *N*-acylhomoserine lactone-synthesizing and-degrading *Acinetobacter* sp. strain GG2. J Bacteriol **194:**6318. http://dx.doi.org/10.1128/JB.01579-12.

Sensors **2014**, *14*, 12104-12113; doi:10.3390/s140712104

OPEN ACCESS

sensors

ISSN 1424-8220

www.mdpi.com/journal/sensors

Article

Characterisation of a Marine Bacterium *Vibrio Brasiliensis* T33 Producing *N*-acyl Homoserine Lactone Quorum Sensing Molecules

Wen-Si Tan, Nina Yusrina Muhamad Yunos, Pui-Wan Tan, Nur Izzati Mohamad, Tan-Guan-Sheng Adrian, Wai-Fong Yin and Kok-Gan Chan *

Division of Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur 50603, Malaysia; E-Mails: tmarilyn36@gmail.com (W.-S.T); ninayusrina@hotmail.com (N.Y.M.Y.); acelinetan38@yahoo.com (P.-W.T.); zetty_mohamad@yahoo.com (N.I.M.); adrian_tan_1991@yahoo.com (T.-G.-S.A); yinwaifong@yahoo.com (W.-F.Y.)

* Author to whom correspondence should be addressed; E-Mail: kokgan@um.edu.my; Tel.: +603-7967-5162; Fax: +603-7967-4509.

Received: 30 March 2014; in revised form: 26 June 2014 / Accepted: 3 July 2014 / Published: 8 July 2014

Abstract: *N*-acylhomoserine lactones (AHL) plays roles as signal molecules in quorum sensing (QS) in most Gram-negative bacteria. QS regulates various physiological activities in relation with population density and concentration of signal molecules. With the aim of isolating marine water-borne bacteria that possess QS properties, we report here the preliminary screening of marine bacteria for AHL production using *Chromobacterium violaceum* CV026 as the AHL biosensor. Strain T33 was isolated based on preliminary AHL screening and further identified by using 16S rDNA sequence analysis as a member of the genus *Vibrio* closely related to *Vibrio brasiliensis*. The isolated *Vibrio* sp. strain T33 was confirmed to produce *N*-hexanoyl-L-homoserine lactone (C6-HSL) and *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10 HSL) through high resolution tandem mass spectrometry analysis. We demonstrated that this isolate formed biofilms which could be inhibited by catechin. To the best of our knowledge, this is the first report that documents the production of these AHLs by *Vibrio brasiliensis* strain T33.

Keywords: 16S rDNA; biosensor; *Chromobacterium violaceum* CV026; marine water-borne; *N*-acyl homoserine lactone (AHL); quorum sensing (QS); *N*-hexanoyl-L-homoserine lactone (C6-HSL); *N*-(3-oxo-decanoyl)-L-homoserine lactone (3-oxo-C10 HSL); *Vibrio brasiliensis*

1. Introduction

The discovery of luminescence in the marine bacterium *Vibrio fischeri* in the late 1970s has paved the way for research into the mechanisms of regulation of bacterial physiological activities by cell-cell communication [1]. It was later shown that the luminescence was initiated not by inhibitor removal, but rather by accumulation of activator molecules, simply known as "autoinducers". The bacteria are able to sense the cell density population by tracking the concentration of signal molecules. This phenomenon is named "quorum sensing (QS)" by Fuqua and co-workers [2,3]. In QS, the concentration of signal molecules plays a vital role that reflects the bacterial population density, when at quorate, specific target genes are activated [4] and a collective behavioral adaptation will occur [5].

There are three documented archetypal QS systems: the *N*-acyl homoserine lactone (AHL)-based signaling system of Gram-negative bacteria, an oligopeptide-based system in Gram-positive bacteria and a furanone-based system that is shared between both [6,7]. The AHL-based QS system has garnered significant interest because of its frequent role in microbial virulence mechanisms. QS is considered to be important as disruption of bacterial communication could be a strategy for developing potential antimicrobial therapeutic targets [3,8]. The cell density sensing apparatus utilized by the AHL-based QS system in most Gram-negative bacteria is the LuxI-LuxR system [9] where "LuxR" is a transcriptional activator protein [10] that functions by binding to a cognate signal molecule produced by "LuxI" autoinducer synthase which will result in specific target gene regulation [11]. The QS system is much more appreciated in the microbial world due to its capability in regulating different physiological activities such as competence, motility, development, antibiotic synthesis, virulence factor induction and cell differentiation [12,13].

To date, different marine bacteria have been studied and their QS properties portrayed and majority of these marine bacteria belongs to the genus *Vibrio* [14]. Several *Vibrio* species can be pathogenic, causes food-borne infections usually related with the consumption of undercooked seafood [15]. Most disease-causing strains are associated with gastroenteritis and it also can infect open wounds and cause septicemia [15]. For example, in the so-called Kanagawa phenomenon, hemolytic *V. parahaemolyticus* strains was isolated from human hosts on blood agar. *Vibrio* is considered as opportunistic pathogen where *V. vulnificus* typically infects the bloodstream and could cause life-threatening illnesses in persons with liver disease or immune-compromising conditions [16]. The pathogenic effect is believed to influenced by regulation of virulence determinants throughout the infection process and QS provide a means for these pathogenic bacteria to make a concerted attack and produce ample virulence factors to overwhelm the host defenses [13]. Research on QS pathogens could be significant in controlling disease besides helping understand their mechanism of pathogenicity. Therefore, this raised significant interest in our group to extend our research on isolating marine bacteria that possess QS properties. In

this study, we investigated the presence of QS bacteria in a Malaysian tropical marine water sample. Here, we report the isolation of a *Vibrio* sp. QS bacterium, and its unique AHL production which has not been reported before.

2. Experimental Section

2.1. Water Sample Collection and Bacterial Strain Isolation

The sampling site chosen for this study was Morib Beach, Malaysia, with GPS coordinates of N 02°45.023' E 101°26.623'. The water sample was collected along the beach coastal area in 2013. The sample was collected at a depth of 20 cm below the water surface and kept in a sterilized plastic bottle that was transferred at 4°C to the laboratory for analysis [17]. A serial dilution of water sample was carried out with sterile saline [18] and it was then spread onto Luria Bertani (LB) agar medium (in grams per 1 L: tryptone, 10; yeast extract, 5; NaCl, 30; Bacto agar, 15). Next, the plates were incubated overnight (24 h) at 28 °C. The observable colonies formed with different morphologies were isolated and pure colonies obtained by repeated streaking on LB medium.

2.2. Bacteria Strains, Culture Conditions and Biosensors Assay

The strain T33 was selected for further work after pure colonies were obtained and it was routinely cultured on the LB medium. The bacterial biosensor chosen for the preliminary screening of AHL in this study was *Chromobacterium violaceum* CV026, which responds by induction of purple violacein pigmentation [19]. The positive and negative controls for the screening were *Erwinia carotovora* GS101 and *E. carotovora* PNP22, respectively. *C. violaceum* CV026, *E. carotovora* GS101 and *E. carotovora* PNP22 were routinely cultured in LB medium. Isolate T33 was screened for AHL production by cross streaking the bacteria cultures with *C. violaceum* CV026 on LB agar plates and incubating overnight (24 h) at 28 °C. After incubation, the observed purple violacein pigmentation indicated the production of AHL by strain T33.

2.3. Bacteria Strain Identification

Amplification of bacterial 16S rDNA genes by polymerase chain reaction (PCR) was carried out to identify the bacteria strain according to a published method [20]. Genomic DNA was extracted using MasterPureTM DNA Purification Kit (Epicentre Inc., Madison, WI, USA). The PCR amplification and purification processes were conducted as described previously [20]. PCR product sequence alignment was done using GenBank BLASTN program, followed by phylogenetic analysis using the Molecular Evolutionary Genetic analysis version 6.0 [21,22].

2.4. AHLs Extraction

Strain T33 was cultured in LB broth buffered with 50 mM of 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 5.5) in an incubator shaker (200 rpm, 28 °C, 18 h). The incubated culture was extracted twice with an equal volume of acidified (0.1% v/v glacial acetic acid) ethyl acetate as previously described [23]. The organic layer was air-dried completely and the extract was resuspended

with 1 mL of acidified ethyl acetate and desiccated completely followed by addition of 200 µL of acetonitrile (HPLC grade) and vortexed to dissolve the dried extracts completely.

2.5. AHL Profiling by Mass Spectrometry (MS)

An Agilent RRLC 1200 system (Agilent Technologies, Agilent Inc., California, CA, USA) was utilized as the liquid chromatography (LC) delivery system with the use of an Agilent ZORBAX Rapid Resolution HT column (2.1 mm × 100 mm, 1.8 µm of particle size) for separation of AHL molecules and an Agilent 6500 Q-TOF LC/MS system was used for MS analysis. The mobile phases, injection volume, parameters for the MS analysis, ESI-positive mode, precursor ion scan mode targeting at the production ion with *m/z* 102, *m/z* value range (*m/z* 150–400) and Agilent MassHunter software for MS spectra analysis were performed essentially as reported [24].

2.6. Biofilm Assay

The biofilm assay was performed as described previously [25,26]. The overnight culture of strain T33 was diluted with LB medium and adjusted to OD_{600} of 0.1. Next, 50 µL of the diluted culture was added to 930 µL of LB medium supplemented with 1, 2, and 3 mg/mL of catechin, an anti-QS compound [25], in a microtitre plate. The T33 cultures were treated with and without DMSO (solvent) and served as negative and positive controls, respectively and were incubated statically for 72 h at 28 °C. The planktonic bacteria were removed by washing three times with sterile distilled water [27] and the plate was air-dried for 15 min and was stained with 0.1% (w/v) crystal violet (200 µL per well) for 30 min. Excess crystal violet was removed and the wells were washed with sterile distilled water twice followed by addition of 95% (v/v) ethanol (200 µL) and 100 µL of the resulting solution was transferred to a new, sterile microtitre plate. The absorbance of the solution was read at OD_{590} with microplate reader. All experiments were repeated twice.

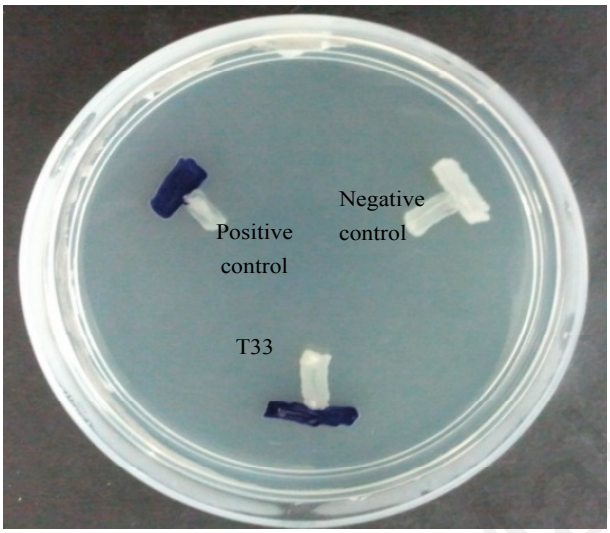
3. Results and Discussion

3.1. Strains Isolation and Preliminary Screening of AHL

The aim of this study was to isolate the AHL-producing bacteria from a Malaysian tropical marine water sample. The sampling spot for this study was Morib Beach, a recreational attraction area. The water sample collected at the sampling spot had a temperature of 27 °C and the pH was 8.0. The marine waster sample was collected near the coastal line to determine the presence of bacteria.

The availability of AHL biosensors increases the capability of researchers to discover samples of QS bacteria present [3,28]. A AHL biosensor such as *C. violaceum* CV026 is a mutant with defective LuxI AHL synthase, that practically depends on the LuxR protein in displaying specificity binding towards the cognate AHL that is able to activate the transcription of the reporter gene [19,29,30]. The biosensor *C. violaceum* CV026, responds to AHLs with C4 to C8 acyl chain length that will induce a purple violacein pigmentation [19]. It is the preferable biosensor for AHL preliminary screening due to the speed and accuracy in AHL detection, hence, we employed *C. violaceum* CV026 for the preliminary screening of AHLs produced by strain T33 (Figure 1).

Figure 1. AHL screening of strain T33 with *C. violaceum* CV026. *E. carotovora* PNP22 (negative control) devoid of QS activity was included and *E. carotovora* GS101 (positive control) that can activate CV026 was included for comparison.

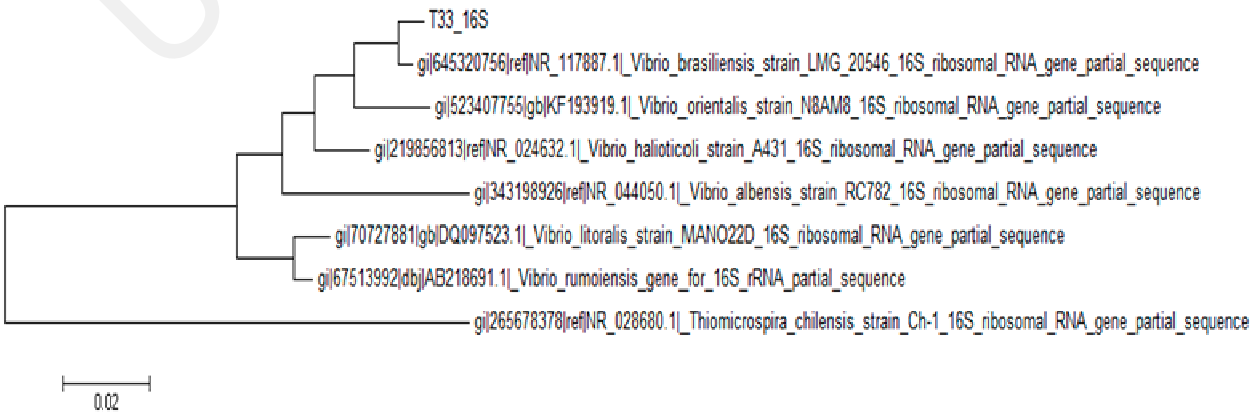


The preliminary screening of strain T33 showed a positive result whereby *C. violaceum* CV026 produced a purple violacein pigmentation. This further indicates that the isolated strain T33 produces short chain AHLs. This strain was then subjected to molecular identification and AHL profiling by mass spectrometry.

3.2. Molecular Identification of Bacterial Strain

The identity of the isolate T33 was confirmed by analysis of its 16S rDNA gene nucleotides sequences showing that it clusters closely to the *Vibrio* genus where the strain shared 99% similarity in the BLAST search.

Figure 2. Phylogenetics analysis of strain T33. The tree is drawn to scale, and branch lengths represents number of base substitutions per site. There were a total of 1244 unambiguous nucleotides used for analysis using MEGA6.



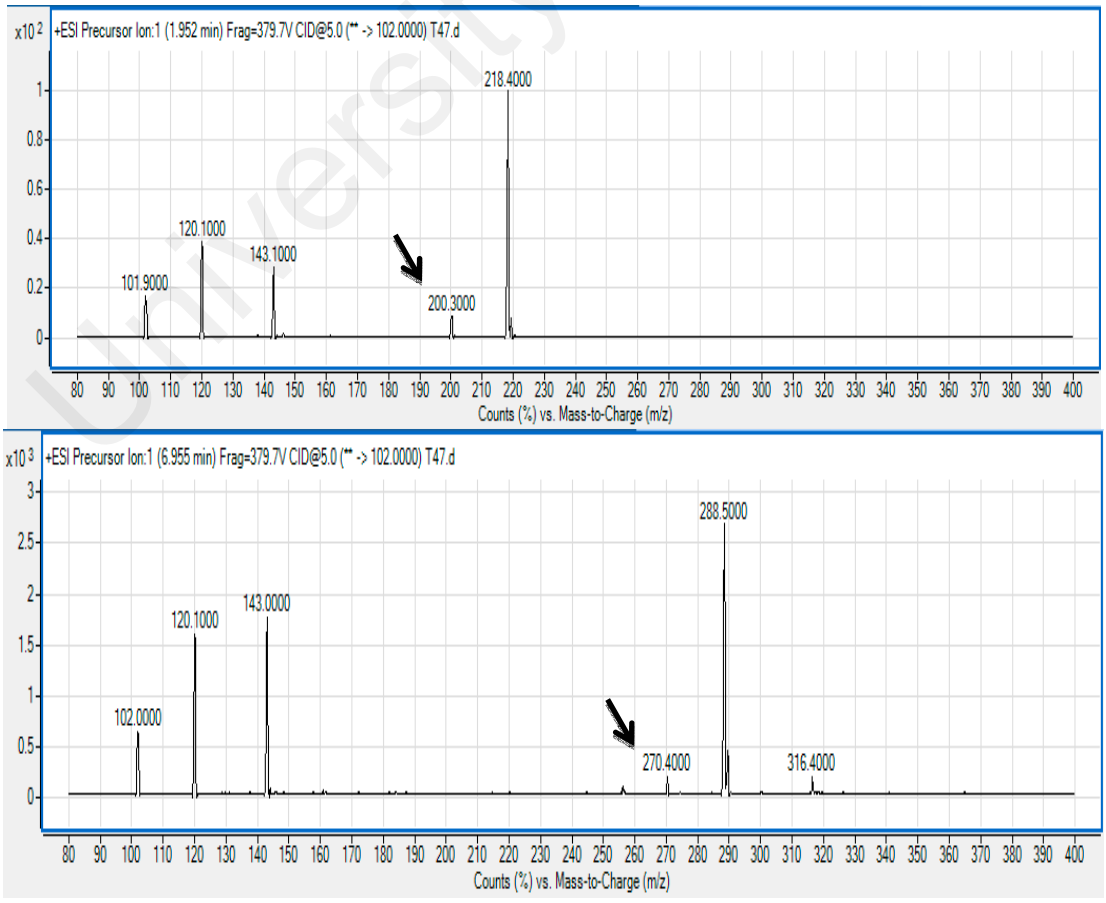
According to the phylogenetic tree constructed (Figure 2), strain T33 was identified as *Vibrio brasiliensis*, a marine bacterium. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model by using Neighbor-Join and BioNJ algorithms.

3.3. Identification of AHL Production

The *Vibrio* genus is a causative agent of different food-borne diseases and in many countries it is a major foodborne pathogen, especially when improperly handled seafood is consumed and mortality related to *Vibrio* sp. has been reported [14]. However, the pathogenicity of *Vibrio brasiliensis* has so far not been documented. Our work has shown for the first time that this bacterium actually exhibits a QS mechanism that regulates certain physiological activities of *V. brasiliensis*.

The spent culture supernatant of *V. brasiliensis* T33 strain was analyzed using the Agilent 6500 Q-TOF LC/MS system and mass spectrometry analysis. The presence of *N*-hexanoyl-L-homoserine lactone (C6-HSL) (*m/z* 200.3000; retention time 1.952 min) and *N*-(3-oxodecanoyl)-L-homoserine lactone (3-oxo-C10 HSL) (*m/z* 200.3000; retention time 6.955 min) was identified (Figure 3) and confirmed by comparing the retention times of the AHLs produced by T33 with the standard retention times as mentioned previously [24].

Figure 3. Mass spectrometry analysis of spent supernatants extract of *V. brasiliensis* strain T33. Upper Panel: mass spectrum of C6-HSL (*m/z* 200.3000; retention time 1.952 min) (marked by arrow); Lower Panel: mass spectrum of 3-oxo-C10 HSL (*m/z* 270.4000; retention time 6.955 min) (marked by arrow).



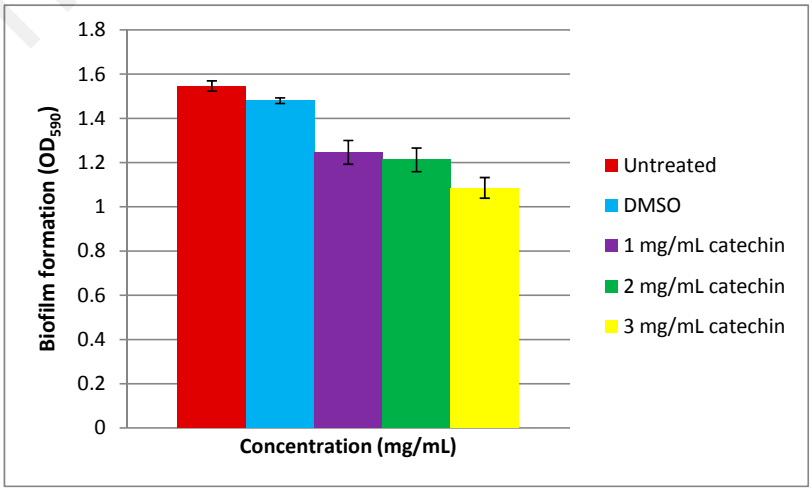
The precursor ion scan mode targeting at the production ion with *m/z* 102 indicates the presence of the core lactone ring moiety [31,32]. To the best of our knowledge, this is the first documentation on AHL profiling of *V. brasiliensis* where it produces these AHL molecules. This result paves the way for research towards a deeper approach into studying the mechanism on QS of *V. brasiliensis* such as characterising the *luxI* and *luxR* homologues of this isolate. We are currently conducting whole genome sequencing on *Vibrio* sp. strain T33 aiming to isolate the AHL synthase and receptor genes that will provide more insight into the QS regulatory system in this bacterium.

A battery of physiological activities such as biofilm formation, virulence factors and motility can be regulated by QS [33] and hence this work provided evidence to illustrate the significance of the research on AHL-producing bacteria present in environmental samples such as marine waters. Isolation of QS bacteria from marine water may indicate that it could be a potential reservoir for QS bacteria and more intense research should be conducted to address this issue.

3.4. Biofilm Formation of Vibrio sp. Strain T33

Biofilm formation is often a QS-regulated phenotype [26,27] that is a multiple-stage process involving initial colonization, attachment to a surface, maturation and occasionally dispersion. The ability of bacteria to form biofilms is often linked to pathogenicity. It has been well-documented that members of *Vibrio* form biofilms [34,35]. In this work, *Vibrio* sp. strain T33 has been shown to form biofilms too (Figure 4). Under our experimental conditions, catechin reduced the biofilm formation of *Vibrio* sp. strain T33 in a dose-dependent manner. In our study, we used the well-known anti-QS compound namely catechin [36] which effectively inhibited the biofilm formation by *Vibrio* sp. strain T33. Since QS is a regulatory system for the expression of myriad virulence factors [15], this work illustrated formation of biofilm in our *Vibrio* isolate is regulated by QS. This work also suggests that marine seawater may be a potential reservoir for QS pathogens that should be given appropriate attention as it might be a possible threat to the aquaculture industry.

Figure 4. Biofilm formation in *Vibrio* sp. strain T33. Qualitative analyses of biofilm formation and inhibition by catechin. Bars represent standard errors of the mean.



4. Conclusions

We have reported the unique AHL profile of *V. brasiliensis* strain T33 isolated from a marine water sample. Two AHLS, namely C6-HSL and 3-oxo-C10 HSL, were extracted and identified from the spent culture supernatant. To the best of our knowledge, this is the first documentation of the fact *Vibrio brasiliensis* produces these two AHLs.

Acknowledgments

This published work was supported by the University of Malaya. Kok-Gan Chan thanks the High Impact Research Grant (UM.C/625/1/HIR/MOHE/CHAN/01, Grant No. A-000001-50001) for the research funding provided.

Author Contributions

W.S.T, T.G.S.A, N.Y.M.Y., W.F.Y., and N.I.M. planed and conducted the experiments related to this study and discussed the obtained results. P.W.T. contributed in analyzed data and experimental test. W.F.Y. coordinated and contributed in research equipment purchasing. All authors wrote the paper together and K.G.C. did the final proofreading. K.G.C. conceived the idea of this work, obtained the funding, and managing the entire project.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Nealson, K.H.; Platt, T.; Hastings, J.W. Cellular control of the synthesis and activity of the bacterial luminescence system. *J. Bacteriol.* **1970**, *104*, 313–322.
2. Fuqua, C.; Greenberg, E.P. Listening in on bacteria: Acyl-homoserine lactone signaling. *Nat. Rev.* **2002**, *3*, 685–695.
3. Williams, P.; Winzer, K.; Chan, W.C.; Camara, M. Look who's talking: Communication and quorum sensing in the bacterial world. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2007**, *362*, 1119–1134.
4. Chen, G.; Malenkos, J.W.; Cha, M.R.; Fuqua, C.; Chen, L. Quorum-sensing antiactivator TraM forms a dimer that associates to inhibit TraR. *Mol. Microbiol.* **2004**, *52*, 1641–1651.
5. Fuqua, W.C.; Winans, S.C.; Greenberg, E.P. Quorum sensing in bacteria—The LuxR-LuxI family of cell density-responsive transcriptional regulators. *J. Bacteriol.* **1994**, *176*, 269–275.
6. Roux, A.; Payne, S.M.; Gilmore, M.S. Microbiol telesensing: Probing the environment for friends, foes, and food. *Cell Host Microbe* **2009**, *6*, 115–124.
7. Yin, W.F.; Purmal, K.; Chin, S.; Chan, X.Y.; Koh, C.L.; Sam, C.K.; Chan, K.G. *N*-acyl homoserine lactone production by *Klebsiella pneumonia* isolated from human tongue surface. *Sensors* **2012**, *12*, 3472–3483.

8. Parsek, M.R.; Greenberg, E.P. Acyl-homoserine lactone quorum sensing in Gram-negative bacteria mechanism involved in association with higher organisms. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 8789–8793.
9. Chong, T.M.; Koh, C.L.; Sam, C.K.; Choo, Y.M.; Yin, W.F.; Chan, K.G. Characterization of quorum sensing and quorum quenching soil bacteria isolated from Malaysian tropical montane forest. *Sensors* **2012**, *12*, 4846–4859.
10. Williams, P. Quorum sensing, communication and cross-kingdom signaling in the bacterial world. *Microbiology* **2007**, *153*, 3923–3938.
11. Bassler, B.L. Small talk: Cell-to-cell communication in bacteria. *Cell* **2002**, *109*, 421–424.
12. Wong, C.S.; Yin, W.F.; Choo, Y.M.; Sam, C.K.; Koh, C.L.; Chan, K.G. Coexistence of quorum-quenching and quorum-sensing in tropical marine *Pseudomonas aeruginosa* strain MW3A. *World J. Microbiol. Biotechnol.* **2012**, *28*, 453–461.
13. Verschuere, L.; Rombaut, G.; Sorgeloos, P.; Verstraete, W. Probiotic bacteria as biological control agents in aquaculture. *Microbiol. Mol. Rev.* **2000**, *64*, 655–671.
14. Thompson, F.L.; Li, Y.; Gomez-Gil, B.; Thompson, C.C.; Hoste, B.; Vandemeulebroecke, K.; Rupp, G.S.; Pereira, A.; de Bern, M.M.; Sorgeloos, P.; *et al*. *Vibrio neptunius* sp. nov., *Vibrio brasiliensis* sp. nov. and *Vibrio xuii* sp. nov., isolated from the marine aquaculture environment (bivalves, fish, rotifers and shrimps). *Int. J. Syst. Evol. Microbiol.* **2003**, *53*, 245–252.
15. Thompson, F.L.; Gevers, D.; Thompson, C.C.; Dawyndt, P.; Naser, S.; Hoste, B.l Munn, C.B.; Swings, J. Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. *Appl. Environ. Microbiol.* **2005**, *71*, 5107–5115.
16. Joseph, S.; Colwell. R.; Kaper, J. *Vibrio parahaemolyticus* and related halophilic *Vibrios*. *Crit. Rev. Microbiol.* **1982**, *10*, 77–124.
17. Wintzingerode, F.V.; Gobel, U.B.; Stackebrandt, E. Determination of microbial diversity in environmental samples: Pitfalls of PCR-based rRNA analysis. *FEMS Microbiol. Rev.* **1997**, *21*, 213–229.
18. Bahgwat, A.A. Rapid detection of *Salmonella* from vegetable rinse-water using real-time PCR. *Food Microbiol.* **2004**, *21*, 73–78.
19. McClean, K.H.; Winson, M.K.; Fish, L.; Taylor, A.; Chhabra, S.R.; Camara, M.; Daykin, M.; Lamb, J.H.; Swift, S.; Brcroft, B.W.; *et al*. Quorum sensing and *Chromobacterium violaceum*: Exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* **1997**, *143*, 3703–3711.
20. Chan, K.G.; Tiew, S.Z.; Ng, C.C. Rapid isolation method of soil bacilli and screening of their quorum quenching activity. *Asia Pac. J. Mol. Biol. Biotechnol.* **2007**, *15*, 153–156.
21. Tamura, K.; Nei, M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Bio. Evol.* **1993**, *10*, 512–526.
22. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **2011**, *28*, 2731–2739.

23. Ortori, C.A.; Dubern, J.F.; Chhabra, S.R.; Camara, M.; Hardie, K.; Williams, P.; Barrett, D.A. Simultaneous quantitative profiling of *N*-acyl-L-homoserine lactone and 2-alkyl-4(1H)-quilonone families of quorum-sensing signaling molecules using LC-MS/MS. *Anal. Bioanal. Chem.* **2011**, *399*, 839–850.
24. Robson, E.H.J.; Yin, W.F.; Chan, K.G. *Pandoraea* sp. RB-44, a novel quorum sensing soil bacterium. *Sensors* **2013**, *13*, 14121–14132.
25. Vandeputte, O.M.; Kiendrebeogo, M.; Rajaonson, S.; Diallo, B.; Mol. A.; El. Jaziri, M.; Baucher, M. Identification of catechin as one of the flavonoids from *Combretum albiflorum* bark extract that reduces the production of quorum-sensing-controlled virulence factors in *Pseudomonas aeruginosa* PAO1. *Appl. Environ. Microbiol.* **2010**, *76*, 243–253.
26. Tan, W.S.; Muhamad, Y.N.Y.; Tan, P.W.; Mohamad, N.I.; Adrian, T.G.S.; Yin, W.F.; Chan, K.G. Freshwater-borne bacteria isolated from a Malaysian rainforest waterfall exhibiting quorum sensing properties. *Sensors* **2014**, *14*, 10527–10537.
27. Lade, H.; Paul, D.; Kweon, J.H. Isolation and molecular characterization of biofouling bacteria and profiling of quorum sensing signal molecules from membrane bioreactor activated sludge. *Int. J. Mol. Sci.* **2014**, *15*, 2255–2273.
28. Steidler, L.; Venturi, V. Detection of quorum sensing *N*-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiol. Lett.* **2007**, *266*, 1–9.
29. De Kievit, T.R.; Iglewski, B.H. Bacterial quorum sensing in pathogenic relationships. *Infect. Immun.* **2000**, *68*, 4839–4849.
30. Frank, J.A.; Reich, C.I.; Sharma, S.; Weisbaum, J.S.; Wilson, B.A.; Olsen, G.J. Critical Evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Appl. Environ. Microbiol.* **2008**, *74*, 2461–2470.
31. Yong, Y.C.; Zhong, J.J. *N*-acylated homoserine lactone production and involvement in the biodegradation of aromatics by an environmental isolate of *Pseudomonas aeruginosa*. *Process Biochem.* **2010**, *45*, 1944–1948.
32. Yong, Y.C.; Zhong, J.J. Regulation of aromatics biodegradation by *rhl* quorum sensing system through induction of catechol meta-cleavage pathway. *Bioresour. Technol.* **2013**, *136*, 761–765.
33. Tan, L.Y.; Yin, W.F.; Chan, K.G. Silencing quorum sensing through extracts of *Melicope lunu-ankenda*. *Sensors* **2012**, *12*, 4339–4351.
34. Chong, Y.M.; Yin, W.F.; Ho, C.Y.; Mustafa, M.R.; Hadi, A.H.A.; Awang, K.; Narrima, P.; Koh, C.L.; Appleton, D.R.; Chan, K.G. Malabaricone C from *Myristica cinnamomea* exhibits anti-quorum sensing activity. *J. Nat. Prod.* **2011**, *74*, 2261–2264.
35. Drenkard, E.; Ausubel, F.M. *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. *Nature* **2002**, *18*, 740–743.
36. Krishnan, T.; Yin, W.F.; Chan, K.G. Inhibition of quorum sensing-controlled virulence factor production in *Pseudomonas aeruginosa* PAO1 by Ayurveda spice clove (*Syzgium Aromaticum*) bud extract. *Sensors* **2012**, *12*, 4016–4030.

Sensors **2014**, *14*, 12958-12967; doi:10.3390/s140712958

OPEN ACCESS

sensors

ISSN 1424-8220

www.mdpi.com/journal/sensors

Article

Short Chain *N*-Acyl Homoserine Lactone Production in Tropical Marine *Vibrio sinaloensis* Strain T47

Pui-Wan Tan, Wen-Si Tan, Nina Yusrina Muhamad Yunos, Nur Izzati Mohamad, Tan-Guan-Sheng Adrian, Wai-Fong Yin and Kok-Gan Chan *

Division of Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur 50603, Malaysia; E-Mails: acelinetan38@yahoo.com (P.-W.T.); tmarilyn36@gmail.com (W.-S.T.); ninayusrina@hotmail.com (N.Y.M.Y.); zetty_mohamad@yahoo.com (N.I.M.); adrian_tan_1991@yahoo.com (T.-G.-S.A.); yinwaifong@yahoo.com (W.-F.Y.)

* Author to whom correspondence should be addressed; E-Mail: kokgan@um.edu.my; Tel.: +60-37967-5162; Fax: +60-37967-4509.

Received: 8 May 2014; in revised form: 30 June 2014 / Accepted: 11 July 2014 / Published: 18 July 2014

Abstract: Quorum sensing (QS), acts as one of the gene regulatory systems that allow bacteria to regulate their physiological activities by sensing the population density with synchronization of the signaling molecules that they produce. Here, we report a marine isolate, namely strain T47, and its unique AHL profile. Strain T47 was identified using 16S rRNA sequence analysis confirming that it is a member of *Vibrio* closely clustered to *Vibrio sinaloensis*. The isolated *V. sinaloensis* strain T47 was confirmed to produce *N*-butanoyl-L-homoserine lactone (C4-HSL) by using high resolution liquid chromatography tandem mass spectrometry. *V. sinaloensis* strain T47 also formed biofilms and its biofilm formation could be affected by anti-QS compound (cathechin) suggesting this is a QS-regulated trait in *V. sinaloensis* strain T47. To our knowledge, this is the first documentation of AHL and biofilm production in *V. sinaloensis* strain T47.

Keywords: 16S rRNA; biofilm; *Chromobacterium violaceum* CV026; marine seawater-borne bacteria; *N*-acylhomoserine lactone (AHL); *N*-butyryl-L-homoserine lactone (C4-HSL); quorum sensing (QS)

1. Introduction

Bacteria are able to communicate in a cell-to-cell manner where they rely on the production and responses to extracellular signaling molecules known as autoinducers to monitor gene expression that is cell density dependent [1,2]. This phenomenon is termed "quorum sensing (QS)" as this system enables bacteria to act in unison by synchronizing their gene expression [3,4]. The most well documented autoinducers are the *N*-acylhomoserine lactones (AHLs) which are used by most Gram-negative bacteria that typically involves the LuxI/LuxR complex in order to regulate the communication [5]. AHLs are synthesized by LuxI homologs (AHL synthases) where AHL will bind to the LuxR receptor to form the AHL-LuxR complex which regulates QS-dependent gene expression [6,7]. The AHL/LuxR complex will then be in an active form that can regulate the QS-dependent genes. Hence, AHLs produced by various microorganisms can differ with respect to the length with various substitutions at the carbon-3 position with the presence or absence of unsaturation within the acyl side chains [8]. Generally, the type of AHL is specific to the target receptor, and hence QS is AHL specific and dependent.

QS microorganisms can be ubiquitous, and they thrive in different habitats. The marine environment could be a habitat for diverse and dense bacterial communities including QS microorganisms. *Vibrio* species usually represent the major microorganisms in the marine environment where they could cause severe infections in many marine organisms such as fish and prawns and the humans that consume them. Most of the pathogenic *Vibrio* studied are normally opportunistic and can pose a threat to aquaculture farming [9,10]. Del Carmen and colleagues showed that infectious diseases, especially those caused by bacterial and viral pathogens, lead to serious losses in shrimp farming where they are hosted in the gut and hepatopancreas of stressed shrimps [10]. This serious disease is caused by *Vibrio sinaloensis* [10]. In this study, we reported the AHL production by this marine isolate.

2. Experimental Section

2.1. Marine Water Sample Collection and Isolation of Bacterial Strain

The sampling site chosen for this study was Morib Beach (GPS coordinates: N02°45.023' E101°26.623') which is located in Selangor, Malaysia in 2013. A water sample was collected in a sterile plastic bottle (50 mL) along the beach coastal area at a depth of 20 cm below water surface. It was then kept at 4 °C before until further analysis [11]. A serial dilution of marine water sample was carried out with sterile saline (0.9% w/v NaCl). Bacterial culture was spread onto Luria Bertani (LB) agar (in grams per 1 L: tryptone, 10; yeast extract, 5; NaCl, 30; Bacto agar, 15) and incubated overnight (24 h) at 28 °C. Colonies of different morphologies were isolated and pure colony was obtained by repeated streaking on LB agar.

2.2. Bacteria Strains, Culture Conditions and Bacterial Biosensor Assay

Colonies obtained were screened by using a AHL biosensor, namely *Chromobacterium violaceum* CV026. Strain T47 activated *C. violaceum* CV026 [12] and was selected for further studies. This

isolate was routinely cultured on LB medium. *Erwinia carotovora* GS 101 producing AHL molecules **(** ***N***-3-oxohexanoyl-homoserine) was used as positive control for AHL production screening involving *C. violaceum* CV026. To further verify the AHL production by strain T47, *Escherichia coli* [pSB 401] was used as another *lux*-based biosensor that will produce bioluminescence in the presence of short chain AHLs [13]. The positive and negative controls for the screening were *Erwinia carotovora* GS101 and *E. carotovora* PNP22, respectively. *C. violaceum* CV026, *E. carotovora* GS101 and *E. carotovora* PNP22 were routinely maintained on LB agar.

2.3. Preliminary Screening of AHLs with Bacterial Biosensors

Isolate T47 was screened for AHL production by cross streaking the bacterial culture with *C. violaceum* CV026 on an LB agar plate and incubating overnight (24 h) at 28 °C. After incubation, purple violacein pigmentation by *C. violaceum* CV026 indicates the production of AHL [12]. For detection of *lux*-based biosensor *E. coli* [pSB401], a photon camera with 60 s of exposure time was used to observe the induced bioluminescence after 24 h incubation at 28 °C. Bioluminescence indicates AHL detection by *E. coli* [pSB401].

2.4. Bacteria Identification

Amplification of bacterial 16S rRNA genes with polymerase chain reaction (PCR) was carried out as previously described [14]. For PCR amplification, we used PCR mix obtained from Promega (Promage Kit, Madison, WI, USA). Genomic DNA extraction was done using MasterPureTM DNA Purification Kit (Epicentre Inc., Madison, WI, USA). PCR amplification and purification process was conducted as described previously [14]. PCR product sequence alignment was done using GenBank BLASTN program followed by phylogenetic analysis using the Molecular Evolutionary Genetic Analysis version 6.0 [15,16].

2.5. AHLs Extraction

Strain T47 was cultured in LB broth buffered with 50 mM of 3-(*N*-morpholino)propanesulfonic acid (MOPS) (pH 5.5) and incubated with shaking (200 rpm, 28 °C, 18 h). Overnight culture supernatant was extracted twice with addition of equal volume of acidified (0.1% v/v glacial acetic acid) ethyl acetate and mixed vigorously [7]. Organic solvent was collected and dried in the fume hood and the extract was resuspended with 1 mL of acidified ethyl acetate and dried again. After that, 200 µL of acetonitrile (HPLC grade) was added to dissolve the AHL extracts for further analysis.

2.6. AHL Profiling by Mass Spectrometry (MS)

An Agilent RRLC 1200 system was utilized as the liquid chromatography (LC) delivery system with the use of Agilent ZORBAX Rapid Resolution HT column and the MS parameters were fixed as reported previously [17]. The high resolution electrospray ionization mass spectrometry (ESI-MS) was performed with an Agilent 6,500 Q-TOF LC/MS system operated in ESI-positive mode. The *m/z* value range to detect the precursor ions was set at *m/z* 150–400. The precursor ion scan mode targeting at the

production ion with *m/z* 102. Analysis of MS spectra generated and AHL profile was performed as described previously [17].

2.7. Biofilm Assay

Biofilm assay was performed as described previously [18]. Overnight culture of strain T47 was diluted with sterile LB medium, and adjusted to OD_{600} of 0.1. Subsequently, diluted culture (50 μL) was dispensed into a microtitre well containing 930 μL of sterile LB medium supplemented with 1 mg/mL of anti-QS compound (catechin) [18]. Strain T47 cultures treated with and without DMSO were used as negative and positive controls, respectively. Strain T47 was incubated statically for 2 days at 28 °C. Unattached planktonic cells were removed by gently washing with sterile distilled water followed by air-dried for 15 min aseptically. To stain the biofilm, the wells were filled with 200 μL of 0.1% (w/v) crystal violet for 30 min, washed twice with sterile distilled water, followed by addition of 200 μL of 95% (v/v) ethanol. Amount of biofilm formed was measured by transferring 100 μL of this ethanol solution to a new microtitre plate and reading the absorbance at OD_{590} with a microplate reader. These biofilm assays were repeated twice.

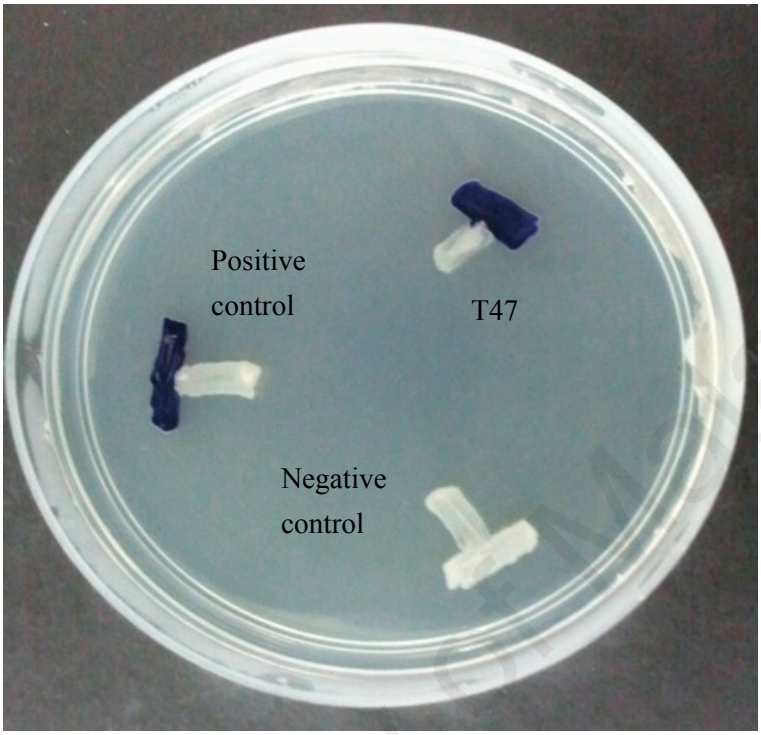
3. Results and Discussion

3.1. Strains Isolation and Preliminary Screening of AHL

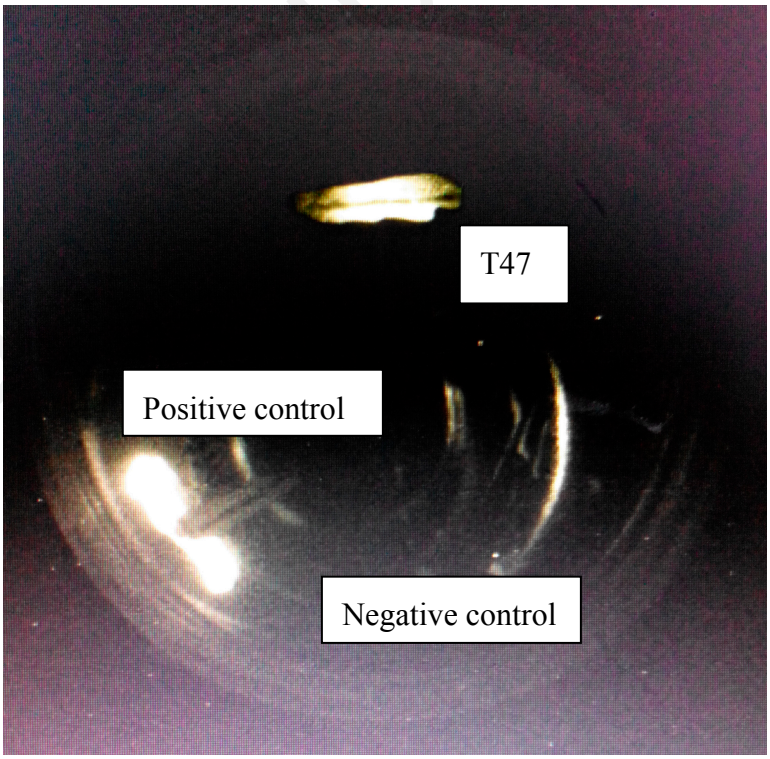
The aim of this study was to isolate AHL-producing bacteria from a marine seawater sample and characterize its AHL profile. The sampling site chosen for this study is Morib Beach which is in an area near a fishing village. The temperature and pH of the water sample collected was recorded at 27 °C and pH 8.0, respectively. The marine water sample was collected near the coastal line to determine whether pathogenic bacteria that could infect either humans or affect fishing activities can be detected.

The availability of the AHL biosensors has led to the discovery of many QS bacteria [19]. AHL biosensors are mutants with defective LuxI AHL synthase, which practically rely on the LuxR protein to display specific binding towards the cognate AHL that is able to activate the transcription of the reporter gene [20]. The biosensor *C. violaceum* CV026 responds to the AHLs with C4 to C8 acyl chain length that will induce a purple violacein pigmentation [12]. It is a preferable biosensor for AHL preliminary screening due to the rapidness and accuracy in its AHL detection. Hence, we used *C. violaceum* CV026 for the preliminary screening of AHLs produced by strain T47 (Figure 1). The induction of purple violacein in the *C. violaceum* CV026 biosensor indicated that the isolated strain T47 produced detectable short chain AHLs. In addition to this, T47 also induced bioluminescence in *E. coli* [pSB401]. Both biosensor tests with *C. violaceum* CV026 and *E. coli* [pSB401] indicated that strain T47 produced short chain AHLs. The strain was next subjected to AHL profiling and molecular identification.

Figure 1. (**a**) AHL screening of strain T47 with *C. violaceum* CV026 and (**b**) with *E. coli* pSB401. *E. carotovora* PNP22 (negative control) devoid of QS activity was included and *E. carotovora* GS101 (positive control) that can activate *C. violaceum* CV026 and *E. coli* pSb401 was included.



(**a**)

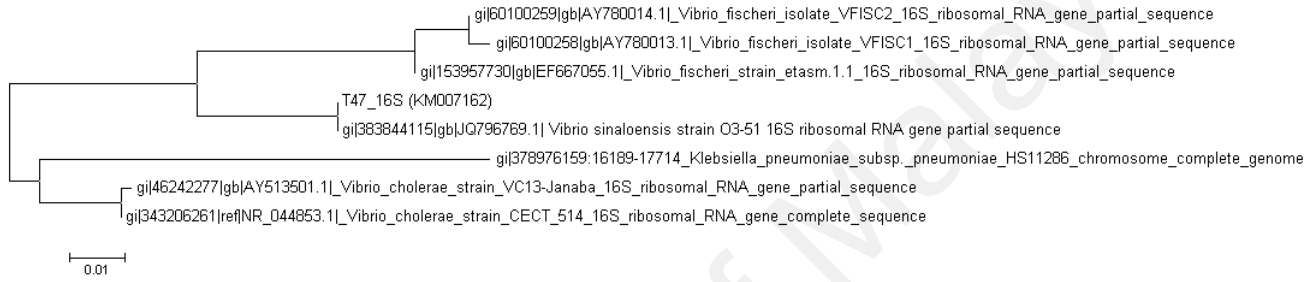


(**b**)

3.2. Molecular Identification of Bacterial Strain

The identity of the strain T47 was confirmed by analysis of its 16S rRNA gene nucleotide sequences showing that it clustered closely within the *Vibrio* genus, where the strain shared 99% similarity in the BLAST search. Based on the phylogenetic tree constructed (Figure 2), strain T47 was identified as *Vibrio sinaloensis*, a marine opportunistic bacterium.

Figure 2. Phylogenetic analysis of strain T47. Phylogenetic tree reconstructed using the Maximum likelihood algorithm showing phylogenetic relationships of strain T47 to members of the other species of the genus *Vibrio*. Bar, 1 substitution per 100 nucleotides positions. Gene accession numbers are indicated.



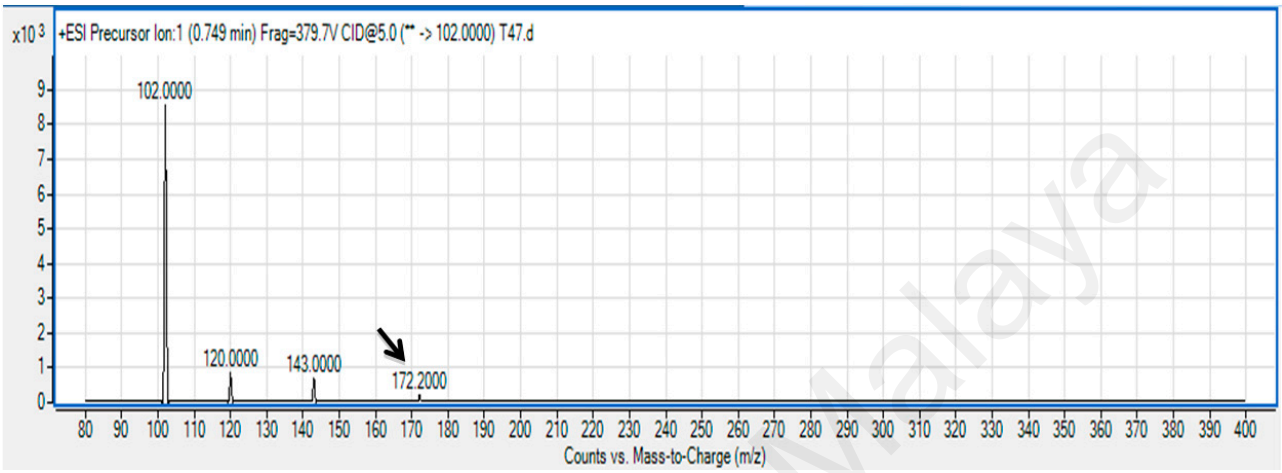
3.3. Analytical Identification of AHL Molecules

Vibrio genus is a causative agent for food-borne diseases and in many countries, it has infected patients who consumed undercooked seafood and mortality has been reported [21]. For example, the species *V. alginolyticus* was isolated in 1997 from patients during an outbreak of acute enteric illness in Vladivostok, Russia [22]. In addition to this, foodborne illness caused by *V. alginolyticus* has been identified in 96 cases after the patients consumed brine shrimp in Chifeng Hongshan, China in 2004 [23]. Although we have reported very detailed QS systems in some of the members of *Vibrio*, this is almost entirely limited to a few model *Vibrio* but little is known about QS in *V. sinaloensis* strain T47. Moreover, the pathogenicity of *V. sinaloensis* is still largely unknown. Interestingly, our work showed that this bacterium produced AHLs which are the vital QS signalling molecules in proteobacteria. It is believed that this AHL is responsible in regulating certain QS physiological activities of *V. sinaloensis* [24]. Thus, the availability of detecting AHL profile of *Vibrio* sp. represents the first step in understanding this bacterium's QS gene regulation.

The spent culture supernatant of *V. sinaloensis* strain T47 was analyzed using mass spectrometry in order to determine the exact AHL produced by *V. sinaloensis* strain T47. The presence of *N*-butanoyl-L-homoserine lactone (C4-HSL) [25,26] with retention time of 0.749 min was identified and confirmed (Figure 3). To our knowledge, this is the first documentation on AHL profiling of *V. sinaloensis* where it produces AHL molecule as part of its QS system. There are three major QS systems in the Vibrionaceae family namely *luxI*, *hdts* and *luxM*. The majority of AHL synthases identified show either formation of 3-hydroxy-HSLs or 3-oxo-HSLs along with or without unsubstituted AHLs of the same or similar acyl chain lengths [26]. Purohit *et al.* [24] reported that they have identified 3-hydroxy-C8-HSL in *Aliivibrio fischeri* ES114, which is produced at a very low concentration. Hence it will not be sufficient to predict the AHL-producing QS system simply by

analyzing the AHLs. This result enables us to take a deeper approach into studying the mechanism of QS of *V. sinaloensis*. Besides that, our group also expanding this research to the whole genome sequence to gain more insights on the *luxI* and *luxR* genes in this strain.

Figure 3. Mass spectrometry analysis of spent supernatants extract of *Vibrio sinaloensis* strain T47. Mass spectrum of C4-HSL (*m/z* 172.2000) (arrow).

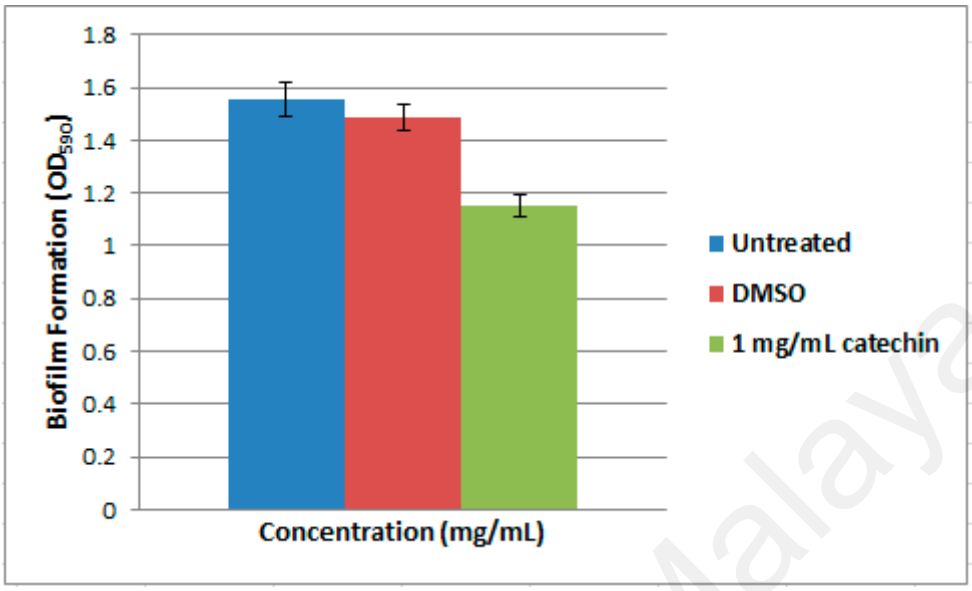


A battery of physiological activities such as biofilm formation, virulence factors and motility can be regulated by QS [1–3] and hence this work provides the basis to illustrate the significance of research on AHL-producing bacteria present in environmental samples. *V. sinaloensis* causes serious losses in shrimp and fish farming. Our work provides valuable input to explore anti-QS [27,28] as a tool to attenuate marine QS pathogens which can form the basis of novel treatments in marine aquaculture.

3.4. Biofilm formation

Numerous studies have investigated biofilm formation in *Vibrio* species such as *V. cholera*, *V. parahaemolyticus*, *V. vulnificus* and *V. fischeri* [29]. Biofilms formed at solid-liquid interfaces have been analyzed under static or flow conditions [29]. In our study, we analyzed biofilm formation of strain T47 under static culture conditions. Bacteria colonization is initiated, followed by surface attachment, resulting in the formation of a mature biofilm. For example, *V. vulnificus* and *V. parahaemolyticus* which are found on surfaces of plankton and colonize shellfish [29]. Strain T47 has been shown to be able to form biofilm (Figure 4). Catechin, an anti-QS compound [30], was used in this study to inhibit the biofilm formation in strain T47. The biofilm formation of strain T47 was reduced in a dose-dependent manner (Figure 4). Since *Vibrio* species use QS to control numerous traits, including bioluminescence, virulence and biofilm formation this work illustrated the significance of expanding the research on AHL-producing bacteria present in environmental samples. Hence, in this work we suggest that sea water may serve as a potential reservoir for QS pathogens that should be given appropriate attention.

Figure 4. Qualitative analyses of biofilm formation and inhibition by anti-QS compound, catechin. Bars: Standard errors of the mean.



4. Conclusions

Here we report the AHL profile of *V. sinaloensis* T47 isolated from tropical marine seawater which confirmed that this isolate produced a short chain AHL *viz.* C4-HSL. This is the first documentation of *Vibrio sinaloensis* producing this AHL.

Acknowledgments

This work was supported by the University of Malaya via High Impact Research Grant (UM.C/625/1/HIR/MOHE/CHAN/01, Grant No. A-000001-50001) which has been awarded to Kok-Gan Chan.

Author Contributions

P.-W. Tan, W.-S. Tan, N.Y. Muhamad Yunos, N.I. Mohamad, T.-G.-S. Adrian performed the experiments. W.-F. Yin analysed the data and proof read the draft. K.-G. Chan applied and managed the funding, conceived the idea, prepared the manuscript and supervised the whole project. All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Williams, P.; Winzer, K.; Chan, W.C.; Camara, M. Look who's talking: Communication and quorum sensing in the bacterial world. *Phil. Trans. R. Soc. B* **2007**, *362*, 1119–1134.

2. Faure, D.; Dessaux, Y. Quorum sensing as a target for developing control strategies for the plant pathogen. *Pectobacterium*. *Eur. J. Plant Pathol.* **2007**, *119*, 353–365.
3. Fuqua, C.; Greenberg, E.P. Listening in on bacteria: Acyl-homoserine lactone signaling. *Nat. Rev.* **2002**, *3*, 685–695.
4. Lau, Y.Y.; Sulaiman, J.; Chen, J.W.; Yin, W.F.; Chan, K.G. Quorum sensing activity of *Enterobacter asburiae* isolated from lettuce leaves. *Sensors* **2013**, *13*, 14189–14199.
5. Ng, W.L.; Bassler, B.L. Bacterial quorum-sensing network architectures. *Annu. Rev. Gen.* **2009**, *43*, 197–222.
6. Williams, P. Quorum sensing, communication and cross-kingdom signaling in the bacterial world. *Microbiology* **2007**, *153*, 3923–3938.
7. Chong, T.M.; Koh, C.L.; Sam, C.K.; Choo, Y.M.; Yin, W.F.; Chan, K.G. Characterization of quorum sensing and quorum quenching soil bacteria isolated from Malaysian tropical montane forest. *Sensors* **2012**, *12*, 4846–4859.
8. Cha, C.; Gao, P.; Chen, Y.C.; Shaw, P.D.; Farrand, S.K. Production of acyl-homoserine lactone quorum sensing signals by Gram-negative plant-associated bacteria. *Mol. Plant Microbe Interact.* **1998**, *11*, 1119–1129.
9. Gil, B.G.; Abila, E.F.; Pascual, J.; Macian, M.C.; Pujalte, M.J.; Garay, E.; Roque, A. *Vibrio sinaloensis* sp. nov., isolated from spotted rose snapper, *Lutjanus guttatus* Sterindachner, 1869. *Int. J. Syst. Evol. Microbiol.* **2009**, *58*, 1621–1624.
10. Del Carmen, F.M.M.; Luna, G.A.; Cordova, A.I.; Fierro, C.J.A.; Partida, A.B.O.; Pintado, J.; Gonzalez, O.H.A. Isolation and characterization of infectious *Vibrio sinaloensis* strains from Pacific shrimp *Litppenaeus vannamei* (Decapoda: Penaeidae). *Rev. Biol. Trop.* **2012**, *60*, 567–576.
11. Wintzingerode, F.V.; Gobel, U.B.; Stackebrandt, E. Determination of microbial diversity in environmental samples: Pitfalls of PCR-based rRNA analysis. *FEMS Microbiol. Rev.* **1997**, *21*, 213–229.
12. McClean, K.H.; Winson, M.K.; Fish, L.; Taylor, A.; Chhabra, S.R.; Camara, M.; Daykin, M.; Lamb, J.H.; Swift, S.; Brcroft, B.W.; Stewart, G.S.; Williams, P. Quorum sensing and *Chromobacterium violaceum*: Exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* **1997**, *143*, 3703–3711.
13. Winson, M.K.; Swift, S.; Fish, L.; Throup, J.P.; Jorgensen, F.; Chhabra, S.R.; Bycroft, B.W.; Williams, P.; Stewart, G.S. Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl homoserine lactone-mediated quorum sensing. *FEMS Microbiol. Lett*. **1998**, *163*, 185–192.
14. Yin, W.F.; Tung, H.J.; Sam, C.K.; Koh, C.L.; Chan, K.G. Quorum quenching *Bacillus sonorensis* isolated from soya sauce fermentation brine. *Sensors* **2012**, *12*, 4065–4073.
15. Tamura, K.; Nei, M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Mol. Biol. Evol.* **1993**, *10*, 512–526.
16. Mandepudi, B.; Mandepudi, D.; Ghanta, V.C. Identification and characterization of novel lipase producing soil bacterial isolates B3 and B4 using 16S rDNA analysis. *Res. J. Pharm. Biol. Chem. Sci.* **2013**, *4*, 149–160.
17. Robson, E.H.J.; Yin, W.F.; Chan, K.G. *Pandoraea* sp. RB-44, a novel quorum sensing soil bacterium. *Sensors* **2013**, *13*, 14121–14132.

18. Cady, N.C.; McKean, K.A.; Behnke, J.; Kubec, R.; Mosier, A.P. Inhibition of biofilm formation, quorum sensing and infection in *pseudomonas aeruginosa* by natural products-inspired organosulfur compounds. *PLoS One* **2012**, *7*, e38492.
19. Steidler, L.; Venturi, V. Detection of quorum sensing *N*-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiol. Lett.* **2007**, *266*, 1–9.
20. De Kievit, T.R.; Iglewski, B.H. Bacterial quorum sensing in pathogenic relationships. *Infect. Immun.* **2000**, *68*, 4839–4849.
21. Thompson, F.L.; Li, Y.; Gomez-Gil, B.; Thompson, C.C.; Hoste, B.; Vandemeulebroecke, K.; Rupp, G.S.; Pereira, A.; de Bern, M.M.; Sorgeloos, P.; Swings, J. *Vibrio neptunius* sp. nov., *Vibrio brasiliensis* sp. nov. and *Vibrio xuii* sp. nov., isolated from the marine aquaculture environment (bivalves, fish, rotifers and shrimps). *Int. J. Syst. Evol. Microbiol.* **2003**, *53*, 245-252.
22. Smolikova, L.M.; Lomov, L.M.; Khomenko, T.V.; Murnachev, G.P.; Kudriakova, T.A.; Fetsailova, O.P.; Sanamiants, E.M.; Makedonova, L.D.; Kachkina, G.V.; Golenishceva, E.N. Studies on halophilic vibrios causing a food poisoning outbreak in the city of Vladivistok. *Mikrobiol. Epidemiol. Immunobiol*. **2001**, *6*, 3–7.
23. Xie, Z.Y.; Hu, C.Q.; Chen, C.; Zhang, L.P.; Ren, C.H. Investigation of seven *Vibrio* virulence genes among *Vibrio alginolyticus* and *Vibrio parahaemolyticus* strains from the coastal mariculture systems in Guangdong, China. *Lett. Appl. Microbiol.* **2005**, *41*, 202–207.
24. Purohit, A.A.; Johansen, J.A.; Hansen, H.; Leiros, H.K.; Kashulin, A.; Karlsen, C.; Smalas, A.; Haugen, P.; Willassen, N.P. Presence of acyl-homoserine lactones in 57 members of the Vibrionaceae family. *J. Appl. Microbiol.* **2013**, *115*, 835–847.
25. Ortori, C.A.; Dubern, J.F.; Chhabra, S.R.; Camara, M.; Hardie, K.; Williams, P.; Barrett, D.A. Simultaneous quantitative profiling of *N*-acyl-L-homoserine lactone and 2-alkyl-4(1H)-quilonone families of quorum-sensing signaling molecules using LC-MS/MS. *Anal. Bioanal. Chem.* **2011**, *399*, 839–850.
26. Churchill, M.E.; Chen, L. Structural basis of acyl-homoserine lactones dependent signaling. *Chem. Rev*. **2011**, *111*, 68–85.
27. Tan, L.Y.; Yin, W.F.; Chan, K.G. Silencing quorum sensing through extracts of *Melicope lunu-ankenda*. *Sensors* **2012**, *12*, 4339–4351.
28. Hong, K.W.; Koh, C.L.; Sam, C.K.; Yin, W.F.; Chan, K.G. Quorum quenching revisited—From signal decays to signalling confusion. *Sensors* **2012**, *12*, 4661–4696.
29. Yildiz, F.H.; Visick, K.L. *Vibrio* biofilms: So much the same yet so different. *Trends Microbiol.* **2009**, *17*, 109–118.
30. Vandeputte, O.M.; Kiendrebeogo, M.; Rajaonson, S.; Diallo, B.; Mol, A.; El Jaziri, M.; Bancher, M. Identification of catechin as one of the flavanoids from *Combretum albiflorum* bark extract that reduce the production of quorum-sensing-controlled virulence factors in *Pseudomonas aeruginosa* PAO1. *Appl. Environ. Microbiol*. **2010**, *76*, 243–253.

APPENDICES

Appendix A: 16S rDNA sequence of *V. variabilis* strain T01

>T01 16S (Genbank accession number : KP329555)

CGAGCGGCGGACGGGTGAGTAATGCCTGGGAAATTGCCCTGATGTGGGGGA
TAACCATTGGAAACGATGGCTAATACCGCATAATAGCTTCGGCTCAAAGAG
GGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCCAGGTGGGATTAGCTAG
TTGGTGAGGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGG
ATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCG
TGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCAGTGAGGAAGGT
GGTGCAGTTAATAGCTGTATCATTTGACGTTAGCTGCAGAAGAAGCACCGG
CTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCG
GAATTACTGGGCGTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAA
AGCCCGGGGCTCAACCTCGGAATTGCATTTGAAACTGGCAGACTAGAGTAC
TGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTG
AAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGA
TGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGATGTCTACTTGGAGGTTGTGGCCTTGAGCCGTGGCTTTCGGAGCTA
ACGCGTTAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAA
TGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATG
CAACGCGAAGAACCTTACCTACTCTTGACATCCTCAGAAGCTTGTAGAGATA
CGAGTGTGCCTTCGGGAACTGAGAGACAGGTGCTGCATGGCTGTCGTCAGC
TCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCT
TGTTTGCCAGCACTTCGGGTGGGAACTCCAGGGAGACTGCCGGTGATAAAC
CGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGGGC
TACACACGTGCTACAATGGCGCATACAGAGGGCGGCCAACTTGCGAGAGTG
AGCGAATCCCAAAAAGTGCGTCGTAGTCCGGATTGGAGTCTGCAACTCGAC
TCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAAT
ACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGCTGC
AAAAGAAGTGGGTAGTTTAACCTTCGGGGGGACGCTCACCACTTTGTGGTTC
ATGACTGGGGTGAAGTCGTAACAAGGTAGCGCTAGGGGAACCTGGCGCTGG
ATC

Appendix B: VanM protein sequence and *vanM* gene sequence of *V. variabilis* strain T01

>T01 VanM

MASYDHTNSVCLSASETYSDVFYRIQSQFGDSANQLFSQCLKSRLEQFHERFPE
KRDDNISSKINSSEFNVSSRTPLTFWPSDWCYYESLLLAHFSHWAEAWTHFQIW
SLEQKYTAVQTRRINLNHWQALPENEAEYDDGLVNDIDQNQKLFFTLHSPFAM
VLKDAVAMINLSTLVLELQWYELLEHIELSKSGTHFLLTYSDPQLEHPLLVSTA
RVNFAVGMQDWLYFSSFFQSKHWRSISHTQQLTTLIQKGLLSAEALLQEPRSQQ
EFDKLVWEHLVCPERSCEVIRLTVSGTKLQKMYYLYLSQKRLMKQLYEQQMM
LSFVVVEQPLMIKYYESLSLGSYCQFGSCTLVGSDNPTYKGAWVVPQMHHALT
SSDYRRYKQQTLQQIKKTRHNELNHA

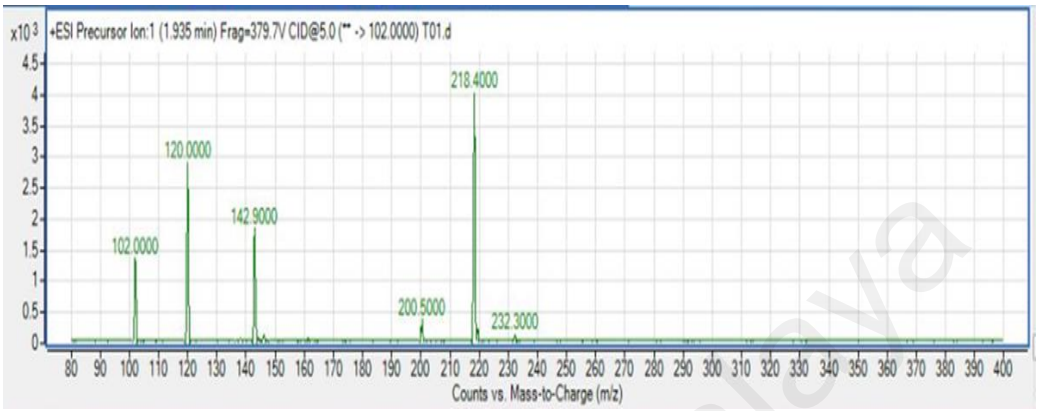
>T01 *vanM* (Genbank accession number : KT258634)

CTAAGCATGGTTGAGCTCATTATGACGTGTCTTTTTTATTTGTTGTAAAGTTT
GTTGCTTGTAGCGGCGATAATCTGAGGATGTTAAAGCGTGGTGCATTTGAGG
AACAACCCAAGCCCCTTTATACGTTGGATTATCTGATCCCACTAACGTGCAT
GAACCAAATTGGCAATAAGATCCCAAAGATAAGCTTTCATAGTACTTGATC
ATCAAAGGTTGTTCGACTACCACGAACGACAGCATCATTTGTTGTTCGTACA
ACTGTTTCATCAAACGCTTTTGCGAAAGGTACAAGTAATACATTTTTTGTAA
TTTGGTTCCGCTCACGGTTAGCCGAATCACTTCACAAGATCTTTCAGGGCAA
ACTAGGTGCTCCCAGACTAATTTATCAAACTCTTGTTGACTCCGTGGCTCTT
GAAGCAATGCTTCCGCAGAGAGTAGCCCCTTTTGTATCAGTGTTGTAAGTTG
CTGTGTGTGACTGATGCTTCGCCAATGTTTGCTTTGGAAAAAAGAGGAGAA
GTAGAGCCAGTCTTGCATCCCGACGGCAAAGTTCACTCTTGCGGTTGAGACG
AGCAGTGGATGCTCAAGCTGTGGATCTGAATAGGTGAGTAAGAAGTGTGTG
CCGGACTTAGACAGTTCGATATGTTCGAGCAGCTCATACCATTGAAGCTCTA
AAACCAAAGTCGACAGATTGATCATCGCCACTGCATCTTTTAGGACCATAGC
GAAAGGAGAGTGAAGCGTGAAAAACAACTTCTGGTTTTGGTCGATATCGTT
GACAAGGCCATCGTCATACTCTGCTTCGTTTTCCGGCAGTGCTTGCCAATGG
TTTAGATTTATACGTCGAGTTTGTACTGCTGTGTATTTTTGCTCTAGCGACCA
AATTTGAAAGTGCGTCCATGCTTCAGCC
CAGTGCGAAAAGTGCGCGAGCAGTAAGGATTCATAGTAACACCAATCTGAA
GGCCGAAGGTTAGGGGCGTGCGCGAGCTAACGTTGAACTCAGAAGAATTGA
TTTTGCTTGAAATATTATCGTCGCGCTTTTCGGGGAATCGCTCGTGAAACTG
TTCGAGCCGGGATTTGAGACATTGAGAAAACAGCTGATTCGCGGAATCGCC
AAATTGACTTTGGATGCGATAAAAGACATCGGAGTAGGTTTCAGACGCTGA
GAGACAAACCGAATTTGTATGATCGTAACTAGCCAT

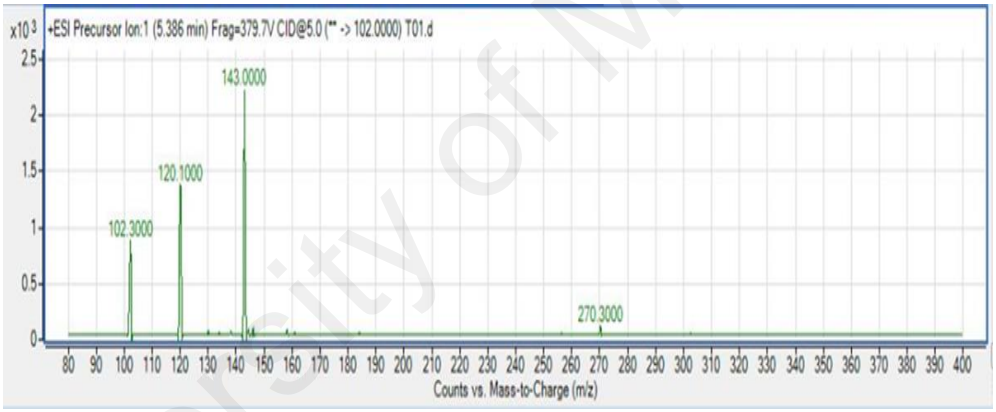
Appendix C: Mass spectra of AHL profiles of five QS strains

AHL profiles of *V. variabilis* strain T01

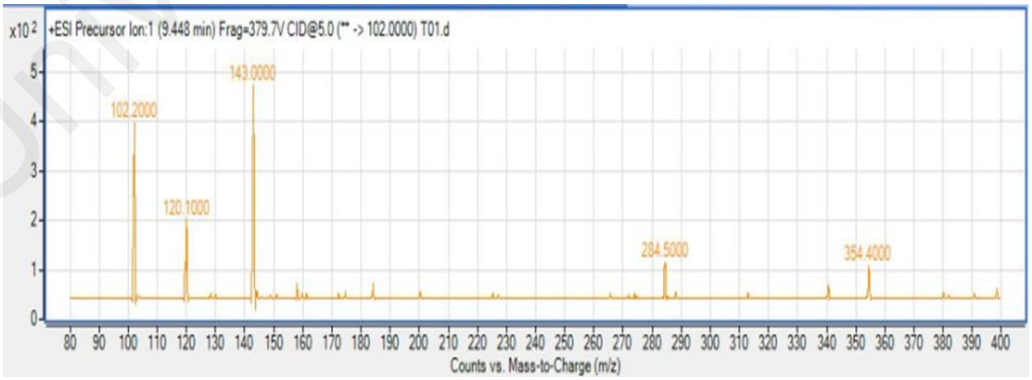
N-hexanoyl-L-homoserine lactone (C6-HSL)



N-(3-oxo-decanoyl)-L-homoserine lactone (OC10-HSL)

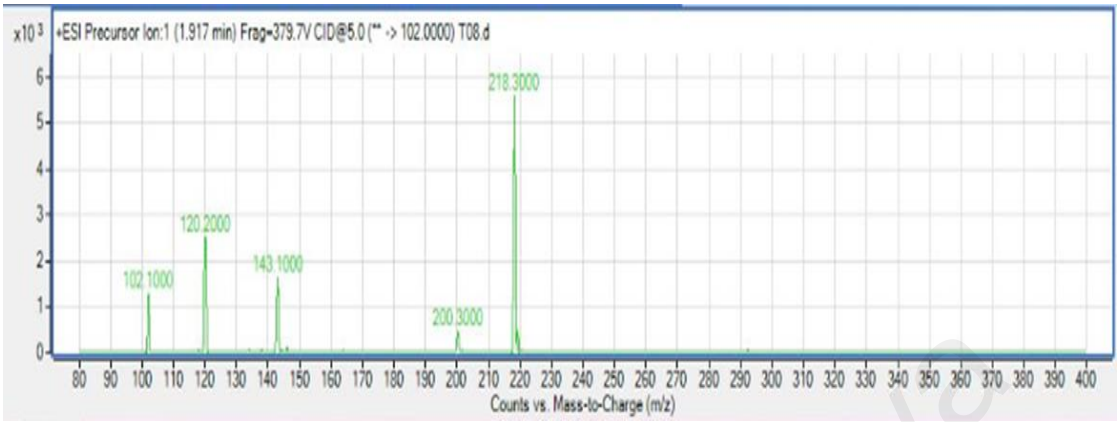


N-dodecanoyl-L-homoserine lactone (C12-HSL)

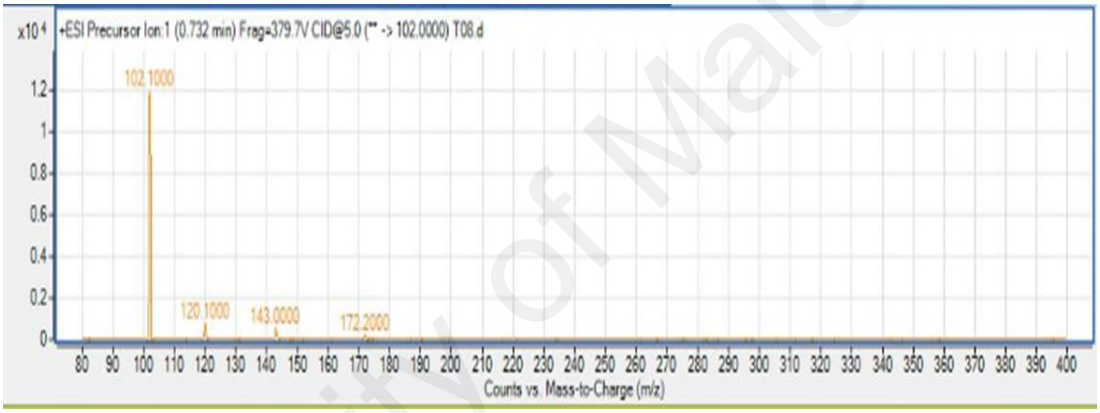


AHL profiles of *V. sinaloensis* strain T08

N-butanoyl-L-homoserine lactone (C4-HSL)

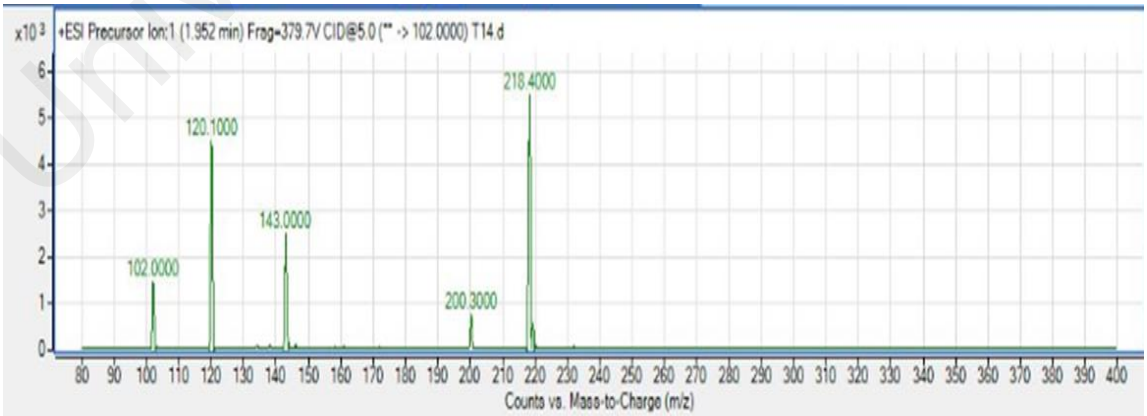


N-hexanoyl-L-homoserine lactone (C6-HSL)



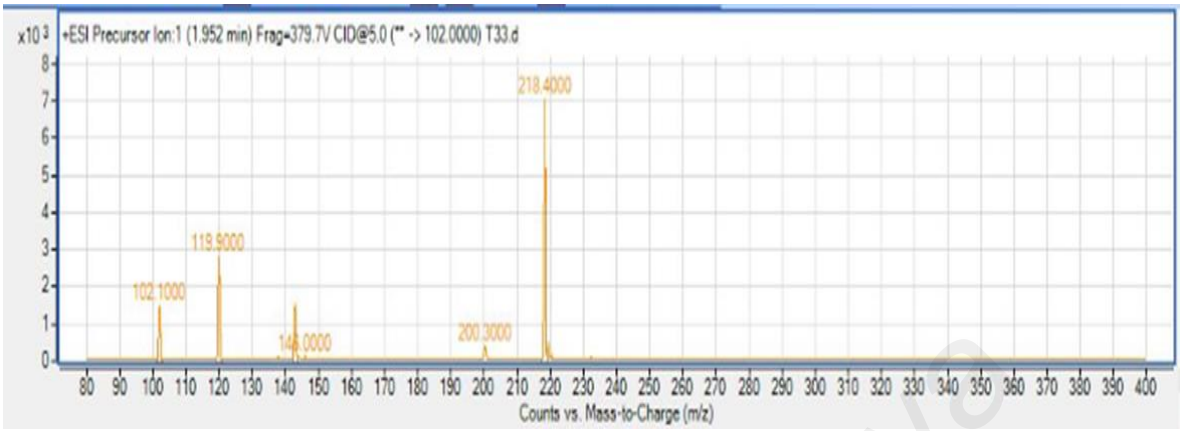
AHL profile of *V. caribbeanicus* strain T14

N-hexanoyl-L-homoserine lactone (C6-HSL)

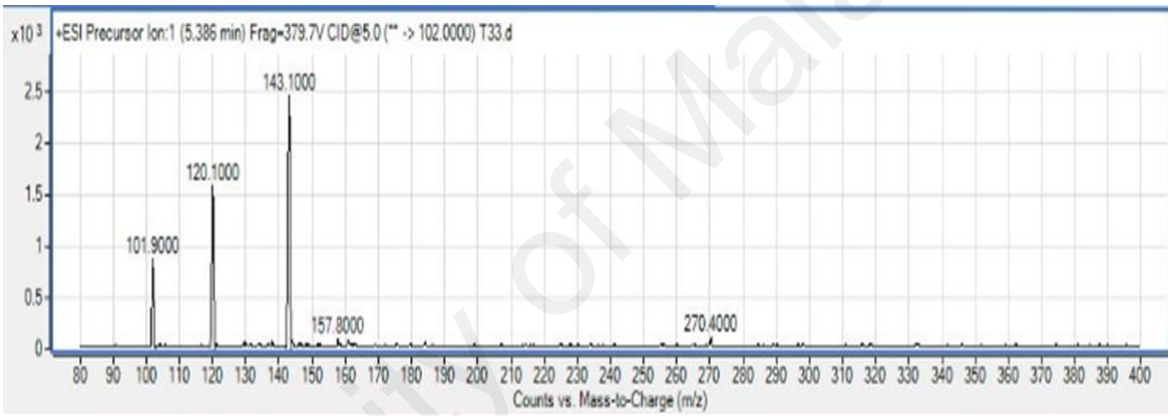


AHL profiles of *V. tubiashii* strain T33

N-hexanoyl-L-homoserine lactone (C6-HSL)

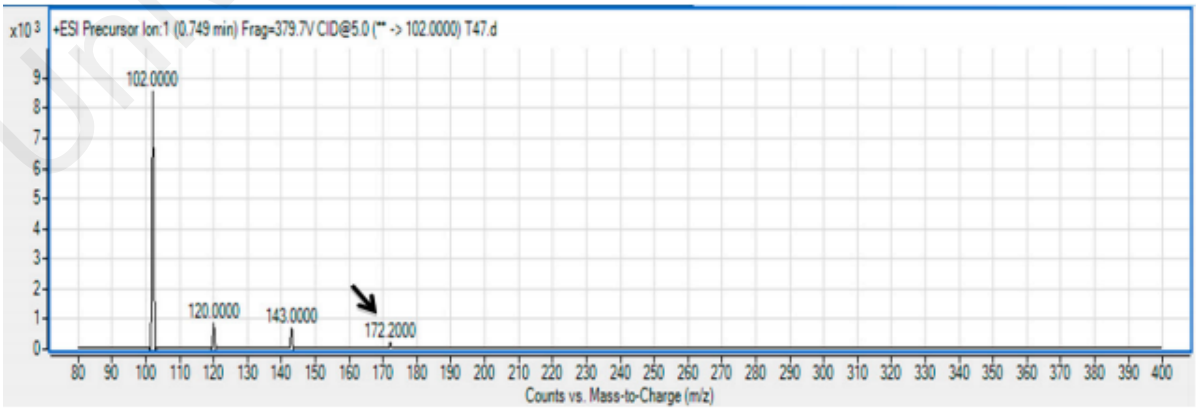


N-(3-oxo-decanoyl)-L-homoserine lactone (OC10-HSL)



AHL profile of *V. sinaloensis* strain T47

N-butanoyl-L-homoserine lactone (C4-HSL)



APPENDIX D: Expression profiling of genes involved in the gallic acid treatment.

Table 4.7: Upregulated and downregulated genes of *V. variabilis* strain T01 affected by gallic acid treatment

Protein Name	p-value(treated vs. untreated)	Log2 value
Upregulated		
hypothetical protein	0.018838	6.935141
Pyridoxamine 5'-phosphate oxidase	0.004772	6.420216
Carbonic anhydrase precursor	0.011732	6.406929
colicin uptake protein TolR	0.002883	6.344678
ChuX-like family protein	0.000551	6.330413
Hemin-binding periplasmic protein HmuT precursor	0.000139	6.090781
biopolymer transport protein ExbB	0.0002	6.011207
Iron-binding protein IscA	0.006046	5.96901
transport protein TonB	0.002392	5.904759
Hemoglobin and hemoglobin-haptoglobin-binding protein A precursor	0.000878	5.808992
Oxygen-independent coproporphyrinogen-III oxidase 1	0.000605	5.791165
hypothetical protein	0.039698	5.646883
putative ABC transporter solute-binding protein YclQ precursor	0.001299	5.516103
Glutaredoxin-like protein NrdH	0.002494	5.37113
Hemin import ATP-binding protein HmuV	0.001674	5.326145
Superoxide dismutase [Mn]	0.002433	5.258217
Fumarate hydratase class II	0.001488	5.185874
N(6)-hydroxylysine O-acetyltransferase	0.000479	5.009213
Putative NrdI-like protein	0.004137	4.851074
Ferric aerobactin receptor precursor	0.000943	4.786602
FeS cluster assembly protein SufD	0.001568	4.772287

Protein Name	p-value(treated vs. untreated)	Log2 value
6-phospho-beta-glucosidase BglB	0.000111	4.769401
Multidrug resistance protein MdtL	0.001698	4.764856
Protein YceI	0.014173	4.694652
hypothetical protein	0.018394	4.645915
hypothetical protein	0.004444	4.592648
hypothetical protein	0.000884	4.572277
N(2)-citryl-N(6)-acetyl-N(6)-hydroxylysine synthase	0.01329	4.547943
Hemin transport system permease protein HmuU	0.00611	4.52095
Succinate-semialdehyde dehydrogenase [NADP(+)] GabD	0.001121	4.505269
Cysteine desulfurase	0.000455	4.38102
hypothetical protein	0.006204	4.335104
Ferrous iron transport protein B	0.003268	4.272777
L-lysine N6-monooxygenase	0.002639	4.26126
Glyoxylate/hydroxypyruvate reductase B	0.003677	4.131186
hypothetical protein	0.008139	4.026632
Ribonucleoside-diphosphate reductase subunit alpha 2	0.003144	3.792939
Aerobactin synthase	0.000354	3.749352
Iron(3+)-hydroxamate import system permease protein FhuB	0.000593	3.743633
Iron(3+)-hydroxamate import ATP-binding protein FhuC	0.000693	3.738022
hypothetical protein	0.006385	3.732519
Bacterial regulatory proteins, tetR family	0.012534	3.700462
EamA-like transporter family protein	0.047809	3.66487
Ribonucleoside-diphosphate reductase subunit beta nrdF2	0.002497	3.635975
Iron(3+)-hydroxamate import ATP-binding protein FhuC	0.002175	3.584205
HTH-type transcriptional regulator CysL	0.005037	3.55508

Protein Name	p-value(treated vs. untreated)	Log2 value
hypothetical protein	0.001539	3.475837
major facilitator superfamily transporter	0.004985	3.446838
Ferrous iron transport protein A	0.004674	3.434215
Iron-uptake system permease protein FeuC	0.001778	3.408793
Iron-uptake system permease protein FeuB	0.005658	3.367609
Alkyl hydroperoxide reductase subunit C	0.002341	3.296362
Leucine carboxyl methyltransferase	0.003572	3.279604
Imelysin	0.001226	3.279381
hypothetical protein	0.000115	3.262835
hypothetical protein	0.000859	3.229651
Quercetin 2,3-dioxygenase	0.018238	3.217949
Glycine dehydrogenase (decarboxylating)	0.018834	3.113442
hypothetical protein	0.001708	3.079649
Cytochrome c	0.000694	2.969624
hypothetical protein	0.000139	2.88543
hypothetical protein	0.000153	2.882858
p-benzoquinone reductase	0.014603	2.872143
hypothetical protein	0.005265	2.797385
Endonuclease/Exonuclease/phosphatase family protein	0.000121	2.793836
Bacterial extracellular solute-binding proteins, family 3	0.000493	2.774429
Dipeptide transport system permease protein DppC	0.014266	2.675333
hypothetical protein	0.000418	2.566482
hypothetical protein	0.018543	2.506965
Fe(3+)-citrate-binding protein YfmC precursor	0.015823	2.474981
hypothetical protein	0.006909	2.405113

Protein Name	p-value(treated vs. untreated)	Log2 value
GMP reductase	0.000838	2.345252
DNA protection during starvation protein	0.000208	2.300112
Protein translocase subunit SecD	0.002541	2.295417
Formate dehydrogenase, cytochrome b556(fdo) subunit	0.019812	2.249324
hypothetical protein	0.000272	2.243511
hypothetical protein	0.000465	2.215756
Inner membrane protein YccF	0.001195	2.187676
Protein of unknown function, DUF	0.002292	2.171463
Dinitrogenase iron-molybdenum cofactor	0.004445	2.170024
Formate dehydrogenase iron-sulfur subunit	0.023871	2.168411
Formate dehydrogenase H	0.02729	2.163682
Protein ViaA	0.009708	2.147297
hypothetical protein	0.02121	2.142315
Fatty acid desaturase	0.004381	2.13586
PBP superfamily domain protein	0.002857	2.117585
CsgBAC operon transcriptional regulatory protein	0.004397	2.103095
Antibiotic biosynthesis monooxygenase	0.017239	2.101052
CTX phage RstB protein	0.017375	2.084421
Oxaloacetate decarboxylase beta chain	0.011191	2.084255
Alcohol dehydrogenase YqhD	0.000443	2.040605
Putative multidrug export ATP-binding/permease protein	0.004452	2.036183
hypothetical protein	0.011857	2.030082
FMN-dependent NADH-azoreductase	0.032756	2.029191
hypothetical protein	0.016847	2.008946
hypothetical protein	0.015651	1.980289

Protein Name	p-value(treated vs. untreated)	Log2 value
Inner membrane protein YebE	0.000236	1.969808
Thioredoxin-2	0.003177	1.942522
Response regulator PleD	0.002535	1.937973
Vitamin B12 transporter BtuB	0.005428	1.928101
Glutathione-regulated potassium-efflux system protein KefC	0.000288	1.8945
Glutathionyl-hydroquinone reductase YqjG	0.008495	1.894146
Tetrathionate sensor histidine kinase TtrS	0.042295	1.880356
Chaperone protein TorD	0.023089	1.879761
von Willebrand factor type A domain protein	0.006353	1.877893
Catalase-peroxidase	0.000352	1.866975
Bacterial OB fold (BOF) protein	0.006274	1.866908
3-hydroxyisobutyrate dehydrogenase	0.015706	1.850699
Tetratricopeptide repeat protein	0.005481	1.847632
ATPase RavA	0.007636	1.841921
Peptidase M15	0.000534	1.841369
D-amino acid dehydrogenase small subunit	0.037999	1.835605
hypothetical protein	0.028474	1.790989
Cyclic di-GMP phosphodiesterase Gmr	0.027895	1.788715
hypothetical protein	0.000891	1.779365
hypothetical protein	0.012136	1.777392
Bacterial extracellular solute-binding proteins, family 3	0.00024	1.777296
hypothetical protein	0.007283	1.763998
hypothetical protein	0.000178	1.757953
hypothetical protein	0.012866	1.747065
NAD(P)H-quinone oxidoreductase subunit I, chloroplastic	0.042385	1.745586

Protein Name	p-value(treated vs. untreated)	Log2 value
putative glutathione S-transferase	0.041809	1.732352
hypothetical protein	0.002834	1.729244
tRNA (cytidine(34)-2'-O)-methyltransferase	0.006196	1.727332
Cation efflux system protein CusB precursor	0.017468	1.723869
inosine 5'-monophosphate dehydrogenase	0.000646	1.712899
hypothetical protein	0.013253	1.708532
TPR repeat-containing protein YfgC precursor	0.002116	1.694595
Cation efflux system protein CusA	0.001429	1.690793
putative ABC transporter solute-binding protein YclQ precursor	0.011195	1.68581
Protein FdhD	0.039658	1.683804
aromatic amino acid exporter	0.008881	1.681773
Methylglyoxal synthase	0.025456	1.676886
hypothetical protein	0.004857	1.676371
Acyl-CoA dehydrogenase	0.002664	1.671638
HTH-type transcriptional activator AllS	0.000207	1.64453
Inner membrane transport protein YdhP	0.000931	1.635583
Carboxymuconolactone decarboxylase family protein	0.001996	1.634412
putative major pilin subunit	0.000335	1.632557
NAD(P)H dehydrogenase (quinone)	0.000989	1.630126
hypothetical protein	0.013757	1.627831
Anti-sigma-E factor RseA	0.004341	1.617665
hypothetical protein	0.000297	1.615882
NADPH-flavin oxidoreductase	0.001419	1.611102
putative MFS-type transporter YcaD	0.014284	1.60827
Cold shock-like protein CspG	0.043795	1.597855

Protein Name	p-value(treated vs. untreated)	Log2 value
hypothetical protein	0.009517	1.597197
Thermostable monoacylglycerol lipase	0.000527	1.589663
General stress protein 14	0.000456	1.578634
Putative phosphoserine phosphatase 2	0.018994	1.569686
Aminopeptidase N	0.000907	1.56773
Peptidase family M3	0.006273	1.564915
hypothetical protein	0.000283	1.562728
hypothetical protein	0.002558	1.561165
Spore coat protein SA	0.005017	1.555168
Virulence protein	0.001785	1.54741
putative peroxiredoxin	0.000375	1.543229
Lysine-arginine-ornithine-binding periplasmic protein precursor	0.044008	1.542734
multidrug resistance protein D	0.035739	1.541748
putative sialic acid transporter	0.029932	1.53309
Branched-chain amino acid transport protein (AzlD)	0.008225	1.530116
3-mercaptopyruvate sulfurtransferase	0.032289	1.529896
Microbial serine proteinase precursor	0.037478	1.529496
Molybdenum transport system permease protein ModB	0.008305	1.521488
aromatic amino acid exporter	0.002854	1.520744
hypothetical protein	0.019298	1.520297
D-lactate dehydrogenase	0.00449	1.517956
Acetolactate synthase isozyme 3 large subunit	0.034121	1.49893
Periplasmic protein TorT precursor	0.031384	1.497648
L-threonine 3-dehydrogenase	0.031118	1.494661
NAD-dependent malic enzyme	0.002099	1.49017

Protein Name	p-value(treated vs. untreated)	Log2 value
Flavohemoprotein	0.001212	1.486632
putative formate transporter 1	0.02978	1.477931
Prolyl endopeptidase	0.002995	1.473397
Arsenate reductase	0.013544	1.472581
Right origin-binding protein	0.000812	1.458844
Acetolactate synthase isozyme 3 small subunit	0.005407	1.449075
Sensory transduction protein LytR	0.005656	1.44579
HTH-type transcriptional regulator BetI	0.001958	1.443012
Pyrimidine 5'-nucleotidase YjjG	0.000722	1.435634
Thioesterase superfamily protein	0.002334	1.432559
Transcriptional activator HlyU	0.001151	1.431265
Phytochrome-like protein cph2	0.000438	1.418244
putative siderophore transport system permease protein YfiZ precursor	0.023748	1.414872
hypothetical protein	0.000214	1.411437
Energy-coupling factor transporter ATP-binding protein EcfA3	0.005039	1.40414
Putative NAD(P)H-dependent FMN-containing oxidoreductase YwqN	0.004622	1.401609
Vibriobactin utilization protein ViuB	0.001182	1.396434
6-aminohexanoate-dimer hydrolase	0.00151	1.39279
Multidrug export protein MepA	0.000709	1.390838
ATP-dependent Clp protease ATP-binding subunit ClpA	0.000261	1.389528
hypothetical protein	0.007506	1.389429
Copper-exporting P-type ATPase A	0.028418	1.389198
Inner membrane protein alx	0.023679	1.373425
C4-dicarboxylate transport sensor protein DctB	0.038614	1.368595
Transposase IS200 like protein	0.004254	1.368489

Protein Name	p-value(treated vs. untreated)	Log2 value
2,3-dehydroadipyl-CoA hydratase	0.008924	1.364399
putative diguanylate cyclase YegE	0.002856	1.345629
Cytochrome oxidase maturation protein cbb3-type	0.025695	1.342651
NAD 5'-nucleotidase precursor	0.004541	1.337591
Nitrilotriacetate monooxygenase component A	0.00941	1.337329
Cytochrome b562	0.007794	1.332674
Phytochrome-like protein cph2	0.003233	1.322701
Acyl-CoA dehydrogenase	0.010007	1.317055
Phosphoglycolate phosphatase	0.019542	1.310486
Fe(3+) dicitrate transport protein FecA precursor	0.025707	1.310189
NMN amidohydrolase-like protein YfaY	0.00261	1.306921
Pyridoxine/pyridoxamine 5'-phosphate oxidase	0.003409	1.302319
L-fuculose phosphate aldolase	0.028593	1.300036
hypothetical protein	0.00044	1.298031
Chloramphenicol acetyltransferase	0.001612	1.292358
Pyruvate dehydrogenase E1 component subunit beta	0.047777	1.292352
hypothetical protein	0.017185	1.272764
hypothetical protein	0.024906	1.266733
MarR family protein	0.007911	1.261567
Spermidine N(1)-acetyltransferase	0.040109	1.256842
putative formate transporter 1	0.00586	1.251725
D-ribose-binding periplasmic protein precursor	0.001222	1.250107
Inner membrane protein YqaA	0.000309	1.248183
Ribosomal protein S6 modification protein	0.02751	1.242468
hypothetical protein	0.040785	1.227864

Protein Name	p-value(treated vs. untreated)	Log2 value
Histidine transport system permease protein HisM	0.04875	1.218236
murein L,D-transpeptidase	0.001637	1.217429
Psp operon transcriptional activator	0.000789	1.212376
hypothetical protein	0.013489	1.202994
Lipase (class 3)	0.015471	1.202869
Regulatory protein UhpC	0.000206	1.19813
Secreted effector protein pipB2	0.047761	1.195133
Microbial collagenase precursor	0.013356	1.19444
hypothetical protein	0.011877	1.18508
2,5-dichloro-2,5-cyclohexadiene-1,4-diol dehydrogenase	0.005179	1.182387
Molybdopterin molybdenumtransferase	0.001753	1.180186
Chaperone protein Skp precursor	0.00865	1.171277
Mechanosensitive channel MscK precursor	0.000762	1.165385
hypothetical protein	0.008472	1.164992
Cold shock-like protein CspD	0.015545	1.164664
putative siderophore transport system ATP-binding protein YusV	0.00273	1.162487
Multidrug resistance protein MexB	0.013078	1.16183
hypothetical protein	0.014891	1.155432
Penicillin-binding protein activator LpoB precursor	0.000305	1.153974
Acyl carrier protein phosphodiesterase	0.006518	1.150248
Outer membrane lipoprotein Blc precursor	0.044318	1.147873
hypothetical protein	0.001112	1.143184
Low conductance mechanosensitive channel YnaI	0.01151	1.141904
Molybdenum cofactor guanylyltransferase	0.014698	1.141524
Cyclic di-GMP phosphodiesterase response regulator RpfG	0.003686	1.13673

Protein Name	p-value(treated vs. untreated)	Log2 value
Phosphate import ATP-binding protein PstB 3	0.010749	1.136401
Oligopeptide transport ATP-binding protein OppF	0.020324	1.135115
Zinc carboxypeptidase	0.024526	1.134655
Thioesterase superfamily protein	0.012866	1.134444
HTH-type transcriptional regulator CueR	0.029067	1.130503
Oligoendopeptidase F, plasmid	0.001021	1.127679
Multidrug resistance protein MdtK	0.004348	1.127039
Chemotaxis protein methyltransferase	0.036377	1.123421
WD domain, G-beta repeat	0.010044	1.121546
hypothetical protein	0.020909	1.11838
Murein DD-endopeptidase MepM	0.000279	1.113694
Oligopeptide transport ATP-binding protein OppF	0.022439	1.107474
Alcohol dehydrogenase 2	0.000413	1.107125
Phosphatidylglycerophosphatase B	0.025233	1.105041
Cytochrome c-type protein TorC	0.01281	1.104223
L-fuculokinase	0.007517	1.103759
Putative oxidoreductase YceM	0.001195	1.089518
Lactoylglutathione lyase	0.017889	1.089234
Ribokinase	0.015275	1.088705
Formate--tetrahydrofolate ligase	0.001837	1.085785
putative chromosome-partitioning protein ParB	0.003632	1.078378
HTH-type transcriptional regulator CysL	0.00303	1.077318
Ribose operon repressor	0.001648	1.076648
putative N-acetyltransferase YafP	0.00302	1.075231
Aspartate-semialdehyde dehydrogenase 1	0.000175	1.074581

Protein Name	p-value(treated vs. untreated)	Log2 value
2-oxoglutarate dehydrogenase E1 component	0.045046	1.068451
Ribosomal large subunit pseudouridine synthase D	0.000896	1.064069
hypothetical protein	0.028914	1.063703
glutaredoxin 2	0.002549	1.063379
Porin-like protein L precursor	0.000412	1.061237
Putative multidrug export ATP-binding/permease protein	0.017828	1.060649
hypothetical protein	0.005197	1.051539
Glutamine transport ATP-binding protein GlnQ	0.025258	1.04466
hypothetical protein	0.005418	1.042665
ABC transporter ATP-binding protein uup	0.003084	1.041117
hypothetical protein	0.007879	1.037171
Bicyclomycin resistance protein	0.011748	1.036123
Inner membrane amino-acid ABC transporter permease protein YhdY	0.0103	1.032997
Aromatic-amino-acid aminotransferase	0.003707	1.030696
hypothetical protein	0.000304	1.02905
Patatin-like phospholipase	0.002345	1.027239
EAL domain protein	0.017716	1.026233
Thiol:disulfide interchange protein DsbL	0.004064	1.021863
Zinc carboxypeptidase	0.001651	1.021032
Peptidoglycan-associated lipoprotein precursor	0.000389	1.020107
Nuclease SbcCD subunit C	0.002468	1.018257
Protoheme IX farnesyltransferase	0.01374	1.015726
Na(+)/H(+) antiporter NhaB	0.043121	1.012218
YhhN-like protein	0.004988	1.010866
hypothetical protein	0.01183	1.006355

Protein Name	p-value(treatedvs. untreated)	Log2 value
Downregulated		
Flp/Fap pilin component	0.00095	4.436428
putative MFS-type transporter YhjX	0.000642	4.434174
hypothetical protein	0.001485	3.60959
Nucleoside permease NupX	0.000884	3.121107
hypothetical protein	0.000632	2.980769
SnoaL-like domain protein	0.033899	2.779718
Periplasmic nitrate reductase, electron transfer subunit precursor	0.000189	2.761006
Cytochrome c-type protein NapC	0.000764	2.662021
putative amino-acid import ATP-binding protein YxeO	0.000247	2.476978
Hypoxanthine phosphoribosyltransferase	0.00011	2.462144
hypothetical protein	0.020624	2.459028
Efflux pump periplasmic linker BepF	0.015216	2.426399
Bicarbonate transporter BicA	0.007881	2.412516
Metallothiol transferase FosB	0.000109	2.412153
hypothetical protein	0.011567	2.408633
hypothetical protein	0.001024	2.344953
HTH-type transcriptional regulator BetI	0.001305	2.341217
hypothetical protein	0.017686	2.262755
flagellar protein FlaG	0.001187	2.199547
tRNA (guanosine(18)-2'-O)-methyltransferase	0.004006	2.186916
hypothetical protein	0.000232	2.186184
putative antibiotic transporter	0.004882	2.151752
Serine/threonine-protein kinase PrkC	0.000144	2.142103
hypothetical protein	0.046676	2.12619

Protein Name	p-value(treated vs. untreated)	Log2 value
hypothetical protein	0.0013	2.113297
putative amino-acid permease protein YxeN	0.004234	2.113204
hypothetical protein	0.000128	2.107062
Siroheme synthase	0.000505	2.093557
Pilus assembly protein, PilP	0.028126	2.051024
Sulfite reductase [NADPH] flavoprotein alpha-component	0.000697	2.043134
Lichenan permease IIC component	0.033603	2.017619
Outer membrane protein A precursor	0.000244	2.010304
Threonine efflux protein	0.006135	1.968345
HTH-type transcriptional activator AllS	0.000386	1.962134
O-acetyltransferase OatA	0.000114	1.95471
Glutamine synthetase	0.00106	1.952505
DNA-binding protein Fis	0.001048	1.94102
Inositol-1-monophosphatase	0.00129	1.93864
hypothetical protein	0.005615	1.937472
putative amino-acid-binding protein YxeM precursor	0.000815	1.936131
tRNA modification GTPase MnmE	0.000138	1.9268
ATP-binding region	0.000354	1.918863
Transketolase 1	0.000102	1.880713
L-2,4-diaminobutyric acid acetyltransferase	0.001298	1.860184
Sulfate adenylyltransferase subunit 2	0.000698	1.85289
Sulfate adenylyltransferase subunit 1	0.000965	1.851215
MltA-interacting protein MipA	0.000646	1.838847
hypothetical protein	0.002213	1.807656
HTH-type transcriptional regulator YidZ	0.001763	1.798187

Protein Name	p-value(treated vs. untreated)	Log2 value
Cytochrome c5	0.003438	1.782593
ECF sigma factor	0.000685	1.777801
Ferredoxin--NADP reductase	0.003382	1.769894
Inner membrane transport protein YdhC	0.000332	1.764703
Phosphate transport system permease protein PstC	0.000421	1.747443
Type IV leader peptidase family protein	0.000834	1.729735
PemK-like protein	0.013064	1.727254
tRNA-dihydrouridine synthase C	0.005913	1.705637
Virulence regulon transcriptional activator VirF	0.000661	1.69643
Flagellar hook-associated protein 3	0.000672	1.694372
NAD/NADP-dependent betaine aldehyde dehydrogenase	0.00017	1.687916
hypothetical protein	0.006968	1.680725
Membrane fusogenic activity	0.000347	1.668087
putative acyltransferase YihG	0.004298	1.657183
HTH-type transcriptional regulator PrtR	0.000743	1.656327
hypothetical protein	0.005816	1.65312
Guanine/hypoxanthine permease PbuG	0.000371	1.65311
Peptidase family U32	0.008593	1.649574
hypothetical protein	0.015199	1.647715
Chromosome initiation inhibitor	0.001705	1.639167
hypothetical protein	0.003189	1.638644
Thiazole synthase	0.002	1.635174
putative glutamine ABC transporter permease protein GlnM	0.019126	1.622073
Fimbrial protein precursor	0.006755	1.621215
HDOD domain protein	0.000813	1.61829

Protein Name	p-value(treated vs. untreated)	Log2 value
Nucleoside recognition	0.00076	1.615605
Sulfite reductase [NADPH] hemoprotein beta-component	0.000185	1.612235
Methyl-accepting chemotaxis protein PctC	0.000236	1.609041
hypothetical protein	0.000218	1.60566
lipopolysaccharide core biosynthesis protein	0.000384	1.59776
Malate-2H(+)/Na(+)-lactate antiporter	0.003736	1.590022
3-dehydroquinate dehydratase	0.006231	1.588378
Acetylglutamate kinase	0.013716	1.585938
hypothetical protein	0.000168	1.585713
Inner membrane protein YjjP	0.025117	1.582672
hypothetical protein	0.003003	1.567117
Peptide deformylase 1	0.000584	1.564729
Zinc import ATP-binding protein ZnuC	0.000152	1.559888
Methyl-accepting chemotaxis protein McpS	0.000572	1.550605
Xanthine phosphoribosyltransferase	0.021371	1.548506
50S ribosomal protein L34	0.011983	1.547721
S-(hydroxymethyl)glutathione dehydrogenase	0.008544	1.542213
Flagellin D	0.006687	1.542184
Aspartate carbamoyltransferase	0.001278	1.541941
putative diguanylate cyclase YcdT	0.000146	1.539313
DNA recombination protein RmuC	0.00217	1.533977
Glucosamine-6-phosphate deaminase	0.000611	1.53299
Met repressor	0.003362	1.532826
Sulfite reductase [NADPH] flavoprotein alpha-component	0.001514	1.525633
hypothetical protein	0.00568	1.522141

Protein Name	p-value(treated vs. untreated)	Log2 value
D-threonine aldolase	0.001066	1.520633
TrkA-C domain protein	0.008226	1.513875
Regulatory protein AsnC	0.024176	1.512004
hypothetical protein	0.005361	1.510876
Putative aminoacrylate hydrolase RutD	0.000135	1.507059
FIST N domain protein	0.000665	1.503339
Uracil permease	0.001812	1.499996
Putative malate transporter YflS	0.004345	1.491053
Phosphoadenosine phosphosulfate reductase	0.002825	1.490678
LexA repressor	0.000184	1.47921
macrolide transporter ATP-binding /permease protein	0.003442	1.479029
Cell division protein ZapB	0.024045	1.476791
S-formylglutathione hydrolase YeiG	0.009819	1.466455
Bacterial extracellular solute-binding proteins, family 3	0.010973	1.465274
Sel1 repeat protein	0.001116	1.462492
Ribosome-binding factor A	0.007396	1.460507
hypothetical protein	0.00219	1.453428
N5-carboxyaminoimidazole ribonucleotide mutase	0.001114	1.451863
Flagellar hook-associated protein 1	0.004045	1.44897
Nitrogen regulation protein NR(I)	0.000326	1.440692
hypothetical protein	0.007393	1.433584
Type II secretion system protein J precursor	0.043302	1.430858
3-oxoadipate enol-lactonase 2	0.000283	1.427719
DTW domain protein	0.006591	1.426463
Glycine--tRNA ligase alpha subunit	0.001493	1.425379

Protein Name	p-value(treated vs. untreated)	Log2 value
hypothetical protein	0.011405	1.41624
Biotin carboxyl carrier protein of acetyl-CoA carboxylase	0.000134	1.415472
Sulfate transport system permease protein CysW	0.023677	1.41478
Flagellar hook-associated protein 2	0.000395	1.411876
putative peptidoglycan biosynthesis protein MurJ	0.006178	1.406586
Methyl-accepting chemotaxis protein PctC	0.000386	1.396927
hypothetical protein	0.039643	1.393608
2-iminoacetate synthase	0.003417	1.391465
Inner membrane protein YhhQ	0.007615	1.387914
Inosine-5'-monophosphate dehydrogenase	0.014335	1.379488
Protein PsiE	0.003737	1.375217
hypothetical protein	0.021389	1.371252
hypothetical protein	0.047935	1.366616
Oxygen-dependent choline dehydrogenase	0.005756	1.363737
Glycine betaine transporter OpuD	0.007005	1.358087
hypothetical protein	0.001927	1.357175
Tyrosine-protein kinase wzc	0.001464	1.354136
hypothetical protein	0.002067	1.353109
Glycerol-3-phosphate regulon repressor	0.000617	1.352058
Undecaprenyl-phosphate alpha-N-acetylglucosaminyl 1-phosphate transferase	0.02171	1.351351
hypothetical protein	0.025967	1.346078
Bifunctional purine biosynthesis protein PurH	0.001567	1.343499
High-affinity zinc uptake system membrane protein ZnuB	0.007676	1.341451
HDOD domain protein	0.000388	1.337374
Acetolactate synthase isozyme 2 large subunit	0.0028	1.336838

Protein Name	p-value(treated vs. untreated)	Log2 value
Flagellar protein FliS	0.000597	1.336632
Shikimate kinase 1	0.001069	1.335312
Cyclodextrin-binding protein precursor	0.01756	1.331957
Methyl-accepting chemotaxis protein PctC	0.003868	1.330283
hypothetical protein	0.004305	1.329835
hypothetical protein	0.008452	1.329761
Glycine cleavage system transcriptional activator	0.00223	1.329669
Thiamine-phosphate synthase	0.002036	1.324125
Ribosomal RNA small subunit methyltransferase D	0.000446	1.323255
Low molecular weight protein-tyrosine-phosphatase wzb	0.004884	1.32142
50S ribosomal protein L33	0.004317	1.321305
O-Antigen ligase	0.006125	1.320132
Ribosomal protein L11 methyltransferase	0.007442	1.319358
hypothetical protein	0.00113	1.317761
Methyl-accepting chemotaxis protein PctB	0.00117	1.314099
50S ribosomal protein L32	0.011957	1.313072
hypothetical protein	0.001485	1.31241
Ribosomal RNA large subunit methyltransferase J	0.000544	1.310753
Lipid A biosynthesis lauroyl acyltransferase	0.003049	1.308891
Methyl-accepting chemotaxis protein PctB	0.008052	1.308774
Inner membrane ABC transporter permease protein YejB	0.017599	1.306624
glycine betaine transporter periplasmic subunit	0.000685	1.304499
DNA utilization protein GntX	0.041376	1.299104
Cyclohexadienyl dehydratase precursor	0.008466	1.298347
DNA-directed RNA polymerase subunit omega	0.003	1.295476

Protein Name	p-value(treated vs. untreated)	Log2 value
hypothetical protein	0.006703	1.294459
Competence protein ComM	0.00407	1.291439
putative inner membrane transporter yiJE	0.027002	1.290053
DNA polymerase I	0.000391	1.283442
Cytochrome bd-I ubiquinol oxidase subunit X	0.020589	1.277854
OpgC protein	0.010839	1.277027
Coproporphyrinogen-III oxidase, aerobic	0.00799	1.276395
putative glycosyltransferase EpsJ	0.001476	1.274286
Elongation factor P	0.014902	1.27382
Murein DD-endopeptidase MepM	0.018937	1.270332
O-Antigen ligase	0.001016	1.269835
Alpha-amylase precursor	0.021109	1.263876
Membrane protein insertase YidC	0.000369	1.261447
hypothetical protein	0.012291	1.261278
Protein TusB	0.027949	1.25967
Flagellar basal-body rod protein FlgF	0.002078	1.259592
hypothetical protein	0.028763	1.256612
hypothetical protein	0.042351	1.254884
hypothetical protein	0.000924	1.253015
N-acetyl-gamma-glutamyl-phosphate reductase	0.022708	1.252882
GTP-binding protein TypA/BipA	0.004441	1.251689
3-deoxy-D-manno-octulosonic acid kinase	0.001245	1.249021
ATP-dependent DNA helicase Rep	0.00241	1.2451
hypothetical protein	0.033846	1.243748
hypothetical protein	0.007154	1.242584

Protein Name	p-value(treated vs. untreated)	Log2 value
alanyl-tRNA synthetase	0.002075	1.242523
hypothetical protein	0.000515	1.242346
RNA-binding protein YhbY	0.002261	1.240876
Peptidase C13 family protein	0.004657	1.239954
hypothetical protein	0.000345	1.238652
Inner membrane protein YrbG	0.011764	1.237319
Inner membrane ABC transporter permease protein YejB	0.010124	1.226909
putative diguanylate cyclase YegE	0.00021	1.225602
Nodulation protein D 2	0.047811	1.223497
Ribonuclease P protein component	0.006446	1.222143
hypothetical protein	0.01197	1.221333
Guanylate kinase	0.04101	1.221221
hypothetical protein	0.003114	1.22119
1,6-anhydro-N-acetylmuramyl-L-alanine amidase AmpD	0.006372	1.219438
1-acyl-sn-glycerol-3-phosphate acyltransferase	0.002468	1.21862
Guanosine-3',5'-bis(diphosphate) 3'-pyrophosphohydrolase	0.005918	1.217944
Sulfur carrier protein ThiS adenylyltransferase	0.002007	1.217553
Ribosomal S4P (gammaproteobacterial)	0.005667	1.21661
hypothetical protein	0.016303	1.215579
hypothetical protein	0.03143	1.213646
Daunorubicin/doxorubicin resistance ATP-binding protein DrrA	0.003316	1.210507
Exodeoxyribonuclease 7 large subunit	0.022285	1.208617
Putative type II secretion system protein I precursor	0.03788	1.206324
hypothetical protein	0.001296	1.203565
Long-chain fatty acid transport protein precursor	0.000833	1.199493

Protein Name	p-value(treated vs. untreated)	Log2 value
Phospho-2-dehydro-3-deoxyheptonate aldolase, Tyr-sensitive	0.000451	1.188781
hypothetical protein	0.006311	1.18579
hypothetical protein	0.000805	1.184991
Nitrogen regulation protein NR(II)	0.000334	1.183067
Ornithine carbamoyltransferase	0.020166	1.182991
GMP synthase [glutamine-hydrolyzing]	0.015589	1.182222
hypothetical protein	0.000742	1.181408
Cysteine synthase A	0.011001	1.178632
tRNA/tmRNA (uracil-C(5))-methyltransferase	0.016707	1.178135
Thymidine kinase	0.000139	1.177573
Methionyl-tRNA formyltransferase	0.000774	1.177567
Type II secretion system protein C	0.012256	1.17571
D-amino acid dehydrogenase small subunit	0.000228	1.17385
Hydrogen peroxide-inducible genes activator	0.027038	1.170899
Iron-sulfur cluster insertion protein ErpA	0.017533	1.168559
HTH-type transcriptional regulator CysL	0.042586	1.167152
Der GTPase-activating protein YihI	0.000455	1.166208
hypothetical protein	0.004744	1.164754
Anaerobic C4-dicarboxylate transporter DcuB	0.006362	1.164722
hypothetical protein	0.0089	1.159112
Transcriptional activator CadC	0.000571	1.158524
50S ribosomal protein L27	0.001809	1.155898
DNA helicase II	0.000933	1.154823
L-threonine dehydratase biosynthetic IlvA	0.005765	1.152443
Putative nucleotidyltransferase substrate binding domain protein	0.001618	1.150969

Protein Name	p-value(treated vs. untreated)	Log2 value
Glycine--tRNA ligase beta subunit	0.000603	1.150404
putative ABC transporter ATP-binding protein YheS	0.002441	1.150176
Cell division ATP-binding protein FtsE	0.017158	1.147834
hypothetical protein	0.009444	1.147046
Sulfur carrier protein ThiS	0.014877	1.146655
Multidrug resistance protein MdtA precursor	0.002567	1.143531
Cyclic di-GMP phosphodiesterase response regulator RpfG	0.014947	1.142884
hypothetical protein	0.014077	1.141223
Argininosuccinate synthase	0.013615	1.139469
putative lipoprotein GfcB precursor	0.00968	1.138218
Ribosomal large subunit pseudouridine synthase A	0.000746	1.137176
Type II secretion system protein M	0.003802	1.13669
dTDP-4-dehydrorhamnose reductase	0.000469	1.136677
General secretion pathway, M protein	0.003846	1.134635
Cobalamin biosynthesis protein CbiB	0.019695	1.134228
hypothetical protein	0.007187	1.13076
Glutamate-pyruvate aminotransferase AlaA	0.000211	1.130285
hypothetical protein	0.002936	1.129059
Aspartate/alanine antiporter	0.015096	1.125869
Sel1 repeat protein	0.000202	1.125201
putative ABC transporter ATP-binding protein/MT1014	0.003868	1.121367
Putative type II secretion system protein K	0.003753	1.120982
Phosphoglycolate phosphatase	0.003716	1.118971
Biotin carboxylase	0.000138	1.116704
ATP-dependent DNA helicase RecQ	0.003055	1.116671

Protein Name	p-value(treated vs. untreated)	Log2 value
Multidrug resistance protein MdtL	0.005266	1.113347
Phosphomethylpyrimidine synthase	0.005582	1.112467
aromatic amino acid exporter	0.003888	1.107775
Flagellar protein FliT	0.004494	1.107674
2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase	0.010403	1.10675
Cytosol aminopeptidase	0.000308	1.105209
Thiamine-monophosphate kinase	0.02994	1.103269
ATP-dependent RNA helicase RhlB	0.000533	1.099861
tRNA N6-adenosine threonylcarbamoyltransferase	0.004057	1.099692
3-deoxy-D-manno-octulosonic acid transferase	0.002043	1.09842
D-methionine transport system permease protein MetI	0.005043	1.097139
PTS system glucose-specific EIICB component	0.000311	1.093506
Na(+)/H(+) antiporter NhaA	0.023012	1.092695
hypothetical protein	0.003873	1.09214
CobQ/CobB/MinD/ParA nucleotide binding domain protein	0.049537	1.091273
putative DNA endonuclease SmrA	0.006342	1.090786
tRNA-(MS[2]IO[6]A)-hydroxylase (MiaE)	0.04517	1.089376
HTH-type transcriptional regulator MhqR	0.035763	1.089139
Insulinase (Peptidase family M16)	0.000794	1.085091
NADPH-dependent 7-cyano-7-deazaguanine reductase	0.003409	1.084146
Dihydroxy-acid dehydratase	0.001582	1.083057
Acetolactate synthase isozyme 2 small subunit	0.016859	1.083002
Translation initiation factor IF-2	0.003798	1.081939
6-phosphofructokinase isozyme 1	0.023688	1.080815
Dipeptide transport system permease protein DppC	0.022312	1.07846

Protein Name	p-value(treated vs. untreated)	Log2 value
Inner membrane protein YbaL	0.014056	1.078056
50S ribosomal protein L31	0.042225	1.076798
Type II secretion system protein E	0.002692	1.076388
Sodium-dependent dicarboxylate transporter SdcS	0.017889	1.07319
putative diguanylate cyclase AdrA	0.031103	1.070671
hypothetical protein	0.001433	1.068967
Monocarboxylate 2-oxoacid-binding periplasmic protein precursor	0.028491	1.068258
hypothetical protein	0.004384	1.067742
Putative gluconeogenesis factor	0.00096	1.063551
Cytoskeleton protein RodZ	0.000308	1.06208
Lipopolysaccharide core biosynthesis protein RfaG	0.000175	1.060123
Flagellar L-ring protein precursor	0.001924	1.057762
N5-carboxyaminoimidazole ribonucleotide synthase	0.013311	1.057208
Sensor protein CpxA	0.001006	1.057034
CDP-diacylglycerol--serine O-phosphatidyltransferase	0.002296	1.055744
Protoporphyrinogen IX dehydrogenase [menaquinone]	0.001187	1.0553
GTPase Obg/CgtA	0.009348	1.054529
putative adenine permease PurP	0.000744	1.052931
tRNA (guanine-N(7)-)-methyltransferase	0.045495	1.051866
hypothetical protein	0.011996	1.050474
Methyl-accepting chemotaxis protein PctB	0.002342	1.050341
hypothetical protein	0.000195	1.050174
hypothetical protein	0.003671	1.049429
Adenylyl-sulfate kinase	0.003774	1.047915
Membrane-bound lytic murein transglycosylase C precursor	0.006463	1.002919