CHARACTERISATION AND GENOME ANALYSIS OF NOVEL QUORUM SENSING SOIL BACTERIA

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Field of Study: Genetics and Molecular Biology

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ABSTRACT

Quorum sensing (QS) describes bacterial communication using signalling molecules. QS is important for regulation of various gene expressions, most notably for the survival and adaptation of a population of bacteria to the surrounding environment. The objective of this study was to isolate and characterise novel QS bacteria. Preliminary screening of QS activity was performed using both *Chromobacterium violeceum* CVO26 and *Escherichia coli* [pSB401] biosensors. Isolates exhibiting QS activity were subsequently identified using polyphasic identification methods and their QS profile was eventually characterised using mass spectrometry. Out of 60 isolates, only two novel QS isolates were selected for further investigation. The first novel QS bacterium was *Chania multitudinisentens* RB-25^T which was a newly proposed genus in the family of *Enterobacteriaceae*. This isolate was found to produce 3 AHLs: C4-HSL, C6-HSL and 3-oxo-C6-HSL. Various taxonomical analyses (for example phenotypic and genotypic characterization) according to Bergey's Manual of Systematic Bacteriology had been conducted to characterise this isolate. The complete genome sequences unveiled presence of two pairs of *luxI/R* in *C. multitudinisentens* RB-25^T and gene cloning performed validated the AHL synthesis activities of these AHL synthases. This discovery added *C. multitudinisentens* RB-25^T to the increasing documentation of QS members in the phylum of *Proteobacteria*. The second novel QS bacterium isolated was strain RB-38 which was later identified as *Pandoraea pnomenusa*. Mass spectrometry analysis revealed that C8-HSL was the only signaling molecules detected from the spent supernatant of *P. pnomenusa* RB38. As this was the first documentation of QS activity in *Pandoraea* genus, we sequenced the complete genome of *P. pnomenusa* RB-38 to study its QS synthase. A set of *luxI/R* (designated as *ppnI/R1*) and an orphan *ppnR2* were identified from the genome in which functional studies of PpnI conducted confirmed its role in the production of C8-HSL.

ABSTRAK

Pengesanan kuorum (QS) menggambarkan komunikasi antara sel-sel bacteria menggunakan molekul isyarat. QS sangat penting untuk mengawal ekspresi pelbagai jenis gen, terutamaya gen yang mengawal kemandirian dan penyesuaian bakteria dalam satu populasi. Objektif kajian ini adalah untuk mencari dan mengkaji ciri-ciri bakteria novel. Pemeriksaan awal aktiviti QS dijalankan dengan menggunakan kedua-dua *Chromobacterium violeceum* CVO26 dan *Escherichia coli* [pSB401] biopenderia. Bacteria yang mempamerkan aktiviti QS kemudiannya dikenal pasti menggunakan kaedah identifikasi polifasa dan profil QS mereka akhirnya dicirikan menggunakan spektrometri jisim. Daripada 60 bakteria, hanya dua bakteria yang novel ditumpukan untuk siasatan lanjutan. Bakteria QS novel yang pertama ialah *Chania multitudinisentens* RB-25^T yang merupakan genus baru dalam keluarga *Enterobacteriaceae*. Isolat ini mampu menghasilkan 3 AHLs: C4-HSL, C6-HSL dan 3-oxo-C6-HSL. Pelbagai analisis taksonomi (sebagai contohnya: pencirian fenotip dan genotip) mengikut Manual Bergey dalam Sistematik Bakteriologi yang telah dijalankan untuk mencirikan isolat ini. Dua pasang *luxI/R* telah dikenal pasti mengesahkan bahawa mereka adalah gen yang menghasikan isyarat molekul. Penemuan ini telah menambah *C. multitudinisentens* RB- 25^T dalam pertumbuhan dokumentasi organisme QS dalam filum *Proteobacteria*. Bakteria QS novel kedua yang diasingkan adalah isolat RB-38 yang kemudiannya dikenal pasti sebagai *Pandoraea pnomenusa*. Analisis spektrometri jisim menujukkan bahawa C8-HSL adalah satu-satunya molekul isyarat dikesan daripada supernatan *P. pnomenusa* RB38. Kemudiannya, kita melangsungkan penjujukan genom *P. pnomenusa* RB-38 untuk mengkaji gen QS-nya kerana ini adalah dokumentasi aktiviti QS yang pertama dalam genus *Pandoraea*. Satu set *luxI/R* (dikenali sebagai *ppnI/R1*) dan satu yatim *ppnR2* telah dikenal pasti di mana fungsi PpnI telah mengesahkan peranannya dalam produksi C8-HSL.

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LIST OF SYMBOLS AND ABBREVATIONS

ACN: Acetonitrile
AGE: Agarose gel electrophoresis
AHL: *N*-acyl homoserine lactone
ANI: Average Nucleotide Identity
Biolog: Biolog Omnilog Identification Sytem
BLAST: Basic local alignment search tool
dH_2O: Distilled water
et. al.: Latin word of "et alia" which means "and others"
FAME: Fatty acid methyl esters
h: Hour
HMDS: Hexamethyldisilazane
LB: Luria-Bertani
LCMS/MS: Triple Quadrupole Liquid Chromatography Mass Spectrometry
m/z: Mass/Charge
MALDI-TOF: Matrix assisted laser desorption ionization time of flight
mL: Millilitre
MLSA: Multi Locus Sequence Analysis
MOPS: 3-[*N*-morpholino]propanesulfonic acid
MS: Mass Spectrometry
NaCl: Sodium Chloride
NCBI: National Center for Biotechnology Information
NGS: Next Generation Sequencing
ORF: Open Reading Frame
PacBio: Pacific Biosciences
PBS: Phosphate Buffer Saline
PCR: Polymerase Chain Reaction
PM: Phenotype Microarray
QS: Quorum Sensing
QQ: Quorum Quenching
SAM: *S*-adenosyl methionine
SB: Sequencing by Synthesis
SMRT: Single Molecule Real Time sequencing
TBE: Tris borate EDTA
TLC: Thin layer chromatography
UHPLC: Ultra High Performance Liquid Chromatography
WGS: Whole genome sequencing
ZMW: Zero-mode wavelength
°C: Degree Celsius

LIST OF APPENDIX

CHAPTER 1: INTRODUCTION

Facilitated by diffusible signaling molecules for "sensing", quorum sensing (QS) is a cell density dependent mechanism when a bacterial population density attained its threshold concentration, it will activate a cascade of various downstream bacterial gene expressions (Fuqua, Parsek, & Greenberg, 2001; Williams, Winzer, Chan, & Camara, 2007). Some prominent phenotypes under QS regulation are production of virulence factors, proteases, exoenzymes, exotoxins, biofilm formation, swarming and swimming motility (Williams, 2007).

The importance of QS research underlies in the QS regulated gene expression of virulence factors. Various human pathogens (for instance *P. aeruginosa*, *Burkholderia cepacia* complex (Bcc), *Burkholderia pseudomallei*) and plant pathogens (for instance *Erwinia carotovora*, *Agrobacterium tumefaciens*) have been documented to employ QS during the course of pathogenicity and infection (de Kievit & Iglewski, 2000). As antibiotic resistance is a growing threat, anti-QS approaches targeting at virulence determinants instead of the viability of the pathogens is an alternative to the exhausting spectrum of antibiotic (Grandclément, Tannières, Moréra, Dessaux, & Faure, 2015).

Hence, more investigations are required to identity and characterize novel QS isolates and to study their QS-regulated phenotypes before the application of anti-QS approaches could be fully exploited. In this study, a former municipal landfill site in Ayer Hitam, Puchong, Malaysia was selected as my sampling site with the objective to isolate novel QS bacteria as this soil habitat has not been well studied.

Two major discoveries in this study were (i) the isolation of a newly proposed genus of *Chania* gen. nov. with QS activities (*Chania multitudinisentens* RB-25^{T}) and (ii) first documentation of QS activities in *Pandoraea* genus (*Pandoraea pnomenusa* RB-38). As *Pandoraea* is an understudied opportunistic pathogen in

immunocompromised and cystic fibrosis patients, we hypothesized that clinical *Pandoraea* spp. exploited QS for infection and pathogenicity. Hence, later part of this study was further advanced into characterizing QS activities in *Pandoraea* genus.

The objectives of this research study are:

1. To isolate novel QS bacteria from a former municipal landfill site.
2. To characterize the QS profile of the isolated novel QS bacteria.
3. To characterize the QS genes (*luxIR*) of the novel QS bacteria.
4. To perform genomic and taxonomic characterization of isolate RB-25.
5. To investigate the QS activity in *Pandoraea* spp.

CHAPTER 2: LITERATURE REVIEW

2.1 Ayer Hitam Sanitary Landfill

Ayer Hitam Sanitary Landfill is a ceased operational sanitary landfill site undergoing landfill closure and post-closure maintenance activity after it reached its maximum capacity in December 2006 (Ayer Hitam Sanitary landfill, 2015). Operated by Worldwide Landfills Sdn. Bhd for 11 years, Ayer Hitam Sanitary Landfill was built on 100 acres area in Air Hitam Forest Reserve, Puchong Selangor and deposited 6.27 million tonne of domestic waste from Klang Valley areas. (Figure 2.1).

Figure 2.1. Google image of Ayer Hitam Sanitary Landfill, retrieved on February 2016. The site is currently transformed into a public recreational park known as Worldwide Landfills Park.

Few studies have been conducted to investigate the soil and leachate composition of Ayer Hitam Sanitary Landfill (Aizat, Roslan, Sulaiman, & Karam, 2014; Salleh & Hamid, 2013). However, there is only one microbial investigation conducted by Tripathi *et al.* (2012) to study the effect of land use and soil pH in shaping the bacterial community in Ayer Hitam Sanitary Landfill in which *Acidobacteria* was found to constitute the most abundant bacterial taxa followed by *Alphaproteobacteria*, *Actinobacteria* and *Gammaproteobacteria*.

2.2 QS

It was a long belief that unicellular bacteria are able to perform a cooperative behaviour for the advantage of adaptation to the surrounding environment. The first evidence was documented by Nealson, Platt, and Hastings (1970) with the description of bioluminescence activity in *Vibrio harveryi* although at the time of writing, the authors could not explained the molecular biology behind the phenomenon. The authors described the finding as:

"The control of the appearance of luminescence during the growth cycle is a spectacular but poorly described and ill-understood feature of the bioluminescent bacteria. The matter of interest in this paper is that the growth of the bacteria and the development of luminescence do not occur in concert in liquid cultures. The development of light emission is delayed and starts only during the middle of the logarithmic phase of growth. Its rise is then far more rapid than is the increase in cell mass during that time."

The generic term QS was later coined to describe this cell density dependent mechanism of communication between unicellular neighbouring cells for an onset of unison and coordinated behaviour using signaling compounds as the modulator (Fuqua, Winans, & Greenberg, 1994). The significance of this communication is crucial to initiate a collective bacterial migration to an environment with better nutrient access or when a specific environment is no longer conducive; for cooperative defence against host's immunity response or competing prokaryotes and to facilitate morphological differentiation to tackle a specific environmental threats (Williams et al., 2007).

QS circuits are governed by two principle protein homologues: autoinducer synthase (also known as LuxI) protein families and transcriptional regulators (also known as LuxR) protein families. Signaling compound is synthesized by LuxI and will

diffuse out of the bacteria cells into the exogenous environment by concentration gradient or active efflux for "sensing" purpose (Figure 2.2). When a bacterial density is high, the accumulation of signaling molecules increases proportionally in which upon achieving a threshold concentration, these signaling compounds will diffuse back into the cells and bind to LuxR communicating within the population that the "quorate" has been attained. The signaling molecule-bound-LuxR protein will subsequently function as cytoplasmic regulators, either as transcriptional activator or repressor to regulate various downstream QS-mediated gene expression leading to a cooperative behaviour of the bacteria population (Cooley, Chhabra, & Williams, 2008; Fuqua et al., 2001; Zhang et al., 2002).

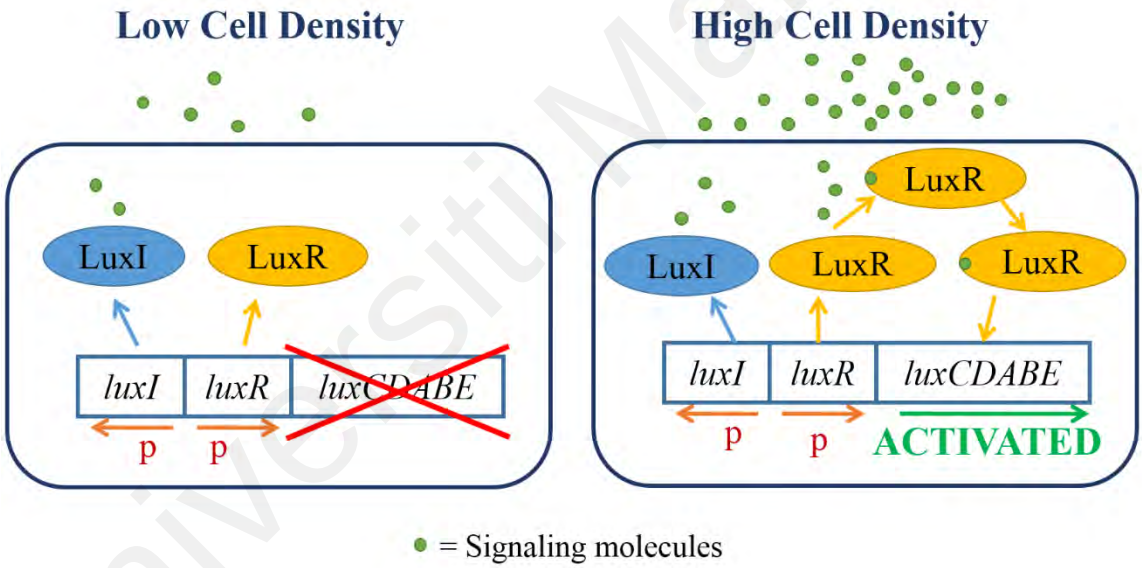

Figure 2.2. Mechanism of QS circuits. At low cell density, signaling molecules synthesized by LuxI diffuse away because the concentration of the signaling molecules is below the required threshold concentration to be detected by LuxR. When the cell density is high, the signaling molecules will diffuse back into the cell upon attaining a threshold concentration and bind to the cognate LuxR mediating a collective behaviour in the bacteria population.

2.3 *N*-Acyl Homoserine Lactone-Mediated QS in Gram Negative Bacteria

To date, *N*-acyl homoserine lactone (AHL) is the most characterized QS signaling molecules produced exclusively by alpha-, beta- and gamma-class of Gram negative *Proteobacteria* (Williams et al., 2007). Structural diversity of AHLs is highly conserved which share a homoserine lactone ring moiety unsubstituted in the *β*- and *γ*-positions with an amide (*N*)-linked acyl side chain at the *α*-position consisting of fatty acids which varies in length (in most cases C4-C18), degree of saturation, and the presence of substituent which could either be hydroxy- or oxo- group at the C3 position (Chhabra et al., 2005) (Figure 2.3). AHL are synthesized by LuxI protein family through a sequential reaction mechanism in which the homoserine lactone ring moiety and acyl side chain was acquired from *S*-adenosylmethionine (SAM) and acylated acyl carrier protein (Acyl-ACP) respectively (Parsek, Val, Hanzelka, Cronan, & Greenberg, 1999) (Figure 2.4).

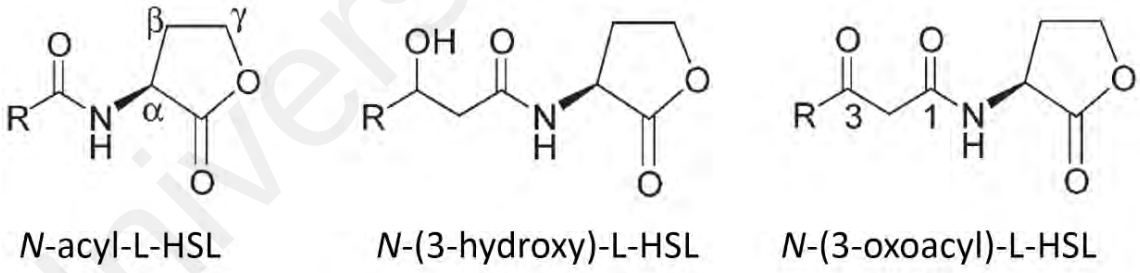

Figure 2.3. Structural diversity of AHL signaling molecules (Chhabra et al., 2005).

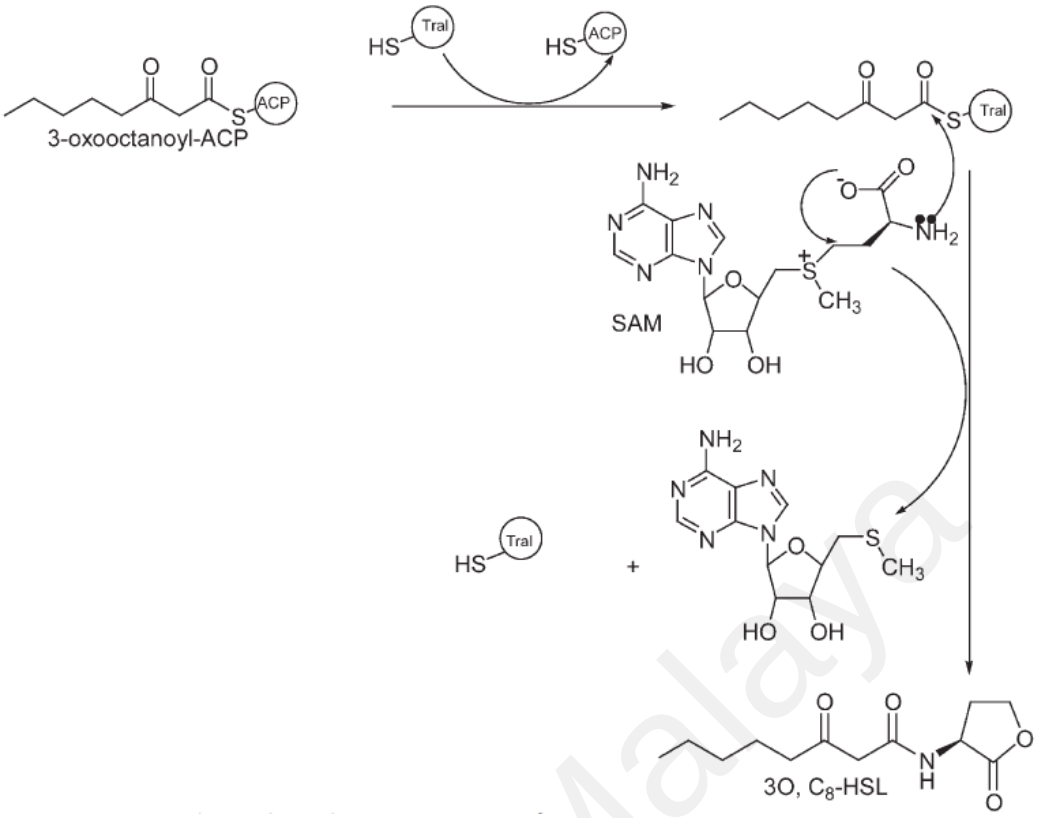

Figure 2.4. Schematic diagram of AHL synthesis by LuxI family protein – Production of 3-oxo-C8-HSL by TraI. TraI binds to 3-oxooctanoyl-ACP to catalyse the donation of acyl group releasing ACP. Subsequently, TraI binds to SAM to catalyse the formation of amide bond between amino group of SAM and the acyl group producing 3-oxo-C8-HSL and 5'-methyladenosine (MTA) as the reaction products (Parsek et al., 1999).

LuxR on the other hand, are members of FixJ-NarL superfamily which are characterized by two distinct components response regulators (Kahn & Ditta, 1991). LuxR is composed of 2 principle conserved domains, a N-terminal AHL binding site and a C-terminal helix-turn-helix (HTH) DNA binding motif to respond to detection of AHL through a series of reactions (Fuqua et al., 2001). The process starts with specific binding of cognate AHL to the N-terminal AHL binding site which leads to LuxR conformation changes and promotion of multimerization. The AHL-bound-LuxR will subsequently bind to the specific regulatory sequences situated upstream of the QS-regulated genes leading to activation or repression the gene transcription (Fuqua et al., 2001). The DNA region which binds to AHL-bound-LuxR is known as *lux* box which is usually a 20bp inverted repeat sequence situated around 40bp upstream of the QS-regulated genes (Parsek & Greenberg, 2000).

Genetic organization of *luxI/R* gene homologues are as diverse as their function where in most cases, they are located adjacently to each other (divergently or convergently) (Gray & Garey, 2001). Example of QS homologues with their functions are summarised in Table 2.1. Some bacteria may even employ multiple pairs of archetypical *luxI/R* to permit production and detection of multiple signals for regulation of a complex intervention of gene expression. For example, a QS model organism, *Pseudomonas aeruginosa* employs a hierarchical circuits of LasI/R and RhlI/R for regulation of various virulence factors with LasI/R on top of the signaling cascade positively regulating RhlI/R (Juhas et al., 2004; Latifi, Foglino, Tanaka, Williams, & Lazdunski, 1996). Synthesis of 3-oxo-C12-HSL is catalysed by LasI for the regulation of elastase, LasA protease and exotoxin A expression (Passador, Cook, Gambello, Rust, & Iglewski, 1993; Pesci, Pearson, Seed, & Iglewski, 1997). The 3-oxo-C12-HSL-bound-LasR subsequently activate transcription of RhlI/R system to synthesize C4-HSL for downstream regulation of rhamnolipid and cyanide expression (Brint & Ohman, 1995; Pearson, Pesci, & Iglewski, 1997).

Burkholderia cenocepacia is another example of QS producer which employs 2 QS circuits (CciI/R and CepI/R) to regulate swarming motility, virulence factors and siderophore production (Huber et al., 2001; Malott, Baldwin, Mahenthiralingam, & Sokol, 2005). As CepI/R is widely distributed in *Bukholderia cepacia* complex (Bcc), CepI/R is predicted to be an ancestral system while CciI/R system is likely a more recently acquired QS system into *B. cenocepacia* by horizontal transfer (Malott et al., 2005).

Table 2.1. Genetic organization of QS homologues with their functions

Organism	QS System	Orientation	AHL	Functions	Reference
α- Proteobacteria					
Agrobacterium tumefaciens	TraIR	Non-adjacent	3-oxo-C8-HSL	Conjugation	(Piper, Beck Von Bodman, Hwang, & Farrand, 1999)
Rhizobium leguminosarum bv. viciae	CinIR	→ →	3OH,C14:1-HSL	Regulation of cell cycle and nodulation in legumes, adaptation to starvation and salt stress	(Danino, Wilkinson, Edwards, & Downie, 2003; Edwards et al., 2009; Lithgow et al., 2000; Rodelas et al., 1999; Wisniewski-Dye, Jones, Chhabra, & Downie, 2002)
	RhiIR	Non-adjacent	C6-HSL, C7-HSL, C8-HSL		
	RaiI/Rl,	→ →	C6-HSL, C7-HSL, C8-HSL, C10-HSL, 3-OH-C8-HSL		
	TraI/R	Non-adjacent	3-oxo-C10-HSL, C8-HSL		
Rhodobacter sphaeroides	CerIR	→ →	C14:1-HSL	Regulation of cell aggregation	(Puskas, Greenberg, Kaplan, & Schaefer, 1997)
β-Proteobacteria					
Burkholderia cepacia	CepIR	← →	C6-HSL, C8-HSL	Swarming motility, biofilm formation and siderosphere production	(Huber et al., 2001; Lewenza, Conway, Greenberg, & Sokol, 1999)
	CciIR	→ →	C6-HSL, C8-HSL	Protease production, Swarming motility	(Holden et al., 2009)
Chromobacterium violaceum	CviIR	→ ←	C10-HSL	Biofilm formation, chitinase and violacein production	(Stauff & Bassler, 2011)
Ralstonia solanacearum	SolIR	← →	C6-HSL, C10-HSL	Improve virulence factors production, but no affect in inactivation of *solIR*	(Flavier, Ganova-Raeva, Schell, & Denny, 1997; Schell, 2000)
Aeromonas hydrophila	AhyIR	← →	C4-HSL, C6-HSL	Biofilm formation and serine protease production	(Lynch et al., 2002; Swift et al., 1997)
Aeromonas salmonicida	AsaIR	← →	C4-HSL	Production of serine protease	(Swift et al., 1997)
γ- Proteobacteria					
Pantoea (*Enterobacter*) *agglomerans*	EagIR	→ ←	3-oxo-C6-HSL	Unclear role	(Swift et al., 1993)
Erwinia carotovora subsp. *carotovora*	ExpIR	→ ←	3-oxo-C6-HSL	Production of carbapenem antibiotic and exoenzymes	(Bainton et al., 1992)

Table 2.1, continued

Erwinia carotovora subsp. *betavasculorum*	EcbIR	→ ←	No characterization	Regulation of antibiotic and exoenzyme production	(Costa & Loper, 1997)
Erwinia chrysanthemi	ExpIR	→ ←	3-oxo-C6-HSL, C6-HSL	Swarming and swimming motility, formation of cell aggregation, virulence factors in potato tuber	(Hussain et al., 2008)
Pantoea (*Erwinia*) *stewartii*	EsaIR	→ ←	3-oxo-C6-HSL	Bacterial adhesion, biofilm formation and host colonization	(Koutsoudis, Tsaltas, Minogue, & von Bodman, 2006)
Serratia liquefaciens	SwrIR	→ ←	C4-HSL, C6-HSL	Biofilm formation	(Labbate et al., 2004)
Serratia marcescens	SmaIR	→ ←	C4-HSL, C6-HSL	Swarming motility and haemolytic activity;	(Coulthurst, Williamson, Harris, Spring, & Salmond, 2006)
Yersinia enterocolitica	YenIR	→ ←	3-oxo-C6-HSL, C6-HSL	Swimming and swarming motility	(Atkinson, Chang, Sockett, Camara, & Williams, 2006)
Pseudomonas aeruginosa	LasIR	→ →	3-oxo-C12-HSL	Production of elastase, protease and exotoxin A	(Passador et al., 1993; Pesci et al., 1997)
	RhlIR	→ →	C4-HSL	regulation of rhamnolipid and cyanide	(Brint & Ohman, 1995; Pearson et al., 1997)
Pseudomonas aureofaciens	PhzIR	→ ←	C6-HSL	Phenazine antibiotic production	(Whistler & Pierson Iii, 2003)
	CsaIR	→ →	Short-chain AHL	Regulation of cell surface properties	(Zhang & Pierson, 2001)
Pseudomonas fuorescens	PhzIR	→ ←	3-OH-C6-HSL, 3-OH-C8-HSL, 3-OH-C10-HSL, C6-HSL, C8-HSL	Production of antibiotics	(Khan et al., 2005)
Pseudomonas syringae pv. syringae	AhlIR	→ ←	3-oxo-C6-HSL	Cell aggregation, epiphytic fitness	(Dumenyo, Mukherjee, Chun, & Chatterjee, 1998; Quiñones, Pujol, & Lindow, 2004)

3OH,C14:1-HSL: Abbreviation of 7,8-cis-*N*-(3-hydroxytetradecenoyl)homoserine lactone or *N*-(3-hydroxy-7-cis-tetradecanoyl)-L-homoserine lactone, is a newly characterized class of AHL which contain double bond in the acyl moiety that was previously thought to be small bacteriocin (Schripsema et al., 1996).
C14:1-HSL: Abbreviation of 7,8-cis-*N*-(tetradecenoyl)-homoserine lactone, is a new member of AHL highly related to 7,8-cis-*N*-(3-hydroxytetradecenoyl)homoserine lactone from *R. leguminosarum* (Puskas et al., 1997)

2.4 LuxR solo

The number of *luxI* and *luxR* presence in a QS bacteria are not always equal (Case, Labbate, & Kjelleberg, 2008). Besides the typical genetically linked canonical *luxI/R* arrangement, presence of unpaired *luxR* without presence of cognate *luxI* homologue (term as *luxR* solo or orphan) are documented in many *Proteobacteria*. These LuxR solos contain typical modular structure of N-terminal AHL binding site and C-terminal HTH DNA binding domain and can be found in both QS or non-QS bacteria (Subramoni & Venturi, 2009) (Table 2.2). Presence additional LuxR solos are highly beneficial to the bearer to response to various endogenous and exogenous signals produced by their neighbours.

The most characterised LuxR solo in QS bacteria is QscR solo in *P. aeruginosa* (Subramoni & Venturi, 2009). Function of QscR solo is to control the timing of production of AHL for the expression of virulence factors in response to 3-oxo-C12-HSL produced by LasI. Previous investigation demonstrated that *qscR* mutant is hypervirulent and indicated that QscR solo is important for an efficient supervision of QS-mediated virulence factors (Chugani et al., 2001; Lequette, Lee, Ledgham, Lazdunski, & Greenberg, 2006).

Examples of LuxR solo in non QS producing bacteria is SdiA which is present in members of *Salmonella*, *Escherichia* and *Klebsiella* genus for detection a wide range of AHLs (Subramoni & Venturi, 2009). Examples of other LuxR solo with their functions are summarised in Table 2.2

Table 2.2. Characterized LuxR solo (Subramoni & Venturi, 2009).

Organism	QS systems	LuxR solo	Ligand	Functions	Reference
Agrobacterium vitis	AvsI/R	AvhR	Not yet determined	Induction of grape necrosis and tobacco hypersensitive response	(Hao, Zhang, Zheng, & Burr, 2005)
Agrobacterium vitis	AvsI/R	AviR	Not yet determined	Induction of grape necrosis and tobacco hypersensitive response	(Zheng et al., 2003)
Rhizobium leguminosarum bv. viciae	CinI/R, RhiI/Rl RaiI/Rl TraI/R	BisR	3OH-C14:1-HSL	Transfer of symbiotic plasmid pRL1J1 and inhibition of growth in the presence of 3OH-C14:1-HSL	(Danino et al., 2003; Wilkinson, Danino, Wisniewski-Dye, Lithgow, & Downie, 2002)
Brucella melitensis	None	BlxR	Not yet determined	Regulation of type IV secretion system and flagella	(Rambow-Larsen, Rajashekara, Petersen, & Splitter, 2008)
Serratia marcescens	SmaI/R	CarR	Ligand independent	Production of carbapenem antibiotic	(Cox et al., 1998)
Sinorhizobium meliloti	SinI/SinR Mel	ExpR	C14-HSL, 3-oxo-C14-HSL, C16:1-HSL, 3-oxo-C16-HSL, C18-HSL	Production of succinoglycan, motility, chemotaxis, cellular proteases, nitrogen fixation, metal transport, etc.	(Bartels et al., 2007; Hoang, Becker, & González, 2004; McIntosh, Krol, & Becker, 2008)
Sinorhizobium meliloti	SinI/SinR Mel	NesR	C14-HSL, 3-oxo-C14-HSL, C16:1-HSL, 3-oxo-C16-HSL, C18-HSL	Influence nutritional and environmental stress response in *S. meliloti*.	(Patankar & González, 2009)
Xanthomonas oryzae pv. *oryzae*	None	OryR	Rice signal molecule	Influence of virulence in rice	(Ferluga, Bigirimana, HÖFte, & Venturi, 2007; Ferluga & Venturi, 2009)
Pseudomonas aeruginosa	LasI/R RhlI/R	QscR	3-oxo-C12-HSL, 3-oxo-C10-HSL	Governing of AHL production and virulence factors	(Chugani et al., 2001; Lequette, Lee, Ledgham, Lazdunski, & Greenberg, 2006)
Escherichia coli, Salmonella enterica serovar Typhimurium	None	SdiA	3-oxo-C8-HSL, 3-oxo-C6-HSL, 3-oxo-C4-HSL, 3-oxo-C10-HSL, 3-oxo-C12-HSL, C6-HSL, C8-HSL	Detection of mixed microbial communities, adhesion and resistance to complement killing	(Ahmer, 2004; Michael, Smith, Swift, Heffron, & Ahmer, 2001; Yao et al., 2006)
Brucella melitensis	None	VjbR	C12-HSL	Regulation of type IV secretion system and flagellar gene	(Delrue et al., 2005)
Xanthomonas campestris pv. *campestris*	None	XccR	Plant signal molecule	Induction of proline iminopeptidase (pip) gene expression and virulence in cabbage	(Zhang, Jia, Wang, & Fang, 2007)

2.5 Social Cheating in QS

Social cheating is a selfish bacterial phenomenon which describe the event of certain bacterial individuals that are living at the benefits of the public resources (known as "public goods") without the paying the full cost of producing the QS signaling molecules (Foster, Parkinson, & Thompson, 2007; Keller & Surette, 2006; West, Griffin, Gardner, & Diggle, 2006). Production of extracellular LasR-induced protease in *P. aeruginosa* is an example of public goods under QS regulation.

In studies conducted using casein as the sole carbon source that requires secretion of protease for survival, researchers have demonstrated that QS mutants have fitness advantage for exploiting the public goods of protease in a mixed population containing both wildtype (QS co-operators) and QS mutants of *P. aeruginosa* (West et al., 2006). The QS mutants eventually outnumber the wildtype and reduce the concentration of QS signaling molecules and thus, deactivate the secretion of QS-mediated protease leading to the collapse of the entire population. In fact, emergence of *P. aeruginosa* LasR mutants were documented to reach a stable equilibrium with the parental wildtype after about 50-100 generation of cultivation on casein supplemented media (Dandekar, Chugani, & Greenberg, 2012; Sandoz, Mitzimberg, & Schuster, 2007).

To control the population of QS cheaters, QS co-operators employed a policing mechanism to control the population of QS cheaters in which the latest mechanism was documented by Wang, Schaefer, Dandekar, and Greenberg (2015). Their study unravelled that there is a cheater control mechanism supervised by the production of RhlR-activated cyanide from the wildtype *P. aeruginosa* (QS co-operators) to control and punish the population of QS cheaters in which the only QS co-operators are able to develop the RhlR induced-cyanide resistance (Wang et al., 2015).

2.6 The *Pandoraea* Genus

The *Pandoraea* genus is made up of nine species and belong to the beta-subclass of *Proteobacteria* family (http://www.bacterio.net/pandoraea.html). Etymology of *Pandoraea* was conceived from "Pandora box" of Greek mythology which describes the origin of all evil (Coenye et al., 2000). *Pandoraea* can be isolated from soil environment and predominantly in immunocompromised and cystic fibrosis (CF) patients (Coenye et al., 2000; Daneshvar et al., 2001; Ee, Lim, Kin, Yin, & Chan, 2014; Sahin et al., 2011).

In 2012, *Pandoraea* was regarded as one of the emerging opportunistic pathogen in CF infections with the pathogenesis of its infection remain a conundrum (Callaghan & McClean, 2012). Infection of *Pandoraea* can lead to chronic bronchopulmonary colonization, degeneration of lung function, multiple organ impairment and even death (Atkinson, Lipuma, Rosenbluth, & Dunne, 2006; Caraher et al., 2008; Costello et al., 2011; Fernandez-Olmos et al., 2012; Jorgensen et al., 2003; Stryjewski, LiPuma, Messier, Reller, & Alexander, 2003). To aggravate the situation, *Pandoraea* species are highly resistance to wide spectrum antibiotics such as ampicillin, carbapenems, cephalosporins, aztreonam, aminoglycosides and meropenem but susceptible to imipenem (Daneshvar et al., 2001; Stryjewski et al., 2003). The most recent report of *Pandoraea* infection was recovered from a deceased CF patient documented by our team in collaboration with University of Tasmania (Ee, Ambrose, Lazenby, Williams, Chan, et al., 2015).

Proper identification of clinical pathogens is very important for effective treatment and clinical management. Previously using conventional phenotypic identification, *Pandoraea* species are often misidentified as their closest neighbours namely, *Burkholderia* and *Ralstonia* because these taxa share high similarity in biochemical, antibiotic susceptibility and morphology resemblance (Aravena-Roman,

2008). In addition, database of most commercial systems do not include some unusual or under studied taxa such *Pandoraea* genus (Fernandez-Olmos et al., 2012). In fact, a quality assurance trial conducted by EuroCareCF in 2009 has demonstrated a high rate of misclassification of *Pandoraea* species in most clinical laboratory indicating that this short-comings should be addressed properly (Hogardt, Ulrich, Riehn-Kopp, & Tummler, 2009). Ambiguity in identifying *Pandoraea* species can be resolved by molecular and genotypic identification methods such as analysis of *gyrB*, 16S rRNA and MALDI-TOF MS analyses (Coenye & LiPuma, 2002; Coenye, Liu, Vandamme, & LiPuma, 2001).

2.7 QS in Cystic Fibrosis Pathogens

Cystic fibrosis (CF) is one of the most life-threatening hereditary autosomal recessive disorder resulting from frameshift mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) gene in chromosome 7 (Rommens et al., 1989). Dysfunctional or absence of CFTR results in aberration of ion flow across the epithelium and hence lead to secretion and accumulation of highly viscous mucus with abnormal composition in the lung which impedes the airway clearance mechanism and weaken the bactericidal activity of the mucus (Winstanley & Fothergill, 2009). This abnormalities consequently increases the susceptibility of CF patients towards lower respiratory tract infections which subsequently advanced into chronic pulmonary infection as a result of impaired eradication of the causative pathogens. For instance *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Bukholderia cepacia* complex (Bcc) represented some of the most prevalent CF lung pathogens (Harrison, 2007).

Interestingly, a common traits shared by these CF lung pathogens is QS which has been discovered to play an active role in coordinating the expression of their virulence factors (Sifri, 2008). Detection of AHLs in CF sputum samples and discovery

QS-regulated transcripts expression in CF airways are among the several experimental evidences which showed the involvement of QS in pathogenesis of CF lung infections. In the example of *P. aeruginosa*, the most predominant CF lung pathogen, QS was reported to play an ongoing role during both initial and subsequent stages of infection. Besides, QS was also demonstrated to coordinate production of crucial virulence factors which contribute to establishment of long-term infections, such as formation and development of biofilm, host tissue destruction and protection of bacterial colonies against host immune attacks (Willcox et al., 2008; Winstanley & Fothergill, 2009).

Cooperative intergeneric and interspecies communication has also been reported to occur between QS pathogens in CF lung to heighten pathogenicity in where the most relevant clinical example was represented by production of mixed biofilms between *B. cepacia* complex (Bcc) and *P. aeruginosa*. This cross-communication was proposed to exacerbate the virulence of the mixed bacterial population involved in CF lung infection (Riedel et al., 2001). In fact, study conducted by Lewenza, Visser, and Sokol (2002) demonstrated that Bcc and *P. aeruginosa* can detect heterologous AHLs however, not all virulence factors are regulated by the heterologous AHLs.

CHAPTER 3: MATERIALS AND METHODS

3.1 Chemical Reagents

Chemical reagents used throughout this study were listed as follow:

1. Agilent Technologies (USA)
2. Amresco® (USA)
3. BDH® (USA)
4. Argus® (OpGen, USA)
5. BD Difco™ Laboratory (USA)
6. Cayman Chemical (USA)
7. First Base Laboratory (Malaysia)
8. Fisher Scientific (UK)
9. Invitrogen™ (USA)
10. Life Technologies™ (USA)
11. Merck Millipore (USA)
12. Scharlau (Spain)
13. Sigma-Aldrich® (USA).

3.2 Commercial Kits

Commercial kits used throughout this study were listed as follows:

1. Agilent High Sensitivity DNA Kit (Agilent, USA)
2. Agilent DNA 1200 Kit (Agilent, USA)
3. Ampure PB MagBead Kit (Pacific Biosciences, USA)
4. Ampure XP Beads (Beckman Coulter, USA)
5. BD™ Gram Stain Kit (Becton, Dickson and Company, USA)
6. DNA Polymerase Binding Reagent Kit (Pacific Biosciences, USA)
7. DNA Sequencing Reagent Kit (Pacific Biosciences, USA)
8. i-Taq™ DNA polymerase (iNTRON Biotechnology, Korea)
9. MasterPure™ DNA Purification Kit (Epicentre, USA)
10. Nextera DNA Sample Preparation Kit (Illumina, USA)
11. Nextera Index Kit (Illumina, USA)
12. SMRTbell Template Preparation Reagent Kits (Pacific Biosciences, USA)
13. QIAquick® Gel Extraction Kit (Qiagen, Germany)
14. QIAquick® Spin Miniprep Kit (Qiagen, Germany)
15. QubitTM dsDNA Broad Range (BR) Assay Kit (Life Technology, USA)
16. QubitTM dsDNA High Sensitivity (HS) Assay Kit (Life Technology, USA)

3.3 Equipment

Equipment used throughout this study were listed as follow:

1. 2100 Bioanalyzer (Agilent, USA)
2. 6400 Series Triple Quadrupole LC/MS (Agilent, USA)
3. Argus Whole Genome Mapping System (OpGen, USA)
4. Autoclave machine (Hirayama, Japan)
5. Benchtop UV Transilluminators (UV Products, Canada)
6. Biolog microplate reader (Biolog, USA)
7. Bottles and conical flasks (Schott, USA)
8. Centrifuge machine (Eppendorf, Germany)
9. Freezer and refrigerator (Sharp, Japan)
10. Gel documentary image analyser (UV Products, Canada)
11. Ice maker (Rinnai, Japan)
12. Inoculating loop (APS, Australia)
13. Inverted microscope (Olympus, Japan)
14. Laboratory fume hood (Labronco, USA)
15. Microflex MALDI-TOF MS (Bruker Daltonik GmbH, Germany)
16. MiSeq Personal Desktop (Illumina, USA)
17. Multimode microplate reader (Tecan, Switzerland)
18. Nanodrop spectrometry (Thermo Scientific, USA)
19. OmniLog reader (Biolog, USA)
20. PCR cabinet (Esco, USA)
21. PCR thermal cycler (Life technologies, USA)
22. Pipettes (Eppendorf, Germany)
23. Pipette tips (Axygen, USA)
24. Qubit 2.0 fluorometer (Life technologies, USA)

25. SC7620 mini sputter coater (Quorum Technologies, England)

26. Shaking incubator (N-Biotek, Korea)

27. Single Molecule Real Time (SMRT) RSII sequencer (Pacific Biosciences, USA)

28. Thermomixer (Eppendorf, Germany)

29. TM3000 Table Top Scanning Electron Microscope (Hitachi, Japan)

30. Ultra-low temperature cascade freezers (Thermo Scientific, USA)

31. White light box (Stratagene, USA)

3.4 Bacterial Strains

All bacterial strains were cultivated routinely in Luria-Bertani (LB) media at 28°C as presented in Table 3.1 unless stated otherwise.

Table 3.1. Bacterial strains used in this study

Strain	Description	Source/reference
Chromobacterium violaceum CV026	Double mini-Tn*5* mutant derived from ATCC31532. Biosensor that synthesize purple violacein pigment in response of short chain exogenous AHL.	(McClean et al., 1997)
Erwinia carotovora GS101	Positive control for QS properties. Capable of producing AHL to activate *C. violaeum* CV026.	Gift from Prof. Paul Williams
Erwinia carotovora PNP22	Negative control for QS properties.	Gift from Prof. Paul Williams
Escherichia coli pSB401	*luxRluxI'* (*Photobacterium fischeri* [ATCC 7744])::*luxCDABE* (*Photorhabdus luminescens* [ATCC 29999]) fusion; pACYC184-derived, Tet^R. Biosensor that produces bioluminescence in the presence of short chain exogenous AHL. Cultured at 37°C.	(Winson et al., 1998)
E. coli BL21(DE3)pLysS	Competent cell for transformation work	Invitrogen
E. coli DSM 30083^T	Type strain of *E. coli* for taxonamical studies purpose	DSMZ culture collection centre
E. coli DH5α	Negative control for AHL inactivation bioassay	(Sambrook, 1989)
Bacillus cereus	Positive control for AHL inactivation bioassay	(Chan, Tiew, & Ng, 2007)
Pandoraea pnomenusa RB-38	Soil isolate	This study
P. pnomenusa DSM 16536^T	Recovered from cystic fibrosis patient, United Kingdom. Cultured at 37°C.	(Coenye et al., 2000)

Table 3.1, continued

Strain	Description	Source/reference
P. pnomenusa 6399	Recovered 11 months before the cystic fibrosis patient deceased, Australia. Cultured at 37°C.	(Ee, Ambrose, Lazenby, Williams, & Chan, 2015)
P. pnomenusa 7641	Recovered from deceased cystic fibrosis patient, Australia. Cultured at 37°C.	(Ee, Ambrose, Lazenby, Williams, Chan, et al., 2015)
P. pulmonicola DSM 16583^T	Recovered from sputum of cystic fibrosis patient, Canada. Cultured at 37°C.	(Coenye et al., 2000)
P. sputorum DSM 21091^T	Recovered from sputum of cystic fibrosis patient USA. Cultured at 37°C.	(Coenye et al., 2000)
P. apista DSM 16535^T	Recovered from sputum of cystic fibrosis patient, Denmark. Cultured at 37°C.	(Coenye et al., 2000)
P. faecigallinarum DSM 23572^T	Isolated from chicken dung, India.	(Sahin et al., 2011)
P. norimbergensis DSM 11628^T	Isolated from oxic water above sulfide containing lake sediment, Germany.	(Coenye et al., 2000)
P. oxalativorans DSM 23570^T	Isolated from soil litter close to *Oxalis* sp., Turkey.	(Sahin et al., 2011)
P. thiooxydans DSM 25325^T	Isolated from rhizosphere soils of sesame *Sesamum indicum* L., Korea.	(Anandham et al., 2010)
P. vervacti DSM 23571^T	Isolated from uncultivated field soil, Turkey	(Sahin et al., 2011)
Serratia marcescens DSM 30121^T	Isolated from pond water	DSMZ culture collection centre
S. glossinae DSM 22080^T	Isolated from the midgut of the tsetse fly *Glossina palpalis gambiensis*	(Geiger, Fardeau, Falsen, Ollivier, & Cuny, 2010)
S. fonticola DSM 4576^T	Isolated from water	(Gavini et al., 1979)

Table 3.1, continued

Strain	Description	Source/reference
S. liquefaciens DSM 4487^T	Isolated from milk, Ireland	(Grimont, Grimont, & Starr, 1978)
S. sakuensis DSM 17174^T	Isolated from an activated sludge of waste water treatment tank, Japan	(Ajithkumar, Ajithkumar, Iriye, Doi, & Sakai, 2003)
S. ureilytic DSM 16952^T	Isolated from river water in Hasimara, Jalpaiguri district, West Bengal, India	(Bhadra, Roy, & Chakraborty, 2005)
S. entomophil DSM 12358^T	Isolated from grass grub *Costelytra zealandica* infected with amber disease, New Zealand	(Grimont, Jackson, Ageron, & Noonan, 1988)

3.5 Buffer Solution

All buffer solutions were sterilized by autoclaving at temperature 121°C and pressure of 15psi for 15mins, unless stated otherwise. Filter sterilization were performed for heat labile solutions and enzymes with a syringe filter of 0.22µm pore size.

3.5.1 Phosphate Buffered Saline (PBS)

PBS (100mM) was prepared with the composition of 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 and 1.8mM KH_2PO_4. All the salts were dissolved in 800mL of dH_2O and adjusted to pH6.5, pH7.2 or pH7.4 using HCl prior to addition of dH_2O to the final volume of 1L.

3.5.2 Tris Borate EDTA (TBE) Solution

To prepare 1×TBE solution (pH8.4), 10× TBE solution (pH8.4) (First Base Laboratory, Malaysia) was diluted 10× by dH_2O. The composition of the 10× TBE solution was 890mM Tris-borate, 890mM boric acid and 20mM EDTA.

3.6 Stock Solution

3.6.1 Synthetic AHL

Synthetic AHL used throughout this was obtained from Sigma-Aldrich® (USA), Cayman Chemical (USA) or gifted from Prof. Paul Williams. Acetonitrile (ACN) was used to dissolve AHL to the desired concentration and stored in 1.5-mL tubes in -20°C freezer.

3.6.2 Antibiotic Stock Solution

Antibiotic stocks were dissolved in either filter sterilized distilled water or absolute ethanol to the desired concentration depending on the solubility of the antibiotic stock.

3.6.3 DNA Ladder Markers

DNA ladder markers used throughout these experiments were GeneRuler™ 100bp DNA Ladder (Fermentas, Canada) and GeneRuler™ 1kb DNA ladder (Fermentas, Canada).

3.7 Soil sampling and Bacteria Isolation

The sampling site is a municipal landfill site in Ayer Hitam, Puchong, Malaysia with the GPS coordinates of 03°00'12.1"N, 101°39'33.1"E with 61 m from sea level. A gouge auger was used drilled the soil surface to the depth of 10cm and a soil sample was collected into a sterile falcon tube. Soil sample was immediately processed upon arrival in the laboratory in which large particles such as stones were removed manually using a sterilized spatula.

3.7.1 KGm Medium

KGm medium was prepared according to Wong et al. (2012). The basal medium consisted of 21.39mM NaCl, 10.06mM KCl, 1.76mM Na_2SO_4, 55.11mM KH_2PO_4, 5.25mM $MgCl_2$, 12.25mM $CaCl_2$, 5.61mM NH_4Cl and 5.12mM 2-(*N*-morpholino)-ethanesulfonic acid (MES) was autoclaved and allowed to cool to room temperature. Then, filter-sterilized (0.22µm pore size) of $FeCl_3$, $MnCl_2$, and $ZnCl_2$ solutions were added to the basal medium to the final concentrations of 30.83µM, 19.86µM, and 4.40µM, respectively. Lastly, 234.52µM of 3-oxo-C6-HSL was added as the sole carbon source.

3.7.2 LB Medium

All strains were routinely maintained aerobically in LB medium with 220rpm agitation at 28°C unless stated otherwise. Composition of LB medium was prepared consisting of 1.0% w/v tryptone, 1.0% w/v NaCl and 0.5% w/v yeast extract in 1L of distilled water.

For preparation of LB agar, 1.5% w/v bacteriological agar was added to the medium for solidification purpose.

For AHL extraction, 50mM of 3-(*N*-morpholino) propanesulfonic acid (MOPS), pH5.5 was added into LB medium to maintain the acidity of the medium to prevent lactonolysis AHL molecules (Yates et al., 2002).

3.7.3 Enrichment Process

Soil sample (1g) was added into 10mL of KGm medium and the mixture was vortexed vigorously. The soil suspension (10% v/v) was subsequently inoculated into fresh 10mL of KGm medium supplemented with 3-oxo-C6-HSL (50mM final concentration) and incubated for 48h in a shaking incubator (28°C and 220rpm). Similar enrichment of soil suspension (10%, v/v) was performed for 4 cycles.

3.7.4 Bacterial Purification and Storage

At the fourth enrichment cycle, ten-fold serial dilution was performed and plated on 3-oxo-C6-HSL-containing KGm and LB agar plates prior to incubation at 28°C for 48h. Single colonies of bacteria with different morphology was selected and purified on LB agar plates.

For storage purpose, all bacterial isolates were kept in LB agar slants at room temperature and in 20% v/v glycerol stock (200µL of sterilised 80% v/v glycerol + 800µL planktonic culture of bacterial isolates) in 1.5-mL tubes in the -80°C freezer.

3.8 Morphological Observation

3.8.1 Gram Staining

Gram staining was performed according to manufacturer's instruction of BD™ Gram Stain Kit. In short, a single colony of bacteria was smeared in a drop of sterile dH_2O on a glass slide and desiccated completely. The slide was initially stained with crystal violet solution before it was washed and drained using dH_2O. Then, iodine solution was used to fix the stain before the iodine solution was washed and drained using dH2O. Acetone solution was subsequently used to decolourize the slide before the solution was immediately washed with dH_2O. Lastly, the slide was counterstained using Safranin solution and washed with dH_2O. The stained slide was allowed to air dried before it was observed under Olympus™ 1X71 inverted research microscope.

3.8.2 Scanning Electron Microscope (SEM)

Sample preparation for SEM was performed according to Fischer, Hansen, Nair, Hoyt, and Dorward (2012) with slight modification. Primary fixation of the bacterial materials was performed with 5% v/v glutaraldehyde in 100mM PBS (pH7.2) and incubated for 24h. The bacteria suspension was then washed twice with 100mM PBS (pH7.2) and secondary fixation was performed using 1% v/v osmium tetraxide (OsO_4) for 1h before the suspension was washed twice again using dH_2O. A 4 steps ethanol dehydration series was then performed using 50%, 75%, 95% and 100% v/v ethanol and lastly followed a complete dehydration process using hexamethyldisilazane (HMDS) for 10mins for each process. The sample was desiccated completely in a desiccator for 24h before it mounted on an aluminium stub using a conductive double sided adhesive tape, gold coated using a SC7620 mini sputter coater and observed using a Table Top SEM.

3.9 Bacterial Identification

3.9.1 MALDI-TOF MS Analysis

MALDI-TOF mass spectrometry analysis was employed for initial and rapid identification of all the isolates exhibiting QS activities. Fresh colonies of isolates on LB agar plates were smeared thinly on the designated wells on the MSP 96 target polished steel BC plate. Subsequently, 1µL of MALDI-TOF matrix (with the composition of 10mg/mL α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/2.5% trifluoroacetic acid) was added to each wells and left for complete desiccation. Mass spectrometry analysis was performed using a Microflex MALDI-TOF (Bruker Daltonik GmbH, Germany) bench-top mass spectrometer equipped with UV laser at wavelength 337nm coupled with Bruker FlexControl software version 3.3 (Build 108).

Each individual well was bombarded with laser shots and the bacterial MS spectra was analysed in the linear positive ion mode with the mass in range of 2 to 20kDa using Bruker MALDI Biotyper Real Time Classification software (Version 3.1, Build 65). The MS spectra was subsequently compared to the available library of bacterial MS spectra in the Bruker database and a dedicated assessment system will be calculated to evaluate the accuracy of the identification which is listed in the Table 3.2. From the assessment system, a dendogram was then constructed using the standard MALDI Biotyper MSP creation method (Bruker Daltonics, Germany) to illustrate the graphical distance values between species constructed from their MALDI-TOF reference spectra.

Table 3.2. Score value of MALDI-TOF MS analysis

Score value	Description
2.3-3.0	Identification is accurate to species level
2.0-2.3	Identification is accurate to genus level
1.7-2.0	Accuracy of identity is lower than genus
<1.7	No reliable identification

3.9.2 16S rRNA Gene Sequence Analysis

3.9.2.1 Extraction of Genomic DNA

Overnight culture (16h) of isolate inoculated in LB broth was harvested by centrifugation at 10,000×*g* for 10mins. Spent supernatant was discarded and the cell pellet was washed twice with 10mL of 100mM PBS, pH7.4. Genomic DNA extraction was performed according to the MasterPureTM DNA Purification Kit (Epicentre, USA) manufacturer's instruction. Quality and quantity of extracted genomic DNA was determined using NanoDrop spectrophotometer (Thermo Scientific, USA) and Qubit 2.0 fluorometer (Life Technologies, USA) respectively.

3.9.2.2 16S rRNA Gene Amplification

Bacterial 16S rDNA was amplified using PCR with 27F forward primer, 1525R reverse primer and PCR mix (Promega Kit, USA) according to manufacturer's instructions. Primers sequences were shown in Table 3.3. PCR amplification was performed at the following conditions which consisted of an initial denaturation at 94°C for 3min, followed by 30 repeated cycles at 94°C for 30s of denaturing, 60°C for 30s of annealing and 72°C for 1min 30s of extension, and a final extension at 72°C for 7min.

Table 3.3. Primers used in this study.

16S rDNA Primers	Sequence	Length (bp)	Reference
27F forward	5'-AGAGTTTGATCMTGGCTCAG-3'	20	(Chan et al., 2011)
515F forward	5'–GTGCCAGCMGCCGCGGTAA-3'	19	(Turner, Pryer, Miao, & Palmer, 1999)
1525R reverse	5'–AAGGAGGTGWTCCARCC-3'	17	(Chan et al., 2011)

3.9.2.3 Agarose Gel Electrophoresis (AGE)

AGE was performed using 1% w/v agarose gel pre-stained with 0.5mg/mL of ethidium bromide. Electrophoresis was performed with in 1×TBE, pH8.4 buffer at 80V, 400mA for 40mins. Visualization of the agarose gel was performed using a UVP Benchtop UV Transilluminators (UV Products, Canada) with comparison to the 1kb DNA ladder marker.

3.9.2.4 Purification of PCR Products

The PCR amplified product was excised from the gel using a clean glass slide and transferred to a 1.5-mL tubes. Purification of the PCR amplified product was performed using a QIAquick® Gel Extraction Kit (Qiagen, Germany) according to the manufacturer's instructions. Purified PCR product was subsequently sent to First Base sequencing company for sequencing purpose.

3.9.2.5 Phylogenetic Analysis

Product sequence alignment was performed by BLAST comparison against GenBank database (http://rdp.cme.msu.edu/) and phylogenetic analysis was performed using Molecular Evolutionary Genetic Analysis (MEGA) version 6.0 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). Bootstrap value was set at 1000 repetition to determine reliability of each phylogeny. Sequence similarity was performed using EzTaxon-e database (http://eztaxon-e.ezbiocloud.net/) (Kim et al., 2012).

3.10 Taxonomical Characterizations for Isolate RB-25

3.10.1 Phenotypic and Biochemical Analysis

3.10.1.1 Phenotypic Identification (GENIII MicroPlate)

Inocula were prepared by inoculating fresh culture of bacteria in IF-A inoculating fluid to the turbidity of 95% T according to manufacturer's instructions. Cell suspensions (100µL) were inoculated into each well of the GENIII MicroPlates and incubated in 28°C for 24h. Results were interpreted using Biolog microplate reader coupled with Biolog's Microbial Identification Systems software version 3.1.

3.10.1.2 Phenotype Microarray (PM1 and PM2 MicroPlate)

Bacterial cells were cultured in LB media. Inoculation and preparation was performed according to manufacturer's instruction (PM procedures for *E. coli* and other GN Bacteria). In short, overnight culture of bacteria on LB agar were collected using sterile cotton swab into IF-0a. The turbidity of the suspension was subsequently adjusted to 85% T before 1% of dye H was added. Cell suspensions (100µL) were then inoculated into each well of the PM1 and PM2 MicroPlates and incubated in OmniLog reader at 28°C for 48h.

3.10.1.3 API Biochemical Assay

Biochemical Assay using API ZYM, API 20NE and API 20E was performed by DSMZ culture collection centre according to the manufacturer's instruction.

3.10.2 Lipid Composition Analysis (performed by DSMZ)

3.10.2.1 Extraction of Respiratory Lipoquinones and Polar Lipid

Extraction of lipid was performed using two stage method as described by (Tindall, 1990a, 1990b). In short, respiratory lipoquinones and relatively non-polar material was extracted from freeze dried cell material using methanol:hexane, (2:1 v/v). The suspension was stirred using a spatula for 30min under N_2 and incubated in an ice bath until the hexane and methanol phases began to separate. Ice cold hexane was subsequently added into the suspension to give a methanol:hexane, (1:1 v/v) ratio biphasis layer. The separated upper hexane phase was then extracted and the methanol phase was further processed by addition of ice cold hexane and 0.3% NaCl to give a hexane: methanol:0.3% NaCL (1:1:1: v/v) ratio. Extraction of hexane was repeated and combined with previous extraction of hexane for analysis of respiratory lipoquinones. The methanolic phase (with mixture of cell materials) was diluted with chloroform and methanol to give a chloroform:methanol:0.3% NaCl (1:2:0.8 v/v) ratio mixture. Polar lipid was then extracted from the mixture as described by Bligh & Dyer, (1959).

3.10.2.2 Respiratory Lipoquinones Analysis

Respiratory lipoquinones were purified into different classes (such as menaquinones and ubiquinones) by thin layer chromatography on silica gel (Macherey-Nagel Art. No. 805 023), using hexane:tert-butylmethylether (9:1 v/v). UV spectra corresponding to the different classes of quinones were extracted from the gel and subsequently analysed using a LDC Analytical (Thermo Separation Products) HPLC coupled with a reverse phase column (Macherey-Nagel, 2mm × 125mm, 3µm, RP18). Methanol:heptane 9:1 (v/v) was used as the eluant and respiratory lipoquinones were detected at 269nm.

3.10.2.3 Polar Lipids Analysis

Polar lipids were separated using two dimensional silica gel thin layer chromatography (Macherey-Nagel Art. No. 818 135). The first direction was established with chloroform:methanol:water (65:25:4, v/v/v) ratio solvent, and the second direction with chloroform:methanol:acetic acid:water (80:12:15:4, v/v/v/v) ratio solvent. Molybdatophosphoric acid was used to detect total lipid material while as described by (Tindall, Sikorski, Smibert, & Krieg, 2007).

3.10.2.4 Extraction of Whole-Cell Fatty Acid Methyl Ester (FAME)

Whole cell FAME were extracted according to manufacturer's instruction and with reference to Kuykendall, Roy, O'Neill, & Devine, 1988; Miller, (1982). Four reagents for extraction of FAME were prepared with composition as listed in Table 3.4. In short, 40mg of bacterial cells was scraped from the third quadrant on the petri dish and transferred into a 13mm × 100mm glass test tube. Saponification was performed by adding 1mL of reagent 1 to each test tube and sealed with Teflon lined screw caps. The test tubes were then vortexed briefly and boiled for 30min. The test tubes were cooled to room temperature before addition of 2mL of reagent 2 for methylation process. During the process, the test tubes were briefly vortexed and heated for 10min at 80°C. Extraction of FAME was performed by addition of 1.25mL of reagent 3 to the rapidly cooled test tubes and mixed for 10min in a clinical rotator. The separated lower aqueous phase was then discarded and 3mL of reagent 4 was added for cleaning up of sample. The test tubes were further mixed for 5min in the rotator before the organic phase was transferred into a chromatography vial for chromatography analysis.

Table 3.4. Reagents preparation for FAME extraction.

Reagent	Process	Composition
Reagent 1	Saponification	45g sodium hydroxide, 150mL methanol, and 150mL distilled water
Reagent 2	Methylation	325mL of 6.0N hydrochloric acid and 275mL methyl alcohol
Reagent 3	Extraction	200mL hexane and 200ml methyl tert-butyl ether
Reagent 4	Sample Clean up	10.8g sodium hydroxide dissolved in 900mL distilled water

3.10.2.5 Analysis of Whole-Cell FAME

The fatty acid methyl esters were analysed using Sherlock Microbial Identification System (MIS) (Microbial Identification Inc., MIDI) which consist of a 6890N gas chromatograph (Agilent Technologies, USA) equipped with a 5% phenyl-methyl silicone capillary column (0.2mm × 25m), a flame ionization detector, a 7683A automatic sampler (Agilent Technologies, USA), and a build in MIDI database (Hewlett-Packard, USA). Peaks were automatically integrated and fatty acid names as well as percentages were calculated by the MIS Standard Software (Microbial Identification, MIDI, USA). Ultra-high purity hydrogen was used as the carrier gas; column head pressure was set at 60kPa; injection volume at 2µL; column split ratio at 100:1; septum purge at 5mL/min; column temperature from 170°C to 270°C at 5°C/min; injection port temperature at 240°C; and detector temperature at 300°C.

3.10.3 Special Characteristic Tests Defining *Serratia* spp.

3.10.3.1 Glucose Oxidation Test

Glucose oxidation test was performed with modification from Lysenko test (Lysenko, 1961) as described by Bouvet, Lenormand, and Grimont (1989). Glucose oxidation medium was prepared with 1L of basal medium with the composition of nutrient broth (8g), bromcresol purple (0.02g) and MgSO4•7H2O (0.62g, final concentration: 2.5mM). Then, 9.2mL of autoclaved basal medium was supplemented with 0.4mL of 1M D-glucose solution (final concentration: 40mM) and 0.4mL of freshly prepared sterile 25mM iodoacetate solution (final concentration: 1mM). The glucose oxidation medium (500µL) is then transferred into each well of a 96 wells plate.

Overnight culture of bacterial cell at 20°C on TSA supplemented with 0.2% (w/v) D-glucose are harvested and immediately suspended into sterile 2.5mM $MgSO_4$ solution and further adjusted to OD_{600nm} of 4.0. Bacterial suspension (50µL) was then inoculated into each well containing glucose oxidation medium and incubated overnight with agitation (270rpm) at 20°C. Positive result is examined with changes of purple-to-yellow colour. If negative, pyrroloquinoline quinone (PQQ) (10µM) was added into the preparation of glucose oxidation medium. Positive control was included with *S. marcescens* (positive without PQQ) and *S. liquefaciens* (positive only with supplement of PQQ).

3.10.3.2 Tetrathionate Reductase Test

Tetrathionate reductase test was conducted as described by Le Minor, Chippaux, Pichinoty, Coynault, & Piechaud, (1970). The reaction medium was prepared with the composition of postassium tetrathionate (5g), 0.2% aqueous solution of bromothymol blue (25mL), peptone water (up to 1L) and adjusted to pH7.4. Positive result was examined with changes of green-to-yellow colour. Positive was included with *S. liquefaciens* and negative control was included with uninoculated medium.

3.10.3.3 Gluconate Dehydrogenase Test

Gluconate dehydrogenase test was performed as described by Bouvet et al., (1989). Briefly, overnight culture of bacterial cells cultivated at 20°C in tryptocasein soy media supplemented with 0.2% w/v of D-gluconate was suspended in sterile dH_2O to OD_{600}=4.0. A reaction medium was prepared with a composition of 0.2M acetate buffer (pH5), 1% (w/v) TritonX-100, 2.5mM $MgSO_4$, 75mM gluconate and 1mM iodoacetate (added immediately before use). A control medium was also prepared containing the same ingredients except for addition of gluconate. Both reaction and control medium (100µL) were subsequently dispensed into each wells in a 96 microtiter plate prior to addition of 10µL of bacterial suspension into each wells. The plate was then incubated for 20°C for 20min. Next, 10µL of 0.1M potassium ferricyanide solution was added into each and the plate was shaken gently prior to a 40min incubation at 20°C.

After incubation period, 50µL of reagent containing 0.6g of $Fe_2(SO_4)_3$, 0.36g of sodium dodecyl sulphate, 11.4mL of 85% phosphoric acid, 11.4mL and distilled water topped up to 100mL was added into each wells and further incubated for 15min in room temperature. Changes from yellow-to-blue colour was examined for positive result with

uninoculated control medium remaining as yellow. Positive and negative control was *S. marcescens* and *E. coli*, respectively.

3.10.3.4 2-Ketogluconate Dehydrogenase Test

2-ketogluconate dehydrogenase test was performed with the same condition as gluconate dehydrogenase test except for the pH of control and reaction medium were adjusted to pH4.0 and 2-ketogluconate was used instead of gluconate in the reaction medium. Positive and negative controls were included with *S. marcescens* (positive at 20°C but not 30°C) and *E. coli*, respectively.

3.10.3.5 Gas Production Test

Gas production test was performed as described by (Grimont, Grimont, & De Rosnay, 1977) with Durham inverted tubes and glucose agar. Glucose agar (pH7.4) was prepared with the composition of meat extract (3g), yeast extract (10g), agar (15g), distilled water to 1L supplemented with 1% w/v glucose. Tubes were subsequently examined for bubbles of gas after few days. Positive and negative controls were *S. liquefaciens* and *S. marcescens*, respectively.

3.11 AHL Inactivation Bioassay

3.11.1 Preparation of Resting Cells

Bacterial cells were cultivated in LB broth at 28°C with 220rpm agitation to approximately 10^9 cfu/mL. Then, 100mL of the bacterial cultures were harvested by centrifugation (10,000×*g*) for 10min at 4°C and washed twice with equal volume of 100mM PBS, pH7.4. Finally, resting cells were prepared by adjusting the pellet of the bacterial cells with 100mM PBS (pH7.4) to OD_{600nm} of 1.0.

3.11.2 AHL Inactivation Bioassay

Resting cells (100mL) were used to resuspend the desiccated 3-oxo-C6-HSL in 1.5mL tubes to the final concentration of 0.5µM. The resting cell suspensions were mixed thoroughly and incubated at 28°C with 220rpm agitation for 0h and 24h. The reactions were stopped with heat inactivation at 95°C for 5min.

Subsequently, 10µL of the supernatant from each reaction were spotted onto the sterile paper discs on the surface of CVO26 biosensor lawn and incubated at 28°C for 24h. *E. coli* DH5α and 100mM PBS (pH 6.5) were included as negative control while *B. cereus* was included as positive control.

3.12 Characterization of QS Profile

3.12.1 Cross-Streak Bioassay (CVO26 Biosensor)

Preliminary screening of QS activity was performed using CVO26 biosensor. All isolates were streaked perpendicularly against the CVO26 biosensor on LB agar plates and incubated in 28°C incubator for 24h. Upon the detection of short chain AHLs in the length of 4-8 carbon side chain, CVO26 biosensor will form purple violacein pigmentation. *E. carotovora* GS101 and *E. carotovora* PNP22 was included as positive and negative controls, respectively.

3.12.2 Extraction of AHL

Pure culture of isolates exhibiting QS activities were cultured in LB media supplemented with 50mM MOPS, pH5.5 and incubated in 28°C shaking incubator for 16h (Ortori et al., 2011). Equal volume of acidified ethyl acetate supplement with 0.1% v/v glacial acetic acid was added into the culture and mixed vigorously before the upper immiscible solvent layer was separated into a sterile beaker. The whole process was repeated twice and the AHL extracts were desiccated.

3.12.3 Bioluminescence Assay (*E. coli* [pSB401] Biosensor)

Expression of bioluminescence activities was performed using *E. coli* [pSB401] biosensor. In short, 250µL of diluted *E. coli* [pSB401] biosensor (OD_{600nm} of 0.1) was used to suspend the extracted AHL before transferred into the wells of 96-well microtiter plates. Optical density of OD_{495nm} and bioluminescence activity were measured for every 60mins intervals for 25cycles using Infinite M200 luminometer (Tecan, Männerdorf, Switzerland). Bioluminescence expression was expressed as relative light unit per OD_{495nm} (RLU/OD_{495nm}) against time (h) (Tan, Yin, & Chan, 2012). Negative control was included with extracted AHL with uninoculated LB media. Experiments were performed in triplicate independent culture.

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3.12.4 C_{18} Reversed-phase UHPLC

An Agilent 1290 Infinity LC system (Agilent Technologies, USA) was used for the purpose of this study. AHLs extract was resuspended in 1mL of ACN, and 1µL was injected into Agilent ZORBAX Rapid Resolution High Definition SB-C18 Threaded Column (2.1mm × 50mm, 1.8µm particle size) operated at a flow rate 500µL/min which maintained at 37ºC. The UPLC system used solvent A (water containing 0.1% v/v formic acid), and solvent B (acetonitrile containing 0.1% formic acid) as the mobile phases. The elution procedure consisted of linear gradient profile from 20% to 70% of solvent B for 7min, followed by isocratic profile of 80% solvent B for 5min, and gradient profile from 80% to 20% solvent B for 3min.

3.12.5 Triple Quadrupole MS Detection

The LC-separated compounds were detected by electrospray ionization trap mass spectrometry (ESI-MS) using Agilent 6490 Triple Quadrupole LC/MS system under positive-ion mode. The electrospray used nitrogen as nebulizing gas (pressure set to 20p.s.i) and drying gas (flow set to 11mL/h). The desolvation temperature was 200ºC and probe capillary voltage set at 3kV. AHL profiles were characterized using both precursor ion scanning mode and multiple reaction monitoring (MRM) (Gould, Herman, Krank, Murphy, & Churchill, 2006) by comparison of retention times and *m/z* transitions with those of the synthetic AHLs (Table 3.5). The ions monitored in Q1 include the AHL precursor ion $[M+H]^+$ whereas both the lactone moiety at *m/z* 102 and the acyl moiety $[M+H-101]^+$ were monitored in Q3. Data analysis was performed using Agilent MassHunter software.

Table 3.5. Precursor and product ion *m/z* values of synthetic AHLs.

Synthetic AHL	Precursor ion, Q1 (*m/z*)	Product ion, Q3 (*m/z*)
C4-HSL	172	102
C6-HSL	200	102
C7-HSL	214	102
C8-HSL	228	102
C10-HSL	256	102
C12-HSL	284	102
3-oxo-C6-HSL	214	102
3-oxo-C8-HSL	242	102
3-oxo-C10-HSL	270	102
3-oxo-C12-HSL	298	102

3.13 Genome Sequencing

3.13.1 Sequencing By Synthesis (SBS) Technology

Template libraries were prepared using Nextera DNA Library Kit and Nextera Index Kit according to manufacturer's instructions (Illumina, USA). Quality and quantity of the template libraries were validated using 2100 Bioanalyzer High Sensitivity Kit (Agilent Technologies, USA), QubitTM dsDNA High Sensitivity (HS) Assay Kit (Life Technology, USA) and Eco Real-Time PCR System (Illumina, USA). Sequencing was performed in a MiSeq (Illumina, USA) desktop sequencer.

3.13.2 SMRT Sequencing Technology

Template libraries were prepared using Pacific Biosciences (Pacific Biosciences, USA) 20-kb SMRTbell library template preparation kit according to the manufacturer's instructions (Pacific Biosciences, USA). Quality and quantity of the template libraries were determined using 2100 bioanalyzer DNA 12000 kit (Agilent Technologies, USA) and QubitTM dsDNA Broad Range (BR) Assay Kit (Life Technology, USA) respectively. Calculator version 2.1.1 (Pacific Biosciences, USA) was used to calculate the loading concentration of the polymerase bound template complex for annealing and binding prior to sequencing in a PacBio RS II sequencer (Pacific Biosciences, USA) coupled with P5C3 sequencing chemistry.

3.14 Genome Assembly and Circularization

FastQC software was employed to determine the quality of the raw sequence data (FASTQ files) from MiSeq sequencer followed by trimming and genome assembly using CLC Genomics Workbench version 7.5 (CLC bio, Denmark). On the other hand, in Hierarchical Genome Assembly Process **(**HGAP) was used to process and assemble the raw output data (bas.h5 files) from PacBio RSII sequencer. In short, initial bas.h5 files was filtered using RS_subreads and error-corrected using long reads self-correction in RS_Preassembler protocol followed by genome assembly using Celera Assembler version 8.1. The assembled contigs were then polished using Quiver to obtain polished complete genome sequences.

Circularization of the complete genome sequences was performed using Gepard dot plot software by detecting the overlapping regions at both ends of the contigs (Krumsiek, Arnold, & Rattei, 2007). The duplicated sequence at the ends of the assembly was then trimmed to form a blunt ended circular sequence.

3.15 Genome Annotation and Bioinformatics Analyses

Genome annotation and bioinformatics analyses was performed using various tools as listed in Table 3.6.

Table 3.6. Various bioinformatics tools used in this study.

Database/Software/Pipeline	Description	References
ANI calculator	ANI calculation software	(Goris et al., 2007)
ARAGORN Database	tRNA and mtRNA detection sofware	(Laslett & Canback, 2004)
Blast2GO Database	Genomic annotation software	(Conesa et al., 2005)
BioCyc Database Collection	Pathways database	(Caspi et al., 2014)
Microbial Genome Annotation & Analysis Platform (MicroScope)	Gene annotation pipeline	(Vallenet et al., 2013)
NCBI Reference Sequence (RefSeq) Database	Genome database	(Pruitt, Tatusova, & Maglott, 2005)
NCBI Prokaryotic Genome Annotation Pipeline (PGAP) version 2.10	Gene annotation pipeline	(Angiuoli et al., 2008)
PHAge Search Tool (PHAST) Database	Prophage prediction software	(Zhou, Liang, Lynch, Dennis, & Wishart, 2011)
PlasmidFinder Database	Plasmid prediction software	(Carattoli et al., 2014)
Prodigal Server	ORF prediction software	(Hyatt et al., 2010)
PROKKA	Gene annotation pipeline	(Seemann, 2014)
Rapid Annotation using Subsystem Technology (RAST) Server	Gene annotation pipeline	(Aziz et al., 2008; Overbeek et al., 2014)

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Table 3.6, continued

Database/Software/Pipeline	Description	References
RNAmmer	rDNA prediction software	(Lagesen et al., 2007)
tRNAscan-SE	Transfer RNA genes prediction software	(Lowe & Eddy, 1997)
Universal Protein Resource (UniProt) Database	Protein sequences and functions database	(Consortium, 2015)
Interproscan identifiers	Protein signature recognition software	(Jones et al., 2014)

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3.16 Multi Locus Sequence Analysis (MLSA)

MLSA for *Enterobacteriaceae* family was performed using 5 concatenated complete gene sequences in the sequence of *fusA*, *pyrG*, *rplB*, *rpoB* and *atpD* obtained from the complete genome sequence of isolate RB-25. Gene sequences for reference strains were obtained from the GenBank. Phylogeny analysis were performed using neighbour joining method (Saitou & Nei, 1987) with a bootstrap value of 1000 in MEGA 6 software (Tamura et al., 2013).

3.17 Whole Genome Optical Mapping

Whole genome optical mapping was performed using OpGen Argus® system (OpGen, USA) in which high molecular weight DNA was extracted using Argus High Molecular Weight (HMW) DNA Isolation Kit. Argus QCard kit was subsequently used to determine the quality and concentration of extracted DNA before the strains of DNA molecules were then flowed through the microfluidic channels formed by Channel Forming Device (CFD).

From the FASTA sequence generated from MiSeq or PacBio RSII sequencing technology, best restriction endonuclease for each isolates were selected using the Enzyme Chooser software. The DNA molecules immobilised on the charged glass surface were subsequently stained with fluorescence dye prior to imaging using fluorescence microscopy and fully automated image-acquisition software. Assembly were performed by overlapping each digitalized captured fragments of DNA molecule to generate a genome map. Using the sequence placement tool in the MapSolver software version 2 (OpGen, USA), the optical map was then aligned with PacBio FASTA-formatted sequences.

3.18 Functional Studies of LuxI

3.18.1 Cloning of Putative *luxI*

For cloning of putative *luxI* gene sequences from strains RB-25 and RB-38, pUC57 vector (GeneScript, USA) was employed as the cloning vector and pGS-21a vector (GeneScript, USA) was employed as the expression vector. pGS-21a harbouring the *luxI* gene sequence was subsequently transformed and cloned into competent *E. coli* BL21(DE3)pLysS. Ampicillin (100µg/mL) and chloramphenicol (34µg/mL) (CalBioChem, USA) were added to the growth medium for antibiotic selection purpose.

3.18.2 Preparation of Competent *E. coli*

Overnight culture of *E. coli* BL21 was inoculated into 250mL of LB broth in a conical flask containing 34µg/mL of chloramphenicol until OD_{600nm} of 0.3-0.4. Pellet of the *E. coli* culture was then collected and washed twice with chilled 0.1M $CaCl_2$ before it is suspended in 20mL of ice-chilled 0.1M $CaCl_2$. The suspension was then incubated in ice bath for 30min before supernatant was discarded. The pellet was subsequently suspended with 0.1M $CaCl_2$ in 85mM of glycerol before 200uL of the cell suspension was transferred into chilled 1.5-mL Eppendorf tubes for transformation purpose. All competent cells were kept in -80°C for storage purpose.

3.18.3 Transformation and selection of transformants

Competent cells in 1.5-mL of Eppendorf tubes was thawed in ice for 5min. Then, 10pg-100ng of the plasmid harbouring the *luxI* was added into the competent cells and incubated for 30min. Heat shock was performed at 42°C for 50s and immediately put back into ice for 2min. Then, 700µL of LB broth was added into each tube and incubated for 1 h at 37°C. Half of supernatant was subsequently discarded and the cell suspension was plated on LB agar plate containing Ampicillin (100µg/mL) and chloramphenicol (34µg/mL). Successful transformants was subjected to screening of QS activity using CVO26 biosensor. QS profile was subsequently characterized using LCMS/MS mass spectrometry.

CHAPTER 4: RESULTS

4.1 Enrichment and Isolation of Bacteria

Turbidity of the KGm medium inoculated with soil suspension was observed to increase in each enrichment cycle indicating growth of fastidious or/and quorum quenching (QQ) bacteria (capable of metabolising 3-oxo-C6-HSL as the sole carbon source). A total of 60 morphologically distinct isolates of bacteria was isolated from the process of purification.

In the screening of QQ activity using AHL inactivation bioassay, none of the 60 isolates demonstrated degradation capability for 3-oxo-C6-HSL indicating that they are all fastidious bacteria. However in the screening of QS activity, 4 isolates (RB-25, RB-38, RB-44 and RB-48) were recovered to exhibit QS activity as represented in section 4.5. Due to time constraint, only isolates RB-25, RB-38, RB-44 and RB-48 were subjected to identification process, characterization of QS signaling profile and genome sequencing.

4.2 Morphology Observation

Morphological characteristics of the four QS isolates (RB-25, RB-38, RB-44 and RB-48) are listed in Table 4.1. SEM observation was performed only for isolate RB-25 and RB-38 (as presented in Figure 4.1) because only isolates RB-25 and RB-38 were selected for further analysis as discussed in section 4.13.

Table 4.1. Morphology and appearance of RB-25, RB-38, RB-44 and RB-48.

Isolate	Pigmentation	Form	Elevation	Margin	Surface	Gram
RB-25	Pale White	Circular	Raised	Entire	Dry	Negative
RB-38	Milky	Circular	Raised	Curled	Smooth	Negative
RB-44	Milky	Circular	Raised	Curled	Smooth	Negative
RB-48	Light green	Irregular	Raised	Lobate	Glittering	Negative

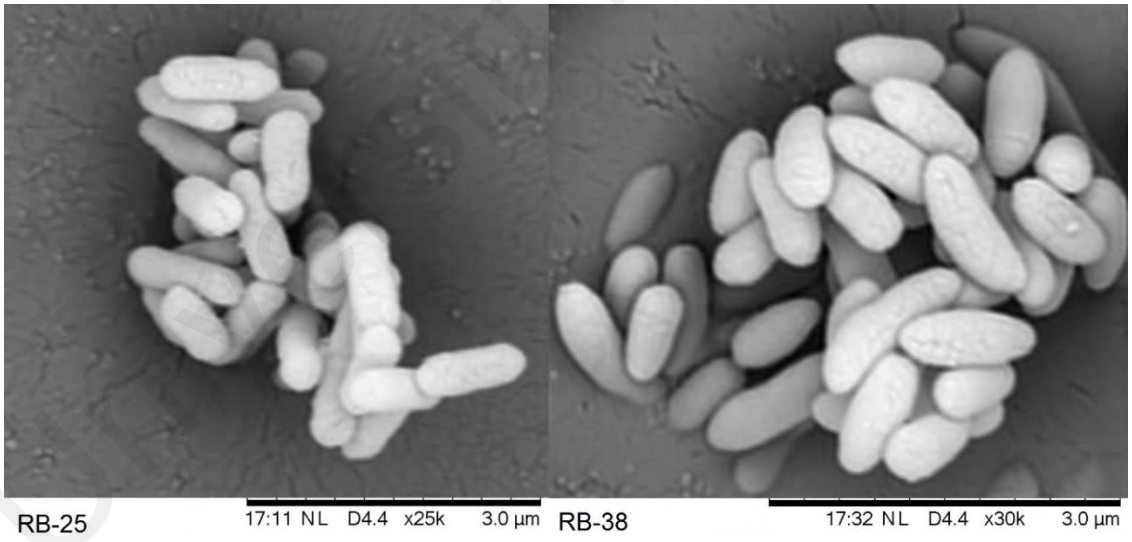


Figure 4.1. SEM image of RB-25 and RB-38. Left: isolate RB-25 was observed to be 3-4 × 7.5-9µm; Right: isolate RB-38 was observed to be 3.5-4.5 × 4.2-5.3µm.

4.3 Bacterial Identification

Initial bacterial identification was performed using MALDI-TOF MS analysis as it provides rapid and accurate identification. However, as MALDI-TOF MS analysis is developed primarily for identification of clinical microorganism whereby this is identification of environmental microorganism, 16S rRNA gene sequence analysis was also performed for verification purpose.

4.3.1 MALDI-TOF MS analysis

Initial identification using MALDI-TOF MS analysis for each the QS isolates are listed in Table 4.2. With score value lower than 1.7, no reliable identification was made for isolate RB-25 indicating possibility of discovery of a new species of bacteria. Meanwhile, isolates RB-38, RB-44 and RB-48 were identified with high confidence level to the species level (score value higher than 2.0) as *P. pnomenusa*, *P. pnomenusa* and *P. aeruginosa* respectively.

Dendogram from MALDI-TOF MS analysis were also generated using MALDI Biotyper MSP creation method (Bruker Daltonics, USA) demonstrating the graphical distance value between the 4 isolates and their closest neighbour (Figure 4.2-4.5).

Table 4.2. Identification of MALDI-TOF MS analysis.

Isolates	Closest Match Identity	Score Value
RB-25	*Serratia fonticola*	1.695
RB-38	*Pandoraea pnomenusa*	2.42
RB-44	*Pandoraea pnomenusa*	2.35
RB-48	*Pseudomonas aeruginosa*	2.512

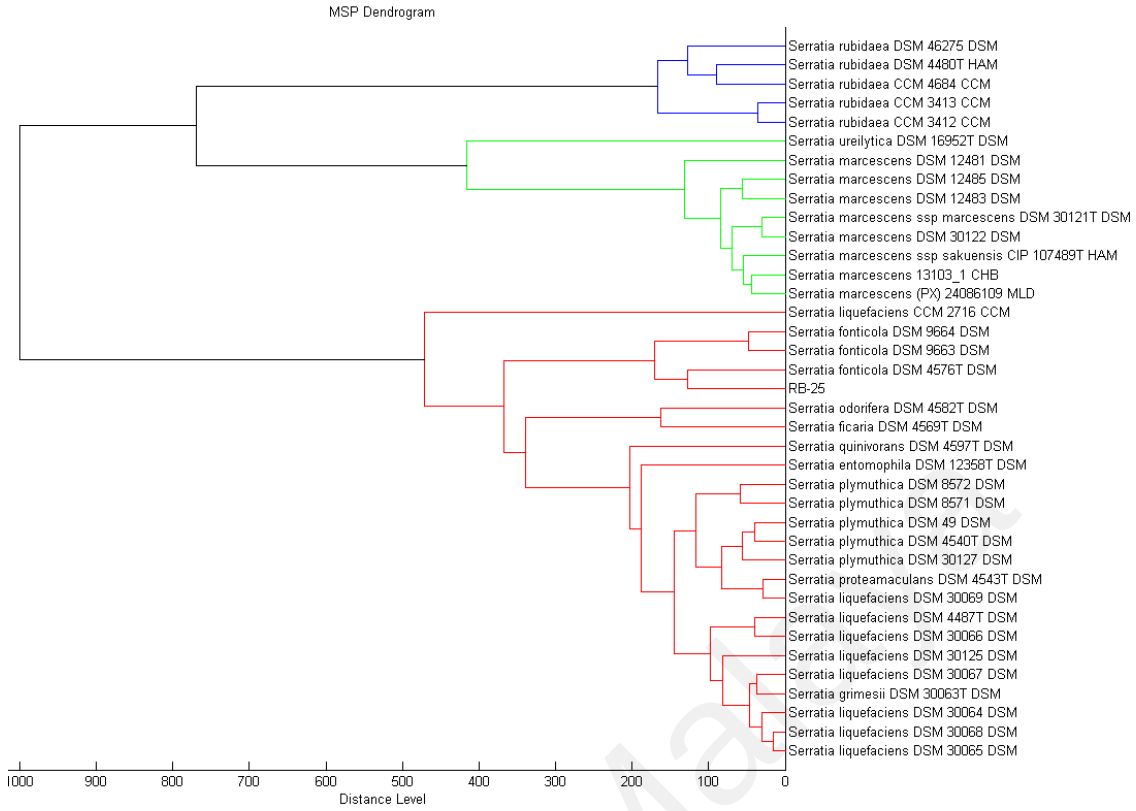


Figure 4.2. Dendogram of isolate RB-25 from MALDI-TOF MS analysis. The phylogeny indicated that RB-25 is clustered within the genus of *Serratia* with a high distance value (distance level of 200) with *S. fonticola* DSM 4576^{T}.

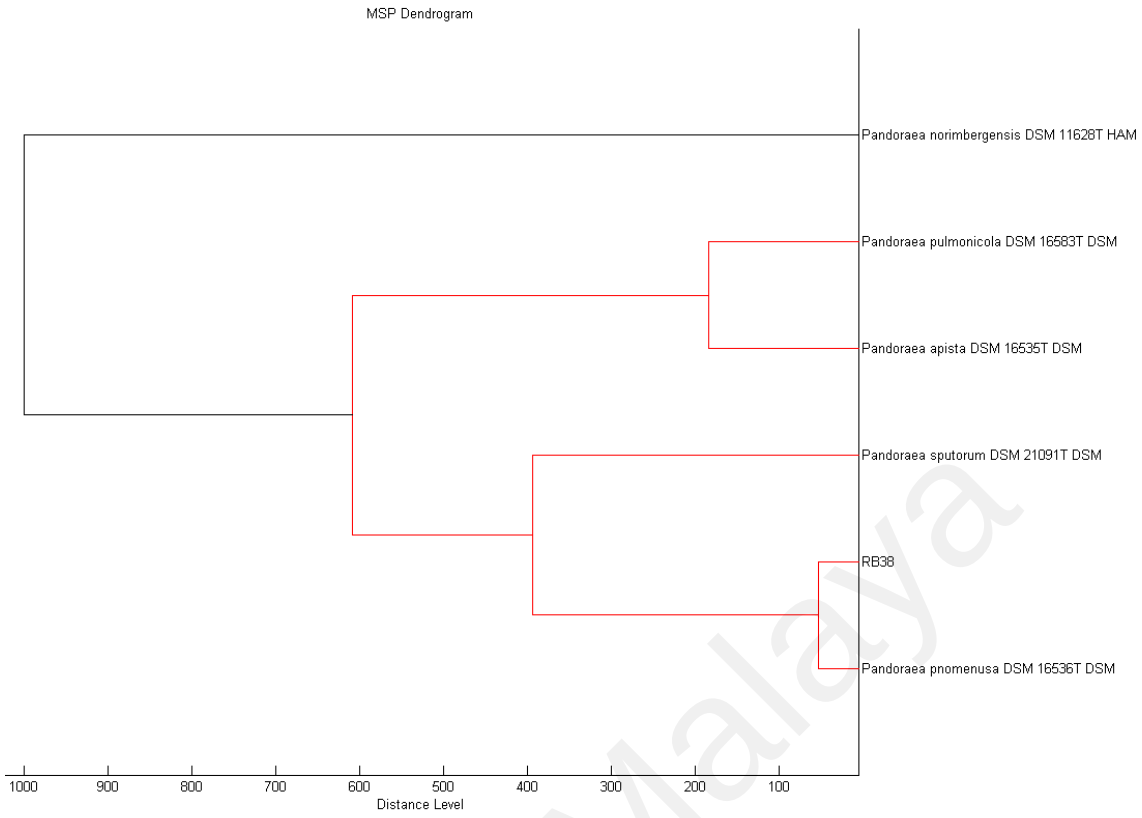


Figure 4.3. Dendogram of isolate RB-38 from MALDI-TOF MS analysis. The phylogeny demonstrated that RB-38 is closely related with *P. pnomenusa* DSM 16536^T with close distance value of 50.

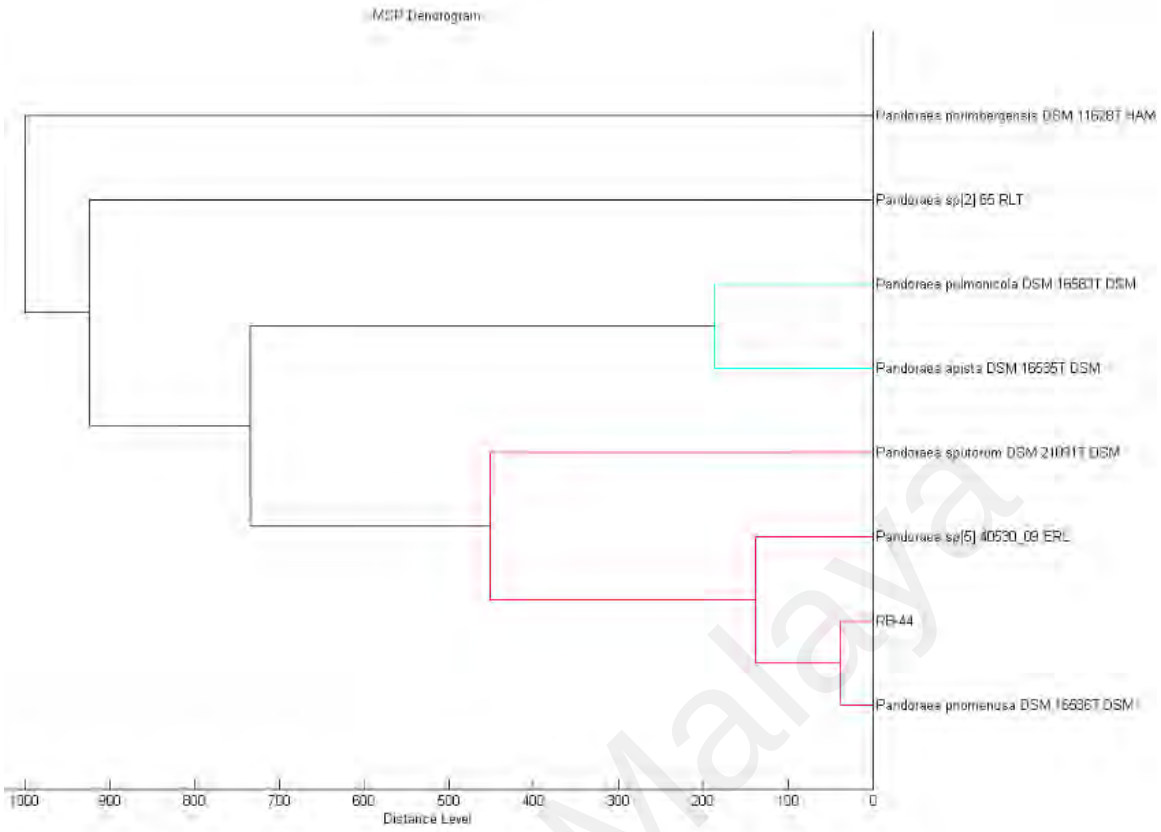


Figure 4.4. Dendogram of isolate of RB-44 from MALDI-TOF MS analysis. The phylogeny demonstrated that RB-44 is closely related with *P. pnomenusa* DSM 16536^{T} with close distance value of 50.

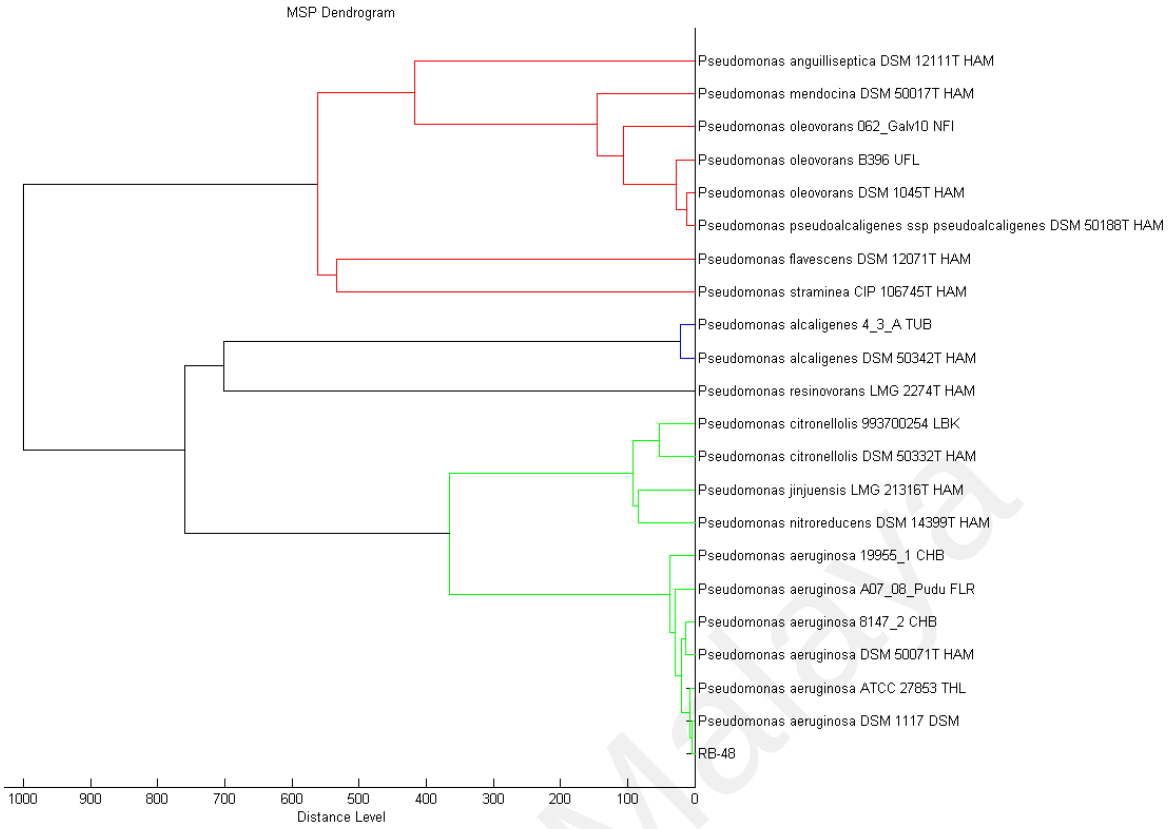


Figure 4.5. Dendogram of isolate of RB-48 from MALDI-TOF MS analysis. The phylogeny demonstrated that RB-48 is very closely related with *P. aeruginosa* DSM 1117 with close distance value (distance value lower than 50).

4.3.2 16S rRNA Gene Sequence Pairwise Similarity Analysis

Bacterial identification was further conducted with amplification and sequencing of 16S rRNA gene. All 16S rRNA gene sequences were initially subjected to comparison against EzTaxon database for identification purpose with results as listed in Table 4.3. Pairwise similarity of 16S rRNA gene sequence of RB-25 in EzTaxon database was lower than 97% indicating that isolate RB-25 was distantly related to all characterized bacteria in the database. Thus, detail taxonomic characterization was required to be conducted for isolate RB-25 in order to determine its status as a novel taxon.

On the other hand, pairwise similarity of RB-38 and RB-44 were higher than 99% securing their identity as *P. pnomenusa*. Lastly, with high confidence value of 100% pairwise similarity, RB-48 was identified as *P. aeruginosa*. Closest relatives of all isolates in comparison with EzTaxon blast results are available in Appendices 1-4.

Table 4.3. BLAST result against EzTaxon database.

Isolate	Closest relative	Pairwise similarity	Completeness
RB-25	*Pantoea rwandensis* LMG 26275^T	96.58%	100%
RB-38	*Pandoraea pnomenusa* CCUG 38742^T	99.86%	95.90%
RB-44	*Pandoraea pnomenusa* CCUG 38742^T	99.79%	96.30%
RB-48	*Pseudomonas aeruginosa* JCM 5962^T	100%	100%

4.4.3 16S rRNA Gene Sequences Phylogenetic Analysis

All 16S rRNA gene sequences has been deposited in NCBI public database with accession number listed in Table 4.4. Phylogenetic analysis of isolate RB-25 demonstrated that RB-25 form a distinct cluster in the *Enterobacteriaceae* family indicated that RB-25 should represent a distinct novel genus (Figure 4.6). Hence taxonomic characterization was performed for isolate RB-25 to identify its position as a new genus.

Phylogenetic analysis of both isolate RB-38 and RB-44 demonstrated that they are closely related to *P. pnomenusa* CCUG 38742^T (Figure 4.7). Phylogenetic analysis of isolate RB-48 demonstrated that it is highly related *P. aeruginosa* JCM 5962^T (Figure 4.8). All 16S rDNA sequences are available in Appendices 5-8.

Table 4.4. GenBank accession number of 16S rRNA gene sequences.

Isolates	Identity	Length (bp)	Accession Number
RB-25	*Chania multitudinesentens*	1611	KJ081442.1
RB-38	*Pandoraea pnomenusa*	1395	KJ507404.1
RB-44	*Pandoraea pnomenusa*	1401	KF648559.1
RB-48	*Pseudomonas aeruginosa*	1153	KF724932.1

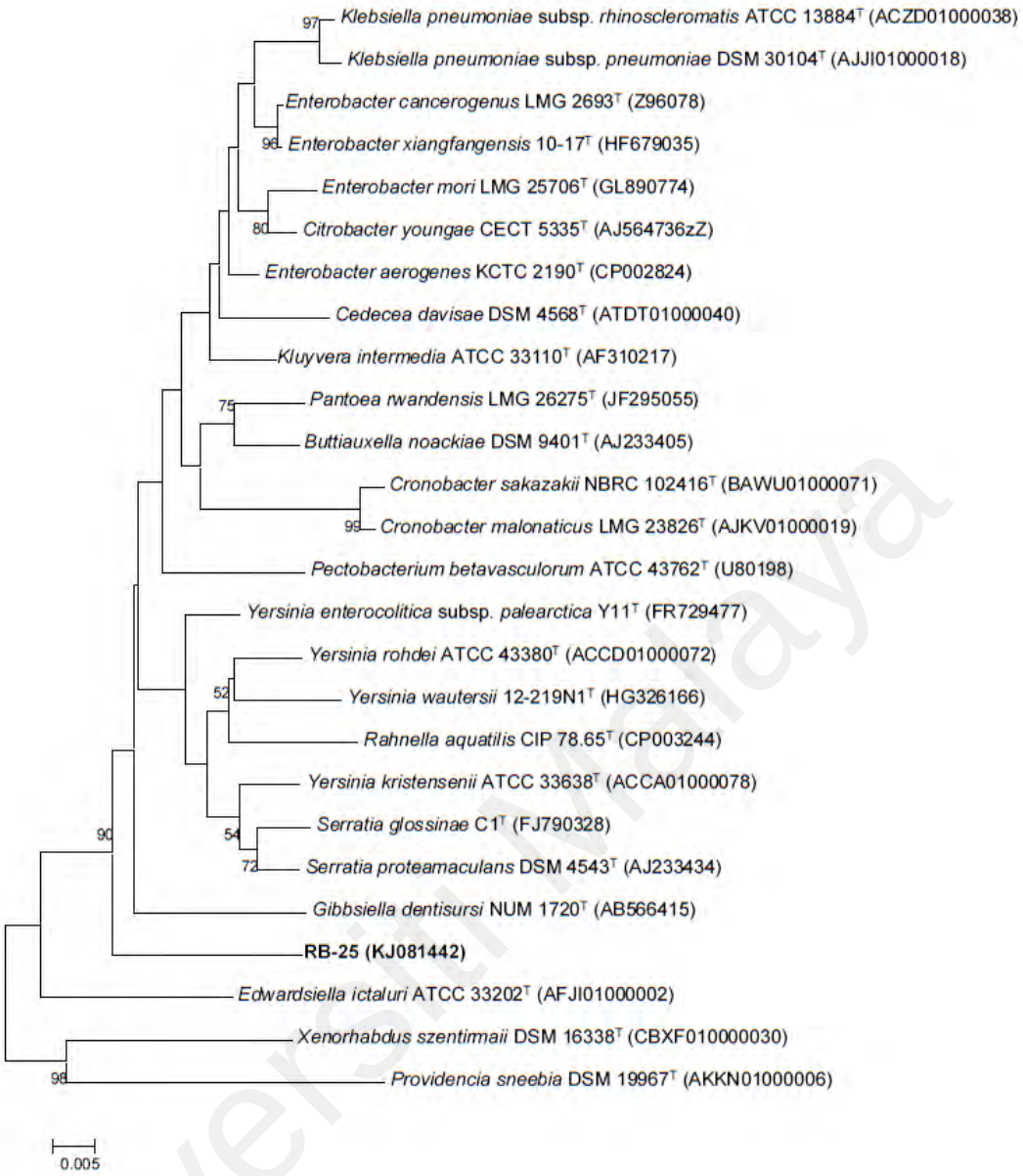


Figure 4.6. 16S rDNA phylogenetic analysis of RB-25. In the phylogeny, RB-25 form a distinct cluster within the family of *Enterobacteriaceae* indicated that RB-25 should represent a distinct novel genus. Bootstrap value (expressed as percentage with 1000 replicates) greater than 50 % are displayed at the branch point. Bar, 0.005 substitution per nucleotide position.

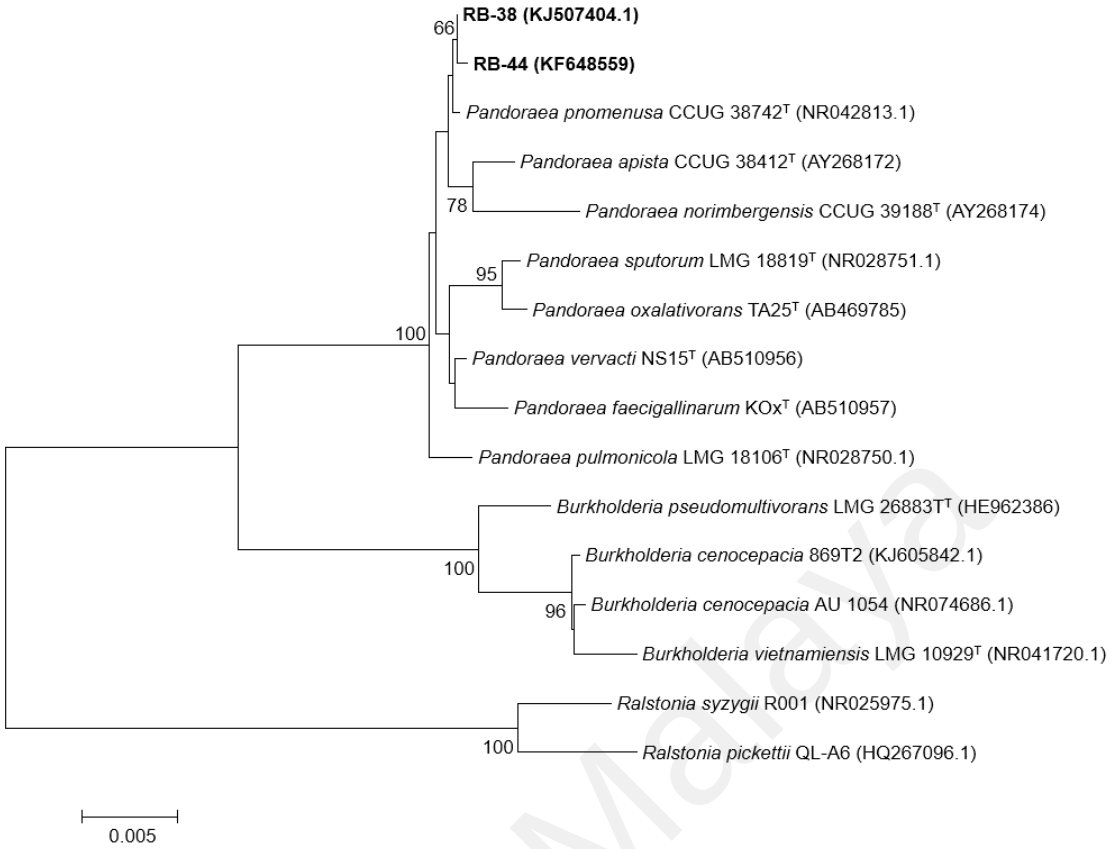


Figure 4.7. 16S rDNA phylogenetic analysis of RB-38 and RB-44. In the phylogeny, RB-38 and RB-44 are closely related to *P. pnomenusa*. Bootstrap value (expressed as percentage with 1000 replicates) greater than 50 % are displayed at the branch point. Bar, 0.005 substitution per nucleotide position

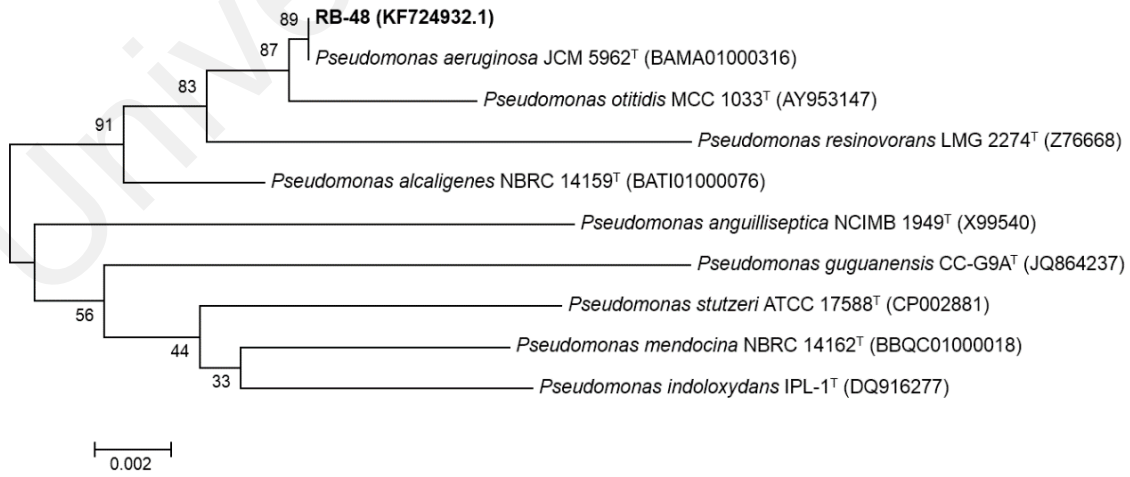


Figure 4.8. 16S rDNA phylogenetic analysis of RB-48. In the phylogeny, RB-48 is closely related to *P. aeruginosa*. Bootstrap value (expressed as percentage with 1000 replicates) greater than 50 % are displayed at the branch point. Bar, 0.005 substitution per nucleotide position.

4.4 Taxonomic Characterisation of Isolate RB-25

Taxonomic characterisation performed for isolate RB-25 confirmed its position as a novel genus in *Enterobacteriaceae* family with the newly proposed identity of *Chania multitudinisentens* RB-25^T gen. nov, sp. nov. Characteristic defining *Serratia* species was performed according to Bergey's Manual of Systematic Bacteriology in section 4.4.3 in which isolate RB-25 showed negative reactions for glucose oxidation test, tetrathionate reductase test, gluconate dehydrogenase test, 2-ketogluconate dehydrogenase test and gas production test suggesting that isolate RB-25 does not belong to *Serratia* genus. Hence, phenotypic characterization analysis using GENIII, PM1, PM2 MicroPlate; biochemical analysis using API 20E, API 20NE and API ZYM; and lipid composition analysis of polar lipid, respiratory lipoquinones and whole-cell FAME were conducted for taxonomical characterisation of isolate RB-25^T. Detailed comparisons against closest genera was discussed in section 4.11. Genomic characterization was subsequently performed after complete genome sequence of RB-25^T was sequenced.

4.4.1 Phenotypic and Biochemical Analysis

4.4.1.1 Phenotypic Identification (GENIII Microplate)

Analysis from phenotypic identification using GENIII microplate identified isolate RB-25 with *Pantoea agglomerans* as the closest relative and *Burkholderia caryophylli* as the second closest relative. Biochemical profile of isolate RB-25 were listed in Appendix 9.

4.4.1.2 Phenotype MicroArrays Analysis (PM1 and PM2 MicroPlate)

In PM1 Microplate, utilization of carbon source L-arabinose, *N*-acetyl-D-glucosamine, D-saccharic acid, succinate acid, D-galactose, L-aspartic acid, D-trehalose, D-mannose, D-sorbitol, glycerol, L-fucose, D-gluconic acid, D,L-α-glycerol-phophate, D-xylose, L-lactic acid, D-mannitol, L-glutamic acid, D-ribose, tween 20, L-rhamnose, D-fructose, acetic acid, α-D-glucose, maltose, L-asparagine, tween 40, sucrose, uridine, D-glucose-1-phosphate, tween 80, *β*-methyl-D-glucosidase, adonitol, maltotriose, 2-deoxy adenosine, adenosine, m-inositol, bromo succinic acid, mucic acid, glycyl-L-glutamic acid, L-serine, methyl pyruvate, glycyl-L-proline, p-hydroxy phenyl acetic acid, D-psicose, L-lyxose, pyruvic acid, L-galactonic acid-*γ*-lactone were positive while L-proline, D-alanine, dulcitol, D-serine, D-gluruconic acid, formic acid, D-glucose-6-phosphate, D-galactonic acid-*γ*-lactone, D,L-malic acid, D-melibiose, thymidine, D-aspartic acid, D-glucosaminic acid, 1,2-propanediol, *α*-keto-glutaric acid, *α*-keto-butyric acid, *α*-methyl-D-galactosidase, *α*-D-lactose, lactulose, L-glutamine, m-tartic acid, D-fructose-6-phosphate, *α*-hydroxy glutaric acid, *α*-hydroxy butyric acid, glycyl-L-aspartic acid, citric acid, D-threonine, fumaric acid, propionic acid, glycolic acid, glyoxylic acid, D-cellobiose, inosine, tricarballytic acid, L-threonine, L-alanine, L-alanyl-glycine,

acetoacetic acid, *N*-acetyl-β-D-mannosamine, mono methyl succinate, D-malic acid, L-malic acid, m-hydroxy phenyl acetic acid, tyramine, glucuronamide, D-galactonic acid, phenylethyl amine, 2-aminoethanol were negative.

In PM2 Microplate, utilization of carbon source Dextrin, pectin, *N*-acetyl-D-galactosamine, *β*-D-allose, D-arabinose, D-arabitol, L-arabitol, arbutin, i-erythritol, 3-methyl glucose, palatinose, salicin, D-tagatose, xylitol, D-glucosamine, β-hydroxy butyric acid, D-lactic acid methyl ester, quinic acid, sebacic acid, L-histadine, hydroxyl-L-proline, putrescine were positive while chondroitin sulfate C, *α*-cyclodextrin, *β*-cyclodextrin, *γ*-cyclodextrin, gelatin, glycogen, inulin, laminarin, mannan, *N*-acetyl-neuraminic acid, amygdalin, 2-deoxy-D-ribose, D-fucose, 3-0-*β*-D-galactopyranosyl-D-arabinose, gentiobiose, L-glucose, lactitol, D-melezitose, maltitol, a-methyl-D-glucoside, *β*-methyl-D-galactoside, *β*-methyl-D-glucuronic acid, *α*-methyl-D-mannoside, *β*-methyl-D-xyloside, D-raffinose, sedoheptulosan, L-sorbose, stachyose, turanose, *N*-acetyl-D-glucosaminotol, *γ*-amino butyric acid, *δ*-amino valeric acid, butyric acid, capric acid, caproic acid, citramalic acid, 2-hydroxy benzoic acid, 4-hydroxy benzoic acid, *γ*-hydroxy butyric acid, a-keto-valeric acid, itaconic acid, 5-keto-D-gluconic acid, malonic acid, melbionic acid, oxalic acid, oxalomalic acid, D-ribono-1,4-lactone, sorbic acid, succinamic acid, D-tartaric acid, L-tartaric acid, acetamide, L-alaninamide, *N*-acetyl-glutamic acid, L-arginine, glycine, L-homoserine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, L-pyroglutamic acid, L-valine, D,L-carnitine, sec-butylamine, D,L-octopamine, dihydroxy acetone, 2,3-butanediol, 2,3-butanone, 3-hydroxy 2-butanone were negative.

4.4.1.3 API Biochemical Assay Analysis

In API-ZYM assays, alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase, leucine arylamidase, acid phosphatase, trypsin, naphthol-As-BI-phosphohydrolase, *β*-galactosidase, *β*-glucuronidase, *β*-glucosidase and *N*-acetyl-*β*-glucosaminidase are present.

In API-20NE assays, nitrate reduction, glucose fermentation, esculin hydrolysis, *β*-galactosidase, glucose assimilation, arabinose assimilation, mannose, mannitol, *N*-acetylglucosamine, maltose and gluconate assimilation were positive. Indol, arginindigydrolase, urease, gelatine hydrolysis, caprate assimilation, adipate assimilation, malate assimilation, citrate assimilation, phenylacetate assimilation were negative.

In API-20E assays, *β*-galactosidase was positive and acid was produced from glucose, mannitol, inositol, sorbitol, rhamnose, saccharose, amygdalin and arabinose. Arigininhydrolase, lysindecarboxylase, ornithindecarboxylase, citrate assimilation, H_2S production, urease, tryptophandesaminase, indole production, VP reaction, gelatinase were negative and acid is not produce from melibiose.

4.4.2 Lipid Composition Analysis

4.4.2.1 Respiratory Lipoquinones, Polar Lipid and FAME Analysis

Ubiquinone-8 (Q-8) was detected as the major isoprenoid quinone of isolate RB-25 for analysis of respiratory lipoquinones. Five polar lipid which includes one unidentified aminolipid, phospholipid, phosphoaminolipid, phosphatidylethanolamine and phosphatidylglycerol were detected from the analysis of polar lipid (Figure 4.9). Fatty acid profile of isolate RB-25 consisted of $C_{12:0}$ (2.1%), $C_{14:0}$ (6.7%), unknown 14.502 (1.0%), $C_{15:0}$ (0.3%), $C_{16:0}$ (30.4%), $C_{16:1}$ ω 5*c* (0.2%), $C_{17:0}$ (0.3%), $C_{17:0}$ cyclo (5.0%), $C_{18:0}$ (0.7%), $C_{18:1}$ ω 7*c* (16.5%), $C_{19:0}$ iso (0.2%), summed feature 2 (comprised of $C_{14:0}$ 3-OH and/or $C_{16:1}$ iso I and/or $C_{12:0}$ aldehyde and/or unknown 10.928) (6.9%) and summed feature 3 (comprised of $C_{16:1}$ ω 7*c* and/or $C_{15:0}$ iso 2-OH) (29.7%).

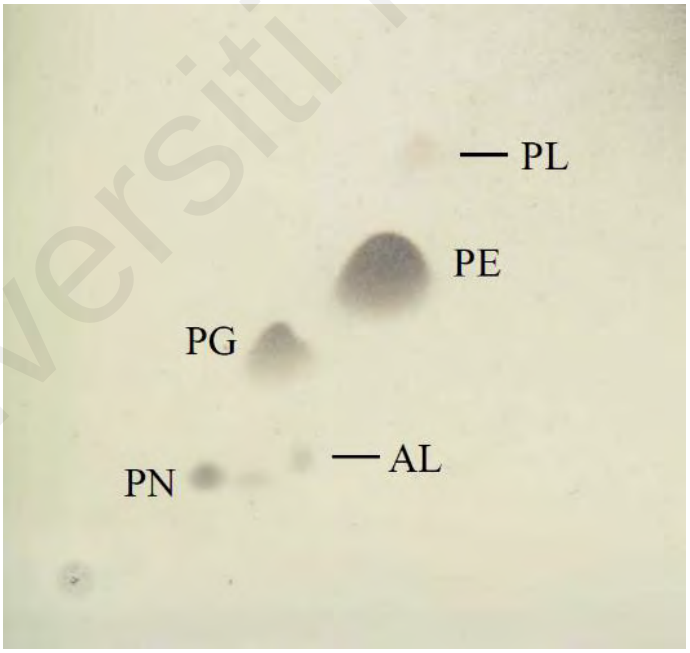


Figure 4.9. Two dimensional silica gel TLC of polar lipid of RB-25. PL: Phospholipid; AL: Unidentified Aminolipid; PN: Phosphoaminolipid; PE: Phosphatidylethanolamine; PG: Phosphatidylglycerol

4.4.3 Special Characteristics Defining *Serratia* species

4.4.3.1 Glucose Oxidation Test Analysis

Isolate RB-25 showed negative reaction (purple colour) with and without PQQ for glucose oxidation test (Figure 4.10). Positive control (yellow colour) was included with *S. marcescens* (positive without PQQ) and *S. liquefaciens* (positive only with supplement of PQQ) while negative control (purple colour) was included with uninoculated medium.

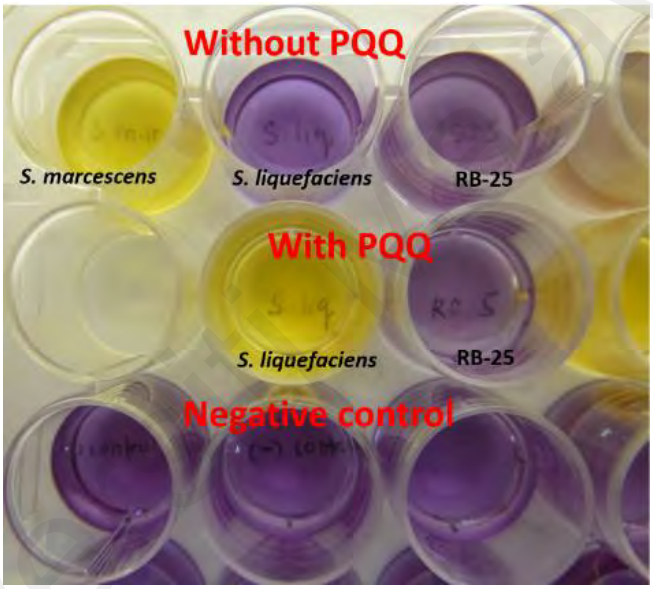


Figure 4.10. Glucose oxidation test. Isolate RB-25 showed negative reaction (purple colour) with and without PQQ. Positive control (yellow colour) was included with *S. marcescens* (positive without PQQ) and *S. liquefaciens* (positive only with supplement of PQQ) while negative control (purple colour) was included with uninoculated medium.

4.4.3.2 Tetrathionate Reductase Test Analysis

Isolate RB-25 showed negative reaction (green colour) for tetrathionate reductase test (Figure 4.11). Positive control (yellow colour) was included with *S liquefaciens* and negative control (green colour) was included with uninoculated medium.

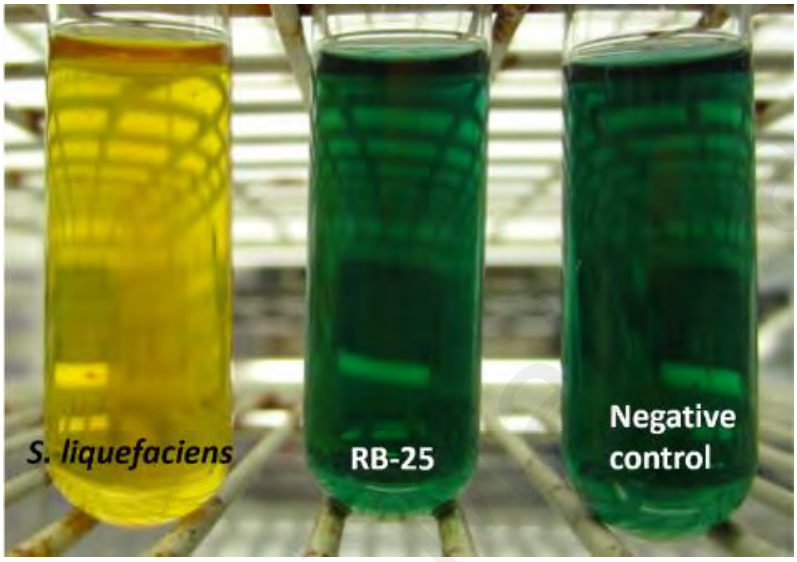


Figure 4.11. Tetrathionate reductase test. Isolate RB-25 showed negative reaction (green colour). *S liquefaciens* and uninoculated medium was included as positive control (yellow colour) and negative control (green colour) respectively.

4.4.3.3 Gluconate Dehydrogenase Test Analysis

Isolate RB-25 showed negative reaction (yellow colour) for gluconate dehydrogenase test (Figure 4.12). Positive control (blue colour) and negative control (yellow colour) was included with *S. marcescens* and *E. coli* respectively.

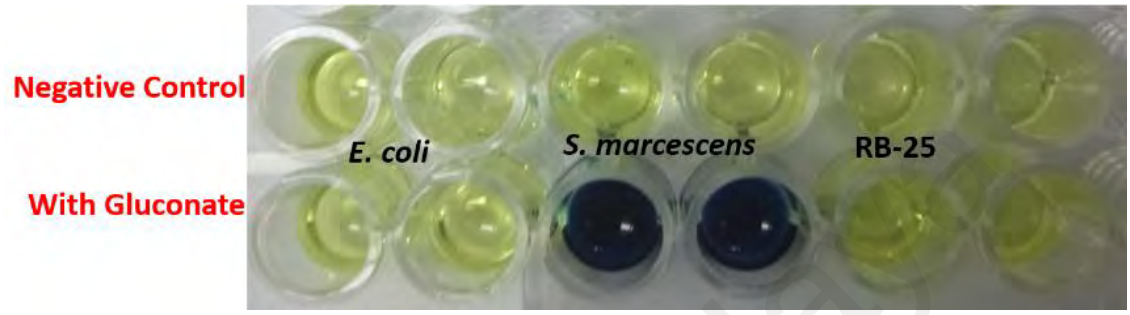


Figure 4.12. Gluconate dehydrogenase test. Isolate RB-25 showed negative reaction (yellow colour) for gluconate dehydrogenase test (Figure 4.18). Positive control (blue colour) and negative control (yellow colour) was included with *S. marcescens* and *E. coli* respectively.

4.4.3.4 2-Ketogluconate Dehydrogenase Test Analysis

Isolate RB-25 showed negative reaction (yellow colour) for 2-ketogluconate dehydrogenase test (Figure 4.13). Positive control (blue colour) and negative control (yellow colour) was included with *S. marcescens* and *E. coli* respectively.

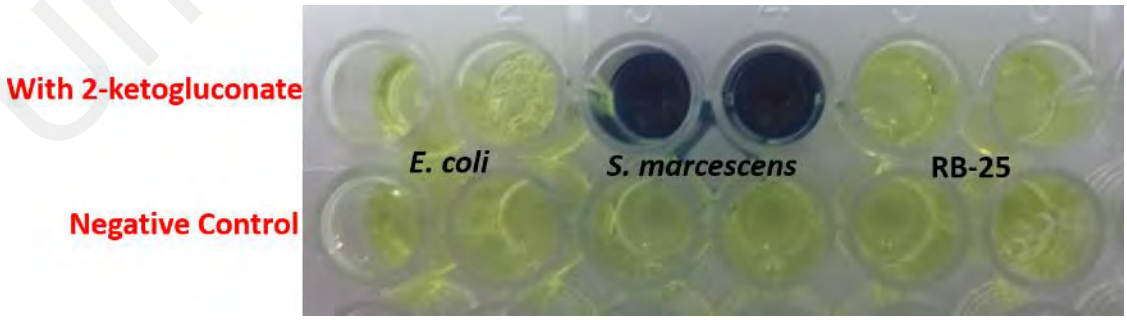


Figure 4.13. 2-ketogluconate dehydrogenase test. Isolate RB-25 showed negative reaction (yellow colour) for 2-ketogluconate dehydrogenase test (Figure 4.19). Positive control (blue colour) and negative control (yellow colour) was included with *S. marcescens* and *E. coli* respectively.

4.4.3.5 Gas Production Test Analysis

Isolate RB-25 did not showed gas production (Figure 4.14). *S. liquefaciens* and *S. marcescens* served as positive and negative control respectively

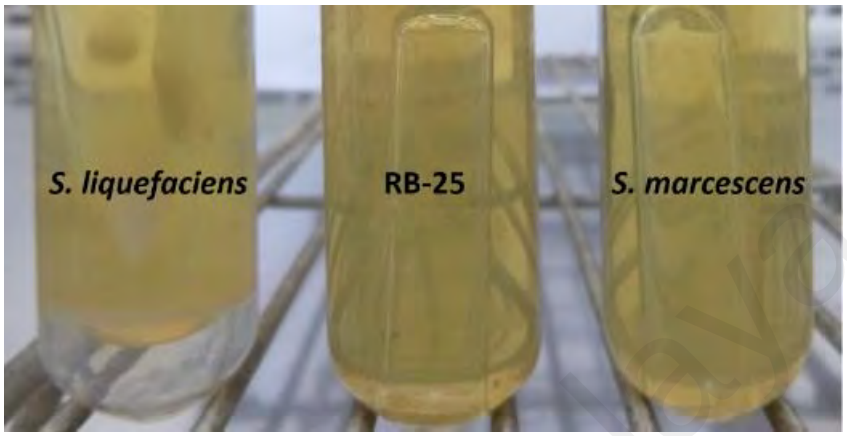


Figure 4.14. Gas production test. Isolate RB-25 did not showed gas production. *S. liquefaciens* and *S. marcescens* served as positive and negative control respectively

4.5 Characterisation of QS Profile

4.5.1 Cross-Streak Bioassay (CVO26 Biosensor)

Out of the 60 isolates, only 4 isolates (RB-25, RB-38, RB-44 and RB-48) induced production of purple pigmentation in CVO26 biosensor which indicates short chain AHL production (Figure 4.15). *E. carotovora* GS101 and *E. carotovora* PNP22 were included as positive and negative controls respectively.

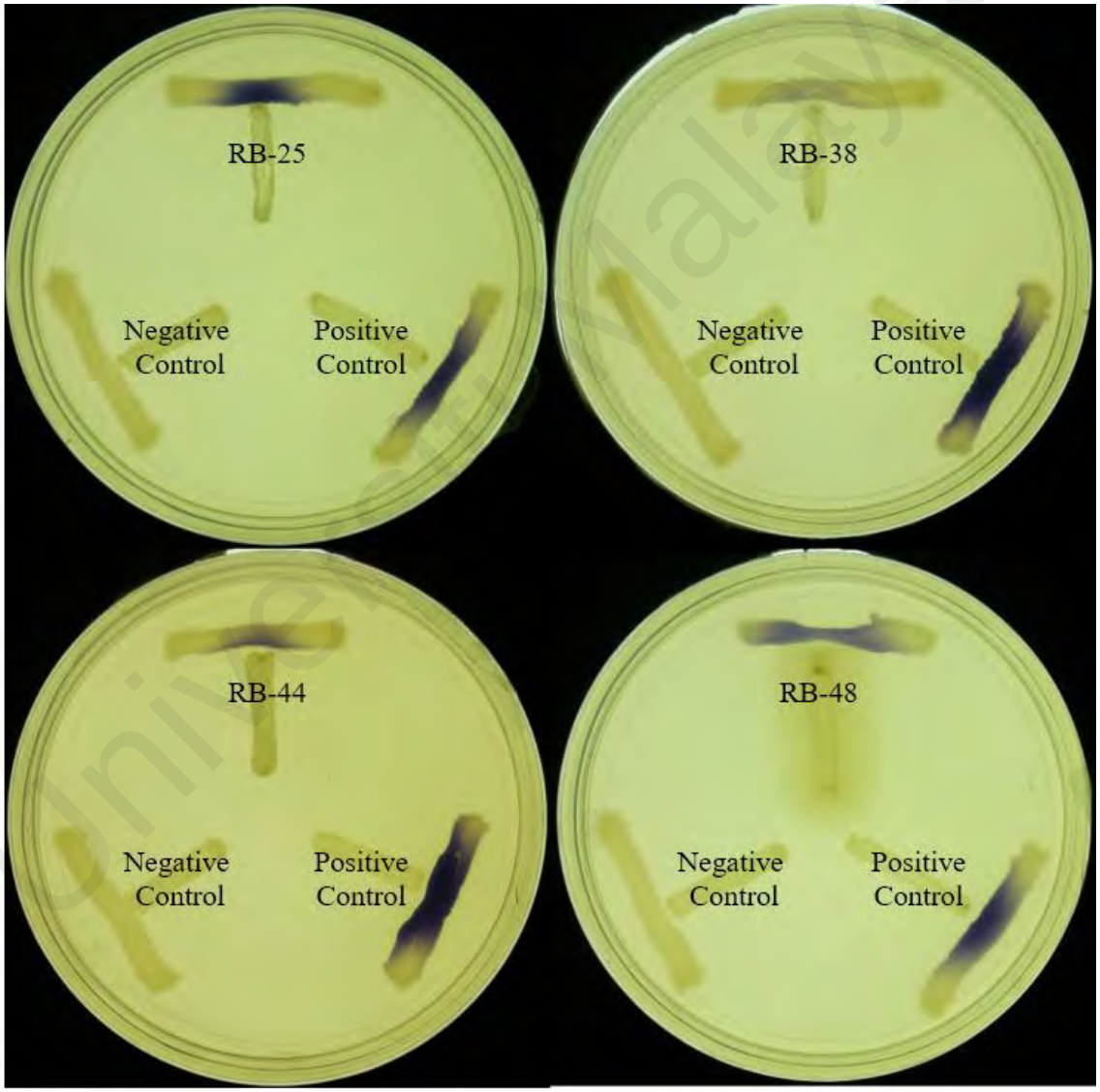


Figure 4.15. Cross-streak bioassay using CVO26 biosensor. *E. carotovora* GS101 and *E. carotovora* PNP22 were included as positive and negative controls, respectively.

4.5.2 Bioluminescence Assay (*E. coli* [pSB401] Biosensor)

QS activity of isolates RB-25, RB-38, RB-44 and RB-48 were confirmed using *E. coli* [pSB401] biosensor. Increment in bioluminescence activity of *E. coli* [pSB401] biosensor supplemented with the AHL extract of isolates RB-25, RB-38, RB-44 and RB-48 indicated for the presence of AHL molecules (Figure 4.16) Positive and negative control were included with LB broth extract inoculated with 3-oxo-C6-HSL and un-inoculated LB broth extract respectively. Raw data reading from luminometer is available in Appendices 10-15.

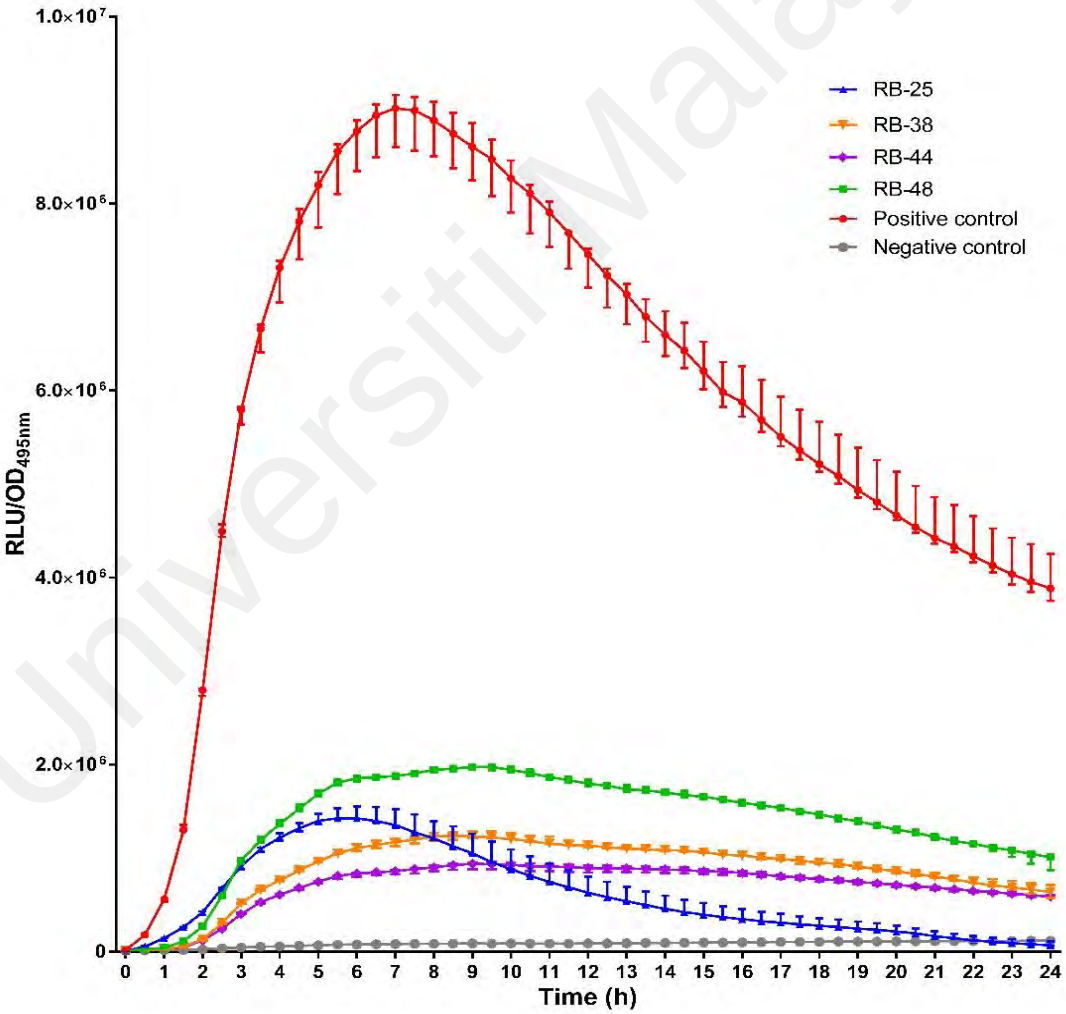


Figure 4.16. Bioluminescence assay using *E. coli* [pSB401] biosensor. Positive and negative controls were included with LB broth extract inoculated with 3-oxo-C6-HSL and un-inoculated LB broth extract, respectively.

4.5.3 Triple Quadrupole MS Analysis

The AHL profile of isolates RB-25, RB-38, RB-44 and RB-48 characterised using MS analysis were summarised in Table 4.5 and chromatogram results were displayed in Figure 4.17-4.20. In brief, isolate RB-25 produced 3 AHLs (C4-HSL, C6-HSL and 3-oxo-C6-HSL), RB-38 and RB-44 produced C8-HSL and RB-48 produced 5 AHLs (C4-HSL, C6-HSL, C8-HSL and 3-oxo-C10-HSL and C12-HSL).

Table 4.5. AHL profile of isolates RB-25, RB-38, RB-44 and RB-48.

Isolate	AHL detected	*m/z* value	Retention time (min)	Abundance
RB-25	C4-HSL	172.0	0.743	556.70
	C6-HSL	200.1	1.985	1198.12
	3-oxo-C6-HSL	214.2	0.940	15,467.14
RB-38	C8-HSL	228.2	4.619	2459.14
RB-44	C8-HSL	228.2	4.633	2459.14
RB-48	C4-HSL	172.0	0.752	1018.00
	C6-HSL	200.3	1.928	1021.42
	C8-HSL	228.4	4.641	706.02
	3-oxo-C10-HSL	270.1	5.385	14,251.16
	C12-HSL	284.2	9.168	514.06

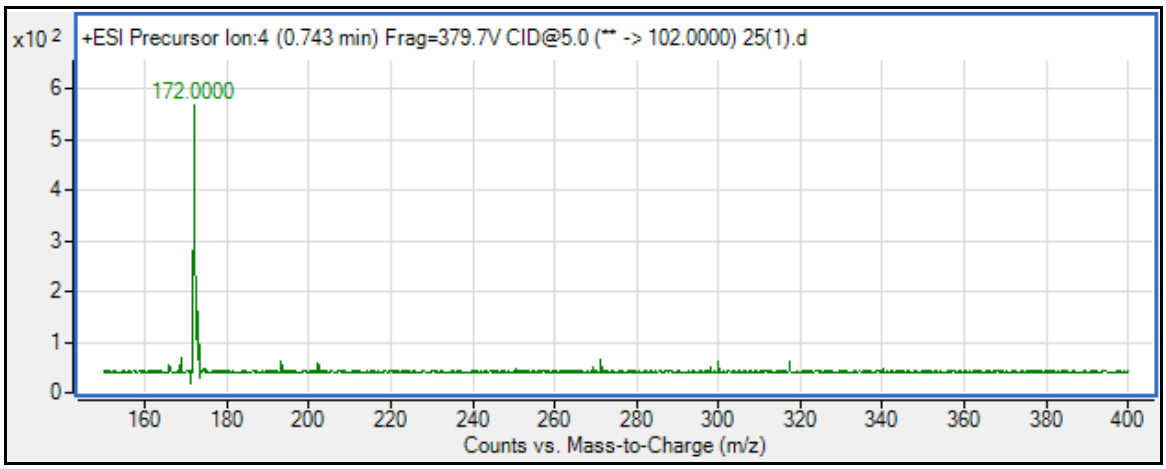


Figure 4.17a

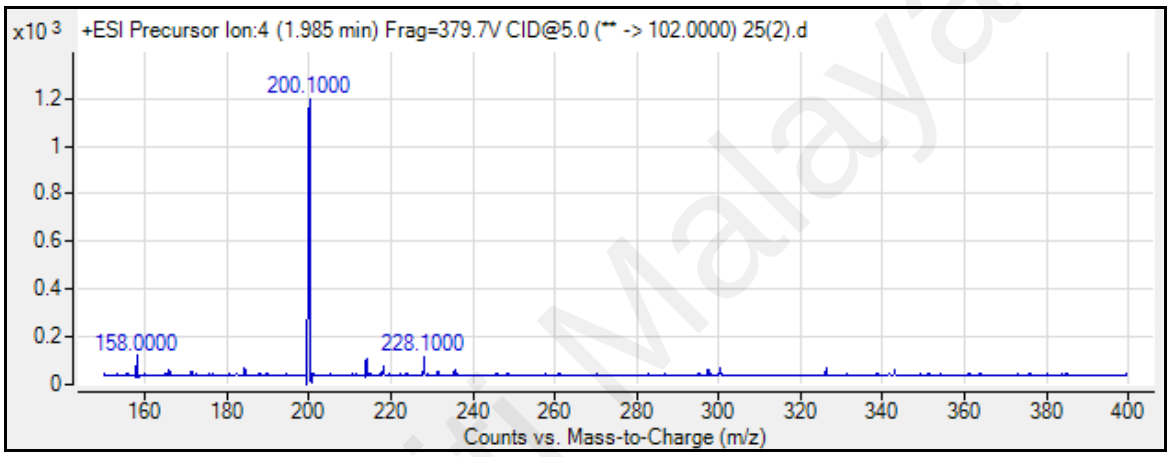


Figure 4.17b

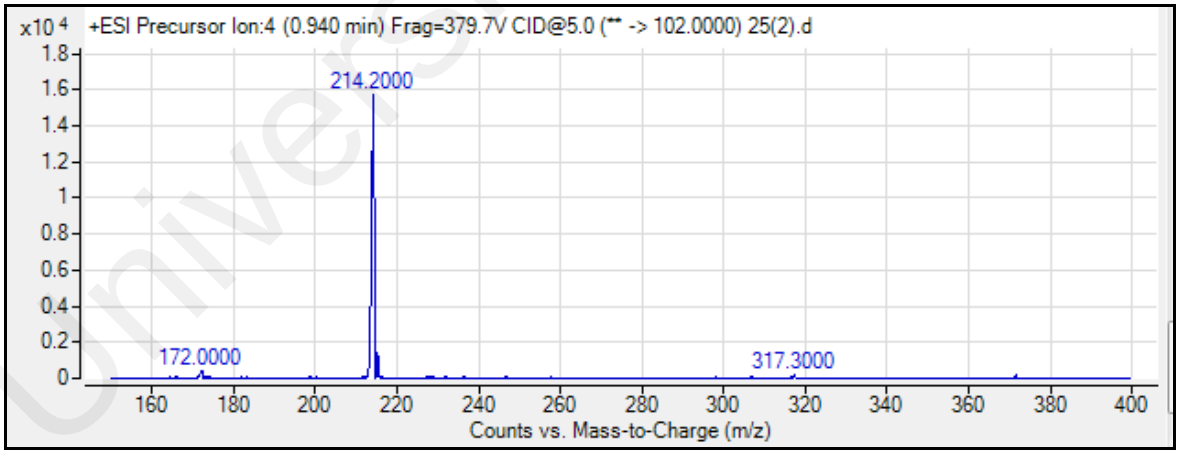


Figure 4.17c

Figure 4.17. MS analysis of RB-25 AHL extract using precursor ion mode. Mass spectrometry analysis showed that RB-25 produces C4-HSL **(Figure 4.17a)** (retention time: 0.743 min; Abundance: 556.7; Abundance %:100; *m/z*: 172.0000); C6-HSL **(Figure 4.17b)** (retention time: 1.985 min; Abundance: 1198.12; Abundance %:100; *m/z*: 200.1000) and 3-oxo-C6-HSL **(Figure 4.17c)** (retention time: 0.940 min; Abundance: 15,467.14; Abundance %:100; *m/z*: 214.2).

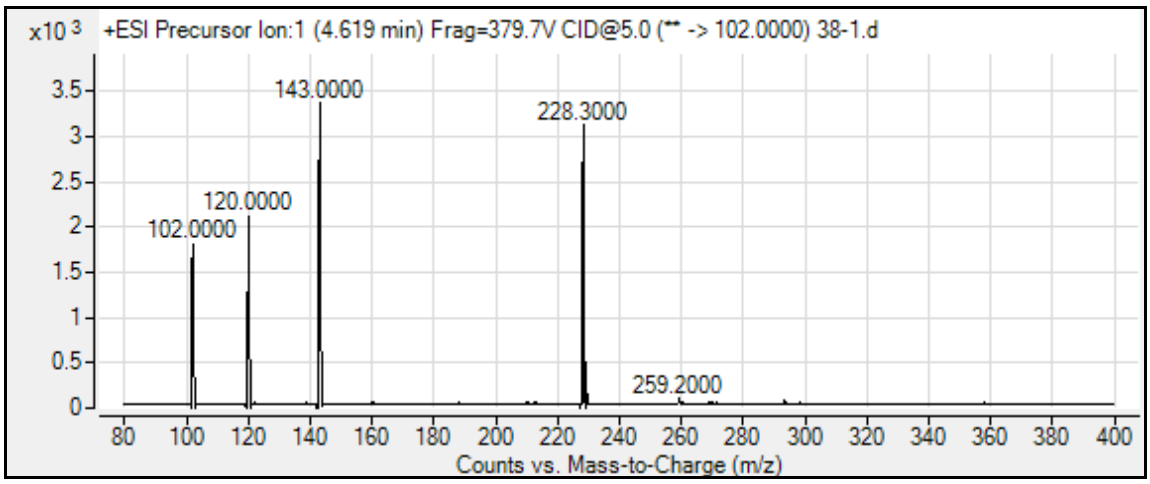


Figure 4.18. MS analysis of RB-38 AHL extract using precursor ion mode. Mass spectrometry analysis showed that RB-38 produces C8-HSL (retention time: 4.633min; Abundance: 2459.14; Abundance %:100; *m/z*: 228.2).

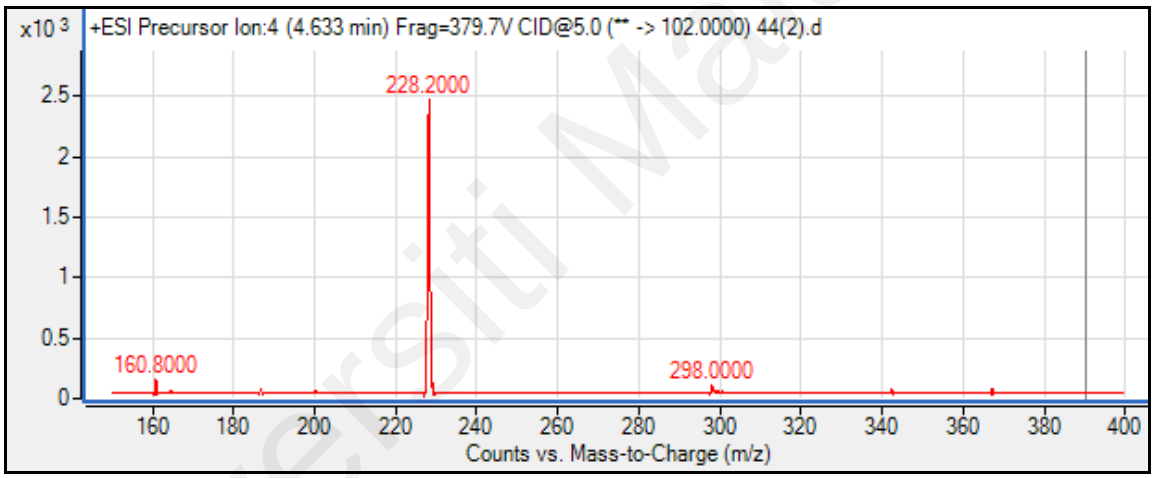


Figure 4.19. MS analysis of RB-44 AHL extract using precursor ion mode. Mass spectrometry analysis showed that RB-44 produces C8-HSL (retention time: 4.633min; Abundance: 2459.14; Abundance %:100; *m/z*: 228.2).

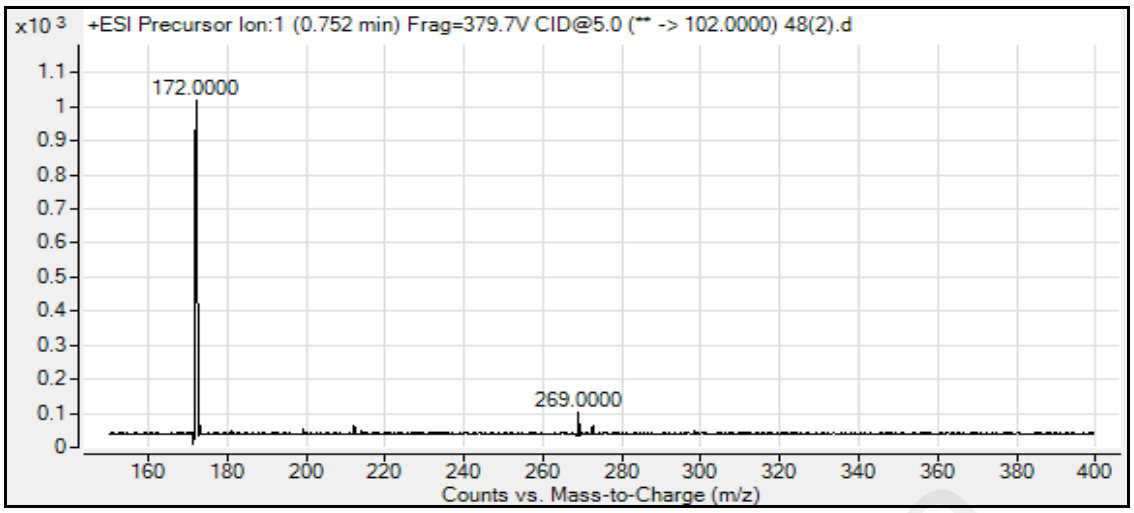


Figure 4.20a

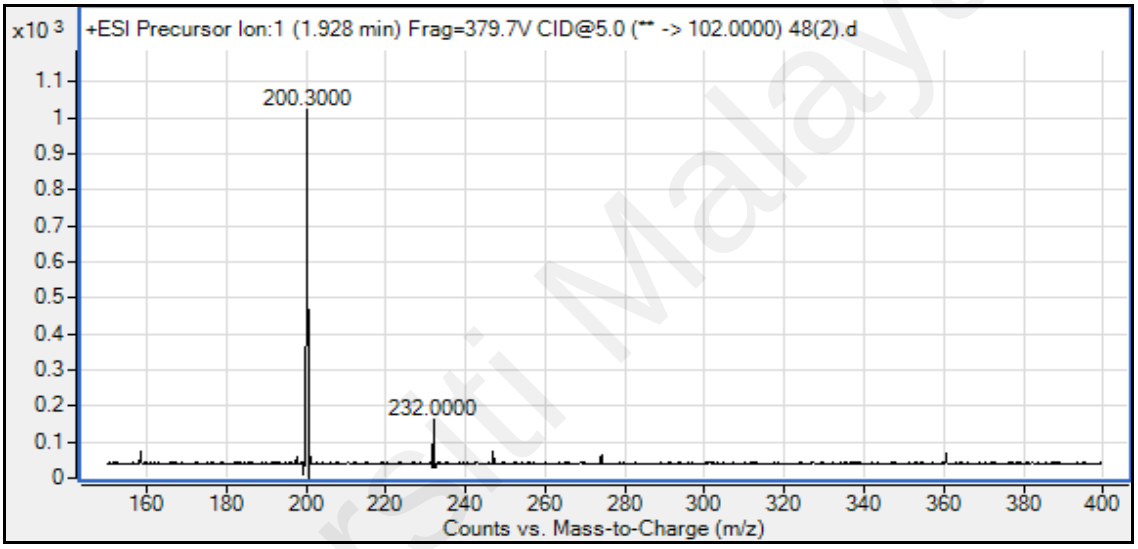


Figure 4.20b

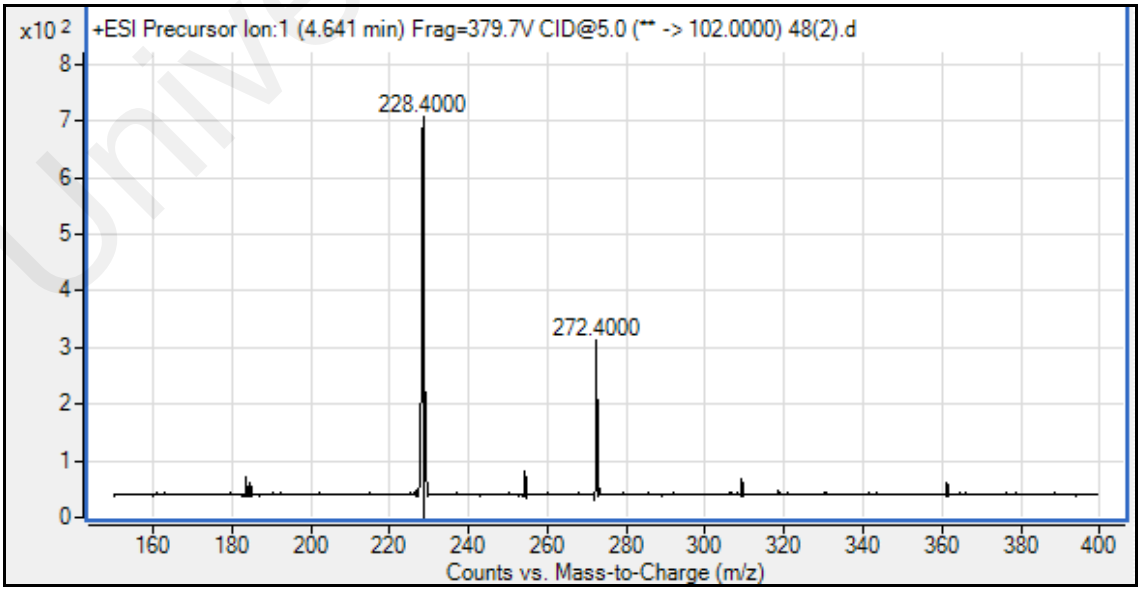


Figure 4.20c

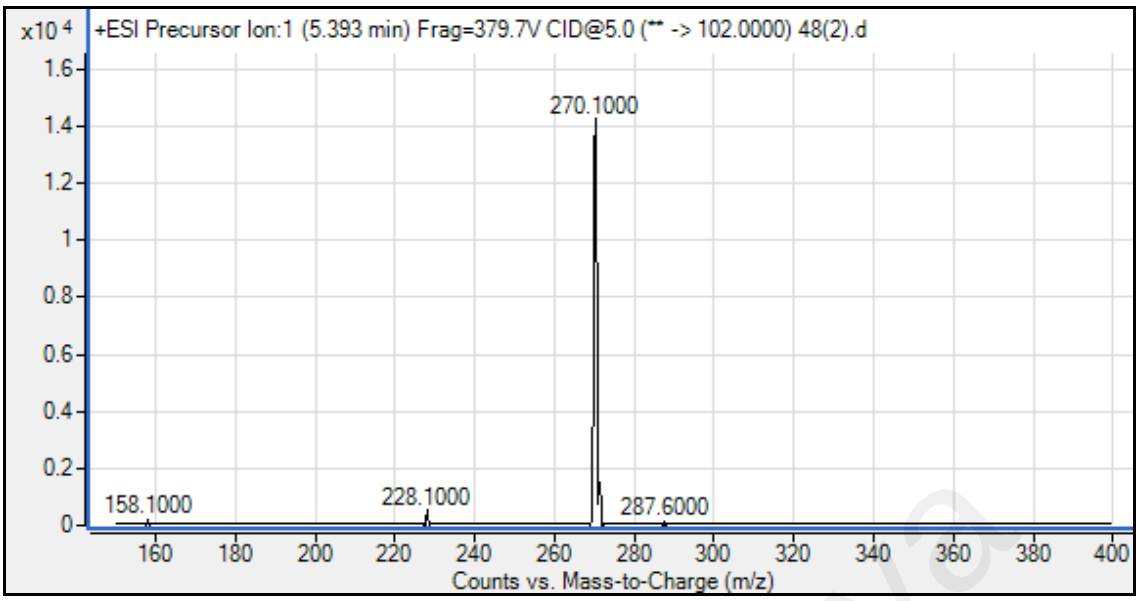


Figure 4.20d

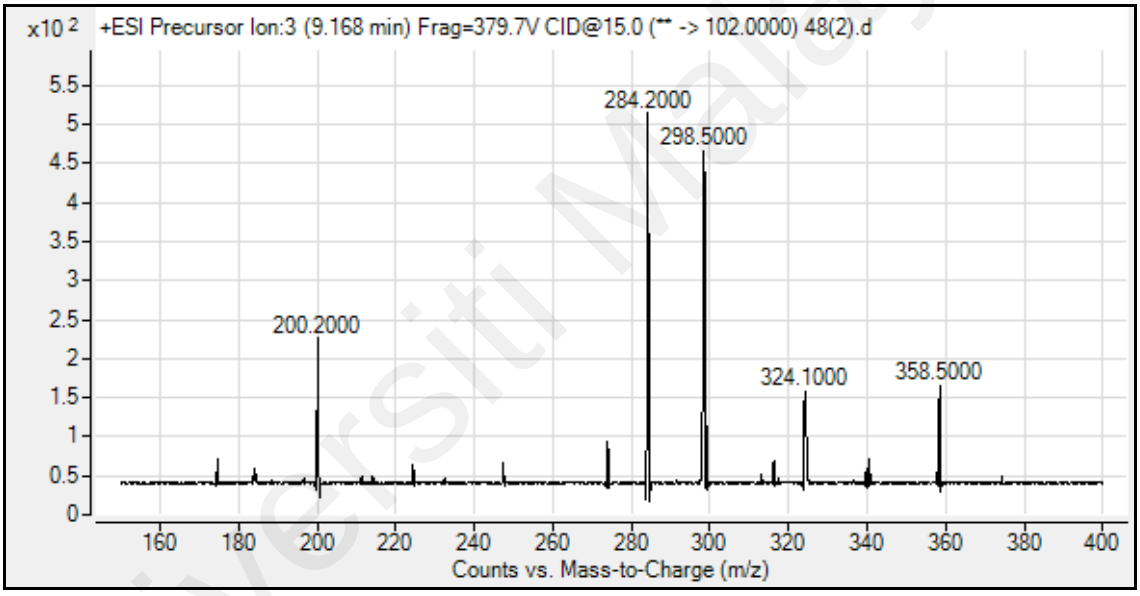


Figure 4.20e

Figure 4.20. MS analysis of RB-48 AHL extract using precursor ion mode**.** Mass spectrometry analysis showed that RB-48 produces 5 AHLs: 4-HSL **(Figure 4.20a)** (retention time: 0.752 min; Abundance: 1018; Abundance %:100; *m/z*: 172.0); C6-HSL **(Figure 4.20b)** (retention time: 1.928 min; Abundance: 1021.42; Abundance %:100; *m/z*: 200.3); C8-HSL **(Figure 4.20c)** (retention time: 4.641 min; Abundance: 706.02; Abundance %:100; *m/z*: 228.4); 3-oxo-C10-HSL **(Figure 4.20d)** (retention time: 5.385 min; Abundance: 14,251.16; Abundance %:100; *m/z*: 270.1); and C12-HSL **(Figure 4.20e)** (retention time: 9.168 min; Abundance: 514.06; Abundance %:100; *m/z*: 284.2)

4.6 Genome Sequencing

Genome sequencing was performed using Sequencing By Synthesis (SBS) technology by Illumina, USA for draft genome sequencing or Single Molecules Real Time (SMRT) sequencing technology by Pacific Biosciences, USA for complete genome sequencing.

4.6.1 Sequencing By Synthesis (SBS) Technology

Summary of genome sequencing information and genome statistics of RB-25, RB-44 and RB-48 using MiSeq platform was summarised in Table 4.7. Genome of RB-38 was not sequenced using MiSeq platform because it was sequenced using SMRT sequencer. Trimming reports and assembly reports are available in Appendices 16-18.

Table 4.7. Information summary and genome statistics of RB-25, RB-44 and RB-48.

Isolate	RB-25	RB-44	RB-48
Finishing quality	Draft genome	Draft genome	Draft genome
Sequencing Platform	MiSeq, Illumina	MiSeq, Illumina	MiSeq, Illumina
Assemblers	CLC Genomics Workbench version 7.5	CLC Genomics Workbench version 7.5	CLC Genomics Workbench version 7.5
Genbank ID	JAJC01	AUZF01	AZYL01
Size (Mb)	5.41319	5.3457	6.17506
Contigs	107	113	183
Fold coverage	101.0×	74.0×	103.0×
GC content	50.80	64.90	66.50
Gene	4831	4787	5717
Protein	4673	4569	5581

4.6.2 SMRT Sequencing Technology

Complete genome of isolate RB-25 was sequenced again using SMRT technology in PacBio (Pacific Biosciences) RSII sequencer as the key gene responsible for the production of 3-oxo-C6-HSL required for downstream analysis was not being sequenced in its draft genome. Detailed explanation has been discussed in section 5.5. Complete genome sequencing information of isolates RB-25 and RB-38 are summarised in Table 4.8. Assembly reports are available in Appendices 19-20.

Table 4.8. Complete genome sequencing information isolates RB-25 and RB-38

	RB-25	**RB-38**
Finishing quality	High quality complete genome	High quality complete genome
Assemblers	HGAP version 3	HGAP version 2
Genbank ID	CP007044.2	CP007506.1
Fold coverage	190×	85×

4.7 Circularity Analysis

Circularisation was performed by trimmed overlapping region in both ends of the genomes manually to provide an accurate genome size. Dot plot analysis (Figure 4.21) and contiguity analysis (Figure 4.22) were subsequently performed to confirm the circularity of complete genome sequences of both RB-25 and RB-38.

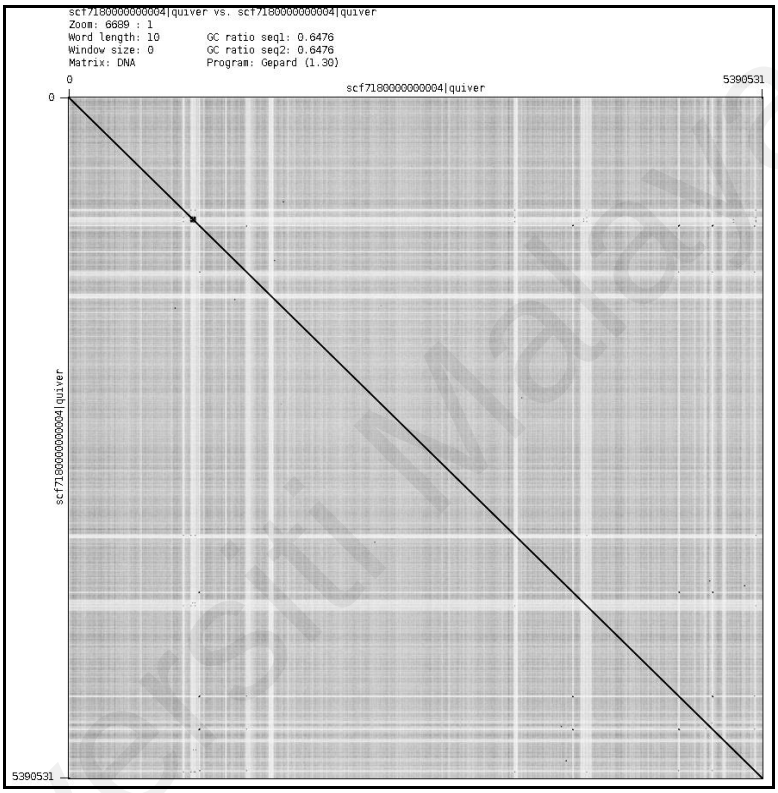


Figure 4.21. Dot plot analysis.

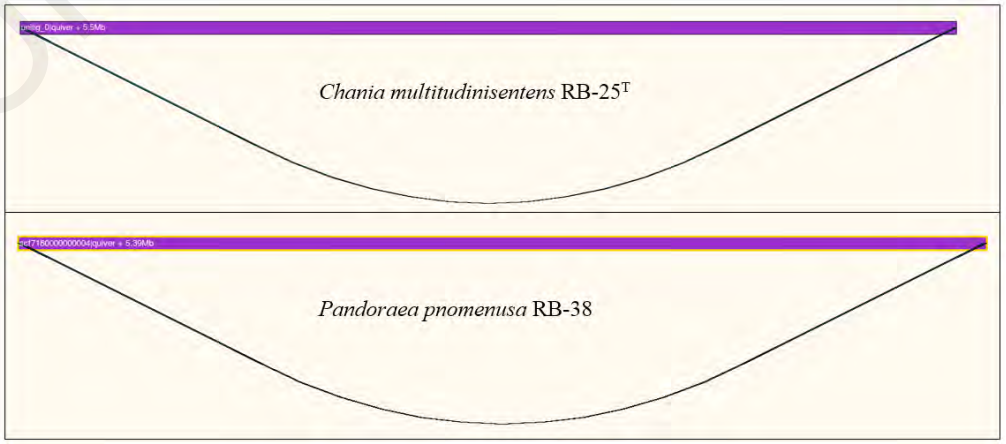


Figure 4.22. Contiguity analysis of RB-25 and RB-38 complete genome sequence.

4.8 Whole Genome Optical Mapping

Whole genome mapping was not performed for complete genome sequences of isolate RB-25 because no optimal restriction endonuclease was able to be determined for the genome by the Enzyme Chooser software. Whole genome optical mapping performed for complete genome sequences of isolate RB-38 using endonuclease BamH1 confirmed the accuracy of its genome assembly (Figure 4.23). Sequence placement analysis performed using MapSolver™ alignment software aligned optical map of isolate RB-38 (top sequence) perfectly to complete genome sequence of isolate RB-38 (bottom sequence) by blue highlights which indicates for similarity found in both two sequences.

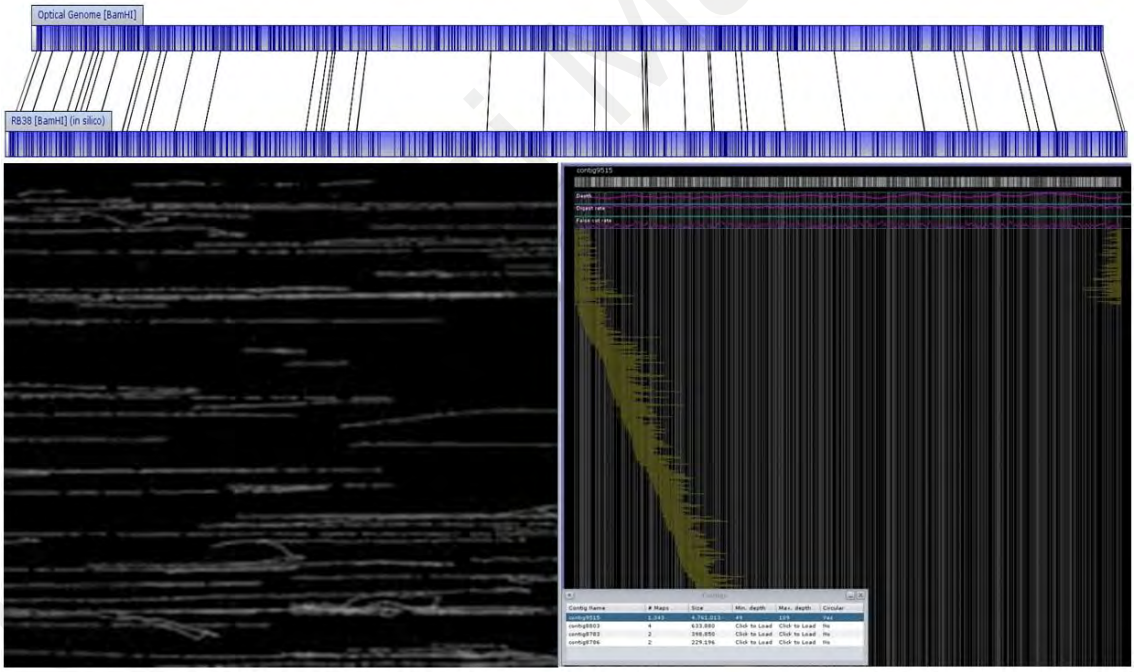


Figure 4.23. Whole genome mapping of isolate RB-38. Top, Sequence placement analysis; bottom left, linearization of high molecular weight DNA; bottom right, optical genome assembly of digitalized DNA fragments.

4.9 Genome Annotation and Bioinformatics Analyses

Genome statistic of complete genome sequences of isolates RB-25 and RB-38 were listed in Table 4.9. Digital representation of circular complete genome sequences of RB-25 and RB-38 were shown in Figure 4.24.

Table 4.9. Genome statistics of complete genome sequence of isolate RB-25 and RB-38

Statistics	Number	
	RB-25	**RB-38**
DNA, total number of bases	5485588	5378872
DNA coding number of bases	4727262	4671993
DNA G+C number of bases	2791193	3483259
DNA scaffolds	1	1
Genes total number	5028	4766
Protein coding genes	4928	4686
Pseudo Genes	103	46
RNA genes	100	80
rRNA genes	22	12
5S rRNA	8	4
16S rRNA	7	4
23S rRNA	7	4
tRNA genes	77	67
Other RNA genes	1	1
Protein coding genes with function prediction	3997	4029
without function prediction	931	657
Protein coding genes with enzymes	1354	1285
w/o enzymes but with candidate KO based enzymes	6	4
Protein coding genes connected to Transporter Classification	732	804
Protein coding genes connected to KEGG pathways3	1634	1489
not connected to KEGG pathways	3294	3197
Protein coding genes connected to KEGG orthology (KO)	2943	2513
not connected to KEGG Orthology (KO)	1985	2173
Protein coding genes connected to MetaCyc pathways	1140	1123
not connected to MetaCyc pathways	3788	3563
Protein coding genes with COGs3	3515	3634
with KOGs3	895	1080
with Pfam3	4168	4121
with TIGRfam3	1742	1532
with InterPro	2797	2834
with IMG Terms	834	426
with IMG Pathways	287	186
with IMG Parts List	290	189
in internal clusters	652	594
in Chromosomal Cassette	4889	4666
Chromosomal Cassettes	609	554
Biosynthetic Clusters	25	36
Genes in Biosynthetic Clusters	212	330
Fused Protein coding genes	121	106
Protein coding genes coding signal peptides	514	379
Protein coding genes coding transmembrane proteins	1097	1129

Table 4.9, continued

Statistics	Number	
	RB-25	**RB-28**
COG clusters	1997	1772
KOG clusters	542	544
Pfam clusters	2578	2226
TIGRfam clusters	1469	1232
Internal clusters	274	248

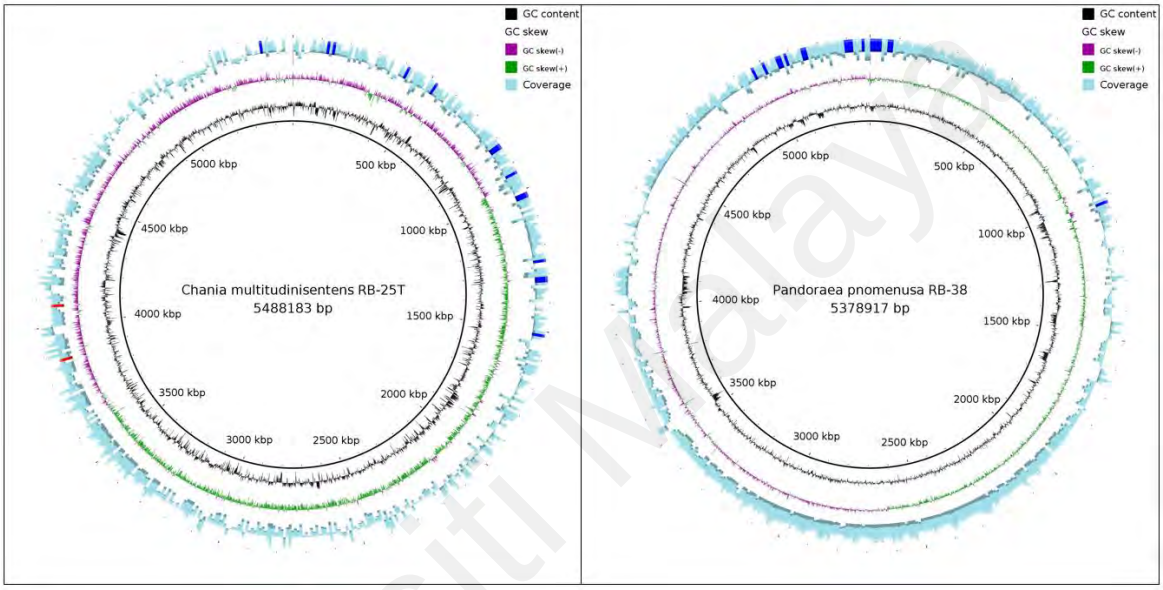


Figure 4.24. Digital representation of complete genome sequences of RB-25 and RB-38.

4.9.1 Annotation Using RAST Server

RAST server was employed as alternative annotation pipeline to annotate both complete genomes of isolates RB-25 and RB-38 were presented in Figure 4.25 and Figure 4.26, respectively.

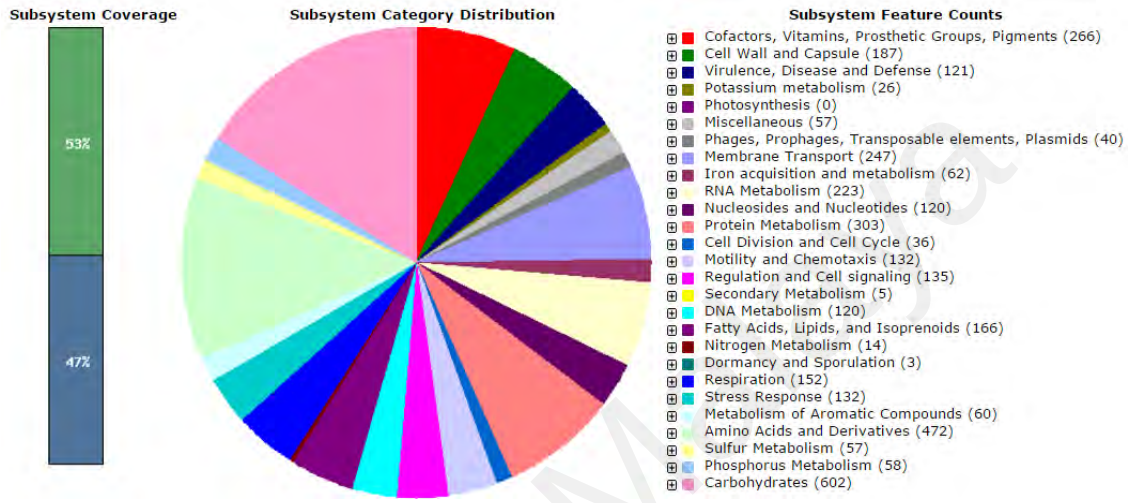


Figure 4.25. Subsystem category distribution statistics for complete genome of RB-25.

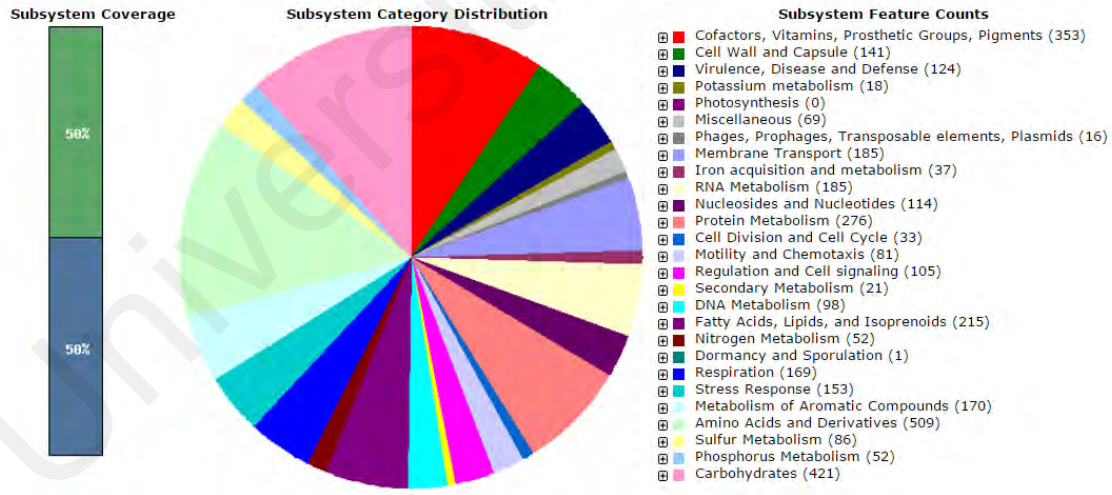


Figure 4.26. Subsystem category distribution statistics for complete genome of RB-38.

4.9.2 KEGG Database Deposition

Both complete genome sequences of isolates RB-25 and RB-38 have been deposited in KEGG database with details as listed in Table 4.10.

Table 4.10. Deposition in KEGG database

	RB-25	**RB-38**
T number	T02987	T03067
Org code	sfo	ppno
Taxonomy	TAX: 1441930	TAX: 93220

4.10 Genotypic Analyses for Isolate RB-25

Additional genotypic analysis consisted of BLAST2GO analysis, ANI analysis, rpoB sequence analysis and MLST analysis was performed to confirm the position of RB-25 as a novel type species.

4.10.1 BLAST2GO Analysis

Whole genome characterization was performed for isolate RB-25 using Blast2Go analysis in which complete genome of RB-25 demonstrated that most of its annotated protein are highly similar to those of *S. fonticola* (Figure 4.27).

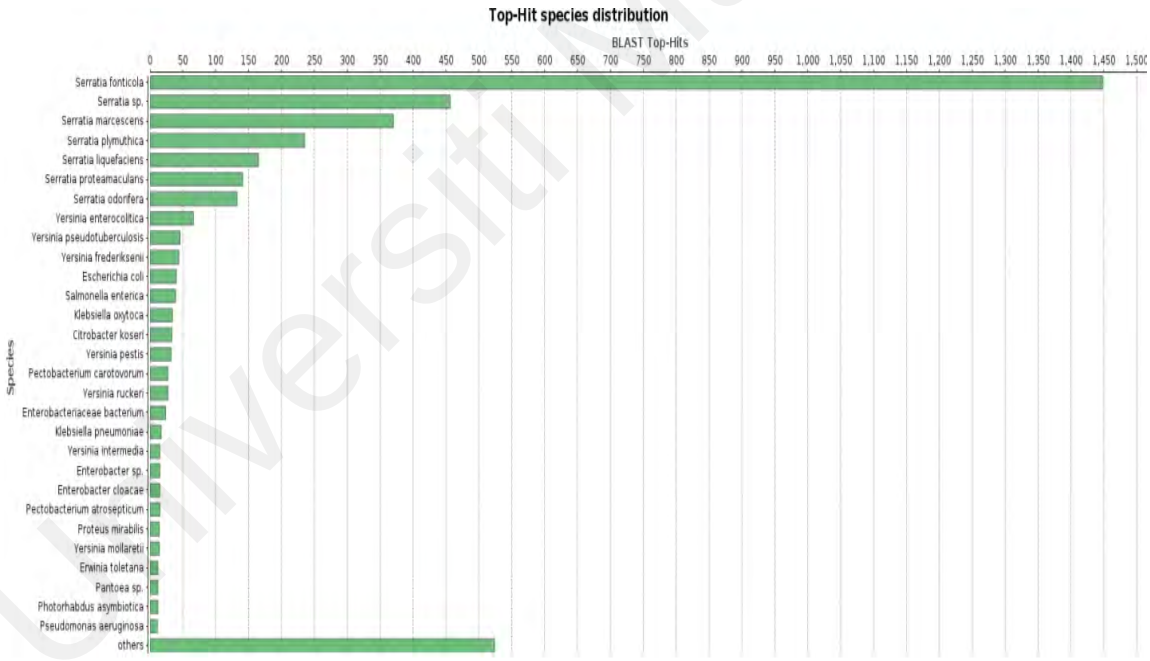


Figure 4.27. Analysis of BLAST2GO for complete genome sequence of RB-25.

4.10.2 Average Nucleotide Identity (ANI) Analysis

ANI analysis was subsequently performed to determine isolate RB-25's position as a novel genus. Identification performed using ANI analysis accurately identified isolate RB-25 as a novel genus with a score value of lesser than 95% (Table 4.11). ANI analysis was not performed for isolate RB-38 because there was no *Pandoraea* genome available in GenBank at the stage of performing this experiment.

Table 4.11. ANI analysis for isolate RB-25.

Close relatives	Isolate RB-25
Serratia fonticola DSM 4576^T (NZ_CP011254)	82.03%
Serratia marcescens Db11 (NZ_HG326223)	81.13%
Yersinia enterocolitica 8081 (NC_00800)	78.96%
Rahnella aquatilis CIP 78.65^T (JMP001)	78.81%
Pantoea rwandensis ND04 (CP009454)	78.52%
Ewingella americana ATCC 33852^T ((JMPJ01)	78.46%
Rouxiella chamberiensis 130333^T (KJ526379)	78.46%
Klebsiella pneumoniae ATCC 13883^T (JOOW01)	78.44%
Pectobacterium betavasculorum NCPPB 2795 (JQHM01)	78.42%
Hafnia alvei ATCC 13337^T (JMPK01)	78.34%
Enterobacter cloacae BIDMC66 (JMUP01)	78.28%
Citrobacter freundii ATCC 8090^T (JMTA01)	78.19%
Pectobacterium carotovorum (ABVX01)	78.14%
Escherichia coli DSM 30083^T (JMST01)	78.12%
Buttiauxella agrestis ATCC 33320^T (JMPI01)	77.94%
Leclercia adecarboxylata ATCC 23216^T (JMPM01)	77.86%
Kluyvera ascorbata ATCC 33433^T (JMPL01)	77.8%
Erwinia amylovora O1SFR-BO (CAPA01)	77.66%
Plesiomonas shigelloides 302-73 (AQQO01)	77.4%
Pantoea agglomerans 299R (ANKX01)	77.13%

4.10.3 *rpoB* Sequence Phylogeny

As *rpoB* gene sequence analysis was reported to provide a better classification resolution in *Enteriobacteriocaea* family, hence *rpoB* phylogeny analysis was performed for further characterization of isolate RB-25 (Mollet, Drancourt, & Raoult, 1997). Phylogeny based on *rpoB* gene sequence showed that isolate RB-25 formed a distinct cluster against other known genus (Figure 4.28).

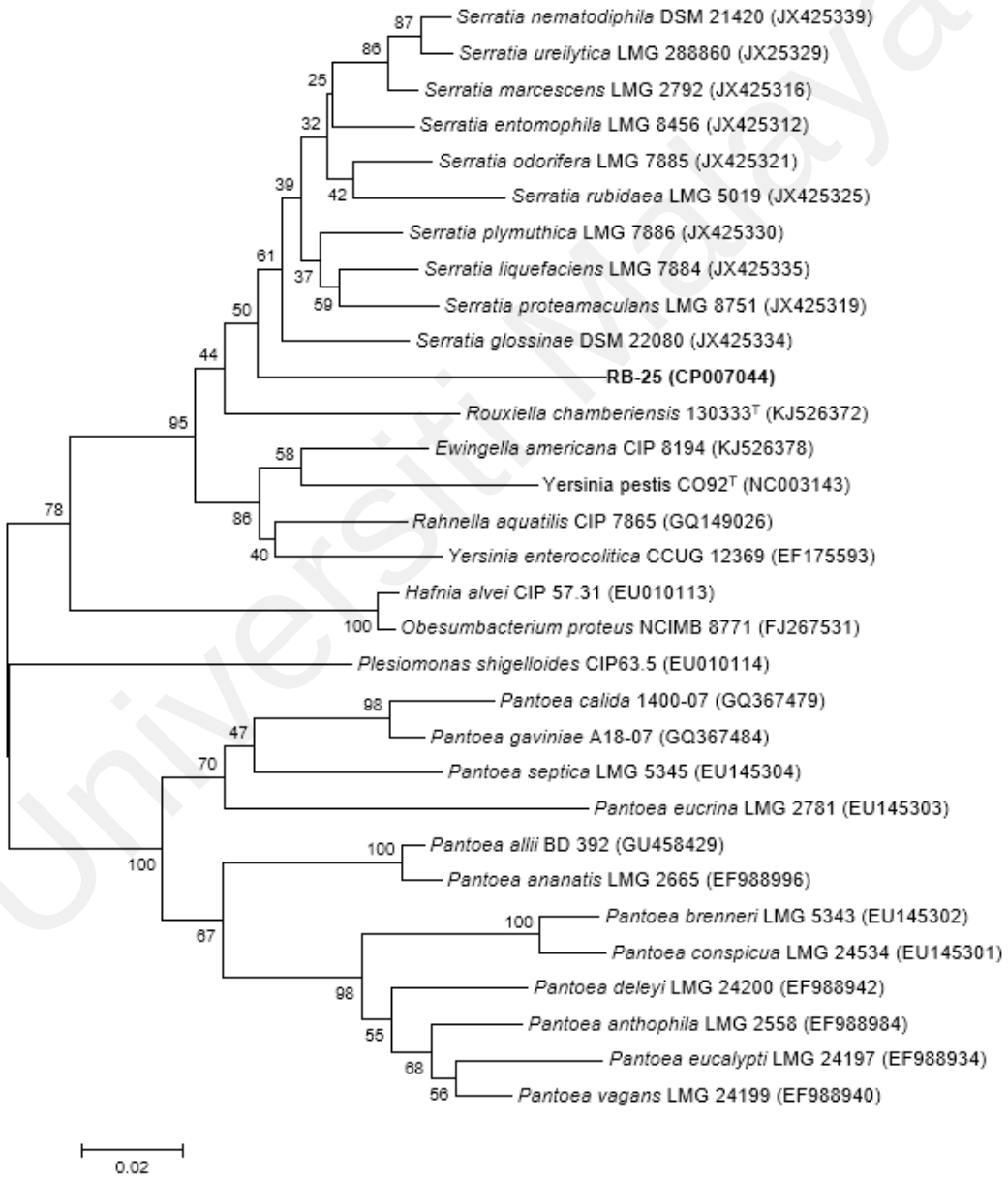


Figure 4.28. Phylogeny *rpoB* analysis for isolate RB-25 constructed using neighbour joining algorithm. Isolate RB-25 formed a distinct cluster against other genera in *Enterobacteriaceae* family. Bootstrap values >50% (based on 1000 replicates) are given at branching points. GenBank accession numbers are provided in parentheses. Bar, 0.02 substitutions per nucleotide position.

4.10.4 MLSA Analysis

Lastly, MLSA was performed according to publication of Le Fleche-Mateos et al. (2015) using concatenated gene sequences in the sequence of *fusA*, *pyrG*, *rplB*, *rpoB* and *atpD* confirmed that isolate RB-25 represents a novel genus in *Enterobacteriaceae* family (Figure 4.29).

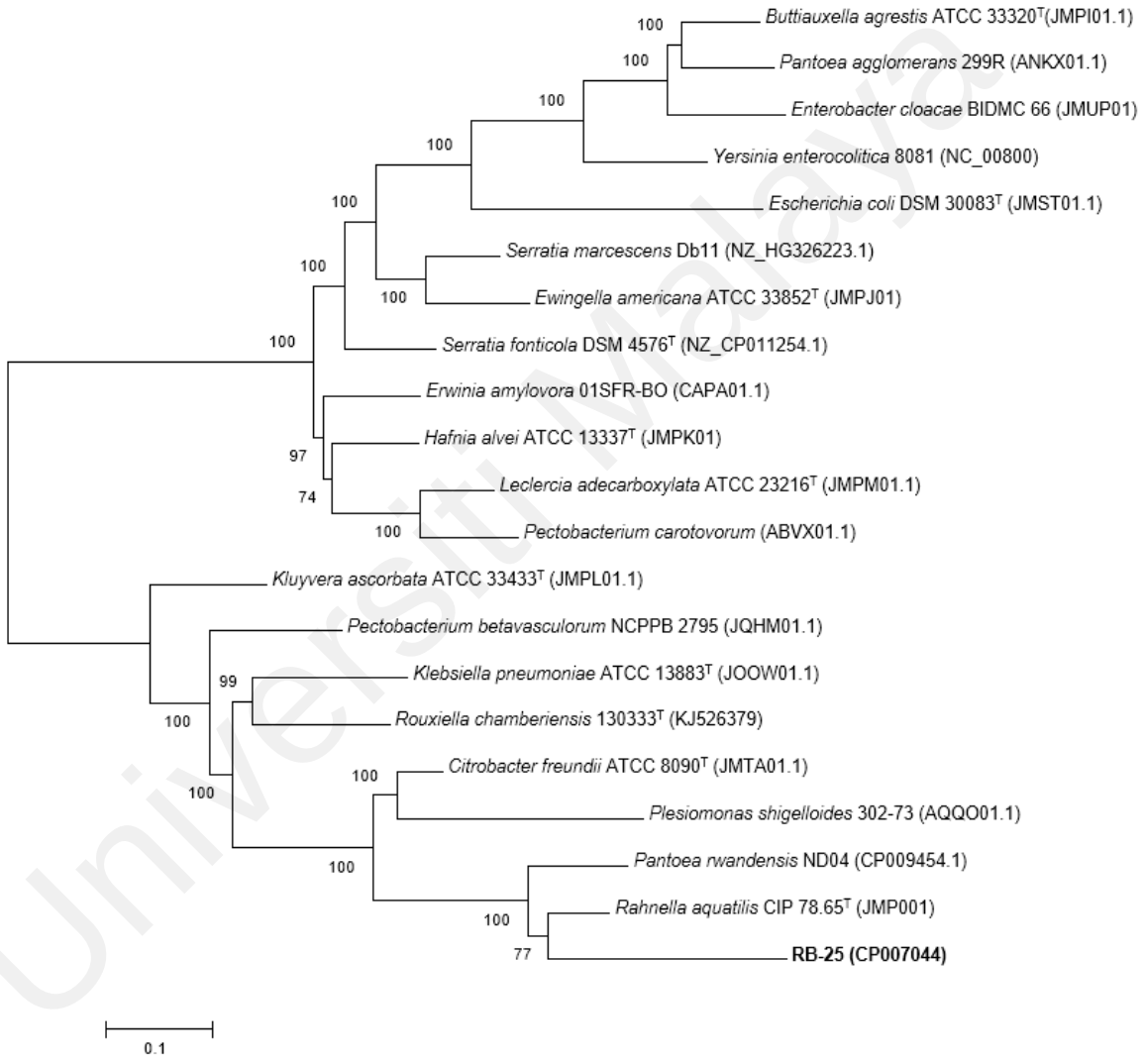


Figure 4.29. MLSA of isolate RB-25 constructed using neighbour joining method. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branch points. GenBank accession numbers are provided in parentheses. Bar, 0.1 substitutions per nucleotide position.

4.11 Description of *Chania multitudinisentens* RB-25^T gen. nov., sp. nov.

Based on various phenotypic, genotypic and chemotaxonomic studies conducted, it is conclusive that isolate RB-25 constitute a novel genus which could not be assigned into any know genus for which the novel genus *Chania* gen. nov. is proposed with *Chania multitudinisentens* sp. nov as the first novel species and isolate RB-25^T as the type strain. The genus of *Chania* belongs to the *Enterobacterioceae* family and is distantly related to the genus of *Rahnella*, *Serratia, Pectobacterium, Pantoea* and *Gibbsiella* (≤96.5% similarity on the basis of 16S rRNA gene sequence analysis).

Chania (Chan'i.a. N.L. fem. n. Chania, named in honour of Professor Chan Kok Gan, a microbiologist in the field of quorum sensing and genomic studies); *multitudinisentens* (*mul.ti.tu.di.ni.sen'tens.* L. fem. n. multitudo -inis a large number, crowd; L. pres. part. sentens feeling; N.L. part. adj. *multitudinisentens* crowd sensing). Differential characteristic of *C. multitudinisentens* RB-25^T were summarised in Table 4.12.

Characteristics of *C. multitudinisentens* RB-25^T are Gram-stain-negative, motile, aerobic, rod shaped (3-4 × 7.5-9µm), non-pigmented, growth occured in LB media at optimum temperature of 28°C, pH, 5.0-8.0 and grow with 1% (w/v) NaCl but does not grow in the presence of 9% (w/v) NaCl or higher. Negative result in glucose oxidation (with and without presence of pyrroloquinoline quinone), tetrathionate reductase, Voges-Proskauer, gluconate dehydrogenase, 2-ketogluconate dehydrogenase tests and gas production. The dominant cellular fatty acids were $C_{16:0}$, $C_{18:1}$ ω 7*c*, summed feature 3 ($C_{16:1}$ ω 7c and/or iso-$C_{15:0}$ 2-OH) whereas the major isoprenoid quinone was Q-8. Phosphoaminolipid, phosphatidylethanolamine and phosphatidylglycerol were the major polar lipids. Alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase, leucine arylamidase, acid phosphatase, trypsin, naphthol-As-BI-phosphohydrolase, β-

galactosidase, *β*-glucuronidase, *β*-glucosidase and *N*-acetyl-*β*-glucosaminidase are present. Nitrate reduction, glucose fermentation, esculin hydrolysis, *β*-galactosidase, glucose assimilation, arabinose assimilation, mannose, mannitol, *N*-acetylglucosamine, maltose and gluconate assimilation were positive. Acid was produced from glucose, mannitol, inositol, sorbitol, rhamnose, saccharose, amygdalin and arabinose. Dextrin, maltose, D-trehalose, sucrose, *β*-methyl-D-glucoside, D-salicin, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, *α*-D-glucose, D-mannose, D-fructose, D-galactose, 3-methtyl glucose, L-fucose, L-rhamnose, D-sorbitol, D-mannitol, D-arabitol, myo-inositol, glycerol, D-glucose-6-phosphate, D-fructose-6-phosphate, glycyl-L-proline, L-aspartic acid, L-glutamic acid, L-histidine, L-serine, pectin, D-galacturonic acid, L-galactonic acid lactone, D-gluconic acid, D-glucuronic acid, glucuronamide, mucic acid, quinic acid, D-saccharic acid, *p*-hydroxy-phenylacetic acid, methyl pyruvate, L-lactic acid, *α*-keto-glutatic acid, L-malic acid, bromo-succinic acid, tween 40, *β*-hydroxy-D,L-butyric acid, acetoacetic acid, acetic acid, L-arabinose, succinic acid, D,L-*α*-glycerol-phophate, D-xylose, D-ribose, tween 20, acetic acid, L-asparagine, uridine, D-glucose-1-phosphate, tween 80, *β*-methyl-D-glucosidase, adonitol, maltotriose, 2-deoxy adenosine, adenosine, m-inositol, bromo succinic acid, glycyl-L-glutamic acid, *p*-hydroxy phenyl acetic acid, D-psicose, L-lyxose, pyruvic acid, L-galactonic acid-*γ*-lactone, *β*-D-allose, D-arabinose, L-arabitol, arbutin, i-erythritol, 3-methyl glucose, palatinose, salicin, D-tagatose, xylitol, D-glucosamine, *β*-hydroxy butyric acid, D-lactic acid methyl ester, sebacic acid, L-histadine, hydroxyl-L-proline, putrescine were utilized.

Table 4.2. Differential characteristics of strain RB-25^T and its closest phylogenetic neighbours.

Strains: 1, *Chania multitudinisentens* gen. nov., sp. nov. strain RB-25^T; 2, *Pantoea rwandensis* LMG 26275^T (Brady et al., 2012); 3, *Rahnella aquatilis* CIP 78.65^T (Brenner et al., 1998); 4, *Pectobacterium betavasculorum* ATCC 43762^T (Nabhan, De Boer, Maiss, & Wydra, 2013; Thompson, 1981); 5, *Pantoea rodasii* LMG 26273^T (Brady et al., 2012); 6, *Gibbsiella dentisursi* NUM 1720^T (Saito, Shinozaki-Kuwahara, & Takada, 2012); 7, *Serratia glossinae* C1^T (Geiger et al., 2010);

Characteristic	1	2	3	4	5	6	7
Optimum NaCl (%)	1-8	nd	nd	nd	nd	nd	5-20
Optimum temperature (°C)	28	28	nd	37	28	37	25-35
Optimum pH range	5-8	nd	nd	nd	-	nd	6.7–8.4
Colony colour	Pale white	Beige	nd	nd	Light beige	Cream	White
Motility	+	–	+	nd	+	–	+
Urease	–	–	-	nd	-	+	+
Catalase	–	+				+	+
Gelatin	–	nd	-	nd	nd	nd	–
Pyruvate	+	+	+	nd	nd	+	+
D-Sorbitol	+	–	+	-	+	+	+
L-Rhamnose	+	+	+	+	+	+	–
Sucrose	+	–	+	+	+	+	–
Melibiose	–	+	+	-	+	+	+
L-Arabinose	+	+	+	+	+	+	+
Cellobiose	–	+	+	-	+	+	–
Dextrin	+	+	nd	-	nd	+	w
D-Galactose	+	+	+	+	+	+	+
Raffinose	–	–	+	-	-	+	+
Polar lipids	Unidentified AL, PL, PN, PE and PG	nd	nd	nd	nd	Two unidentified lipids, three unidentified AP, PE and PG.	nd

Note:
+, Positive; w, weakly positive; -, negative; nd, no data
PL, phospholipid; AL, Aminolipid; PN, phosphoaminolipid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; AP, aminophospholipid
SF-3, $C_{16:1}$ ω 7c and/or iso-$C_{15:0}$ 2-OH

Table 4.2, continued

Characteristic	1	2	3	4	5	6	7
Predominant fatty acid (>15 %)	$C_{16:0}$, $C_{18:1}$ ω 7*c*, SF-3	$C_{16:0}$, SF-3	$C_{16:0}$, $C_{17:0}$ cyclo	$C_{16:0}$, $C_{18:1}$ ω 7*c*, SF-3	$C_{16:0}$, SF-3	$C_{16:0}$, $C_{17:0}$ cyclo	$C_{16:0}$, $C_{16:1}$ ω 7c
Quinone	Q-8	nd	nd	MK-7, DMK-8, Q-7, Q-8	nd	Q-8 and MK-8(H4)	nd
G+C content (mol %)	50.9	51.2	52.1	54.4	53.2	58.7	53.6

Note:
+, Positive; w, weakly positive; -, negative; nd, no data.
PL, phospholipid; AL, Aminolipid; PN, phosphoaminolipid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; AP, aminophospholipid
SF-3, $C_{16:1}$ ω 7c and/or iso-$C_{15:0}$ 2-OH

4.11.1 *Enterobacteriaceae* Family Living Tree

To demonstrate the phylogenetic affiliation of *C. multitudinisentens* RB-25^T with other genera in the family of *Enterobacteriaceae*, an *Enterobacteriaceae* family living tree was constructed from a total of 268 curated 16S rDNA sequences in *Enterobacteriaceae* family (Munoz et al., 2011; Yarza et al., 2008). All curated 16S rRNA gene sequences were retrieved from LTP release 123 (https://www.arb-silva.de/browser/). *C. multitudinisentens* clustered within the family of *Enterobacteriaceae* but formed a distinct genus with *Edwardsiella* genus as the closest relative (Figure 4.30).

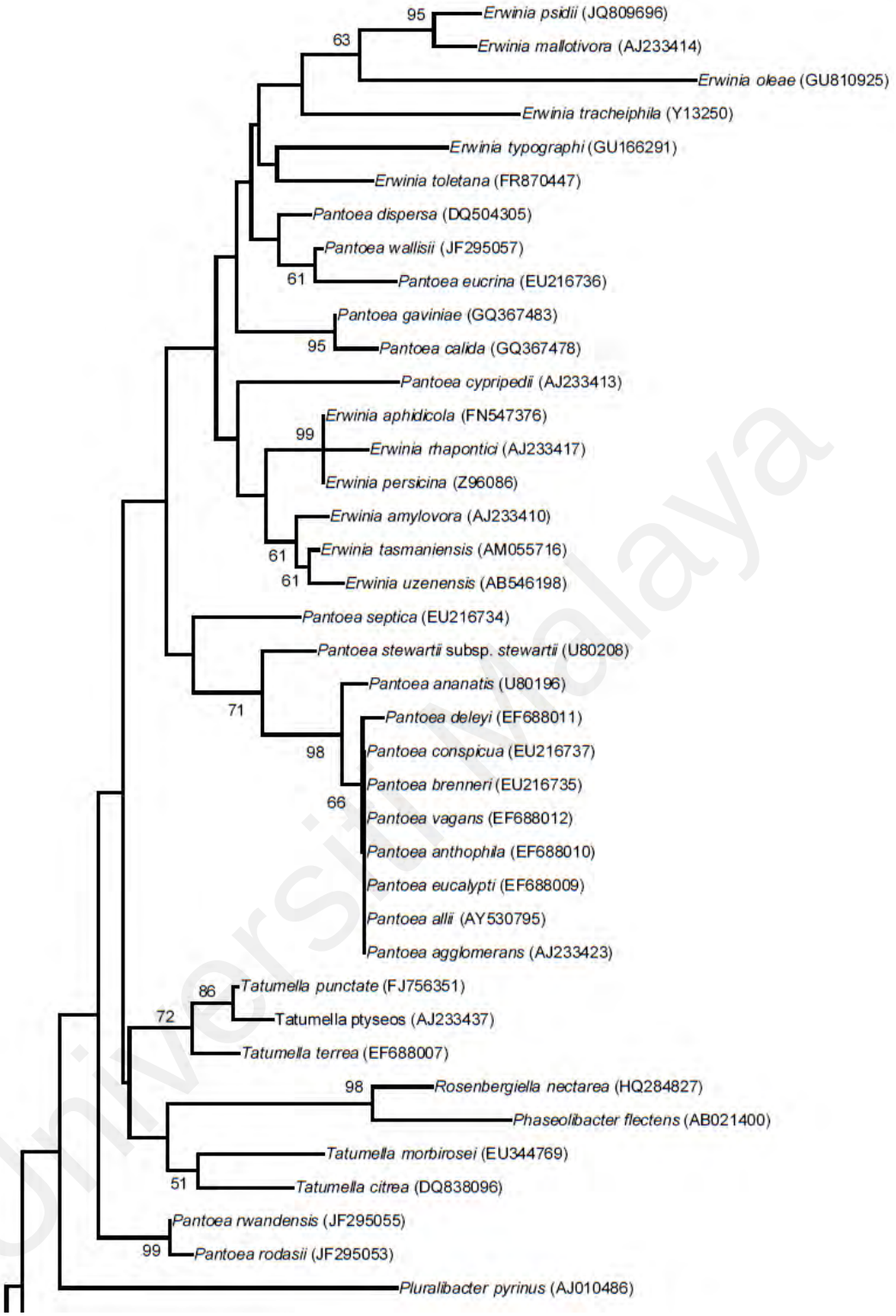


Figure 4.30, continued

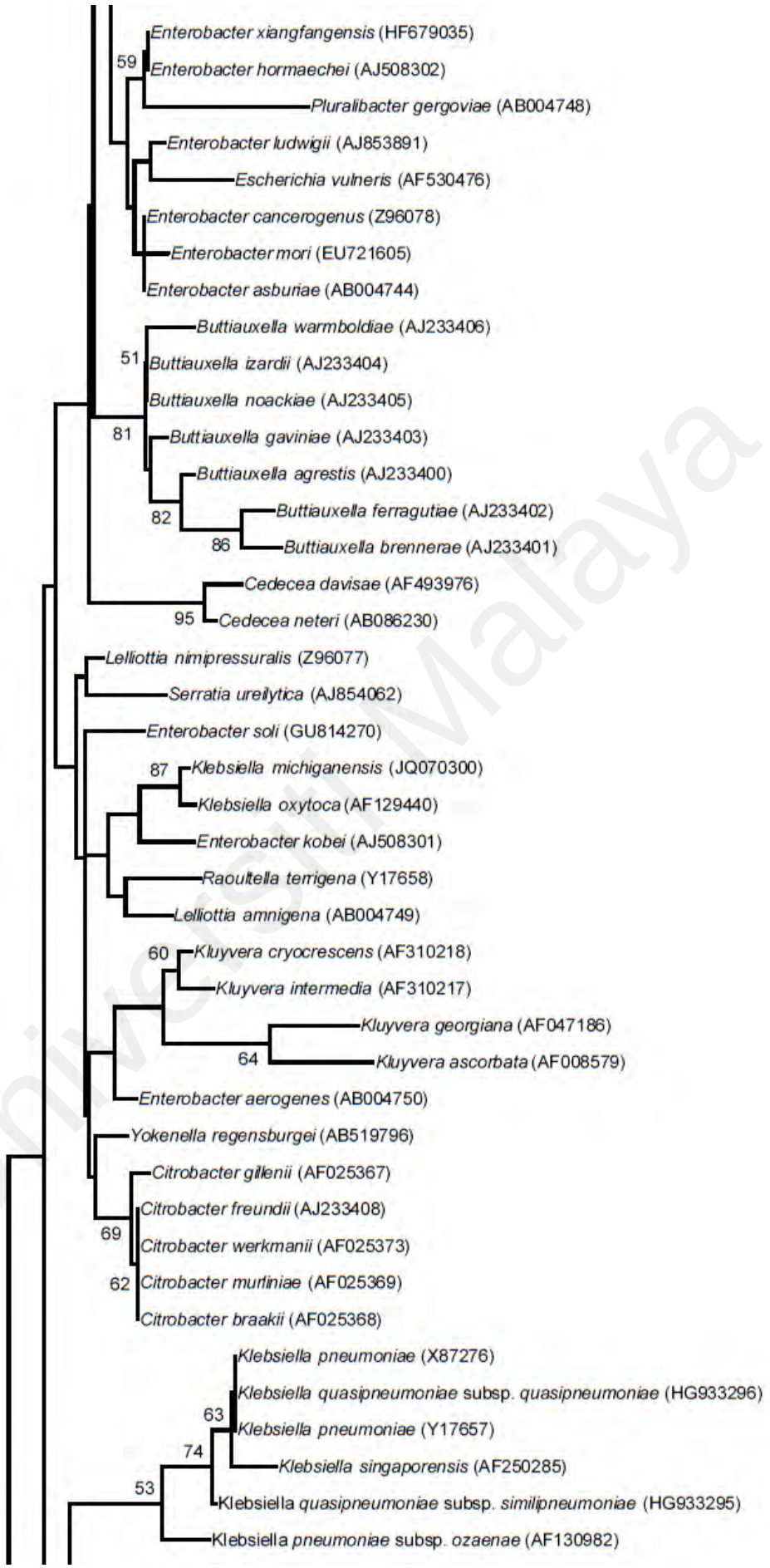


Figure 4.30, continued

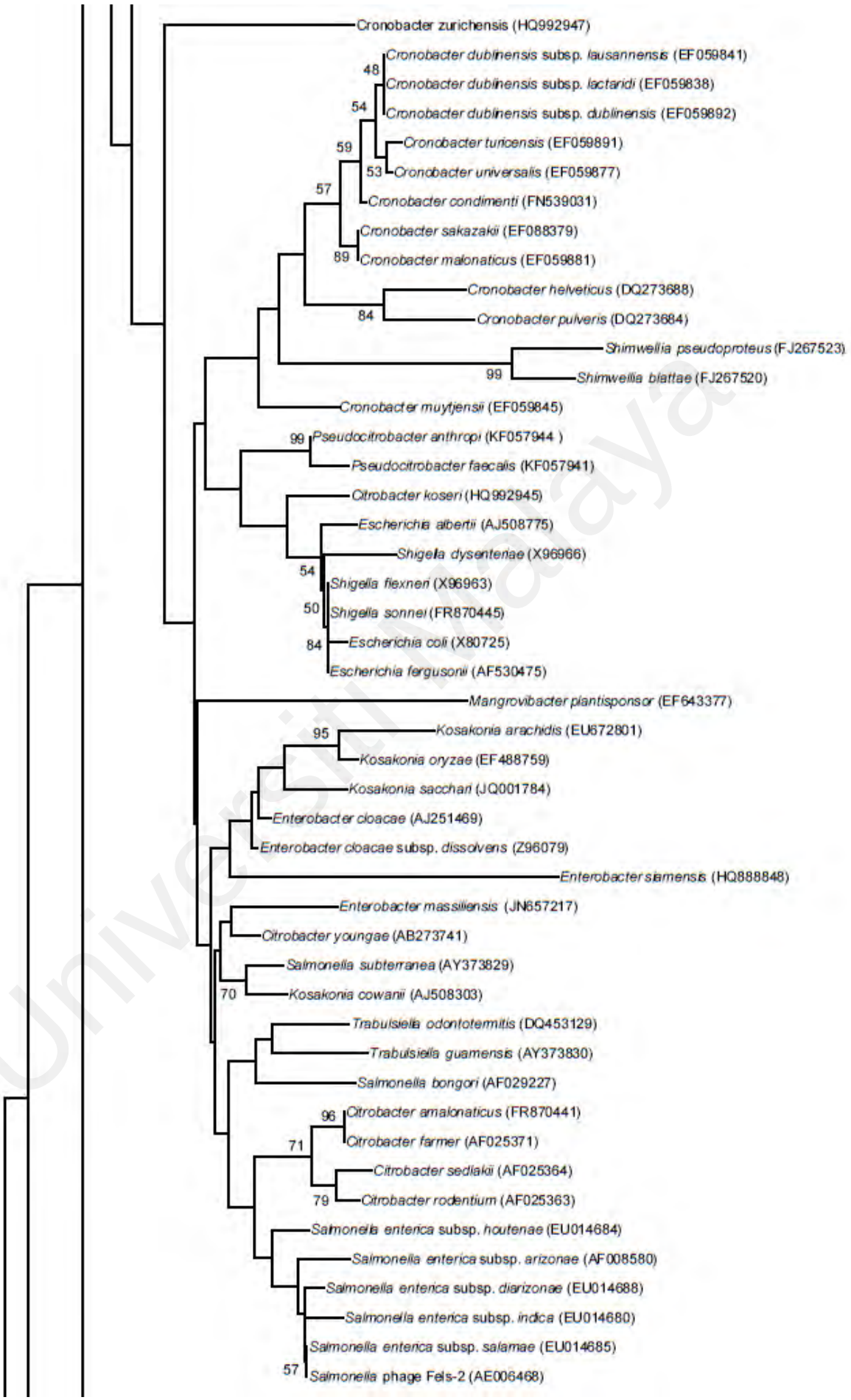


Figure 4.30, continued

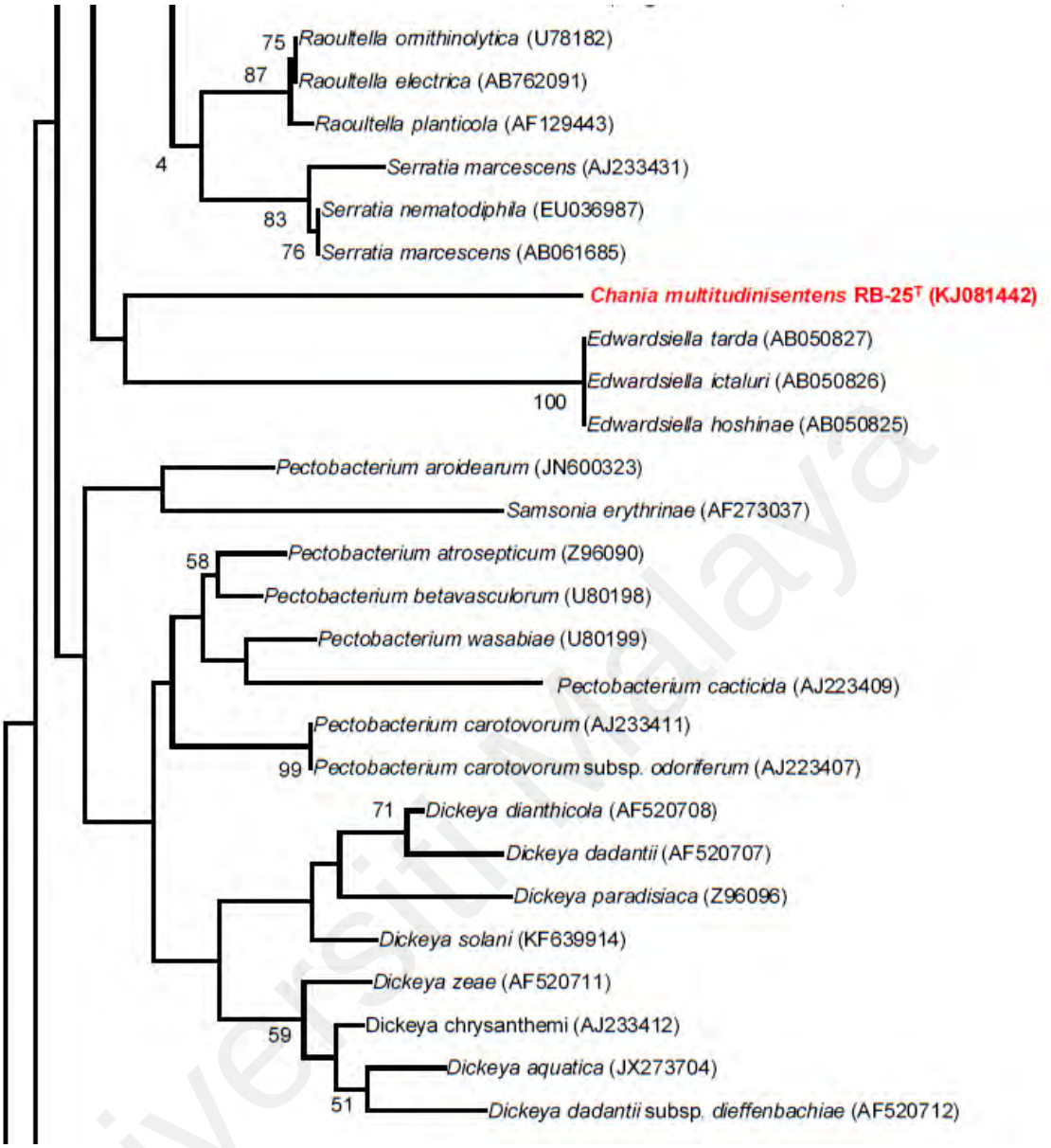


Figure 4.30, continued

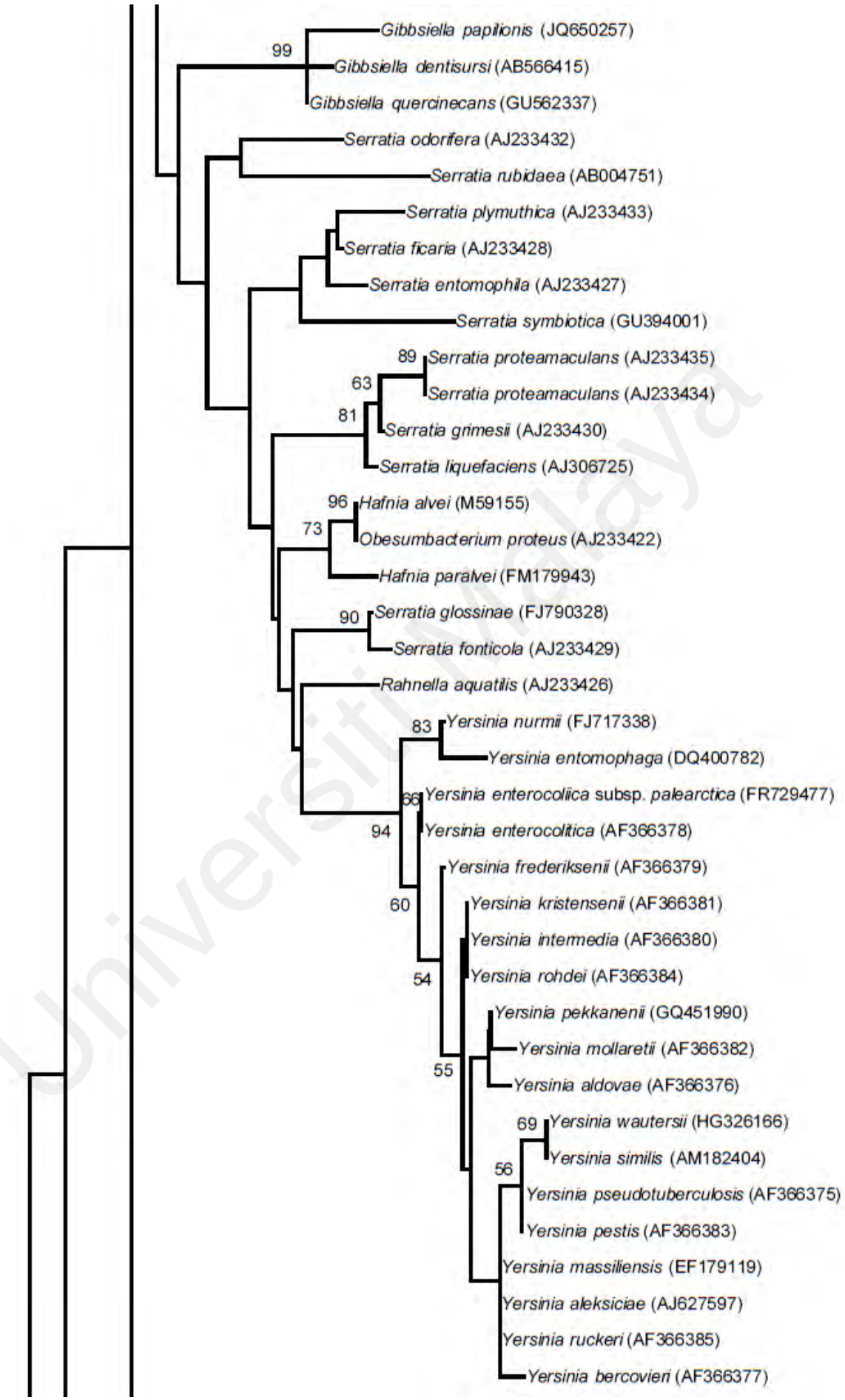


Figure 4.30, continued

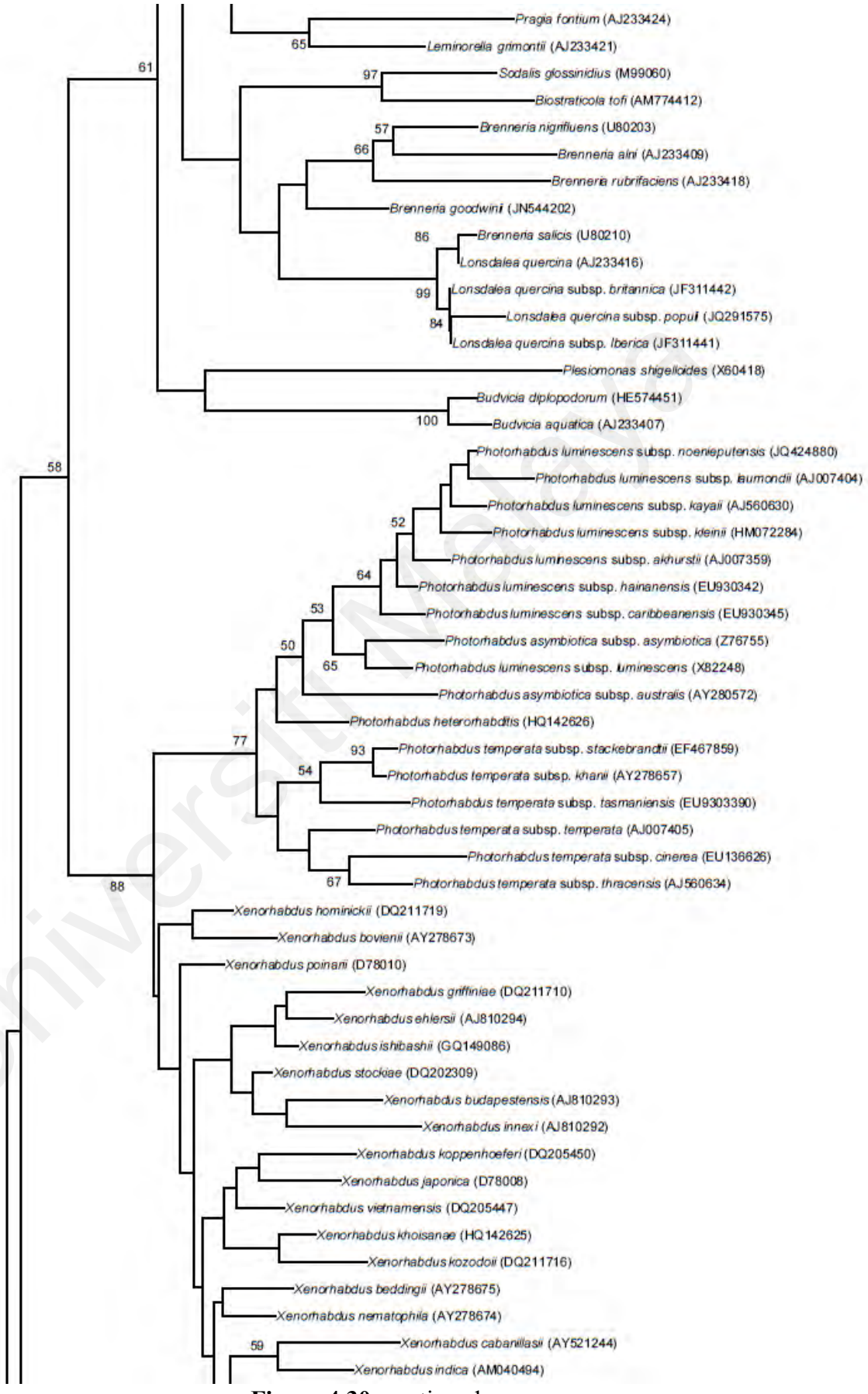


Figure 4.30, continued

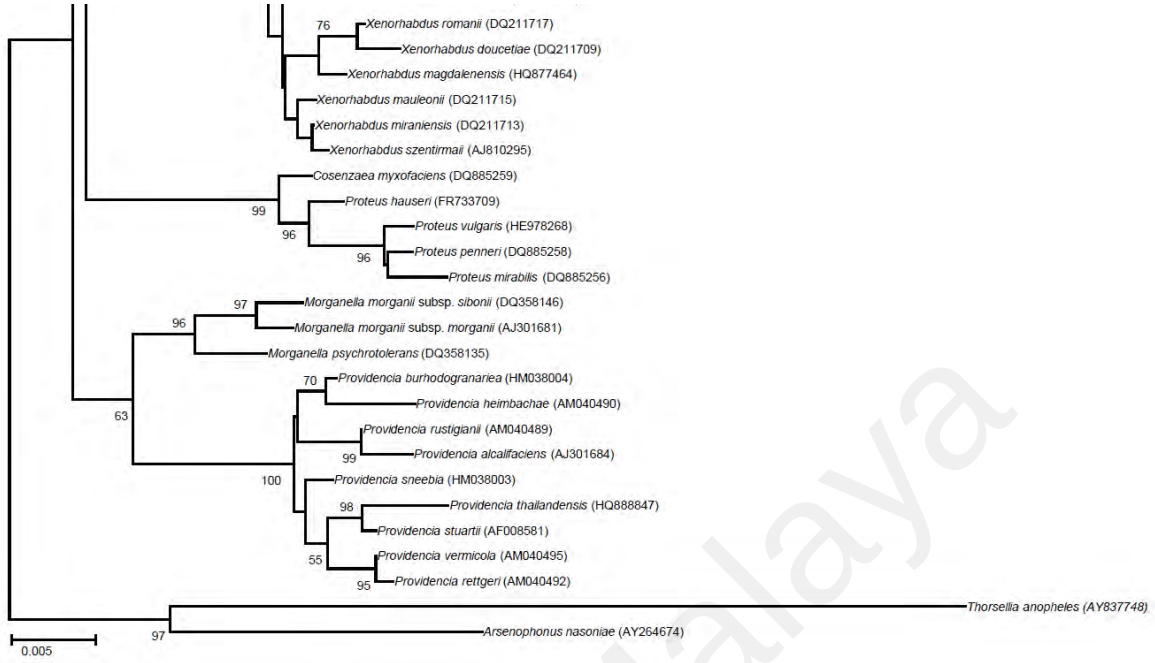


Figure 4.30. *Enterobacteriaceae* family living tree. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branch points. Bar, 0.005 substitutions per nucleotide position.

4.12 *In Silico* Analysis of Putative QS Genes

Various annotations were performed to identify the *luxI* and *luxR* of isolates RB-25, RB-38, RB-44 and RB-48. All putative LuxI were screened for signature autoinducer synthase domain (PFAM00765) presence in all LuxI protein (Figure 4.31). To quality as an authentic LuxI protein, all short listed candidates of LuxI were subsequently screened for presence of autoinducer synthesis protein (IPR001690) using Interproscan identifiers.

All putative LuxR were screened for signature autoinducer binding domain (PFAM03472) present in all LuxR protein (Figure 4.32). Then, all short listed candidates of LuxR were subsequently screened for presence of autoinducer binding (IPR005143), signal transduction response regulator (IPR016032), C-terminal effector), winged helix-turn-helix DNA-binding domain (IPR011991), and C-terminal transcription regulator LuxR, (IPR000792) using Interproscan identifiers

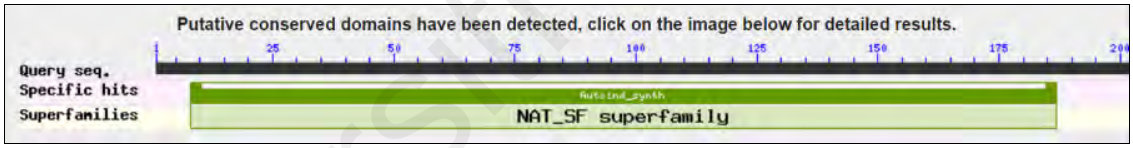


Figure 4.31. Putative conserved domain of LuxI. Autoinducer synthases (accession number: pfam00765) is a member of *N*-acyltransferase superfamily (accession number: cl17182) which catalyse the transfer of an acyl group to a substrate.

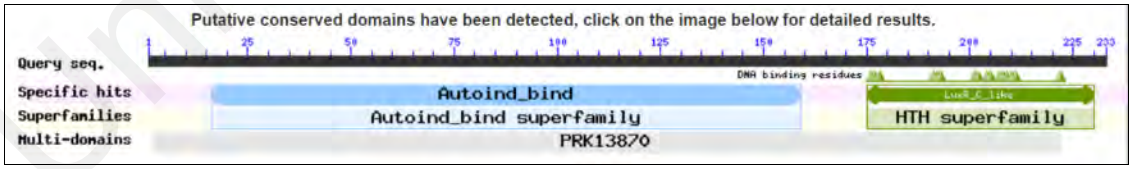


Figure 4.32. Putative conserved domain of LuxR. Two conserved domains for a functional LuxR are autoinducer binding domain and C-terminal DNA-binding domain of LuxR-like proteins. Autoinducer binding domain (accession number: pfam03472) which is a member of autoinducer binding domain superfamily (accession number: cl04096) for binding of QS signaling molecules. C-terminal DNA-binding domain of LuxR-like proteins (accession number: cd06170), is a member of helix-turn-helix domain family (accession number: cl21459) which contain helix-turn-helix motif to bind to DNA and acting as response regulators (either transcriptional activators or transcriptional repressors).

4.12.1 QS genes of *C. multitudinisentens* RB-25^{T}

Two set of *luxI/R* (designated as *cmuI1/R1* and *cmuI2/R2*) were identified in convergent orientation from the genome sequence of *C. multitudinisentens* RB-25^{T} (Figure 4.33). Phylogeny of CmuI and CmuR was presented in Figure 4.34 and Figure 4.35 respectively in which CmuI1/R1 and CmuI2/R2 were clustered closely with LuxI/R of *Serratia* spp. but into two groups (Group A and Group B) which was reported previously by Liu et al. (2011).

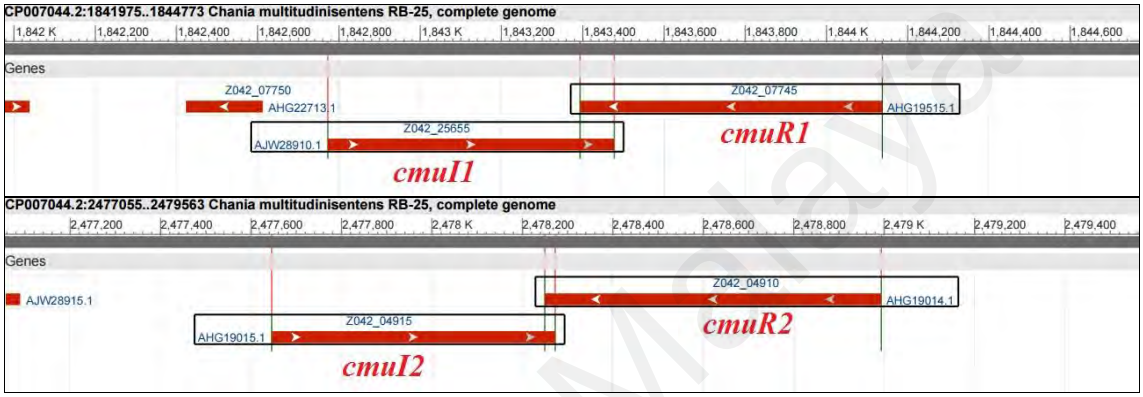


Figure 4.33. Genetic organization of *cmuI1/R2* and *cmuI2/R2*.

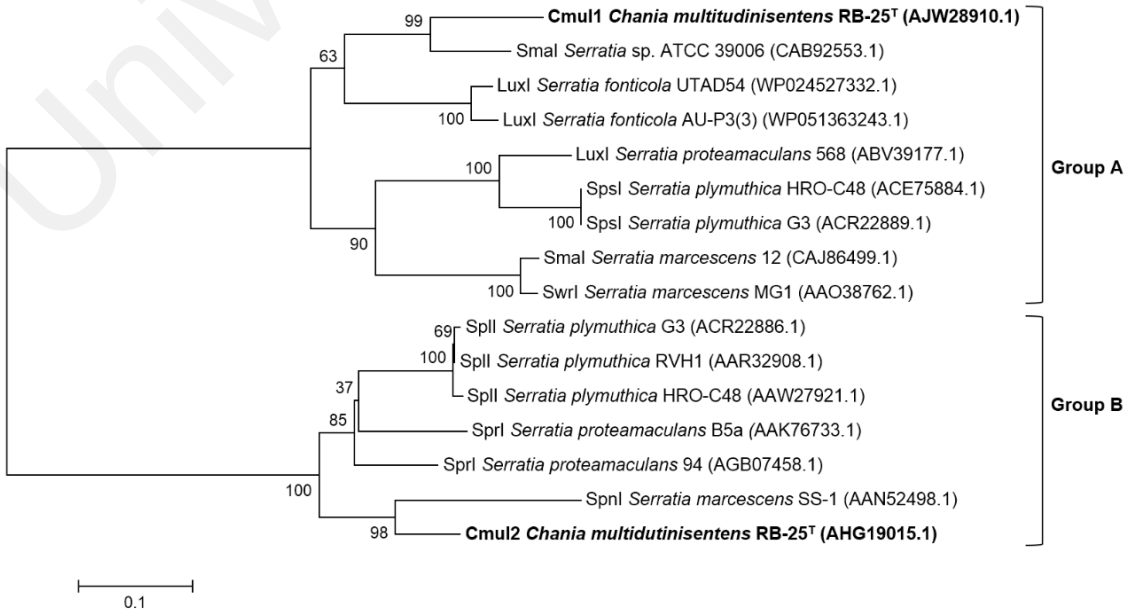
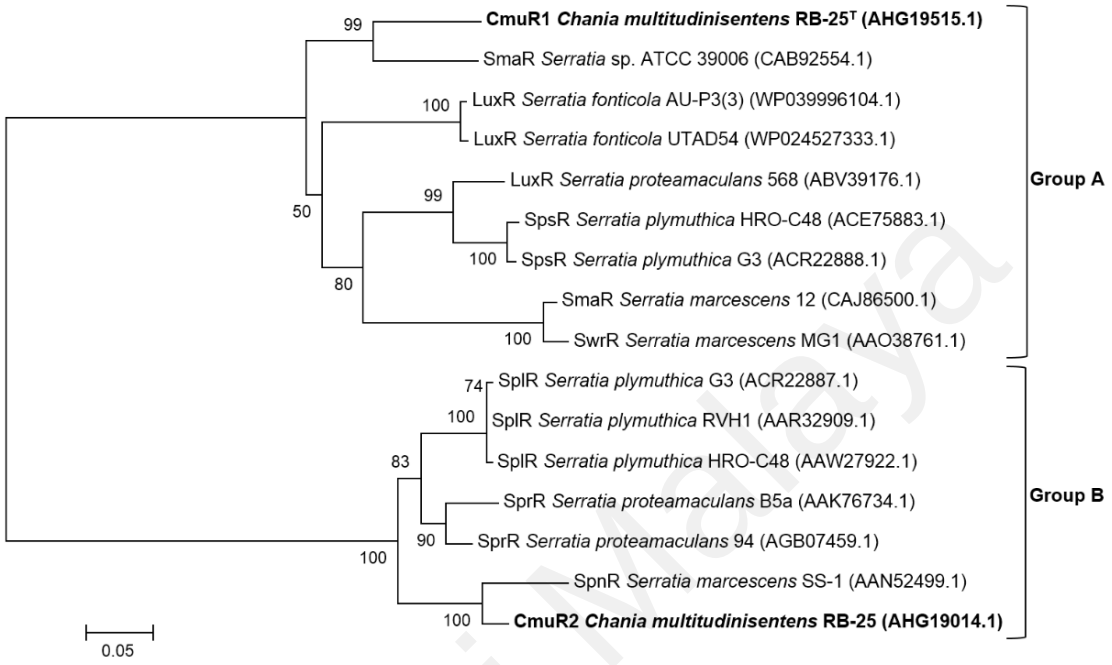


Figure 4.34. Phylogeny of CmuI1 and CmuI2 of *C. multitudinisentens* RB-25^{T}. In the phylogeny, CmuI1 and CmuI2 are separated into two group within the LuxI of Serratia genus. Bootstrap value (expressed as percentage with 1000 replicates) greater than 50 % are displayed at the branch point. Bar, 0.1 substitution per nucleotide position.



Figur 4.35. Phylogeny of of both CmuR1 and CmuR2 of *C. multitudinisentens* RB-25^{T}. In the phylogeny, CmuR1 and CmuR2 are separated into two group within the LuxI of *Serratia* genus similar to phylogeny of CmuI. Bootstrap value (expressed as percentage with 1000 replicates) greater than 50 % are displayed at the branch point. Bar, 0.1 substitution per nucleotide position.

4.12.2 QS genes of *P. pnomenusa* RB-38 and RB-44

One set of *luxI/R* (designated as *ppnI/R1*) was identified in convergent orientation in the genomes of *P. pnomenusa* RB-38 and RB-44 (Figure 3.36 and Figure 3.37). A *luxR* solo (designated as *ppnR2* solo) was also identified from both genomes.

Phylogeny of PpnI demonstrated that PpnI formed a unique cluster in LuxI of *Burkholderia* spp. with CciI *B. cenocepacia* J2315 as the outgroup (Figure 4.38). Phylogeny of PpnR demonstrated that PpnR1 was clustered within the LuxR of *Burkholderia* spp. while PpnR2 solo was clustered within LuxR of *P. aeruginosa*

(Figure 4.39). Since PpnIR of *P. pnomenusa* RB-38 and RB-44 shared 100% amino acid similarity, multiple alignment and functional studies was only performed for QS genes of *P. pnomenusa* RB-38.

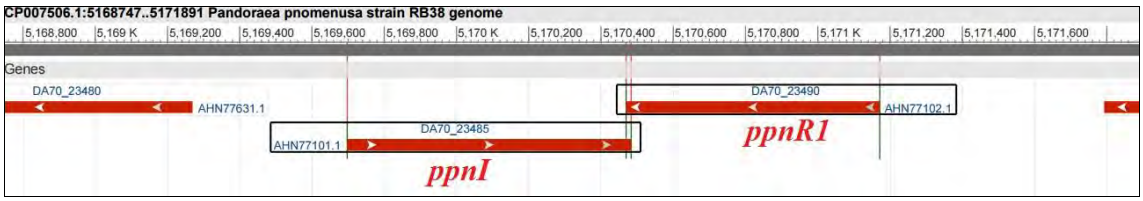


Figure 3.36. Genetic organization of *ppnI/R1* of *P. pnomenusa* RB-38.

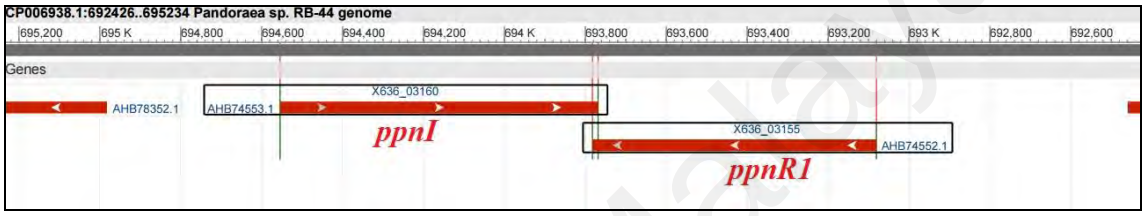


Figure 3.37. Genetic organization of *ppnI/R1* of *P. pnomenusa* RB-44.

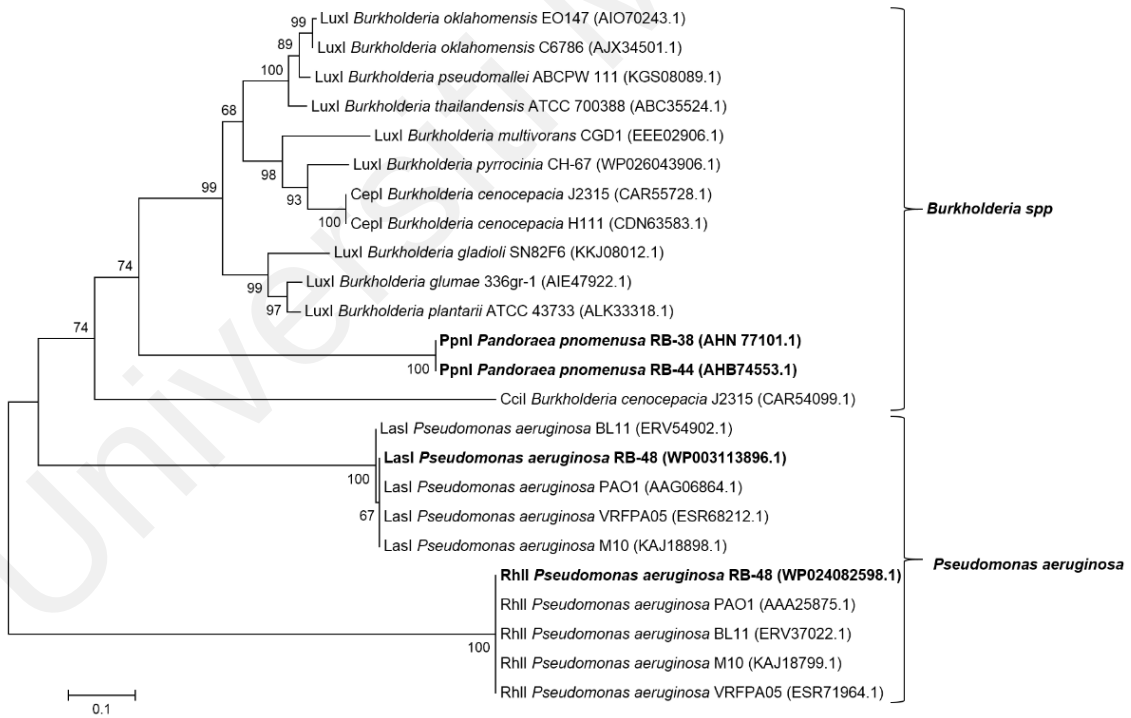


Figure 4.38. Phylogeny of of PpnI of *P. pnomenusa* RB-38 and RB-44 and LasI and RhlI of *P. aeruginosa* RB-48. In the phylogeny, both PpnI formed a distinct cluster of a novel class of LuxI within LuxI of *Burkholderia* spp. while LasI and RhlI were closely related to LuxI of *P. aeruginosa*. Bootstrap value (expressed as percentage with 1000 replicates) greater than 50% are displayed at the branch point. Bar, 0.01 substitution per nucleotide position.

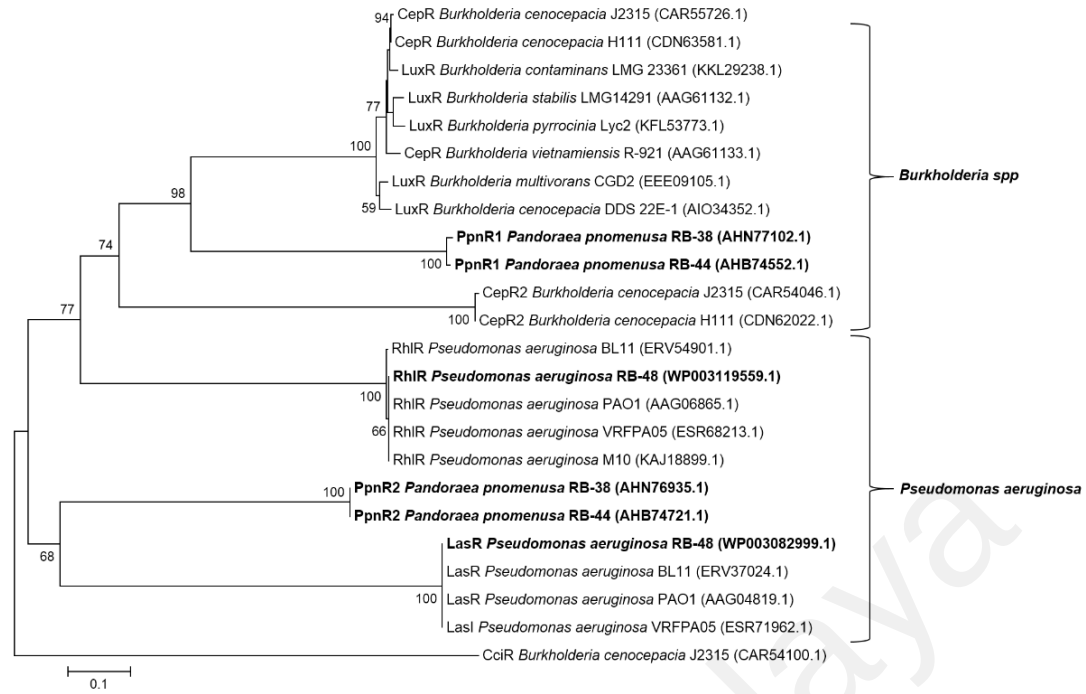


Figure 4.39. Phylogeny of of both PpnR1 and PpnR2 of *P. pnomenusa* RB-38 and RB-44 and LasR and RhlR of *P. aeruginosa* RB-48. In the phylogeny, both PpnR1 and PpnR2 formed a distinct cluster of novel class of LuxR against other documented LuxI. Bootstrap value (expressed as percentage with 1000 replicates) greater than 50% are displayed at the branch point. Bar, 0.1 substitution per nucleotide position.

4.12.3 QS genes of *P. aeruginosa* RB-48

A set of LasI/R and RhlI/R was identified in the genome of *P. aeruginosa* RB-48 which shared 100% similarity and identity with previously documented LasI/R and RhlI/R of *P. aeruginosa*. Genetic organization of *lasI/R* and *rhlI/R* was demonstrated in Figure 4.40 and Figure 4.41, respectively. Phylogeny of LasI/R and RhlI/R was demonstrated in Figure 4.37 and Figure 4.38, respectively. *P. aeruginosa* was not selected for further study since *P. aeruginosa* is a well-studied QS model organism and LasI/R and RhlI/R are well characterized.

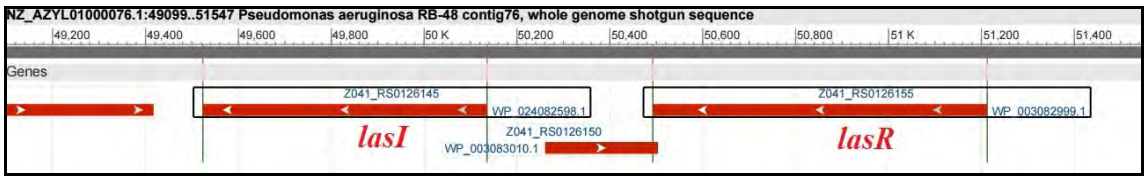


Figure 4.40. Genetic organization of *lasI/R* of *P. aeruginosa* RB-48

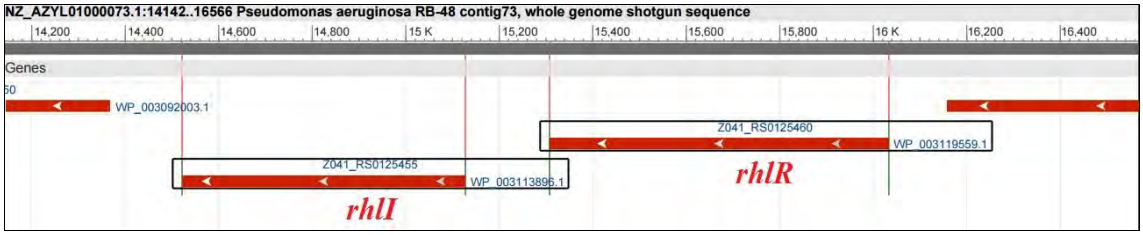


Figure 4.41. Genetic organization of *rhlI/R* of *P. aeruginosa* RB-48.

4.12.4 Multiple Alignment Analysis

Multiple alignment analysis of LuxI was performed against 10 conserved key amino acids (R25, F29, W35, E44, D46, D49, R70, F84, E101 and R104) reported by Fuqua and Greenberg (2002) in which CmuI1, CmuI2, PpnI, LasR and RhlI identified in this study contained similar conserved amino acid profile (Table 4.13).

On the other hand, multiple alignment analysis of LuxR performed against 6 key amino acid in autoinducer binding domains (W57, Y61, D70, P71, W85, G113) and 3 key amino acid in DNA-binding domains (E178, L182, G188) of TraR also demonstrated that CmuR1, CmuR2, PpnR1, PpnR2, LasR and RhlR contained similar conserved amino acid profile as reported by (Table 4.14) (Subramoni, Florez-Salcedo, & Suarez-Moreno, 2015).

Table 4.13. Multiple alignment analysis of LuxI

LuxI	Key amino acid conserved in all LuxI-type protein									
	R25	**F29**	**W35**	**E44**	**D46**	**D49**	**R70**	**F84**	**E101**	**R104**
CmuI1	R	F	W	E	D	D	R	F	E	R
CmuI2	R	F	W	E	D	D	R	F	E	R
Ppn1	R	F	W	E	D	D	R	F	E	R
LasR	R	F	W	E	D	D	R	F	E	R
RhlR	R	F	W	E	D	D	R	F	E	R

Table 4.14. Multiple alignment analysis of LuxI

LuxR	Key amino acid in autoinducer binding domain						Key amino acid in DNA binding domain		
	W57	**Y61**	**D70**	**P71**	**W85**	**G113**	**E178**	**L182**	**G188**
CmuR1	W	Y	D	P	W	G	E	L	G
CmuR2	W	Y	D	P	W	G	E	L	G
PpnR1	W	Y	D	P	W	G	E	L	G
PpnR2	W	Y	D	P	W	G	E	L	G
LasR	W	Y	D	P	W	G	E	L	G
RhlR	W	Y	D	P	W	G	E	L	G

4.13 Functional Studies of LuxI

Due to time constraint, only LuxI with novelty were selected for functional studies and these are CmuI1 and CmuI2 of *C. multitudinisentens* RB-25^{T} (novel species) and PpnI of *P. pnomenusa* RB-38 (first documentation of QS activity in *Pandoraea* genus).

4.13.1 Functional Studies of CmuI1 and CmuI2

CmuI1 and CmuI2 were heterologously expressed in *E. coli* BL21(DE3)pLysS::*cmuI1* and *E. coli* BL21(DE3)pLysS::*cmuI2* and screened using CVO26 biosensor. Formation of purple pigmentation in CVO26 biosensor confirmed that CmuI1 and CmuI2 are functional AHL synthases for *C. multitudinisentens* RB-25^{T}. Negative control was

included with *E. coli* BL21(DE3)pLysS harbouring an empty vector did not induced formation of purple pigmentation in CVO26 biosensor (Figure 4.42).

Parental wildtype of *C. multitudinisentens* RB-25^T produces 3 AHLs: C4-HSL, C6-HSL and 3-oxo-C6-HSL. Summary of MS analysis was summarised in Table 4.15 in which CmuI1 produced 2 AHLs (C4-HSL and C6-HSL) (Figure 4.43) while CmuI2 produced 3 AHLs (C4-HSL, C6-HSL and 3-oxo-C6-HSL) (Figure 4.44).

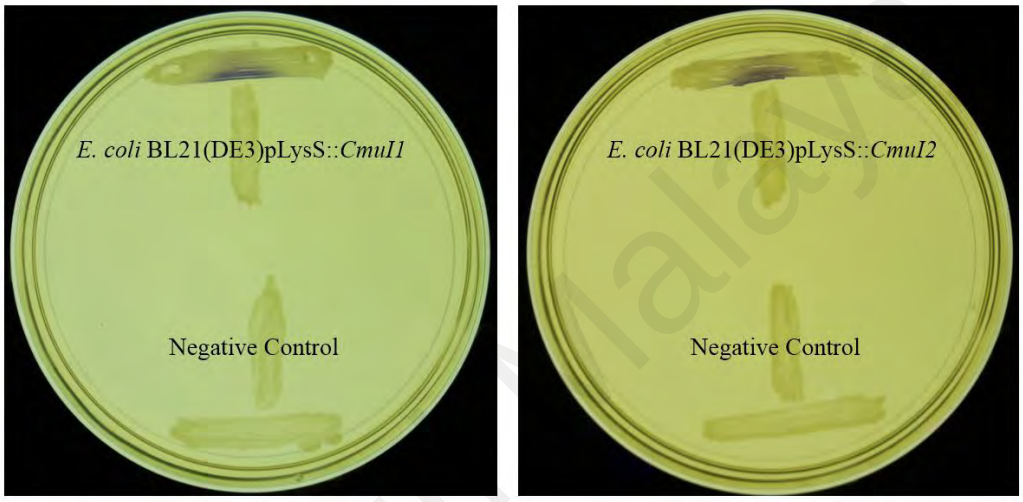


Figure 4.42. Cross streak bioassay of *E. coli* BL21(DE3)pLysS::*cmuI1* and *E. coli* BL21(DE3)pLysS::*cmuI2*

Table 4.15. AHL profile of CmuI1 and CmuI2.

Strain	AHL profile				
	AHL detected	***m/z* value**	**Retention time**	**Abundance**	**Abundance %**
C. multitudinisentens RB-25^T (Parental wildtype)	C4-HSL	172.1	0.445 min	194.18	4.4
	C6-HSL	200.2	1.219 min	200.58	2.48
	3-oxo-C6-HSL	214.2	0.572 min	12448.28	100
E. coli BL21(DE3)pLysS::*CmuI1*	C4-HSL	172.1	0.445 min	9784.14	17.76
	C6-HSL	200.2	1.219 min	4333.06	51.26
E. coli BL21(DE3)pLysS::*CmuI2*	C4-HSL	172.0	0.445 min	255.88	3.06
	C6-HSL	200.3	1.219 min	7420.24	69.23
	3-oxo-C6-HSL	214.1	0.572 min	306117.03	100

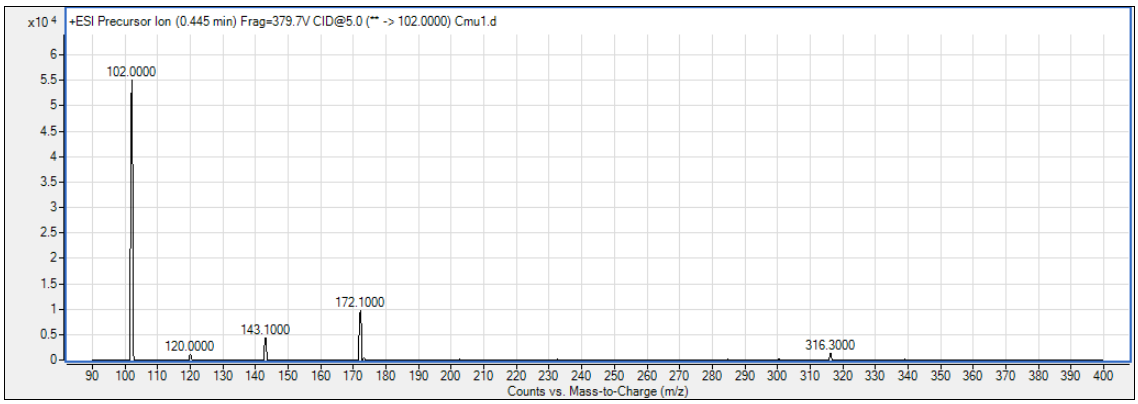


Figure 4.43a

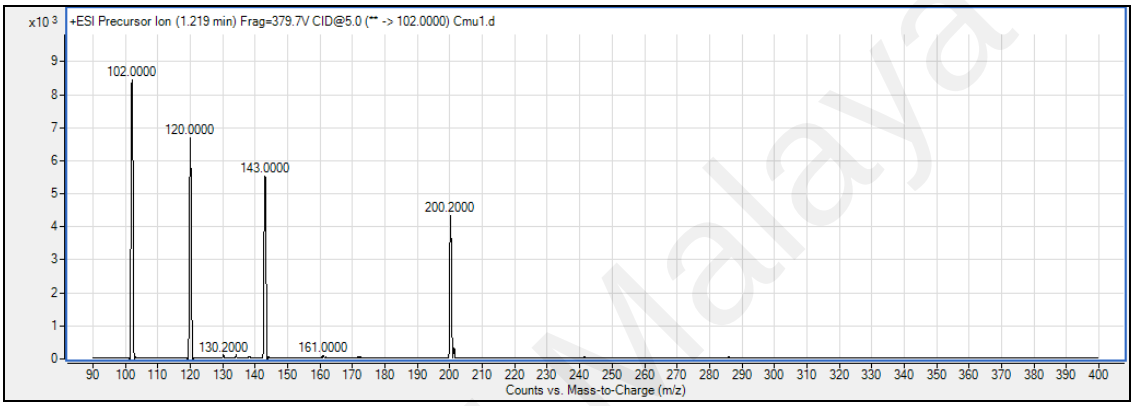


Figure 4.43b

Figure 4.43. MS analysis of spent supernatant of *E. coli* BL21(DE3)pLysS::*cmuI1*. MS analysis showed that CmuI2 produced C4-HSL (**Figure 4.43a**) (retention time: 0.445 min; Abundance: 9784.14; Abundance %: 17.76; *m/z*: 172.1) and C6-HSL (**Figure 4.43b**) (retention time: 1.219 min; Abundance: 4333.06; Abundance %: 61.26; *m/z*: 200.3)

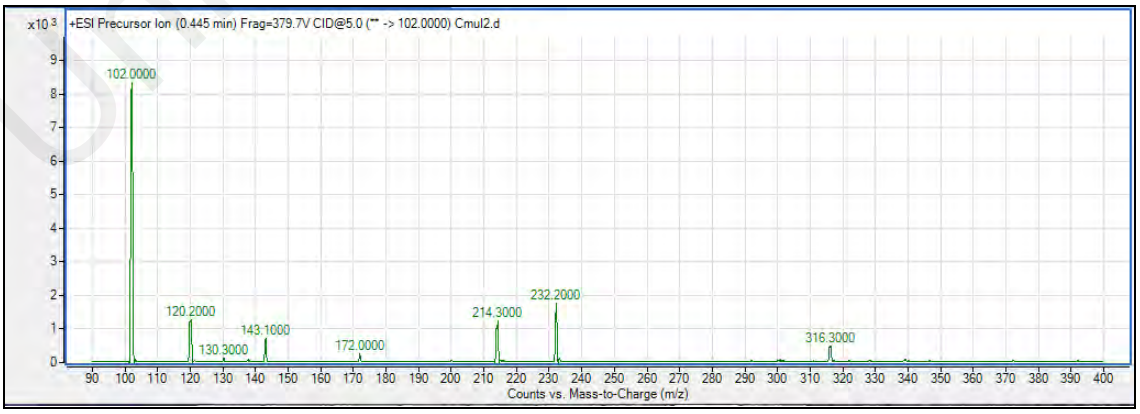


Figure 4.44a

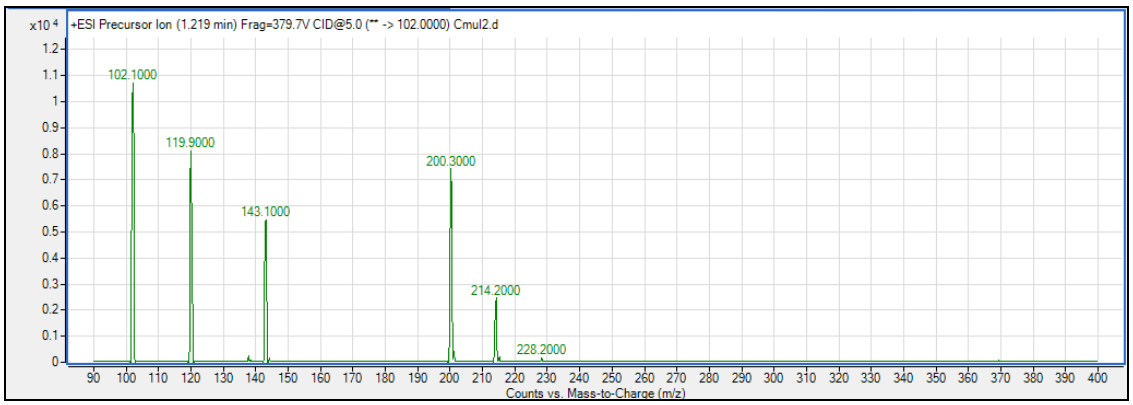


Figure 4.44b

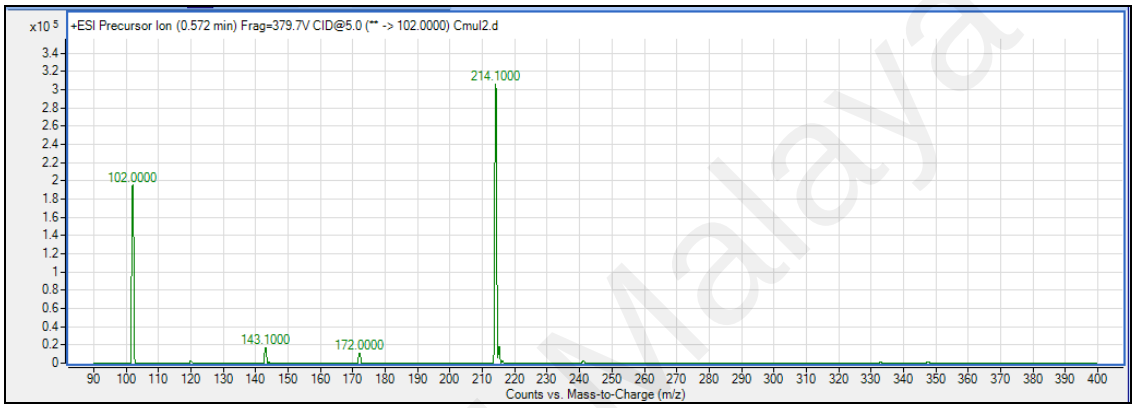


Figure 4.44c

Figure 4.44. MS analysis of spent supernatant of *E. coli* BL21(DE3)pLysS::*cmuI2*. MS analysis showed that CmuI1 produced 3 AHLs: C4-HSL (**Figure 4.34a**) (retention time: 0.445 min; Abundance: 255.88; Abundance %: 3.06; *m/z*: 172.0), C6-HSL (**Figure 4.34b**) (retention time: 1.219 min; Abundance: 7420.24; Abundance %: 69.23; *m/z*: 200.3) and 3-oxo-C6-HSL (**Figure 4.34c**) (retention time: 0.572 min; Abundance: 306117.03; Abundance %: 100; *m/z*: 214.1).

4.13.2 Functional Studies of PpnI

Expression of PpnI was also screened using *E. coli* BL21(DE3)pLysS::*ppnI* against CVO26 biosensor in which formation of purple pigmentation in CVO26 biosensor confirmed that PpnI is a functional AHL synthase for *P. pnomenusa* RB-38 (Figure 4.45). Negative control included with *E. coli* BL21(DE3)pLysS harbouring an empty vector did not induced formation of purple pigmentation in CVO26 biosensor.

Mass spectrometry analysis performed confirmed for the production of C8-HSL (retention time: 3.430 min; abundance: 26515.2; abundance %:100; *m/z*: 228.1) from the spent supernatant of *E. coli* BL21(DE3)pLysS::*ppnI* which is an identical AHL profile with the parental wildtype of *P. pnomenusa* RB-38 (Figure 4.46).

Figure 4.45. Cross streak bioassay of *E. coli* BL21(DE3)pLysS::*ppnI*

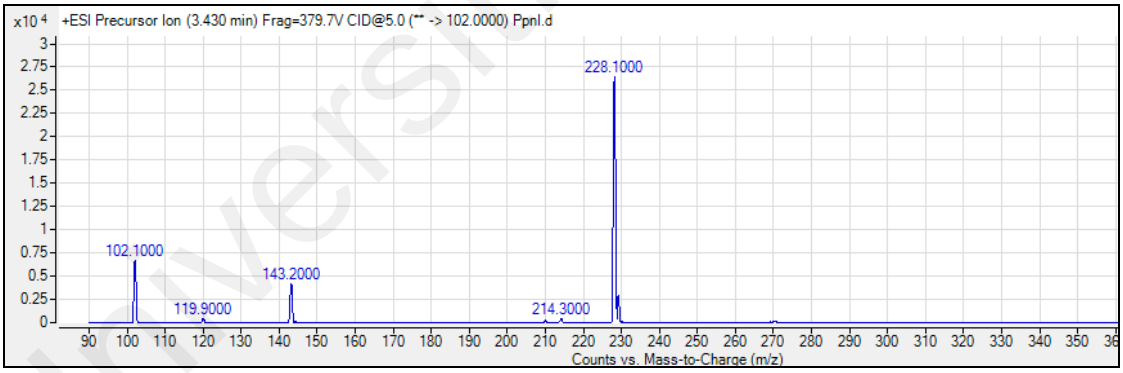


Figure 4.46. MS analysis of spent supernatant of *E. coli* BL21(DE3)pLysS::*ppnI*.

4.14 QS Activity of *Pandoraea* Genus

4.14.1 Screening of QS Activity in *Pandoraea* spp.

To my knowledge, *P. pnomenusa* RB-38 and RB-44 are the firstly reported to show QS activity (Ee et al., 2014). Since *Pandoraea* are opportunistic pathogens in CF patients, it is hypothesized that like other CF pathogens (such as *P. aeruginosa* and Bcc employing

QS for pathogenicity), QS is a homogenous behaviour in *Pandoraea* spp.. Hence, further analysis on QS activities in *Pandoraea* genus was carried out.

A total of 11 *Pandoraea* strains (9 species) was screened for QS activity. Only 4 species (*P. pnomenusa* DSM 16536^T, *P. sputorum* DSM 21091^T, *P. oxalativorans* DSM 23570^T and *P. vervacti* DSM 23571^T) demonstrated QS activity (Table 4.16). In MS analysis, C8-HSL was detected as the only signaling molecule present in the spent supernatant of these 4 *Pandoraea* species (Figure 4.47).

Table 4.16. QS activity of *Pandoraea* genus. QS activity of *P. pnomenusa* DSM 16536^T, *P. sputorum* DSM 21091^T, *P. oxalativorans* DSM 23570^T and *P. vervacti* DSM 23571^T were determined using CVO26 biosensor and C8-HSL was the only AHL detected from mass spectrometry analysis.

Strain	QS activity	AHL Profile	Source
P. pnomenusa DSM 16536^T	Positive	C8-HSL	Clinical
P. pnomenusa 6399	-	-	Clinical
P. pnomenusa 7641	-	-	Clinical
P. pulmonicola DSM 16583^T	-	-	Clinical
P. sputorum DSM 21091^T	Positive	C8-HSL	Clinical
P. apista DSM 16535^T	-	-	Clinical
P. norimbergensis DSM 11628^T	-	-	Clinical
P. faecigallinarum DSM 23572^T	-	-	Environmental
P. oxalativorans DSM 23570^T	Positive	C8-HSL	Environmental
P. thiooxydans DSM 25325^T	-	-	Environmental
P. vervacti DSM 23571^T	Positive	C8-HSL	Environmental

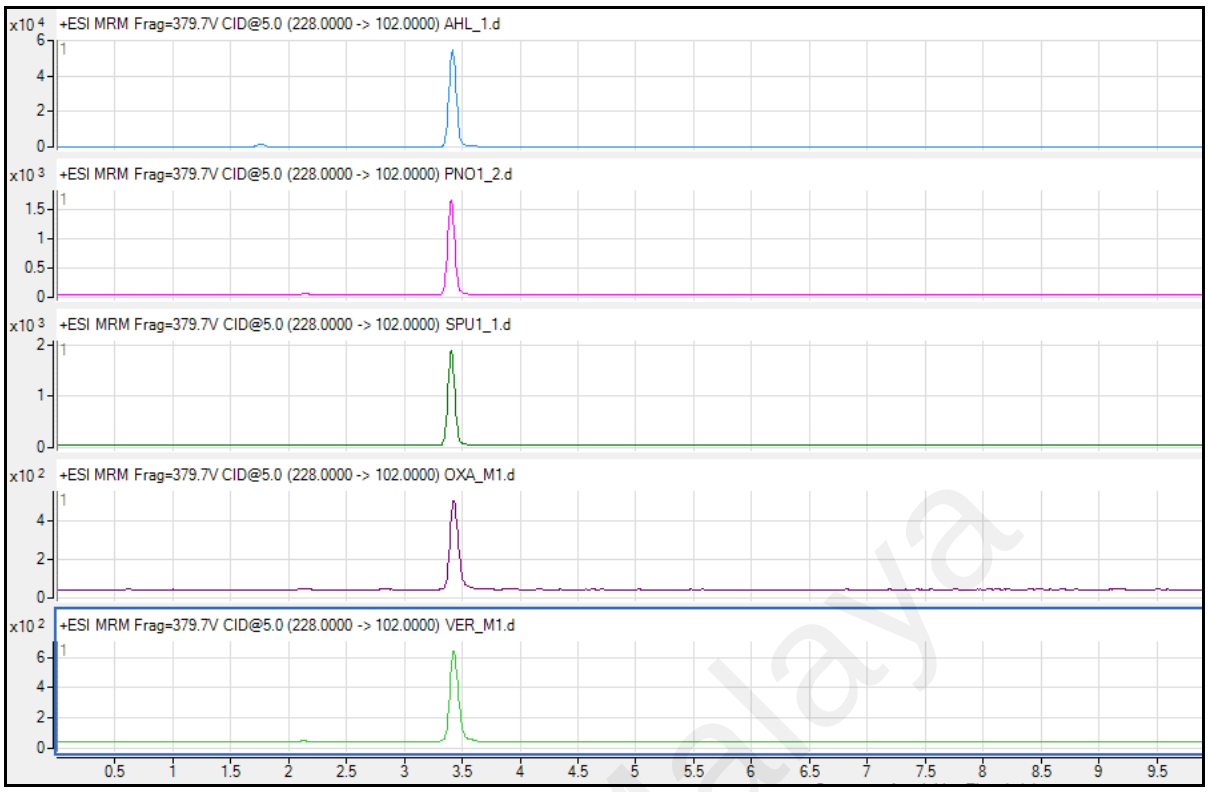


Figure 4.47. MS analysis using MRM mode for *Pandoraea* genus. C8-HSL (*m/z*: 228) was detected as the only signaling molecule produced by *P. pnomenusa* DSM 16536^{T}(2nd chromatogram from top), *P. sputorum* DSM 21091^{T} (3rd chrmotogram from top), *P. oxalativorans* DSM 23570^{T} (4th chromatogram from top) and *P. vervacti* DSM 23571^{T} (bottom chromatogram) with synthetic C8-HSL as a reference (top chromatogram).

4.14.2 Genome Sequencing of *Pandoraea* Genus

Since QS is not a common properties in *Pandoraea* genus, it is intriguing if QS negative species of *Pandoraea* is actually QS cheater harbouring LuxR solo for "listening" to ride on the benefits of "public good". To prove this hypothesis, the genome of all type strain of *Pandoraea* species was sequenced in the effort to search for the presence of LuxR solos in these QS negative species of *Pandoraea*.

All *Pandoraea* strains in our collection were sequenced by PacBio RSII SMRT Technology except *P. pnomenusa* 6399 and *P. pnomenusa* 7641 which were sequenced using MiSeq, Illumina. All PacBio sequenced genomes were subsequently assembled into complete circular genomes after assembly using Hierarchical Genome Assembly Process (HGAP) and circularization as illustrated previously in section 4.7. Presence of plasmids were differentiated from the chromosomal DNA using contiguity analysis.

Genomes of *P. pnomenusa* 6399 and *P. pnomenusa* 7641 sequenced by MiSeq were assembled into 70 and 66 contigs, respectively, using CLC Genomics Workbench version 7.5. Genome sequencing information for all *Pandoraea* species were listed in Table 4.17. Appendices of all HGAP and CLC assembly report are available in Appendices 21-31.

Table 4.17. Genome sequencing information for all *Pandoraea* species**.** SMRT, Single Molecules Real Time (SMRT) technology by PacBio RSII SMRT sequencer; SDS, Sequencing By Synthesis (SDS) technology by MiSeq sequencer.

Strain	Technology	Fold coverage	GenBank ID	
			Chromosome	Plasmid
P. pnomenusa DSM 16536^T	SMRT	171.03×	CP009553.1	-
P. pnomenusa 6399	SDS	208.87×	JTCR01	-
P. pnomenusa 7641	SDS	246.96×	JTCS01	-
P. pulmonicola DSM 16583^T	SMRT	110.5×	CP010310.1	-
P. sputorum DSM 21091^T	SMRT	215.16×	CP010431.1	-
P. apista DSM 16535^T	SMRT	253.76x	CP013481.1	CP013482.1
P. norimbergensis DSM 11628^T	SMRT	165.09x	CP013480.1	-
P. faecigallinarum DSM 23572^T	SMRT	72×	CP011807.1	CP011808.1, CP011809.1
P. oxalativorans DSM 23570^T	SMRT	215.16×	CP011253.2	CP011518.1, CP011519.1, CP011520.1, CP011521.1
P. thiooxydans DSM 25325^T	SMRT	144×	CP011568.1	-
P. vervacti DSM 23571^T	SMRT	185×	CP010897.1	CP010898.1

4.14.3 Genome Annotation for *Pandoraea* Genomes.

All *Pandoraea* eleven genomes were annotated using various database as listed in section 3.2.9 and curated manually. General genome annotation information for all *Pandoraea* spp. were summarised in Table 4.18.

Table 4.18. General genome annotation information for all *Pandoraea* genomes. 1, *P. pnomenusa* RB-38; 2, *P. pnomenusa* DSM 16536^T; 3, *P. pnomenusa* 6399; 4, *P. pnomenusa* 7641; 5, *P. pulmonicola* DSM 16583^T; 6, *P. sputorum* DSM 21091^T; 7, *P. apista* DSM 16535^T; 8, *P. norimbergensis* DSM 11628^T; 9, *P. faecigallinarum* DSM 23572^T; 10, *P. oxalativorans* DSM 23570^T; 11, *P. thiooxydans* DSM 25325^T; 12, *P. vervacti* DSM 23571^T.

Strain	Size (Mb)	GC %	Genes	Protein	Pseudogenes	rDNA	tRNA	ncRNA
1	5.38	64.8	4767	4562	46	12	67	1
2	5.40	64.9	4819	4617	124	12	65	1
3	5.57	62.8	4880	724	472	3	59	1
4	5.57	62.8	4882	4723	469	3	59	1
5	5.87	64.3	5,022	4,820	113	12	75	1
6	5.75	62.8	5,037	4,859	100	12	65	1
7	5.59	62.6	5042	4864	100	12	65	1
8	6.17	63.1	5417	5195	144	12	65	1
9	5.26	63.7	4619	4412	128	12	65	1
10	5.64	63.1	4,983	4,711	194	12	65	1
11	4.48	63.2	4,134	3,988	90	6	49	1
12	5.66	63.5	4912	4697	136	12	65	1

4.14.4 ANI Analysis for *Pandoraea* Genomes

With the availability of all *Pandoraea* type species genomes, ANI analysis was performed (1) to investigate if ANI can provide a better species delineation resolution among *Pandoraea* species and (2) to confirm the identity of isolate RB-38 and RB-44 as *P. pnomenusa*.

Cut-off value for species delineation in ANI analysis is 95% which is correlated with 70% of DNA-DNA hybridization analysis (Goris et al., 2007). ANI value of *P. pnomenusa* RB-38 and RB-44 against *P. pnomenusa* DSM 16536^T is 99.32% and 99.34% respectively, hence reconfirmed their identity as *P. pnomenusa* (Table 4.19).

ANI value of *P. pnomenusa* 6399 and 7641, *Pandoraea* sp. B-6 and *Pandoraea* sp. SD6-2 which demonstrated ANI value lesser than 95% against all characterized *Pandoraea* type species indicating possibility of representing novel species.

ANI phylogeny was also constructed to study the taxonomic evolution of *Pandoraea* species (Figure 4.48). In short, *Pandoraea* genus was separated into 5 clusters: cluster 1 (*P. pnomenusa*, *P. pulmonicola*), cluster 2 (*P. sputorum*, *P. oxalativorans*, *P. faecigallinarum*, *and P. vervacti*), cluster 3 (*P. apista*), cluster 4 (*P. norimbergensis*) and cluster 5 (*P. thiooxydans*).

Table 4.19. ANI analysis between genomes of *Pandoraea* species. Ppn DSM, *P. pnomenusa* DSM 16536^T (CP009553.2)*;* Ppn RB38, *P. pnomenusa* RB-38 (CP007506.1); Ppn RB44, *P. pnomenusa* RB-44 (CP006938.1); Ppn 3kgm, *P. pnomenusa* 3kgm (CP006900.2); Ppn 6399, *P. pnomenusa* 6399 (JTCR01); Ppn 7641, *P. pnomenusa* 7641 (JTCS01); Ppu DSM, *P.* pulmonicola DSM 16583^T (CP010310.1); Psp DSM, *P. sputorum* DSM 21091^T (CP010431.1); Pap DSM, *P. apista* DSM 16535^T (CP013481.1, CP013482.1); Pap AU21, *P. apista* AU2161 (CP011501.1); Pap TF80, *P. apista* TF80G25 (CP011279.1); Pap TF81, *P. apista* TF81F4 (CP010518.3); Pno DSM, *P. norimbergensis* DSM 11628^T (CP013480.1); Pfa DSM, *P. faecigallinarum* DSM 23572^T (CP011807.1, CP011808.1, CP011809.1); Pox, *P. oxalativorans* DSM 23570^T (CP011253.2, CP011518.1, CP011519.1, CP011520.1, CP011521.1); Pthi DSM, *P. thiooxydans* DSM 25325^T (CP011568.1)); Pve, *P. vervacti* DSM 23571^T (CP010897.1, CP010898.1); sp. B-6, *Pandoraea* sp. B-6 (AKXS01); sp. E26, *Pandoraea* sp. E26 (AYXJ01); sp. SD6-2, *Pandoraea* sp. SD6-2 (AQOU01)

		Ppn						Ppu	Psp	Pap				Pno	Pfa	Pox	Pth	Pve	sp.		
		DSM	RB38	RB44	3kgm	6399	7641	DSM	DSM	DSM	AU21	TF80	TF81	DSM	DSM	DSM	DSM	DSM	B-6	E26	SD6-2
Ppn	DSM	100	99.32	99.34	99.31	85.04	85.14	85.49	84.50	84.55	84.469	84.50	84.55	83.92	84.66	84.61	78.62	84.61	84.81	99.35	84.65
	RB38	99.32	100	99.41	99.44	84.66	84.59	85.06	84.50	84.43	84.48	84.46	84.40	83.97	84.52	84.71	78.57	84.67	84.77	99.41	84.67
	RB44	99.34	99.41	100	99.43	84.64	84.63	85.07	84.48	84.51	84.47	84.46	84.46	83.96	84.49	84.63	78.68	84.61	84.79	99.97	84.61
	3kgm	99.31	99.44	99.43	100	84.61	84.58	85.07	84.43	84.47	84.48	84.45	84.49	83.94	84.54	84.64	78.54	84.67	84.85	99.44	84.67
	6399	85.04	84.66	84.64	84.61	100	100	84.78	84.66	88.56	88.55	88.51	88.55	84.14	84.66	84.78	78.14	84.60	85.49	84.61	87.89
	7641	85.14	84.59	84.63	84.58	100	100	84.77	84.64	88.46	88.55	88.53	88.48	84.12	84.58	84.79	78.21	84.60	85.45	84.65	87.86
Ppu DSM		85.49	85.06	85.07	85.07	84.78	84.77	100	84.68	84.60	84.55	84.58	84.60	84.33	84.63	84.80	78.79	84.73	85.05	85.10	84.75
Psp DSM		84.50	84.50	84.48	84.43	84.66	84.64	84.68	100	84.55	84.54	84.64	84.55	83.80	85.85	92.39	78.37	85.56	84.60	84.41	84.71
Pap	DSM	84.55	84.43	84.51	84.47	88.56	88.46	84.60	84.55	100	99.31	99.31	99.31	84.15	84.58	84.68	78.19	84.44	85.32	84.44	87.77
	AU21	84.46	84.48	84.47	84.48	88.55	88.55	84.55	84.54	99.31	100	100	100	84.10	84.46	84.59	78.11	84.42	85.32	84.42	87.78
	TF80	84.50	84.46	84.46	84.45	88.51	88.53	84.58	84.64	99.31	100	100	100	84.10	84.48	84.72	78.11	84.48	84.30	84.48	87.77
	TF81	84.55	84.40	84.46	84.49	88.55	88.48	84.60	84.55	99.31	100	100	100	84.15	84.50	84.68	78.19	84.44	85.29	84.41	87.74
Pno DSM		83.92	83.97	83.96	83.94	84.14	84.12	84.33	83.80	84.15	84.10	84.10	84.15	100	83.88	83.99	78.24	83.90	84.09	83.92	84.05
Pfa DSM		84.66	84.52	84.49	84.54	84.66	84.58	84.63	85.80	84.58	84.46	84.48	84.50	83.8	100	8.72	78.47	86.70	84.54	84.47	84.62
Pox DSM		84.61	84.71	84.63	84.64	84.78	84.79	84.80	92.39	84.68	84.59	84.72	84.68	83.99	85.72	100	78.35	85.90	84.71	84.60	84.79
Pth DSM		78.62	78.57	78.68	78.54	78.14	78.21	78.79	78.37	78.19	78.11	78.11	78.19	78.24	78.47	78.35	100	78.25	78.08	78.42	77.85
Pve DSM		84.61	84.67	84.61	84.67	84.60	84.60	84.73	85.56	84.44	84.42	84.48	84.44	83.90	86.70	85.90	78.25	100	84.53	84.49	84.59
sp.	B-6	84.81	84.77	84.79	84.85	85.49	85.45	85.05	84.60	85.32	85.32	84.30	85.29	84.09	84.54	84.71	78.08	84.53	100	85.05	85.81
	E26	99.35	99.41	99.97	99.44	84.61	84.65	85.10	84.41	84.44	84.42	84.48	84.41	83.92	84.47	84.60	78.42	84.49	85.05	100	84.67
	SD6-2	84.65	84.67	84.61	84.67	87.89	87.86	84.75	84.71	87.77	87.78	87.77	87.74	84.05	84.62	84.79	77.85	84.59	85.81	84.67	100

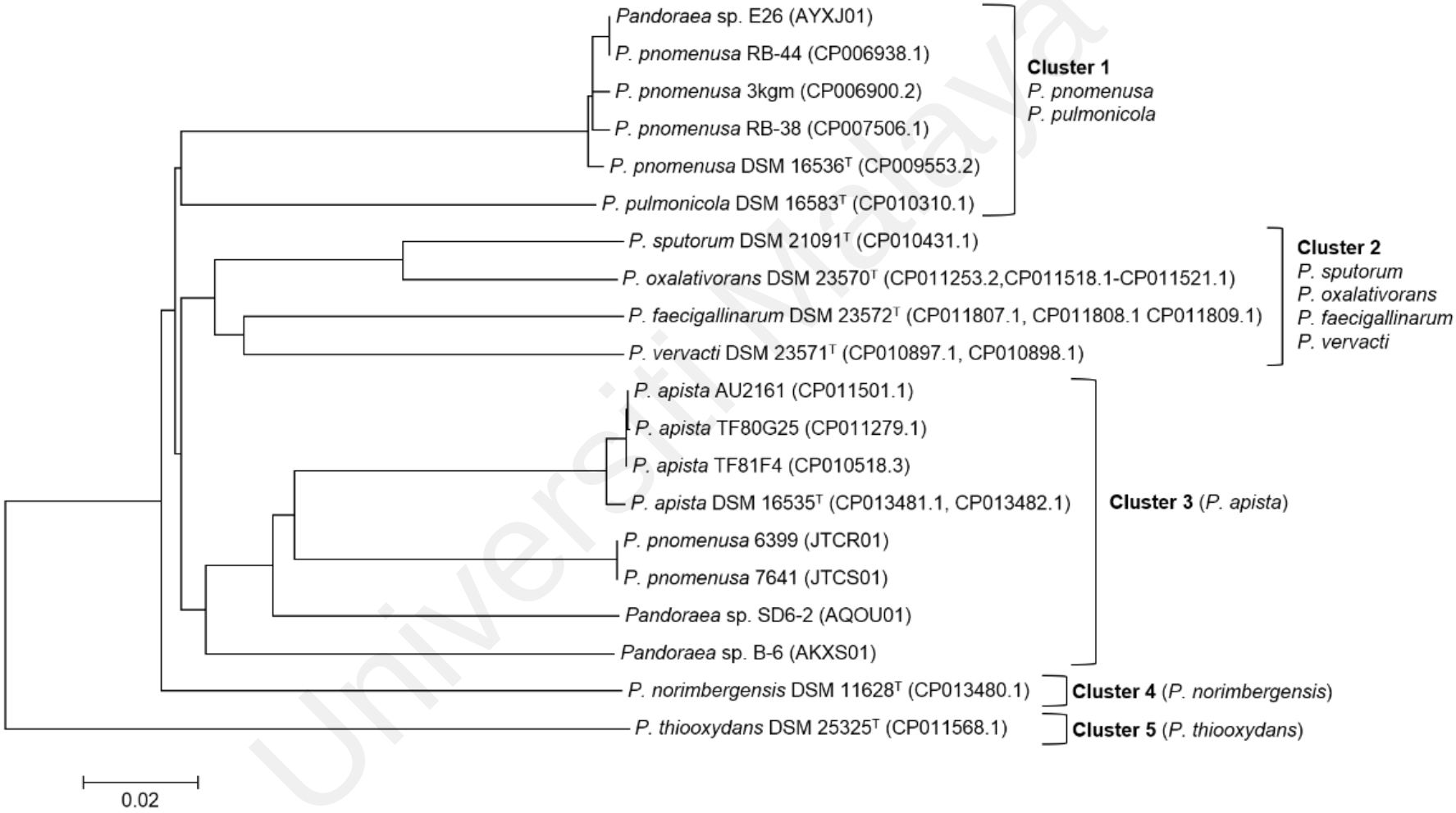


Figure 4.48. ANI phylogeny of all *Pandoraea* genomes.

4.14.5 QS Genes in *Pandoraea* Genomes

QS genes in all *Pandoraea* genomes were identified by searching for the signature domains of QS genes using HMM search and Interproscan identifiers as described in section 4.9.7. An additional LuxR3 solo (designated as PpnR3, sharing only 26% and 33% amino acid identity from PpnR1 and PpnR2) of *P. pnomenusa* RB-38 which was not predicted as an ORF in previous analysis was discovered in this analysis. All QS synthases (LuxI) and transcriptional regulators (LuxR) identified from *Pandoraea* genomes were summarised in Table 4.20.

Genetic nomenclature of all *luxI* and *luxR* of *Pandoraea* species were proposed with the first alphabet of the genus followed by first two alphabet of species name in combination of I indicating for QS synthase, R1 indicating for paired LuxR or R2 and R3 indicating for LuxR2 solo and LuxR3 solo (For example: *ppnI* encodes for *luxI* of *P. pnomenusa*; *ppnR1* encodes for cognate *luxR* of *P. pnomenusa*; *ppnR2* and *ppnR3* encodes for *luxR* solo of *P. pnomenusa*).

Interestingly, all *Pandoraea* species (except for *P. thiooxydans*) harboured both LuxR2 solo and LuxR3 solo. This demonstrated that QS negative species of *Pandoraea* are capable to detecting QS signaling molecules in the exogenous environment hence reaffirmed our hypothesis about presence of LuxR solo in QS negative species of *Pandoraea*.

Table 4.20. LuxI/R of *Pandoraea* species. Ppn RB38, *P. pnomenusa* RB-38; Ppn DSM, *P. pnomenusa* DSM 16536^T; Ppn 6399, *P. pnomenusa* 6399; Ppn 7641, *P. pnomenusa* 7641; Ppu DSM, *P. pulmonicola* DSM 16583^T; Psp DSM, *P. sputorum* DSM 21091^T; Pap DSM, *P. apista* DSM 16535^T; Pno DSM, *P. norimbergensis* DSM 11628^T; Pfa DSM, *P. faecigallinarum* DSM 23572^T; Pox DSM, *P. oxalativorans* DSM 23570^T; Pth DSM, *P. thiooxydans* DSM 25325^T; Pve DSM, *P. vervacti* DSM 23571^T.

Strain	QS	LuxI	LuxR1	LuxR2 solo	LuxR3 solo
Ppn RB38	Positive	PpnI (261 aa) AHN77101.1	PpnR1 (233 aa) AHN77102.1	PpnR2 (255 aa) AHN76935.1	PpnR3 (331 aa) CP007506.2, 4853186-4854178
Ppn DSM	Positive	PpnI (245 aa) ALR35832.1	PpnR1 (233 aa) ALR35833.1	PpnR2 (255 aa) AIU26521.1	PpnR3 (377 aa) CP009553.1, 3683069- 3684199
Ppn 6399	-	-	-	PpnR2 (255 aa) KHQ92691.1	PpnR3 (274 aa) WP_052240333.1
Ppn 7641	-	-	-	PpnR2 (255 aa) KHQ93427.1	PpnR3 (274 aa) WP_052240333.1
Ppu DSM	-	-	-	PpuR2 (255 aa) AJC19606.1	PpuR3 (271 aa) WP_052266958.1
Psp DSM	Positive	PspI (261 aa) WP_052252565.1	PspR1 (237 aa) WP_052252566.1	PspR2 (255 aa) AJC18152.1	PspR3 (304 aa) AJC15852.1
Pap DSM	-	-	-	PapR2 (255 aa) ALS67327.1	PapR3 (275 aa) ALS65841.1
Pno DSM	-	-	-	PnoR2 (275 aa) ALS58731.1	PnoR3 (246 aa) ALS60193.1
Pfe DSM	-	-	-	PfeR2 (255 aa) AKM29693.1	PfeR3(310 aa) WP_053059535.1
Pox DSM	Positive	PoxI (222 aa) AKC71607.1	PoxR1 (233 aa) AKC72674.1	PoxR2 (255 aa) AKC71755.1	PoxR3 (304 aa) AKC69307.1
Pth DSM	-	-	-	-	-
Pve DSM	Positive	PveI (217 aa) AJP59868.1	PveR1 (233 aa) AJP59867.1	PveR2 (255 aa) AJP58402.1	PveR3 (286 aa) AJP56205.1

4.14.6 Phylogeny and Multiple Alignment of QS genes in *Pandoraea* spp.

Phylogeny of LuxI and LuxR were constructed against *B. cenocepacia* and *P. aeruginosa* to investigate their evolutionary relationship. LuxI phylogeny of *Pandoraea* spp. was presented in Figure 4.49 in which all LuxI of *Pandoraea* spp. form a monophyletic lineage with LuxI of Bcc and *P. aeruginosa* as the sister clade. Multiple alignment analysis performed demonstrated that LuxI of *Pandoraea* spp. contained all 9 conserved residues (R25, F29, W35, E44, D46, D49, R70, F84, E101 and R104) (Table 4.21).

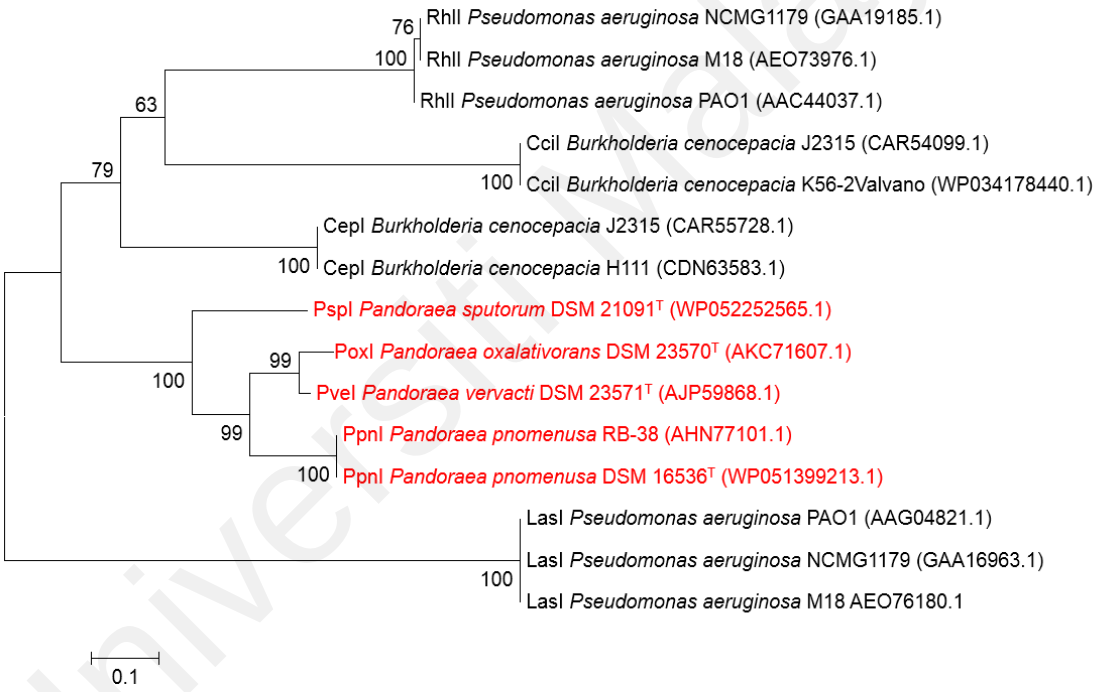


Figure 4.49. Phylogeny of LuxI of *Pandoraea* spp.

Table 4.21. Multiple alignment analysis of LuxI of *Pandoraea* species.

LuxI	Key amino acid conserved in all LuxI-type protein									
	R25	**F29**	**W35**	**E44**	**D46**	**D49**	**R70**	**F84**	**E101**	**R104**
PpnI RB38	R	F	W	E	D	D	R	F	E	R
PpnI DSM	R	F	W	E	D	D	R	F	E	R
PspI	R	F	W	E	D	D	R	F	E	R
PoxI	R	F	W	E	D	D	R	F	E	R
PveI	R	F	W	E	D	D	R	F	E	R

Phylogeny of LuxR of *Pandoraea* spp. against LuxR of *B. cenocepacia* and *P. aeruginosa* was demonstrated in Figure 4.50. All cognate LuxR1 of *Pandoraea* spp. formed a paraphyletic lineage with CepR with strong nodal support of 97% bootstrap value but monophyletic lineage with RhlR, QscR and CepR2. Meanwhile, LuxR2 solo of *Pandoraea* spp. formed a paraphyletic lineage with CciR (55% bootstrap value) and monophyletic lineage with LasR and LuxR3.

Multiple alignment analysis of LuxR of *Pandoraea* spp. demonstrated they contained similar 6 key amino acid in autoinducer binding domain (W57, Y61, D70, P71, W85, G113) and 3 key amino acids in DNA-binding domain (E178, L182, G188) as TraR (Table 4.22)

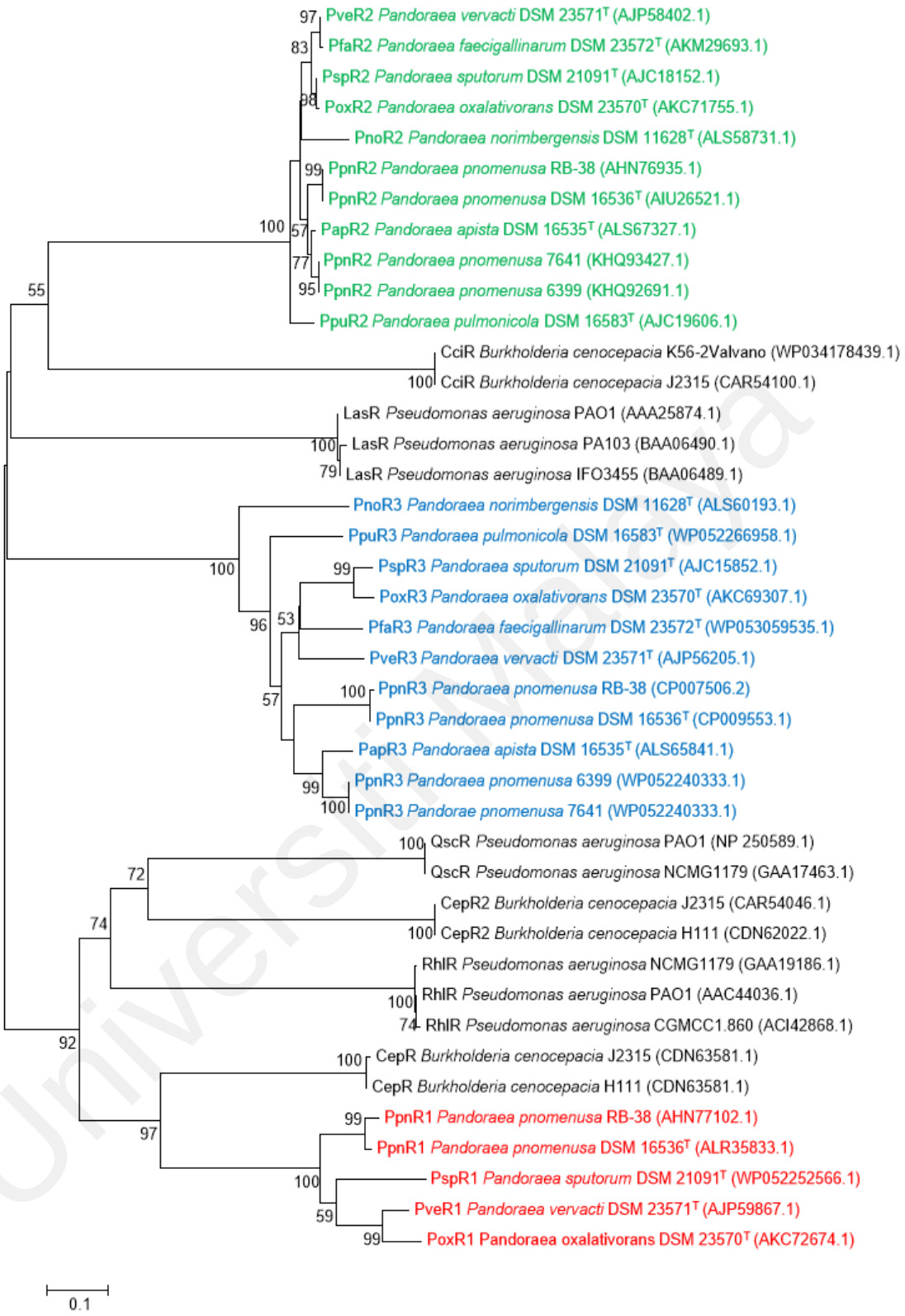


Figure 4.50. Phylogeny of LuxR of *Pandoraea* spp.

Table 4.22. Multiple alignment analysis of LuxR of *Pandoraea* species.

LuxR		Key amino acid in autoinducer binding domain						Key amino acid in DNA binding domain		
		W57	**Y61**	**D70**	**P71**	**W85**	**G113**	**E178**	**L182**	**G188**
LuxR1	PpnR1 DSM	W	Y	D	P	W	G	E	L	G
	PpnR1 RB38	W	Y	D	P	W	G	E	L	G
	PspR1	W	Y	D	P	W	G	E	L	G
	PoxR1	W	Y	D	P	W	G	E	L	G
	PveR1	W	Y	D	P	W	G	E	L	G
LuxR2	PpnR2 DSM	W	Y	D	P	W	G	E	L	G
	PpnR2 RB38	W	Y	D	P	W	G	E	L	G
	PpnR2 6399	W	Y	D	P	W	G	E	L	G
	PpnR2 7641	W	Y	D	P	W	G	E	L	G
	PpuR2	W	Y	D	P	W	G	E	L	G
	pspR2	W	Y	D	P	W	G	E	L	G
	PapR2	W	Y	D	P	W	G	E	L	G
	PnoR2	W	Y	D	P	W	G	E	L	G
	PfaR2	W	Y	D	P	W	G	E	L	G
	PoxR2	W	Y	D	P	W	G	E	L	G
	PveR2	W	Y	D	P	W	G	E	L	G
LuxR3	PpnR3 DSM	W	Y	D	P	W	G	E	L	G
	PpnR3 RB38	W	Y	D	P	W	G	E	L	G
	PpnR3 6399	W	Y	D	P	W	G	E	L	G
	PpnR3 7641	W	Y	D	P	W	G	E	L	G
	PpuR3	W	Y	D	P	W	G	E	L	G
	PspR3	W	Y	D	P	W	G	E	L	G
	PapR3	W	Y	D	P	W	G	E	L	G
	PnoR3	W	Y	D	P	W	G	E	L	G
	PfaR3	W	Y	D	P	W	G	E	L	G
	PoxR3	W	Y	D	P	W	G	E	L	G
	PveR3	W	Y	D	P	W	G	E	L	G

CHAPTER 5: DISCUSSION

5.1 Isolation of QS Bacterium From KGm medium

KGm medium is a chemically defined novel medium supplemented with AHL as the sole carbon and nitrogen source designed for isolation of AHL degrading bacteria (Chan, Yin, Sam, & Koh, 2009; Wong et al., 2012). Although the objective of this study was set out to isolate bacteria with QQ and/or QS activity, all 60 morphologically distinct bacterial isolates purified from KGm enrichment did not demonstrated QQ activity against 3-oxo-C6-HSL but instead only 4 isolates were found to exhibit QS activity.

This could be explained by the preparation of KGm medium which contained only minimum of essential growth nutrient that could also support the thriving of other non fastidious bacteria with QS activity, especially in *Pseudomonas* and *Burkolderia* genus. This finding could be supported by previous documentation of QS bacteria being isolated from KGm medium, for instances, *Pseudomonas aeruginosa* MW3A (Wong et al., 2012), *Bukholderia* sp. A9 (Chen, Koh, Sam, Yin, & Chan, 2013) and *Burkholderia cepacia* GG4 (Chan et al., 2011).

For screening of QS activity, two biosensors (a violacein-based *C. violaceum* CV026 and a bioluminecence-based *E. coli* [pSB401] bionsensor) with different sensitivity and reporter system were selected in this study for rapid screening of QS activity. Only 4 isolates which were later identified as *C. multitudinisentens* RB-25^{T} (a newly propsed novel genus in *Enterbacteriocaea* family), *P. pnomenusa* RB-38 and *P. pnomenusa* RB-44 (two newly documented QS members which are closely related to *Burkholderia*) and *P. aeruginosa* RB-48 activated both AHL biosensors suggesting presence of QS activity.

To identify QS profile of these 4 isolates, triple quadropole MS analysis using precursor ion mode was performed with reference chromatogram of 10 synthetic AHLs (inclusive of short and long chain AHLs, with and without 3-oxo-substituted at C3 of the acyl side chain) as listed in Table 3.5. Three AHLs (C4-HSL, C6-HSL and 3-oxo-C6-HSL) was detected from spent supernatant of *C. multitudinisentens* RB-25^{T}; C8-HSL was detected as the only signaling molecule produced by *P. pnomenusa* RB-38 and RB-44 which to our best knowledge, this are the first documentation of QS activity in *Pandoraea* spp; and lastly, five AHLs (C4-HSL, C6-HSL, C8-HSL, 3-oxo-C10-HSL and C12-HSL) was detected in spent supernatant of *P. pseudomonas* RB-48.

5.2 Bacterial Identification and Classification

Definitive bacterial identification and taxonomical studies is important to enhance our understanding about microorganism biodiversity, their functional role in the ecosystem and to establish effective clinical diagnostic and treatment (Das, Dash, Mangwani, Chakraborty, & Kumari, 2014; Gevers et al., 2005). Despite the advancement of various molecular techniques for microbial identification, and development of various commercial identification system (such as API® and VITEK® identification system, Phenotypic MicroArrays, MALDI-TOF MS Biotyper system and Sherlock Microbial ID System), no single identification technique is effective to be relied upon solely as a source of taxonomic information (Das et al., 2014; Janda & Abbott, 2002). Hence, in this study, a systematic bacterial polyphasic identification was designed involving a combination of phenotypic, genotypic and phylogenetic approaches.

5.2.1 Systematic Bacterial Identification

The systematic bacterial identification employed in this study involved initially with a peptide mass fingerprinting using MALDI-TOF MS analysis, pairwise similarity and phylogeny analysis of 16S rDNA sequence as presented in Figure 5.1. Only bacteria isolate that was identified as putative novel species will be studied further with a complete taxonomic characterization such as isolate RB-25 in which no realiable identification was made in MALDI-TOF MS analysis (≤ 2.0) indicating for a possibility of new bacterial species discovery. Meanwhile, comparison against EzTaxon database generated 96.58% pairwise similarity value for isolate RB-25 in which sequence similarity value of <97% is the cut off value indicating for isolation of a new species over full pairwaise comparison (Kim et al., 2012; Tindall, Rosselló-Móra, Busse,

Ludwig, & Kämpfer, 2010). Thus, complete taxanomical characterisation was performed for isolate RB-25 which later confirmed its position as a novel genus in *Enterobactericeae* family.

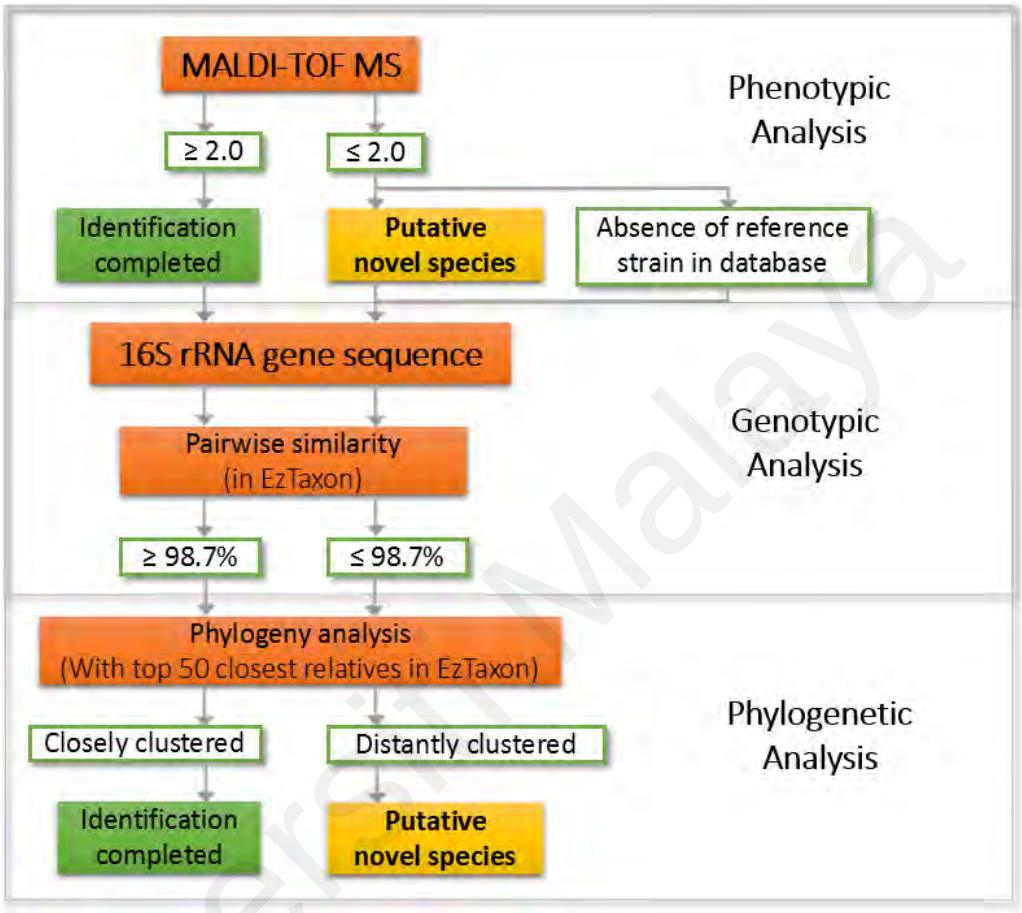


Figure 5.1 Outline of systematic bacterial identification

Isolates RB-38 and RB-44 were identified as *P. pnomenusa* with high confidence level in MALDI-TOF MS analysis (≥ 2.0), pairwise similarity analysis (99.9%) and 16S rDNA phylogeny analysis. Isolate RB-48 was also identified with high confidence level as *P. aeruginosa* in this outline of systematic bacterial identification.

5.2.2 Discovery of *C. multitudinisentens* RB-25^{T} gen. nov., sp. nov.

Since initial identification using MALDI-TOF and 16S rRNA gene sequence analysis could not classified the identity of isolate RB-25, various phenotypic and genotypic taxonomic characterisations were subsequently performed according to Tindall et al. (2010) and Bergey's Manual of Systematic Bacteriology as presented in Figure 5.2.

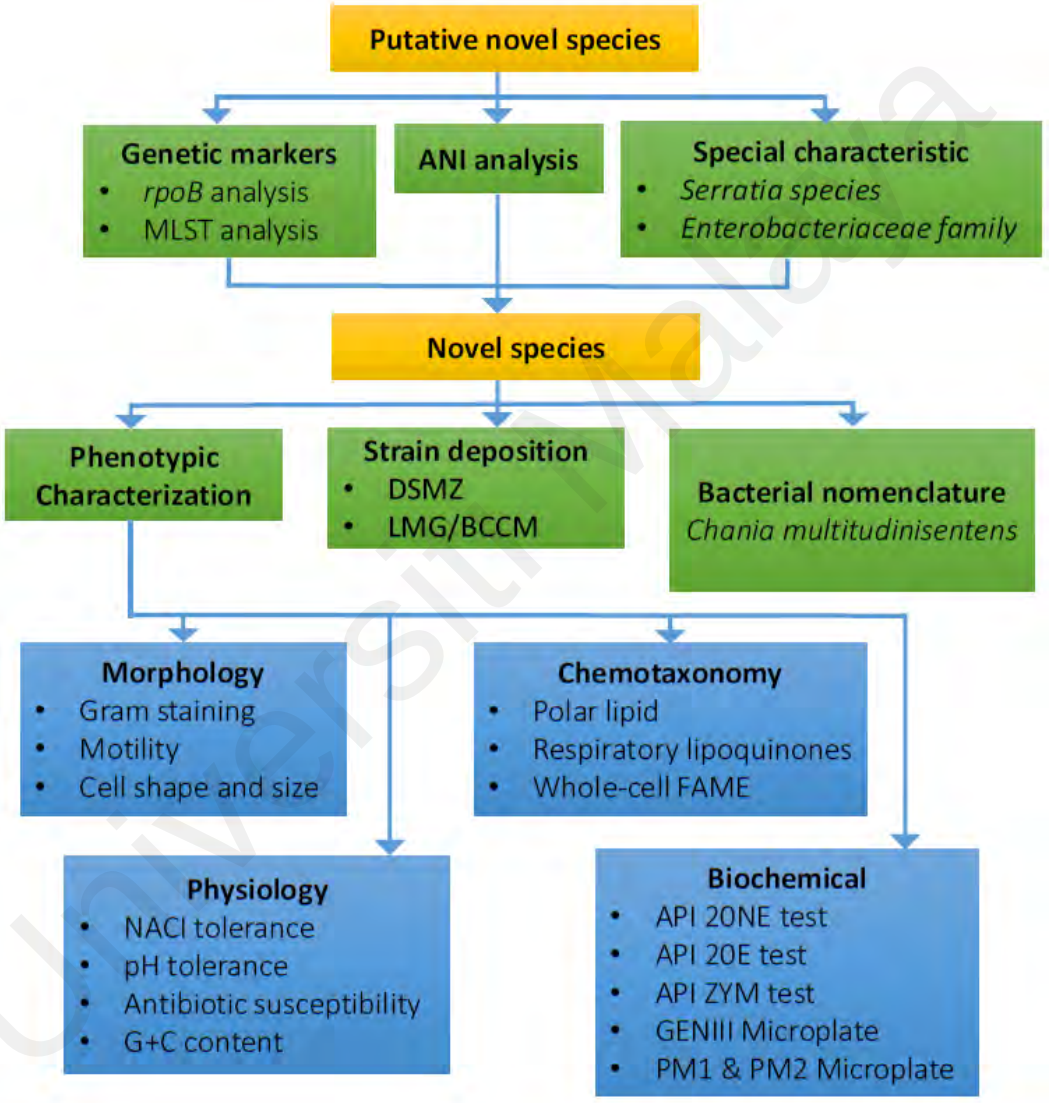


Figure 5.2. Flowchart of the complete taxonomic characterisation for isolate RB-25.

DNA-DNA hybridization (DDH) is the golden standard for species delineation using genome wide comparisons between organisms since 1960 (Goris et al., 2007), However, DDH was not performed for isolate RB-25 since its pairwise similarity value is lower than 97%. Instead, ANI analysis was performed with the cut off value of 95%

in ANI for delineation of bacteria species corresponding to 70% in DDH (Goris et al., 2007). ANI analysis of isolate RB-25 demonstrated an ANI value of <83% to other *Enterobacteriaceae* members, thus confirming that isolate RB-25 should represent a novel genus (section 4.9.4).

As initial MALDI-TOF (section 4.3.1) and Blast2GO (section 4.10.1) analysis demonstrated that isolate RB-25 could be related to *Serratia* genus, various identification tests for characterization of *Serratia* spp. (for instances glucose oxidation tests with and without presence of pyrroloquinoline quinone, tetrathionate reductase, Voges-Proskauer, gluconate dehydrogenase, 2-ketogluconate dehydrogenase and gas production tests) were conducted (Grimont & Grimont, 2006). Isolate RB-25 showed all negative results for all characteristic defining *Serratia* spp. which confirmed that the isolate RB-25 does not belong to the *Serratia* genus.

Classification of *Enterobacteriaceae* members based on 16S rRNA gene sequence has been proven to be difficult due to the polyphyletic nature in *Enterobacteriaceae* family (Brady, Cleenwerck, Venter, Coutinho, & De Vos, 2013). As such, *rpoB* sequence phylogeny and MLSA based on five concatenated housekeeping genes (*fusA*, *pyrG*, *rplB*, *rpoB* and *atpD*) was performed to provide a better phylogenetic resolution (Brady et al., 2013; Le Fleche-Mateos et al., 2015). Both analysis unanimously support that isolate RB-25 could not be assigned into any documented genus (section 4.95 and section 4.9.6).

Chania multitudinisentens sp. nov. can be easily differentiated from the genus of *Rahnella* with the opposing result on several phenotypic tests, including: positive result in acid production from sucrose, L-arabinose; negative result for urease and catalase activities and in acid production from melibiose, cellobiose and raffinose.

Chania multitudinisentens sp. nov. can be easily differentiated from the genus of *Rouxiella* with the opposing result on several phenotypic tests, including: pale white colour; growth at 37°C; positive result in motility and acid production from D-sorbitol, sucrose; negative result for catalase activity and in acid production of melibiose.

Chania multitudinisentens sp. nov. can be easily differentiated from the genus of *Pantoea* with the opposing result on several phenotypic tests, including: positive result in motility and acid production from D-sorbitol, sucrose; negative result for catalase activity and in acid production from melibiose and cellobiose.

Chania multitudinisentens sp. nov. can be easily differentiated from the genus of *Pectobacterium* with the opposing result on several phenotypic tests, including: positive result in acid production from D-sorbitol; negative result in acid production from melibiose, cellobiose and raffinose; absence of MK-8 quinone.

Chania multitudinisentens sp. nov. can be easily differentiated from the genus of *Gibbsiella* with the opposing result on several phenotypic tests, including: positive result in motility; negative result for urease and catalase; negative result in acid production from melibiose, cellobiose and raffinose.

Chania multitudinisentens sp. nov. can be easily differentiated from the genus of *Serratia* with the opposing result on several phenotypic tests, including: positive result in acid production from L-rhamnose; sucrose; negative result for urease and catalase activities and in acid production from melibiose and raffinose.

5.3 Genome Sequencing Technologies Comparison

In the effort to identify *luxI/R*, genomes sequencing was performed using either MiSeq sequencing by synthesis (SBS) technology or PacBio Single Molecule Real Time (SMRT) sequencing technology depending on the importance of the genome. SBS technology are generally more cost effective but the genomes assembled usually contain many gaps (also known as "draft genomes") which is less valuable as compared to complete genome from SMRT technology. DNA template libraries of SBS technology are usually small fragment libraries of about ~150-300bp amplified using more than one step amplification process to provide abundant coverage during the sequencing process. During sequencing of GC bias (GC rich or GC poor) genomes, this short and amplified fragments typically leads to a huge drop in sequencing quality as GC bias repeats are usually poorly amplified and thus, resulted in incomplete closure of a genome (draft genome).

Overall, SMRT approach provides more advantages as it employed parental long read of 10-20kb of amplification free insert template library (Roberts, Carneiro, & Schatz, 2013). The advantage of the long seed read is to span through GC bias repeats and increase the possibility of complete genome closure. With the availability of complete genome, the genome was circularised (by trimming off repeated region at both ends) and search for the site of origin of replication in the genome to provide the ultimate polishing of a genome for downstream bioinformatics analysis. Besides, plasmids can also be differentiated from the bacterial chromosome and harness the bacteria methylation profile from the polymerase kinetics from the single run of SMRT sequencing (Flusberg et al., 2010).

5.4 Systematic Bioinformatics Prediction of LuxI and LuxR

To effective identify all LuxI and LuxR in this study, a systematic bioinformatics prediction and identifcation of LuxI and LuxR was employed in this study as presented in Figure 5.3.

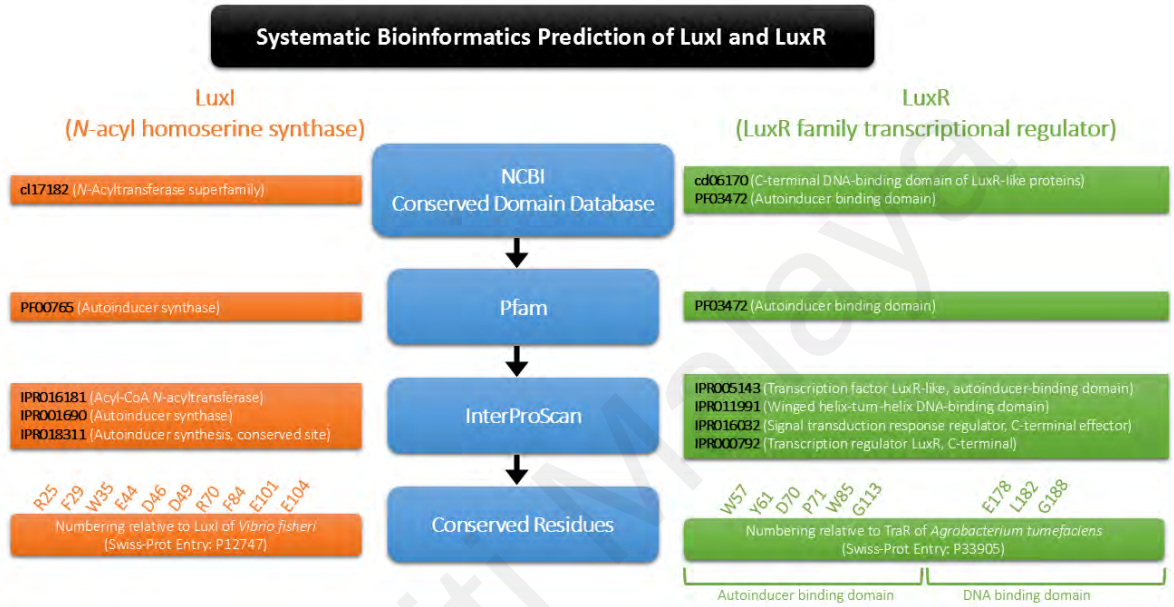


Figure 5.3 Systematic Bioinformatics Prediction of LuxI and LuxR

5.5 CmuIR of *C. multitudinisentens* RB-25^T

A set of *cmuI1/R1* was initially identified from the draft genomes of *C. multitudinisentens* RB-25^T in which 3-oxo-C6-HSL was not detected from the expression of CmuI1 (section 4.6.2). As parental wildtype of *C. multitudinisentens* RB-25^T was known to produce C4-HSL, C6-HSL and 3-oxo-C6-HSL, it is hypothesized that there might be another set of *luxI/R* that was missing in the draft genome. Thus, complete genome of *C. multitudinisentens* RB-25^T was sequenced again using SMRT technology which leads to the discovery of the *cmuI2/R2* which produced all 3 AHLs (C4-HSL, C6-HSL and 3-oxo-C6-HSL) identical to the QS profile of its parental wildtype.

In silico analysis performed demonstrated that CmuIR1 and CmuIR2 were classified into group A and group B within the LuxIR of *Serratia* (section 4.12.1) as reported by Xiaoguang Liu et al. (2011). The AHLs commonly produced by LuxI of Group A is C4-HSL and C6-HSL and functional studies performed demonstrated that CmuI1 produced similar AHL profile in LuxI of *Serratia* spp. in Group A. CmuI1 was clustered in the group A with high similarity to SmaI of *Serratia* sp. ATCC 39006 (99% similarity and 81% identity) which produces C4-HSL and C6-HSL for regulation of the production of carbapenem, pectate lyase, prodiogiosin and cellulase (Thomson, Crow, McGowan, Cox, & Salmond, 2000). Meanwhile, other LuxI synthase which were also documented in Group A are SwrI of *S. marcescens* MG1 and SmaI of *S. marcescens* strain 12 which also produce C4-HSL and C6-HSL for regulation of biofilm formation and swarming motility (Coulthurst et al., 2006; Eberl, Christiansen, Molin, & Givskov, 1996; Eberl et al., 1996).

On the other hand, CmuI2 was clustered in the Group B with SpnI of *S. marcescens* SS-1 (98% similarity and 79% identity). LuxI in Group B were reported to produce C4-HSL, C6-HSL, 3-oxo-C6-HSL however SpnI was produces an additional of C7-HSL and C8-HSL but not C4-HSL (Horng et al., 2002). Nonetheless, SpnI of *S. plymuthica* RHVI (79% identity) and *S. plymuthica* HRO-C48 (78% identity) produce similar QS profile (C4-HSL, C6-HSL, 3-oxo-C6-HSL) as CmuI2 (Liu et al., 2007; Van Houdt et al., 2007). Since SpnI of *S. plymuthica* RHVI is known to regulate for the production of extracellular chitinase, we hypothesize that CmuI2 could also play similar regulation for the expression of chitinase in which a complete set of chitinase operon was discovered in genome of *C. multitudinisentens* RB-25^{T} (Lim, Yong, et al., 2015).

5.6 PpnIR of *P. pnomenusa* RB-38 and RB-44

In silico analysis of PpnI demonstrated that PpnI formed a distinct cluster against LuxI of *Burkholderia* and *Pseudomonas* (section 4.12.2). As PpnI of *P. pnomenusa* RB-38 and RB-44 shared 100% amino acid similarity. Functional studies was only performed for PpnI of *P. pnomenusa* RB-38 in which C8-HSL was detected as the only signaling molecule produced from the recombinant PpnI (Lim, Ee, et al., 2015). Since PpnR1 was located adjacently to PpnI, this data suggested that PpnR1 is the transcriptional regulator for binding of C8-HSL from its cognate PpnI. Multiple alignment of PpnR1 also indicated that PpnR1 contain the similar 9 conserved amino acid as TraR crucial for formation of hydrogen bond against AHL.

On the other hand, PpnR2 solo forms a cluster with LasR suggesting that PpnR2 could be the LuxR for binding of 3-oxo-C12-HSL from LasI for interspecies communication. Since CepI of *Burkholderia cepacia* complex (Bcc) is known to produce C8-HSL for regulation of various virulence factors such as protease, siderophore, polygalacturonase, swarming motility, and biofilm formation (Aguilar, Bertani, & Venturi, 2003; Huber et al., 2001; Lewenza et al., 1999; Lewenza & Sokol, 2001). Thus, we hypothesized that C8-HSL could also play an important in regulation of various virulence factors in *P. pnomenusa* RB-38 and RB-44.

5.7 QS Activity in *Pandoraea* Genus

In this study, this thesis presented the first comprehensive QS activity profiling, genome sequencing and identification of all QS genes in all *Pandoraea* species. For the first time with the availability of these genomic data, this work managed to classify *Pandoraea* species with high resolution using ANI analysis and confirm the identity of *Pandoraea* sp. E26 as *P. pnomenusa* besides pointed out that *P. pnomenusa* 6399 and 7641 should represent novel species in *Pandoraea* genus. More interestingly, this shown that all *Pandoraea* species (except for *P. thiooxydans*) contained 2 LuxR solo for detecting exogenous QS signaling molecules while C8-HSL is the only signaling molecules produced by QS species of *Pandoraea*.

5.8 Future Work

Future investigation will be conducted to identify the QS-mediated genes expression in *Pandoraea* species especially virulence factors in clinical isolates. This can be achieved firstly by knocking out the QS genes follow by global transcriptomic studies. To provide the ultimate confirmation of QS-regulation genes, chromatin immunoprecipitation sequencing (ChiP) can be performed to identity the DNA-binding site (*lux* box) of the AHL-bounded LuxR. Subsequently, phenotype microarray can be performed to study the phenotypic changes between wildtype and mutants.

Next, to prove the theory that LuxR solo in *Pandoraea* species can response to AHLs produced other organisms, isothermal titration calorimetry can be performed to study the interaction of various signaling molecules with the cognate LuxR1, LuxR2 solo and LuxR3 solo. Lastly, X-ray crystallography can be performed to identify the site interaction between these LuxR and signaling molecules.

CHAPTER 6: CONCLUSION

In this study, four QS isolates (*C. multitudinisentens* RB-25^T, *P. pnomenusa* RB-38, *P. pnomenusa* RB-44 and *P. aeruginosa* RB-48) were isolated from the soil sample of a former municipal landfill site. Genome sequencing were subsequently performed to identify novel QS genes.

This work reports the complete characterization of *C. multitudinisentens* RB-25^T gen., nov., sp. nov. as a novel taxon. Two pairs of *luxI/R* (designated as *cmuI1/R*2 and *cmuI2/R2*) were found in the genome of *C. multitudinisentens* RB-25^T. Expression of CmuI1 and CmuI2 confirmed that these are indeed the functional LuxI in *C. multitudinisentens* RB-25^T that produced C4-HSL, C6-HSL and 3-oxo-C6-HSL.

P. pnomenusa RB-38 and its *luxI/R* (designated as *ppnI/R1*) and a *luxR2* solo (designated as *ppnR2* solo) were identified from the genomes. Expression of PpnI confirmed its role in producing C8-HSL as the only signaling molecule. Intriguingly, all *Pandoraea* species showed presence of LuxR2 solo and LuxR3 solo, except for *P. thiooxydans*. This study presented the first comprehensive QS characterization and genome analysis in *Pandoraea* genus.

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