POLYPHASIC AND OMICS INVESTIGATION ON QUORUM SENSING MECHANISMS IN AQUATIC BACTERIA FROM TROPICAL RAINFOREST WATERFALL

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FACULTY OF SCIENCE
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KUALA LUMPUR

2017

POLYPHASIC AND OMICS INVESTIGATION ON QUORUM SENSING MECHANISMS IN AQUATIC BACTERIA FROM TROPICAL RAINFOREST WATERFALL

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THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF SCIENCE
UNIVERSITY OF MALAYA
KUALA LUMPUR

2017

UNIVERSITY OF MALAYA
ORIGINAL LITERARY WORK DECLARATION

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ABSTRACT

Quorum sensing (QS) regulates bacterial gene expressions correlating at bacterial cell population density. In this study, a total of 13 isolates were found to show QS activity with preliminary screening using bacterial QS biosensor. These 13 isolates were identified that belonged to 7 genera (*Aeromonas, Cedecea, Dickeya, Enterobacter, Erwinia, Pantoea and Pectobacterium*). They were identified using MALDI-TOF mass spectrometry and 16S rDNA sequence-based identification via EzTaxon database. Next, high tandem mass spectrometry was used to profile *N*-acylhomoserine lactone (AHL) signalling molecules produced and 11 out of 13 isolates were able to produce AHLs. Of these isolates, *Enterobacter cancerogenus* strain M004 was selected for further analyses due to its novelty in QS. *E. cancerogenus* strain M004 was found to produce 3-oxo-C6 HSL and 3-oxo-C8 HSL via mass spectrometry analysis. *E. cancerogenus* strain M004 was whole genome sequenced where the draft genome was 5.67 Mbp in size. Its genome data showed two QS synthase genes which were named as *ecnI*-1 and *ecnI*-2; found located at contig 7 (633 bp) and contig 2 (615 bp) respectively. Functionality of these genes was studied via heterologous gene cloning into *Escherichia coli*. BL21(DE3)pLysS. The *ecnI*-2 was found to be a non-functional QS synthase gene. The findings indicated that *ecnI*-1 is responsible in the production of both *N*-3-oxo-acylhomoserine lactones in strain M004. One of the known anti-QS compounds, gallic acid was used to treat *E. cancerogenus* strain M004 in the transcriptome study and several genes related with QS such as *fliH* and *betI* were differential expressed. Furthermore, the biofilm assay conducted with *E. cancerogenus* strain M004 treated with gallic acid showed significant reduction as compared to the control (without gallic acid). To further study the functionality of QS related *ecnI*-1 gene, an *ecnI*-1 knock-out mutant was constructed using the λ red system through single-gene knock-out principle. The *E. cancerogenus* strain M004 *luxI* mutant (Δ*ecnI-1::Kan*^{r}) was then verified using bacterial

QS biosensors followed by high resolution tandem mass spectrometry; no AHLs was detected from Δ*ecnI-1::Kan*^{r} mutant. Loss of function in the mutant in antibiotic resistance on chlortetracycline, erythromycin and lomeflaxin were significant as compared to wildtype via OmniLog Phenotype Microarray (PM) technology. Transcriptomic profiling was conducted to study the effect of *ecnI*-1 knock-out from *E. cancerogenus* strain M004; some genes such as cell wall/membrane/envelope biogenesis (*epsJ*) was down-regulated and carbohydrate transport and metabolism (*glpT*) was up-regulated. Notable reduction in biofilm formation was observed in M004 Δ*ecnI-1::Kan*^{r} as compared to wildtype that further showed that *ecnI*-1 was involved in biofilm development. Rapid plate assay for auxin production showed a halo reddish-pink formation indicated *E. cancerogenus* strain M004 produced auxin. This assay was repeated with M004 *ecnI*-1 mutant and reduction in reddish-pink halo formation was observed. These preliminary findings indicated that QS modulate auxin production in *E. cancerogenus* strain M004.

ABSTRAK

Penderiaan kuorum mengawal pertukaran ekspresi gen bakteria mengikut kepadatan populasi. Dalam kajian ini, sejumlah 13 pencilan bakteria ditemui menggambarkan aktiviti penderiaan kuorum dengan saringan awal yang menggunakan biosensor QS bakteria. Kemudian, 13 pencilan bakteria yang dikenal pasti tergolong dalam 7 genus berlainan (*Aeromonas*, *Cedecea*, *Dickeya*, *Enterobacter*, *Erwinia*, *Pantoea* dan *Pectobacterium*) dengan menggunakan MALDI-TOF jisim spektroskopi dan 16 rDNA berasaskan jujukan pengenalan melalui pangkalan data EzTaxon. Seterusnya, analisis mengenai pengeluaran molekul isyarat telah dijalankan dengan spektroskopi jisim selaras resolusi tinggi dan mendedahkan bahawa 11 daripada 13 pencilan bakteria menghasilkan molekul isyarat *N*-acylhomoserine lactone (AHL). Daripada 13 pencilan, *Enterobacter cancerogenus* strain M004 dipilih untuk analisis lanjut kerana baru sebagai QS bakteria. Dari analisis spektroskopi jisim selaras resolusi tinggi, terikan M004 telah ditemui untuk menghasilkan 3-oxo-C6 HSL dan 3-oxo-C8 HSL. Dari penjujukan genom *E. cancerogenus* M004, saiz genom draf adalah 5.67 Mbp. Dua gen penderiaan kuorum synthase ini dinamakan melalui kajian ini sebagai *ecnI*-1 dan *ecnI*-2 dan didapati terletak di contig 7 (633 bp) dan contig 2 (615 bp) masing-masing. Ia kemudian diikuti dengan pengesahan fungsi gen ini melalui pengklonan gen heterologous kepada *Escherichia coli* BL21(DE3)pLysS. Gen *ecnI*-2 didapati menjadi satu gen penderiaan kuorum synthase yang tidak berfungsi. Penemuan jugak menunjukkan bahawa *ecnI*-1 bertanggungjawab dalam pengeluaran kedua-dua *N*-3-oxo-acylhomoserine lactones dalam *E. cancerogenus* strain M004. Salah satu daripada anti-penderiaan kuorum kompaun yang dikenali, asid gallic telah digunakan dalam kajian transcriptomic ini untuk merawat *E. cancerogenus* strain M004 dan gen-gen yang berkaitan dengan penderiaan kuorum seperti *fliH* dan *betI* diexprestasi pengkamiran. Selain itu, cerakin biofilm yang dijalankan dengan *E. cancerogenus* strain M004 dirawat dengan asid gallic menunjukkan penurunan ketara

berbanding kawalan (tanpa asid gallic). Knock-out *ecnI*-1 mutan telah dibina menggunakan sistem λ merah melalui prinsip knock-out single-gen untuk mengkaji fungsi gen *ecnI*-1 berkaitan penderiaan kuorum. Mutan Δ*ecnI-1::Kan*^{r} kemudian disahkan menggunakan biosensors QS baktera dan spektroskopi jisim selaras resolusi tinggi; AHLs tidak dikesan daripada mutan. Perbezaan phenotypes diperhatikan rintangan antibiotik chlortetracycline, erythromycin dan lomeflaxin didapati hilang dengan ketara dalam mutant berbanding wildtype melalui teknologi OmniLog Phenotype Microarray (PM). Kajian transcriptomic juga dijalankan untuk menyelidik kesan knock-out gen *ecnI*-1 dan keputusan ketara diperhatikan berkaitan dengan biogenesis dinding sel/membran/sampul (*epsJ*) dalam keadaan down-regulated dan up-regulated pengangkutan dan metabolisme karbohidrate (*glpT*). Pengurangan ketara pada pembentukan biofilm diperhatikan dalam M004 Δ*ecnI-1::Kan*^{r}. dan mengenalpasti hubungan *ecnI*-1 dengan pembentukan biofilm *E. cancerogenus* strain M004. Cerakin plat pantas pengeluaran auxin dipakai dan pembentukan lingkaran kemerahan memberikan petunjuk awal yang *E. cancerogenus* strain M004 mampu mehasilkan auxin. Seterusnya, cerakin plat yang pantas telah diulang dengan M004 Δ*ecnI-1::Kan*^{r} mutant dan pengurangan ketara lingkaran kemerahan telah diperhatikan. Ini menunjukkan bahawa penderiaan kuorum memodulasi pengeluaran auxin oleh *E. cancerogenus* strain M004.

ACKNOWLEDGEMENTS

It is my honored to have many people that I am thankful for making this thesis possible.

I must offer my profoundest gratitude to my Ph.D. supervisor, Associate Professor Dr. Chan Kok-Gan. From accepting me to be a student supervised under him, to finding an appropriate subject for the research till completion of thesis writing, Dr. Chan offers his unreserved help and guidance for me to finish my entire research and thesis step-by-step. His enthusiasm, efforts and kind words always inspire me to achieve dreams and goals that I set for life. He not only taught me on research and thesis writing, but how to view the world from a new perspective. Without his kind and patient guidance, it is impossible for me to finish this thesis. I could not have imagined having a better supervisor and mentor for my Ph.D. study. Moreover, I would also like to express my gratitude to Dr. How Kah-Yan for guiding me throughout my research without showing any frustration. Her patience and sound advice are one of the contributing factors that I am able to finish this thesis. I would also like to extend my gratitude to my undergraduate supervisor, Dr. Winnie Yap for her insightful comments and constructive advice during my final year project and she is the mentor that built my research foundation.

I am indebted to my friends who are also my student colleagues for providing stimulating, motivating and fun environment in which to learn and grow. I am especially grateful to Nur Izzati Mohamad, Kumutha Priya and Tan Kian Hin that give me constant encouragement and motivation for me to complete my thesis. Without them, life seems to be less fun and exciting. Writing thesis is like running a long marathon. Thanks to three of them, I know I always have someone to turn to and I am not at all alone. Their spiritual support is indispensable to motivate me in completing this thesis. Special gratitude extends to Izzati, besides being my best friend; she is always willing to give her helping

hands despite her busy schedule. She is the best consultant that I can always pursue whenever I faced difficulties in research or real-life matters. A simple phrase “thank you”, would not justify how much the friendships mean to me. I wish to thank the secretaries and librarians in the Faculty of Science of University of Malaya, for helping me in many different ways. Puan Hapizah binti Nor Annis deserves special mention.

I also thank my brother, Tan Wen Keat for being so supportive and keep pushing me towards my goals. Deepest gratitude extends to my fiancé, Derrick Tee and his family for being so supportive along my Ph.D. journey. I am thankful for having him as my future spouse. He is always there supporting me emotionally and always cared for me especially during the time when I felt depressed. Lastly, and most importantly, I wish to thank my parents, Tan Soon Teck and Kang Hwa Chaen for supported me and loved me. They always tell me that all I need to do is to focus on my study and leave the rest to them. I am so lucky to be their daughter. To them, I dedicate this thesis.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percent
×	:	Times
× *g*	:	Gravity
°C	:	Degree Celsius
µg	:	Microgram
µL	:	Microlitre
µm		Micron
µM		Micromolar
3-hydroxy-C4-HSL		*N*-(3-hydroxybutanoyl)-L-homoserine lactone
3-hydroxy-C8-HSL		*N*-(3-hydroxyoctanoyl)-L-homoserine lactone
3-hydroxy-C10-HSL		*N*-(3-hydroxydecanoyl)-L-homoserine lactone
3-hydroxy-C12-HSL		*N*-(3-hydroxydodecanoyl)-L-homoserine lactone
3-oxo-C6-HSL		*N*-(3-oxo-hexanoyl)-L-homoserine lactone
3-oxo-C8-HSL		*N*-(3-oxo-octanoyl)-L-homoserine lactone
3-oxo-C10-HSL		*N*-(3-oxo-decanoyl)-L-homoserine lactone
3-oxo-C12-HSL		*N*-(3-oxo-dodecanoyl)-L-homoserine lactone
3-oxo-C14-HSL		*N*-(3-oxo-tetradecanoyl)-L-homoserine lactone
ACN		Acetonitrile
AGE		Agarose gel electrophoresis
AHL		*N*-acyl homoserine lactone
AI-2		Autoinducer-2
AIP		Autoinducer peptide
bp		Basepair
C4-HSL		*N*-butanoyl-L-homoserine lactone
C6-HSL		*N*-hexanoyl-L-homoserine lactone
C7-HSL		*N*-heptanoyl-L-homoserine lactone
C8-HSL		*N*-octanoyl-L-homoserine lactone
C10-HSL		*N*-decanoyl-L-homoserine lactone
C12-HSL		*N*-dodecanoyl-L-homoserine lactone
COG		Clusters of Orthologous Groups
dH_2O		Distilled water
dsDNA		Double-stranded deoxyribonucleic acid
DKP		Diketopiperazines
DNA		Deoxyribonucleic acid
dNTP		Deoxyribonucleotide triphosphate
DSF		Diffusible signal factor
EDTA		Ethylenediaminetetraacetic acid
g		Gram
HAQ		4-hydroxy-2-alkylquinolines
HMDS		Hexamethydisilazane
HHQ		2-heptyl-4-hydroxyquinolone
Hr		Hour
HS		High sensitivity

IAA	Indole-3-acetic-acid
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
Kb	Kilobase pair
L	Litre
LB	Luria-Bertani
LC-MS/MS	Triple quadrupole liquid chromatography mass spectrometry
M	Molarity
m/z	Mass to charge ration
mA	Miliampere
MEGA	Molecular Evolutionary Genetic Analysis
MIGS	Minimum Information about the Genome Sequence
min	Minute
mg	Milligram
mL	Mililitre
mm	Milimetre
mM	Milimolar
MOPS	3-(*N*-morpholino) propanesulfonic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
MTA	Methylthioadenosine
NA	Not applicable
NCBI	National Center for Biotechnology Information
n	Number
ng	Nanogram
nM	Nanomolar
PAME	Palmitate methyl ester
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PGAP	Prokaryotic Genome Annotation Pipeline
PGPB	Plant growth promoting bacteria
PM	Phenotypic Microarray
PQS	*Pseudomonas* quinolone signal
psi	Pounds per square rich
qRT-PCR	Quantitative real-time polymerase chain reaction
QQ	Quorum quenching
QS	Quorum sensing
QSI	Quorum sensing inhibitors
R2A	Reasoner's 2A
RAST	Rapid Annotation using Subsystem Technology
RNA	Ribonucleic acid
TLC	Thin-layer chromatography
s	Second
SAM	*S*-adenosyl-L-methionine
SEM	Scanning electron microscope
SOC	Super optimum broth

ssDNA	Single-stranded deoxyribonucleic acid
TBE	Tris-boric acid ethylenediaminetetraacetic acid
TOF	Time of flight
V	Voltage
VRE	Vancomycin resistance enterococci
v/v	Volume per volume
w/v	Weight per volume
X-gal	5-bromo-4-chloro-indoyl-galactopyranoside

LIST OF APPENDICES

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CHAPTER 1: INTRODUCTION

1.1 Research Background

Equatorial regions of west-central equatorial Africa, Central and South America and South and Southeast Asia through the New Guinea and the north-eastern coast of Australia (Bruenig & Geldenhuys, 1996) are rich in distribution of tropical rainforest. Malaysia has been declared as one of the most complex ecosystem tropical rainforests, with astounding biodiversity of species and community types of living organisms (D. M. Olson et al., 2001; Trier, 2008). Hence allowing tropical rainforest to represent the pinnacle of ecosystem development on earth due to its complex characteristics. Clearly, tropical rainforests are extremely rich in flora, fauna and microorganisms; each type of organism has its own niche. To date, microbial diversity is still the least well understood component of biodiversity (Ysabel & Kaspari, 2007). Bacteria are the most abundant microorganisms where most species are often found ubiquitous (Finlay & Clarke, 1999). Tropical rainforests serve as a reservoir for dispersal of microorganism and hence making it rich in microbial physiological activities (Finlay & Esteban, 2001). Microorganisms such as bacteria are diverse in their impacts such as in spreading of infectious diseases or play a valuable role in biotechnological purposes. Hence, it is interesting to gain a look upon the ways where bacteria regulates their daily processes in the environment.

Bacteria communicate with each other through extracellular signalling molecules or also known as autoinducers (AIs) that are produced, detected and show response (Withers, Swift, & Williams, 2001). This process is termed as quorum sensing (QS) which indicates that bacteria do communicate in order to perform various physiological activities (Fuqua, Winans, & Greenberg, 1996). QS enable bacteria to have the advantages that are unattainable as individual bacterial cell. Thus, study on QS will increase our understanding of bacterial QS-dependent activities and further in hindering or enhancing the bacterial activities by targeting their communication circuit.

1.2 Hypothesis

It is hypothesized that tropical fresh water is a reservoir for QS bacteria which may regulate novel phenotypic activities. Anti-QS compounds could be used to interfere bacterial QS activities which may have implications on controlling bacterial phenotypes.

1.3 Research Objectives

The objectives for this research are:

1. To screen bacteria from tropical fresh water that exhibit QS properties and further identify their signalling molecules
2. To understand the genetic makeup of genes related to quorum sensing using whole genome approach
3. To construct QS mutant among isolated QS bacterial strains

CHAPTER 2: LITERATURE REVIEW

2.1 Tropical Rainforest Waterfall

Water is essential to life and it is of fundamental importance to all living organisms. Water constitutes approximately 90% of the body weight in the human body (Vitousek, Mooney, Lubchenco, & Melillo, 1997). The availability of drinking water is an important ingredient for preventing endemic disease besides improving life quality. Waterfall serves as major attraction for recreational purposes and there are some "urban survival myths" where running water and water found in natural depressions are safe to drink (Hudson, 1998; Jakariya & Ahmed). In recreational waterfall, tourists may tend to fill their water bottles with the up-stream flowing water. Fast-moving or natural occurring water could allow waterborne disease carrying bacteria to thrive and could cause infections once consumed.

Sungai Tua waterfall as the selection in this research has yet to be documented with any microbiological research but is one of the favorite recreational attractions for tourists and locals (Tan et al., 2014). There are several microbiological research studies that provide a foundation vital to study the microbiology spread in water. For example, the findings of environmental chemoheterotrophic bacteria and *E. coli* in the falls of Northern Alabama and Northwestern Georgia (Campbell, Watson, Watson, Ball, & Pirkle, 2011). Next, psychrotolerant heterotrophic bacteria were discovered from the waterfall in Finnish Lapland (Mannisto & Haggblom, 2006) and presence of *Legionella* bacterium was detected in waterfall of New Zealand (Robert & David, 2007). The research suggests that fresh water features could serve as a potential reservoir for microorganisms and effective in the local spread of bacteria. Research into it can establish a roadmap in tracing

pathogenic bacteria besides understanding the beneficial bacteria distribution that enable plant growth improvement.

2.2 Tropical Aquatic Bacteria

Water act as a reservoir that contains different microorganisms and one of them is bacteria. In freshwater environment, microbial communities play the central roles and key components for the ecological cycles (Hayden & Beman, 2015). Bacteria can be present as free-living, associated with decaying material or attached to surfaces of rocks, stones, sand grains or aquatic animals as part of biofilm. Aquatic bacteria also display wide range of ability in their metabolism where they are both autotrophic and heterotrophic. Water serves as a vehicle for bacteria to move from one place to other. The greatest microbial risks would be ingestion of contaminated water by human or animal feces. The emerging pathogenic bacteria have recorded to be potentially spread through water. For example, *Aeromonas hydrophila* is well-known to be water-borne pathogen where it is publicly acknowledged as an opportunistic pathogen (Cabral, 2010; Daslakov, 2006). As reported by Cabral (2010), *Aeromonas* concentration is commonly found to be approximate 10^2 colony-forming units (CFU/mL). In water systems, their concentration could grow higher due to growth of biofilms. Bacteria could transmit diseases where living organisms ingest contaminated water or by bacterial contact with wound on skin. Another common water-borne bacterium is the *Klebsiella* which is also known as nosocomial pathogen. They often recovered from aquatic environments that associated with plant products, wastewater from industries and living trees (Grimont & Grimont, 2005). *Klebsiella* is a commensal pathogen in nasopharynx and in the intestinal tract of human body and could lead to fatal septicaemia. As waterfall are majorly the water source in tropical region, more researches should be dedicated to understand the ecology and behavior of aquatic bacteria in the waters (Martin et al., 2007).

2.3 Quorum Sensing (QS)

Bacteria are always recognized to exist as isolated and anti-social lives. Researches over the past decades had disclosed that bacteria are able to communicate via chemical signalling system in order to communicate within species had led to the realization that bacteria are able to behave in a much more complex patterns (Kaufmann et al., 2005). Specifically bacteria release signaling molecules, which are also known as autoinducers; then detect and respond to the accumulation of those molecules (More et al., 1996). Bacterial communication or coiled as "quorum sensing (QS)" by Fuqua and colleagues to describe the process where bacterial communication achieved depending on the density of bacterial cell population where it is synchronize with the concentration of signal molecules in the extracellular environment (Fuqua, et al., 1996; Swift, et al., 1993). In a simplest guise, QS benefits bacteria as a community rather than as an individual.

The paradigm shift of understanding the ability of bacteria in conducting complex patterns of co-operative behaviors had lead us into translating QS into four essential steps (P. Williams, 2007; Atkinson & Williams, 2009). First, bacterial cell population density and concentration of autoinducers in the external environment increase simultaneously. Once the bacteria sense the threshold, the signal will diffuse into bacterial cells and bind by receptor proteins which hence causing an activation of signal transduction cascade (Figure 2.1). The autoinducer-receptor protein complex will bind with targeted promoter to induce an auto-regulatory mechanism to either up-regulate or down-regulate certain bacterial phenotypes (Atkinson & Williams, 2009; P. Williams, 2007; Bassler & Losick, 2006).

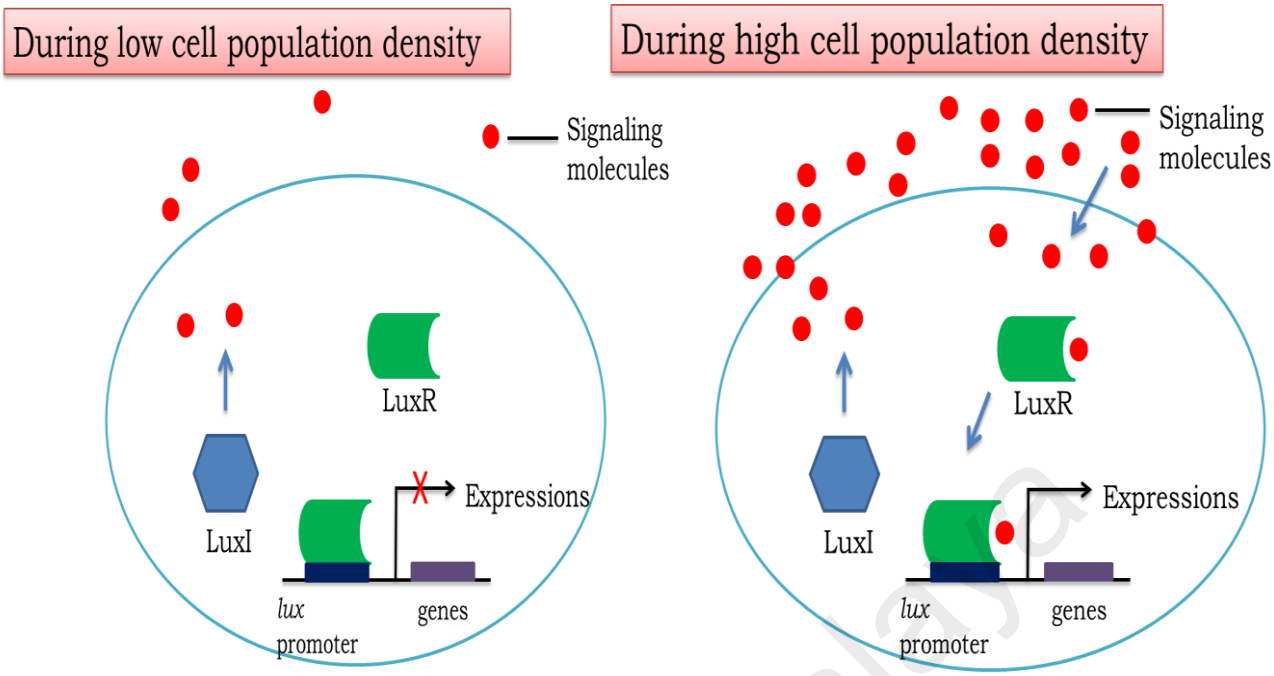


Figure 2.1: Two QS regulatory components in Gram-negative bacteria: LuxR (transcriptional activator protein) and LuxI (autoinducer synthase). Signal molecules accumulated in a cell-density-dependent manner until a threshold level is reached. No gene expression is driven at low bacterial cell density but at high bacterial cell density, gene expression will be activated.

The evolution of QS was originated from the discovery of controlling luminescence by *Vibrio fischeri*, a bacterium that forms a mutualistic light organ symbiosis with Hawaiian bobtail squid (*Eupryma scolope*) (Eberhard, 1972; Nealson, Patt, & Hastings, 1970; Ruby & Lee, 1998). The cell-population density influenced luminescence portrayed by *V. fischeri* have been convincing many scientists to pursue on QS research for the past decade in defining the details of quorum regulation in this bacterium. Experimental analysis on dramatic pattern of light production in the squid portray the findings that autoinducer played a role in induction of luciferase (Eberhard, 1972; Nealson et al., 1970; P. Williams, 2007). A minute amount of *V. fischeri* is harbored in the light organ of the squid during day time. As bacterial incubation hour increases, the production of signalling molecules at its concentration threshold triggered the luciferase expression. This bioluminescence was needed by the squid to counter eliminate its shadow and avoid predation at night. "Switching off" of luminescence occurred when the squid pumped out the bacteria pool from its light organ, hence bacterial population decreases discouraging the triggering of luciferase production due to insufficient signalling molecules production. The information emerged from *V. fisheri* system serves as a model for further discovery of quorum circuit in other species (Ruby & Lee, 1998).

Autoinduction of luminescence is now recognized as a QS model with wide applicability in applied research on gene regulation and host association of bacteria. QS indeed not only involved in luminescence but also regulates a vast array of phenotypes. QS facilitates bacteria for adaptability and survival where it also appears as bridge for interaction of several different bacterial species with eukaryotic hosts. However, the life-threatening ability phenotypes that are regulated by QS causes many concerns. Biofilm formation, production of virulence factors and antibiotic resistance in several notorious pathogens such as *Pseudomonas aeruginosa* (de Kievit, 2009), *Burkholderia cepacia*

(Suppiger, Schmid, Aguilar, Pessi, & Eberl, 2013), *Vibrio cholera* (Camara, Hardman, Williams, & Milton, 2002), *Streptococcus mutans (*Senadheera & Cvitkovitch, 2008), *Clostridium difficile* (Carter, Purdy, & Minton, 2005), and *Erwinia carotovora* (Andersson et al., 2000) were reported to be regulated by QS to attack different hosts ranging from human, plants and aquatic animals. Subsequently, the study into both QS systems and QS signal molecules are essential in various field from biotechnology, pharmaceutical and to agricultural industries, particularly targeting QS for establishment of novel antibacterial measures (P. Williams, 2007).

QS mechanisms have derived to three tracks; (i) *N*-acylhomoserine lactone (AHL)-based signalling system of Gram-negative bacteria (ii) oligopeptide-based system in Gram-positive bacteria and (iii) shared furanone-based system between Gram-negative and Gram-positive bacteria (Goh et al., 2014). QS signal molecules are structurally diverse and ranged according to the needs of the bacterium itself. There are various types of QS signalling molecules discovered and documented such as the AHL, 2-alkyl-4(1H)-quinolones (AHQs), autoinducer peptides (AIP), DSF, palmitate methyl ester (PAME) and diketopiperazines (DKP) but they did share similarity such as small (< 1000Da) organic molecules or peptides with 5-20 amino acids and are highly diffusible (Deng et al., 2014; Dong & Zhang, 2005; K. Kim et al., 2010; M. E. Olson et al., 2014; P. Williams, 2007). Of all the signalling molecules documented and reported, AHL received the utmost attention by most research institutions. AHL is basically a group of signalling molecules which employed by most Gram-negative bacteria (Bassler & Losick, 2006).

2.3.1 QS of Gram-negative Bacteria

Quorum size is sensed by Gram-negative bacteria through AHLs production that accumulates in their surroundings as the cell population increases. To date, there are more than 100 species of bacteria reported to portray QS properties are found ranging from marine, soil and freshwater environment to plants and animals. Their presence play roles involving pathogens, symbiont, extremophiles and plant-growth promoting bacteria that varies according to its environment (Joint, 2006). Many of these bacteria are able to produce multiple AHLs and contain more than one AHL synthases (Swift et al., 1993; P. Williams, 2007). Some of the examples of AHL-dependent QS systems with the phenotypes controlled are summarized in Table 1. However, there are still an astronomical numbers of bacteria that are yet to be discovered and characterized whether they do communicate inter- or intra- species. Many researches should be carried on to understanding QS deeply, it will be the foundation for various areas in biotechnology, pharmaceutical and agricultural industries in such that QS is particularly the target of interest in antimicrobial purposes.

Table 2.1: Some examples of phenotypes controlled in AHLs-dependent QS systems in Gram-negative bacteria.

Microorganisms	Major AHL(s)	Phenotypes	References
Acinetobacter baumannii	3-hydroxy-C12-HSL	Biofilm, virulence	(Niu, Clemmer, Bonomo, & Rather, 2009)
Aeromonas hydrophila	C4-HSL	Biofilms, exoproteases, motility, virulence	(Jahid, Lee, & Ha, 2013)
Aeromonas salmonicida	C4-HSL	Extracellular protease	(Simon Swift et al., 1997)
Agrobacterium tumefaciens	3-oxo-C8-HSL	Plasmid conjugation	(C. Wang, Yan, Fuqua, & Zhang, 2014)
Burkholderia cepacia	C6-HSL, C8-HSL	Biofilms, virulence	(Riedel et al., 2001)
Burkholderia glumae	C8-HSL	Protein secretion, oxalate production, swarming, virulence	(Nickzad, Lepine, & Deziel, 2015)
Burkholderia pseudomallei	C8-HSL, C10-HSL, 3-hydroxy-C8-HSL, 3-hydroxy-C10-HSL, 3-oxo-C14-HSL	Virulence, exoproteases	(Ulrich et al., 2004)
Chromobacterium violaceum	C6-HSL	Exoenzymes, cyanide, pigment	(McClean et al., 1997)

Table 2.1: continued.

Microorganisms	Major AHL(s)	Phenotypes	References
Enterobacter agglomerans	3-oxo-C6-HSL	Pectinase expression	(Chalupowicz, Barash, Panijel, Sessa, & Manulis-Sasson, 2009)
Erwinia carotovora	3-oxo-C6-HSL	Biofilm, virulence	(Joe, Benson, Saravanan, & Sa, 2015)
Nitrosomonas europaea	3-oxo-C6-HSL	Emergence from lag phase	(Burton, Read, Pellitteri, & Hickey, 2005)
Pantoea stewartii	3-oxo-C6-HSL	Exopolysaccharides	(Chug, Khosla, & Singh, 2015)
Pseudomonas aeruginosa	C4-HSL, C6-HSL, 3-oxo-C12-HSL	Biofilms, exoenzymes, exotoxins, swarming, virulence	(Jakobsen, Bjarnsholt, Jensen, Givskov, & Hoiby, 2014; Jimenez et al., 2012; P. Williams, 2007)
Rhizobium leguminosarum	C6-HSL, C8-HSL	Rhizome interaction	(Lithgow et al., 2000)
Serratia marcescens	3-oxo-C6-HSL, C6-HSL, C7-HSL, C8-HSL	Biofilm formation, sliding motility and prodigiosin produciton	(Horng et al., 2002; Rice et al., 2005)
Vibrio fischeri	3-oxo-C6-HSL	Bioluminescence	(Ruby & Lee, 1998)
Vibrio harveyi	3-hydroxy-C4-HSL	Bioluminescence, virulence	(Cao & Meighen, 1989; Manefield, Harris, Rice, de Nys, & Kjelleberg, 2000)
Yersinia pseudotuberculosis	3-oxo-C6-HSL	Motility and clumping	(Atkinson et al., 2008)

2.3.1.1 AHL as Signalling Molecules

AHL signalling is obviously to be highly conserved among the Proteobacteria and received the absolute attention in which intensive studies had been carried out (Chan, Cheng, Chen, Yin, & Ngeow, 2014). A homoserine lactone ring with its β- and γ-positions remained unsubstituted but its α-position is *N*-acylated with a fatty acid chain; this ring is highly conserved in all the AHLs documented and characterized (Figure 2.2) (Chan et al., 2014). There are several structural differences that influence the characteristics of an AHL molecule which are (i) acyl side chain range commonly from 4 to 18 carbons where AHL usually carries an even number carbon chain (ii) reduction or oxidation carbonyl or presence of hydroxyl group at third carbon of the acyl side chain (iii) there is also possible for presence of unsaturated AHLs where double bond occurred in the 5 and 7 positions of a long acyl chain (12-14 carbons) (Churchill, Sibhatu, & Uhlson, 2011; P. Williams, 2007). The minimum acyl chain to be function as signal molecule is 4 carbons as in existence with lactone ring itself will be hydrolysed at pHs above 2 where 70% of *N*-propionyl-homoserine lactone is hydrolysed at pH 6 (Churchill et al., 2011). The shortest naturally occurring AHLs were found to be produced by several Gram-negative bacteria such as *Vibrio harveyi*, *Pseudomonas aeruginosa* and *Aeromonas hydrophila*. AHL molecules also found to interact with some other molecules such as cysteine, biotin and fluorescence that bring an effect on the binding affinity of modified AHL to its native AHL-receptor (Fuqua & Greenberg, 2002). Besides that, alkali-driven rearrangement reaction can occur in 3-oxo-AHLs that lead to formation of corresponding tetramic acids, iron chelating compounds and antibacterial activities. In short, differences in substitution at predetermined sites on the AHL molecule confer its specificity and affect the functions to the cells.

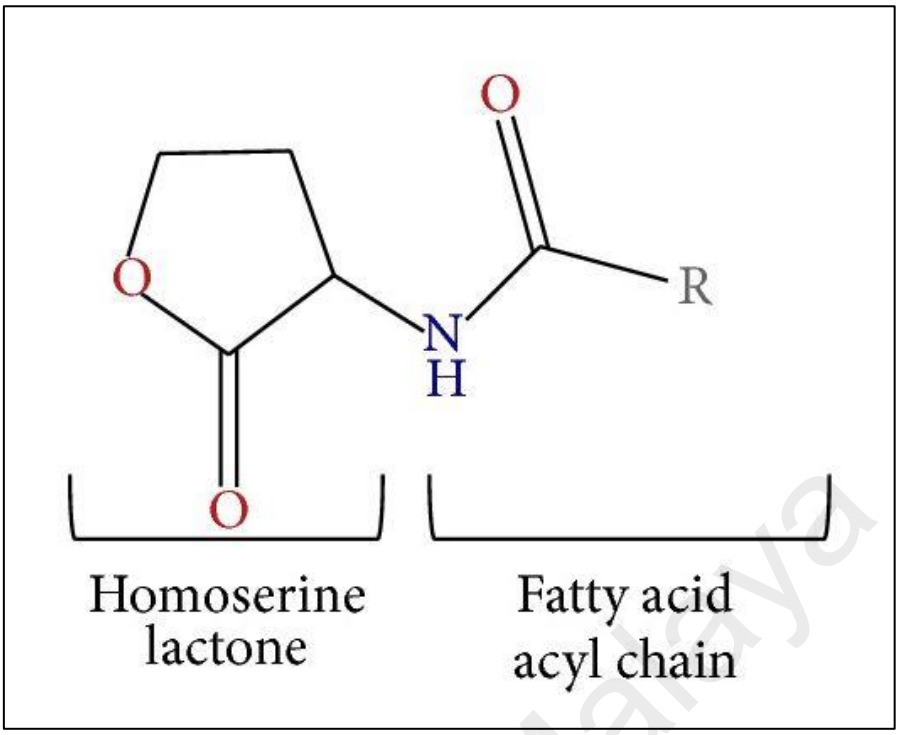


Figure 2.2: General structure of *N*-acyl homoserine lactone (AHL). R represents the fatty acid acyl side chains (Chan et al., 2014).

AHL diffusion and transportation through membrane is highly related with its structural features. AHLs tagged as amphipathic molecules, are wonted to diffuse freely from the internal cell environment to the external cell environment, and vice versa, demonstrated with *V. fischeri* and *E. coli* using a ^{3}H-labelled derivative (Kaplan & Greenberg, 1985). However, this demonstration only proved using a short chain AHL, 3-oxo-C6-HSL. The hydrophobic characteristic is influence by the acyl side chain length, the number of in-saturations and the nature of the C3 substituent (H, O or OH). On the other hand, rate of AHL diffusion is correlated with the nature of the acyl chain and if long acyl chain AHLs can diffuse through cell membrane, it would diffuse slower as compared with short acyl chain AHLs (Boyer & Wisniewski-Dye, 2009). Commonly, short acyl side chain are usually those AHL with acyl side chain lesser than C8 can passive diffused in and out the bacterial cell but for to aid in facilitate the transport of long acyl side chain AHL, an active transport mechanism is needed. The presence of an active

efflux of 3-oxo-C12-HSL in P. aeruginosa was evidenced, whereas the short-chain C4-HSL freely diffuses across the cell membrane (Evans et al., 1998; Pearson, Feldman, Iglewski, & Prince, 2000). Another active efflux is evidenced in *Burkholderia pseudomallei*.

2.3.1.2 Gram-negative QS Circuits

The QS system in Gram-negative bacteria consists of signalling molecules (autoinducers), autoinducer synthase (LuxI), Lux-R type regulators and target genes (Myszka & Czaczyk, 2012). The signaling circuits composed of LuxI/LuxR appears to be the standard communication mechanism in Gram-negative bacteria, where QS system resembling the canonical *V. fischeri* circuit have been shown to be the model system in controlling gene expression in over 100 species of Gram-negative bacteria (Schaeffer, Val, Hanzelka, Cronan, & Greenberg, 1996). Commonly, the acylated HSL synthesized by the responsible enzyme LuxI-like protein; a cognate LuxR-like protein will then recognize the HSL autoinducer and activation of transcription downstream target genes occurred subsequently (Schauder & Bassler, 2001). The mode of action of the LuxI/LuxR pairs is highly conserved across all cases. The coupling of acyl-side chain of a specific acyl-acyl carrier protein (acyl-ACP) from the fatty acid biosynthetic machinery with the homocysteine moiety of *S*-adenosylmethionine (SAM) is produced by LuxI homologue. The coupling process forms a ligated intermediate which then convert to form acyl-HSL and methylthioadenosine (MTA). The LuxR homologue, on the other hand function by binding their substrate, autoinducer and activating the transcription of targeted DNA. The LuxR homologue consists of an amino-terminal region that binds to autoinducers and the C-terminal domains responsible for oligomerization and promoter DNA binding (Choi & Greenberg, 1991; Schauder & Bassler, 2001; Slock, VanRiet, Kolibachuk, & Greenberg 1990).

Rather delicate signalling specificity exists in LuxI/LuxR type circuits and specificity inherent stems from a high selectivity of the LuxR proteins to its signalling molecules. As evolutionary goes by, more regulatory complexity has been added to the basic backbone of QS circuit, such as the use of multiple AHL autoinducers and LuxR proteins that can act either parallel or in series (Ng & Bassler, 2009). This can be seen in the plant phytopathogen, the gene regulation of *Ralstonia solaacearum* LuxI/LuxR like autoinduction system (SolI/SolR) are regulated by PhcA and also RpoS (Flavier, Ganova-Raeva, & Denny, 1997). Next, the opportunistic pathogen *P. aeruginosa* employ two pairs of LuxI/LuxR homologues (LasI/LasR, RhlI/RhlR) and function in tandem to control the virulence factors production (Pearson, Pesci, & Iglewski, 1997). Recently QscR, identified as third LuxR homologue was found from the complete genome sequence of *P. aeruginosa*. However, there are yet indication of cognate LuxI homologue that could be responsible in producing the autoinducer in which QscR can respond (Chugani & Greenberg, 2014). In fact, level of communication complexity layered to the LuxI/LuxR backbone circuit highly dependent on the nature of bacteria. Apparently the complex interconnected network could serve for precise timing of the expression of various QS controlled phenotypes (Schauder & Bassler, 2001). QS has been potentially responsible in managing various bacterial physiological activities such as expression of virulence factors, biofilm formation and swarming. Hence, by aiming bacterial communication circuit would be a novel way either in interfering its virulence or to enhance them for biotechnological purposes.

2.3.1.3 Employment of AHL Biosensors

Discovery of vast diverse AHL QS system has been rendered possible by adopting bacterial biosensors capable in sensing the AHLs production in a rapid manner. The bacterial biosensors contain defective LuxI protein which led them in disability in producing their own AHLs (Steindler & Venturi, 2007). However, these biosensors carry a functional LuxR-family protein cloned with a cognate target promoter that up-regulates the transcription of reporter genes that exhibits phenotypes such as green-fluorescent protein, bioluminescence and purple violacein pigmentation. Specificity in sensing exogenous AHL by bacterial biosensors strongly relies on the LuxR family protein and hence it is essential to carry out the detection with several biosensors.

There are several biosensors available in detecting short and medium acyl chain AHLs (acyl chain range within C4 to C8 in length). *Chromobacterium violaceum* (CV026), a Gram-negative water and soil bacterium is commonly employed to serve this type of detection (McClean et al., 1997). CV026 was developed after mini-Tn5 transposons insertion into *cviI* AHL synthase gene while the ability to induce purple pigmentation via *cviR* was retained. This mini-Tn5 mutant forms white colony and will only be able to turn purple in the presence of exogenous AHLs. CV026 is incapable to detect any AHLs with acyl chains of C10 or longer and all 3-hydroxy-AHLs. Several biosensors available in detecting short and medium acyl chain AHLs rely on a plasmid construct harboring *luxCDABE* operon and the host *E. coli* was commonly used for the cloning of plasmids because *E. coli* is not able to produce any AHLs. Genetically modified *E. coli* carrying AHL sensors plasmids; pSB401, pSB536 and pAL101 containing fusion of *luxRI'::luxCDABE, ahyRI'::luxCDABE, rhlRI'::luxCDABE* respectively are able to exert bioluminescence in presence of AHL molecules (Winson et al., 1996).

Other than short and medium length acyl chain AHLs, there are also some biosensors available in detecting long acyl chain AHLs (length of C10 and above). One of the biosensors is genetically modified *E. coli* harboring pSB1075 and pKDT17 both containing same fusion of *lasRI'::luxCDABE* but pSB1075 luminesce (Winson et al., 1996) while pKDT17 responds through standard β-galactosidase activity upon exposure of exogenous AHLs (Pearson et al., 1994). On the other hand, the *P. aeruginosa* PAO1 M71LZ could be utilized in detecting particularly C12 3-oxo-HSL and this biosensor is a *lasI* genomic knock-out mutant under control of *rsaL* promoter with transcriptional fusion of *lasR::lacZ* (Dong, Zhang, Soo, Greenberg, & Zhang, 2005).

In order to detect AHLs with 3-hydroxyl group attached, a bacterial biosensor known as *P. fluorescens* 2-79 could be employed. Strain 2-79 employed genetically linked PhzI/R QS system that regulates expression of *phzABCDEFG* operon (S. R. Khan et al., 2005). The strain 2-79 biosensor basically was developed from wildtype *P. fluorescens* 1855 that harbors two plasmid system; (i) pSF105 carrying *phzR* gene regulates by *trc* promoter (ii) pSF107 harbors *phzR-phzA* divergent that regulates by dual promoter region and fuse with two different reporters, *uidA* and *lacZ*. The sensing of exogenous AHLs could be easily detected via β-glucuronidase and β-galactosidase activity.

There are also some other biosensors developed to detect broad range of AHLs such as *Agrobacterium tumefaciens* WCF47, biosensors to detect uncommon AHLs, such as SinI/R-based biosensors to detect any AHLs with longer than 12 carbon length acyl chain, and there is also biosensors with gene encoding for the green fluorescent protein allowing detection of AHLs at single-cell level (Steindler & Venturi, 2007). Although a negative results usually indicates that the no AHLs are produced by the tested bacterial strain, but this could be due to the biosensors used could not detect novel AHLs or the AHLs

produced are in low concentration and below a threshold that biosensors could barely detect (Steindler & Venturi, 2007). Hence other approaches such as thin layer chromatography (TLC) or high resolution tandem-mass spectrophotometry could be used in detecting the AHLs.

2.3.2 Other Signalling Molecules

Besides AHLs as QS signalling molecules, there is presence of other signalling molecules being reported. Some of the well-documented intercellular signalling molecules are the members of a family of quinolone compounds termed 4-hydroxy-2-alkylquinolines (HAQs) (Sifri, 2008). Transcriptional regulator MvfR controls the synthesis of HAQs, which leads to modulation of several genes expression in the production of anthranilic acid and its conversion to 4-hydroxy-2-heptylquinoline (HHQ). The molecule 3,4-dihydroxy-2-heptylquinoline or known as *Pseudomonas* quinolone signal (PQS) will be produced from conversion of HHQ via PqsH action. However, the production of MvfR and PqsH are tightly control by LasR to intertwine with AHL-based pathway. The signalling of PQS is incorporated in the AHL QS pathway that is governed by Las and Rhl systems and known to be upregulated in cystic fibrosis patients during lung infections (J. Lee & Zhang, 2015).

Plant pathogen *Xanthomonas campestris* and *X. fastidiosa* utilize new type of communication language in regulating their virulence factors (Deng, Wu, & Zhang, 2011). The signalling molecule is known as diffusible signalling factor (DSF) which was later identified as unsaturated fatty acid, cis-11-methyl-2-dodecenoic acid. Three major QS components are needed in this QS pathway: RpfF, RpfC and RpfG where they are involve in catalyzes, perception and transduction of the signalling molecules. The DSF-based QS mechanisms have been expanding and found to be utilized in other microorganisms such

as *Xylella fastidiosa* (Ionescu et al., 2013), *Stenotrophomonas maltophila* (Ryan, An, Allan, McCarthy, & Dow, 2015) and *Burkholderia cepacia* (Ryan et al., 2015). Gram-negative bacteria are also capable in producing other types of signaling molecules; the iron mediated oxetane ring containing bradyoxetin namely 2-4-[[4-(3-aminooxetan2-yl)phenyl](imino)methyl]phenyl oxetane-3ylamine and 3-hydroxypalmitic acid methyl ester (3-OH PAME) by *Bradyrhizobium japonicum* (Bogino, Nievas, & Giordano, 2015) and *Ralstonia solanacearum* (Kai et al., 2015). Both these signalling molecules are involved in protruding symbiotic relationship with higher organism, the plant.

While majority Gram-negative bacteria uses AHLs as autoinducers, Gram-positive bacteria use post-translationally modified autoinducing peptide (AIP) molecules for QS (Ng & Bassler, 2009). The AIP is secreted through an ATP Binding cassette (ABC) transporter protein (Ng & Bassler, 2009). Gram-positive bacteria employ the two-component QS systems for AIP detection where it involved a membrane-bound histidine kinase receptor and a cognate cytoplasmic response regulator. The two component system is regulated by a series of auto-phosphorylation cascade. Similar with the AHL-based QS, the concentration of secreted AIP increases parallel with increasing cell density. Several peptide-based QS systems include the AgrC/AgrA system of *Staphylococcus aureus* in regulating virulence (Queck et al., 2008), the ComD/ComE system of *Streptococcus pneumoniae* in controlling bacterial competence (Pestova, Håvarstein, & Morrison, 1996), the ComP/ComA system of *Bacillus subtilis* in regulating DNA uptake and sporulation (Nakano, Xia, & Zuber, 1991) and FsrB/FsrD system of *Enterococcus faecilis* for conjugation (Del Papa & Perego, 2011).

2.3.3 QS and Bacterial Physiology

In the natural environment, bacteria utilizes the ability to sense cell population density in order to regulate essential phenotypes such as bioluminescence, biofilm formation, virulence factor production, swarming motility, chemotaxis, toxin secretion and antibiotic resistance (Decho, Norman, & Visscher, 2010). These QS-regulated phenotypes are essential for bacteria in order to perform successful establishment of symbiotic (beneficial or pathogenic) relationship with higher organisms. Same AHL could be produced by a vast diverse of bacterial where this molecule could be involved in controlling different phenotypes which is strain-dependent (Decho et al., 2010; Galloway, Hodgkinson, Bowden, Welch, & Spring, 2010).

2.3.3.1 Development of Biofilm

Attachment to surfaces usually the first step for bacteria before forming communities (known as biofilm) that enmeshed in a self-produced polymeric matrix (Nadell, Xavier, Levin, & Foster, 2008; Parsek & Greenberg, 2000). Biofilm formation is one of the known phenotypes to be closely related with QS. The development of biofilm *in vitro* involves five stages where first the reversible attachment of bacterial cells to surface turned into irreversible attachment mediated by production of exopolymeric material (Annous, Fratamico, & Smith, 2009; Parsek & Greenberg, 2005). Fibrinogen- and fibronectin-binding proteins are usually found to play a role in the attachment process. Next, microcolonies are formed and this indicates the beginning of biofilm maturation. The mature biofilms engineered varies; from flat, homogenous biofilms to highly structured 3-dimensional biofilms. The matured biofilm contains cells that are packed in clusters with channels in between to allow water and nutrient transportation and waste removal. The architecture of matured biofilm often influence by motility, rhamnolipid production and the production of extracellular polymeric substance matrix. AHL based

QS have shown to influence the biofilm formation at maturation stage. In the study done by Labbate and colleagues (2004) proven that a mutation in *S. liquefaciens* acyl-synthase gene, *swrI* resulted in thin biofilms that lacked aggregates and filaments as compared to its wildtype's biofilm which is heterogenous that consist of aggregation of long filaments of cells (Labbate et al., 2004). This is further substantiated by work in *Burkholderia cepacia* H111 with mutations in either *cepI* or *cepR* (Huber et al., 2001). Both mutants showed defective in biofilm maturation and were only arrested at the microcolony stage of growth as compared to the robust biofilms covered with attachment surface formed by the wildtype. Besides the AHL-dependent pathway, maturation of biofilm is also influenced by the LuxS-based QS (Merritt, Qi, Goodman, Anderson, & Shi, 2003; Yoshida, Ansai, Takehara, & Kuramitsu, 2005). In *Streptococcus mutans*, mutation in *luxS* resulted in mature biofilm with decreased biomass as compared with its wildtype. The final stage of biofilm involved aggregation and detachment, dissolution or dispersal of cells from the biofilm to initiate a new biofilm formation. The dispersed cells showed similarity with planktonic cells which is non-adherent. This dispersal process allows bacteria to colonize new surfaces and spread its virulence effectively within a closed environment. This final stage of biofilm formation, the cell dispersal was also found to be QS controlled. In *Rhodobacter sphaeroides*, mutation in its AHL synthase resulted in hyper-aggregation of cells; but role of QS in this bacteria is still remain unknown (Puskas, Greenberg, Kaplan, & Schaefer, 1997). Other than that, *yspR* mutant of *Yersinia pseudotuberculosis* resulted in increased swimming motility (Parsek & Greenberg, 2005).

The complex formation of biofilm provide a "room" with hydrated matrix of microbially produced proteins, nucleic acids and polysaccharides that allows the cells to act less as individual entities but more as a collective living systems (Annous et al., 2009). Biofilm shields the bacteria by significantly increased in resistant to environmental

stresses (pH fluctuation, high salt, and nutrient fluctuation) or microbially deleterious particles (antibiotics and biocides). The interesting point arise is the criteria in determining the role of QS that plays in biofilm formation (Parsek & Greenberg, 2005). Perhaps it is not surprising that QS indeed play a major role in biofilm formation evident by increasing study of mutant construction studies that produce pleiotropic phenotypes that affect motility, surface attachment expression or chemistry of cell surface which is later translated into biofilm formation. However, it would be best if the role of QS could be evaluated by monitoring the signalling process in situ in a developing biofilm in the parental strain and determine if the onset of QS corresponds to any observable transition in bacterial biofilm development that relates with other phenotypes such as incline of antimicrobial tolerance.

2.3.3.2 Production of Plant-growth Promoting Elements

One of the interesting fact regarding QS is eukaryotes are able to recognize bacterial QS molecules and cross-kingdom interaction alters the physiological adaptation in colonized eukaryotes that modify their defense system, immune responses, hormonal responses or growth responses (A. Hartmann & Schikora, 2012). Besides creating pathogenic relationship with higher organisms, the fairly interesting interaction is signalling molecules (AHLs); reported to mediate root growth through biosynthesis of phytohormones. Indole-3-acetic acid (IAA), or known as auxin is a crucial phytohormone that enhance different developmental processes in plants; in which IAA production is widely spread among plant associated bacteria and they are able to play critical role in promoting plants' growth and development especially root elongation (Ali, Sabri, Ljung, & Hasnain, 2009; Spaepen, Vanderleyden, & Remans, 2007). Plant growth promoting bacteria (PGPB) receive extensive studies as potential bio-fertilizers due to increasing pollution by over-usage of chemical fertilizers (Tu, Zheng, & Chen, 2000). Biosynthesis

of IAA by microbial strain is consider as one of the basic criterions to be selected as efficient PGPB. To date, there are increasing number of reports stating that QS facilitate the PGPB in enhancing plant growth. As previously reported, treatment of *Arabidopsis thaliana* roots with 1 – 10 µM of C4- and C6-HSL increased the ratio of IAA/cytosine that led to promoted root growth (von Rad et al., 2008). In their study, they found out that the introduction of C6-HSL did not induced the systemic resistance and priming effect of *A. thaliana*. They further stated that short chain AHLs might play better role in promoting plant growth due to the hydrophobicity of long chain AHLs. This fact is substantiated by the study that revealed that C6-HSL was transported to the leaves of yam beans and barley leaves but not the C10-HSL (Götz et al., 2007). Various studies also showed that *Rhizobium* mutants that was unable to produce AHLs were unable to nodulate legume plants as compared to the wildtype strain (Daniels et al., 2002; Rosemeyer, Michiels, Verreth, & Vanderleyden, 1998; Zheng et al., 2006). These also further support the idea that AHLs could be participating in beneficial plant-bacteria interactions. Poonguzhali and colleagues also proven that the AHL-producing *Burkholderia* spp. strains CBMB40, CBPB-HOD and CBPB-HIM stimulated root elongation in canola via seed bacterization (Poonguzhali, Madhaiyan, & Sa, 2006). They also further hypothesized that signalling molecules by PGPB *in planta* could play a substantial role in enhancing the plants' pathogenic resistance. In the model legume *Medicago truncatula*, significant changes in the protein accumulation in axenically grown roots were found by responding to 3-oxo-C12-HSL and 3-oxo-C16-HSL; in which the proteins were found to involve in plant hormone responses, metabolic processes and host defense (Mathesius et al., 2003). These researches had paved the potential of QS-dependent PGPB. Although not fully understood, molecular communication in the rhizosphere indeed plays an important in regulated beneficial plant-bacteria interaction. Thus far, the study of QS could lead us into

different dimension in searching the potential of QS bacteria to contribute in a beneficial way.

2.4 Quorum Sensing Inhibitors (QSIs)

The pathogenesis portray by bacteria is a multi-factorial process that is regulated by production of virulence factors, which causes variation of bacterial infectious diseases (Joint, Downie, & Williams, 2007). The dedication of antibiotics in the early 20th century initiated a new era into treating microbial infections and they were the most rewarding drug that save myriad lives (Ivanova, Fernandes, & Tzanov, 2013). However, usage of antibiotics over a long period causing a substantial evolutionary stress on bacterial population and emergence of multi-drug resistance. Methicillin-resistant *Staphylococcus aureus* (MRSA) (DeLeo & Chambers, 2009), vancomycin resistance enterococci (VRE) (Monroe & Polk, 2000) and multi-drug resistance *Mycobacterium tuberculosis* (Komolafe, 2004) are some dangerous bacterial species that have emerged due to over usage of antibiotic. Emergence of multi-drug resistant bacteria caught particular attention and approaches are now taken to look into alternative antimicrobials. In recent years, research into QS linking to bacterial pathogenicity had been evidently strong because virulence is greatly reduced in mutants that are defective in QS (Joint et al., 2007). Therefore, by targeting QS pathway could be a new way into attenuating pathogenicity.

Halogenated furanone compounds or known as fimbrolides are intensively studied as group of QS inhibitors (Ivanova, Fernandes, & Tzanov, 2013). They are isolated from red microalga *D. pulchra*, an alga which is able to produce secondary metabolites that are made up of more than 30 types of furanones. Previous studies had shown that these secondary metabolites could interfere the AHL-based QS communication circuit. Study by Janseens and colleagues (2008) showed that brominated furanones have potential in

preventing biofilm formation of *Salmonella* serovar *Typhimurium* at a non-growth inhibiting concentrations (Janssens et al., 2008). Brominated furanones also found to meddle with biofilm formation of several other bacterial species, *E. coli*, *B. subtilis*, *P. aeruginosa* and *Streptococcus spp*. (Janssens et al., 2008). Study by Givskov and colleagues (1996) evident that 100 µg/mL of furanone extracted from *D. pulchra* could inhibit swarming abilities of *Serratia liquefaciens* (Givskov et al., 1996). Another study also showed that furanone is capable in inhibiting bioluminescence of *Vibrio harveyi* strain JMH597 at concentration of 100 mg/L (Defoirdt et al., 2006). However, drawbacks of halogenated furanones are too reactive and could cause toxicity towards human cells.

Thus, researches exert into finding potential quorum sensing inhibitors (QSI) from various natural sources and it has been proposed that a potential QSI should fulfill certain criteria (V. C. Kalia, 2013); (i) small molecule with high efficiency in reducing QS regulated genes (ii) high degree of specificity with no adverse effect (iii) chemically stable and resist to host metabolic system (iv) longer than AHLs to prevent bacteria resistance (v) do not affect the host microbiome (vi) show no toxicity effects towards host. To date, there are a large number of natural occurring QSI well established and are grouped into various categories. There are quorum quenching (QQ) enzymes that is produced by prokaryotes and from animal sources. One of the QQ enzymes is AHL-acylase that cleaves acyl side chain. Acylase produces by *Streptomyces* sp. is similar with acylase I produces by porcine kidney where both cleaves the acyl chain longer than 6 carbons (Park et al., 2005; F. Xu, Byun, Dussen, & Duke, 2003). Some other QQ enzymes are AHL lactonases by *Bacillus* spp. (Thomas, Stone, Costello, Tierney, & Fast, 2005) and mammalian paraoxonases (Romero, Acuña, & Otero, 2012) that function in hydrolyzing the AHL lactone ring. However, focuses have been placed on exploring potential QSIs from plant extract because the plant source are safe for human

consumption and the active compounds extracted should be regarded as safe and cause no toxicity towards human cells (Table 2). Still, toxicology study should be performed on the extracted compounds. These compounds are known as secondary metabolites (or phytochemicals) and many classes of these phytochemicals demonstrated their potential as antimicrobials or synergists of other products (Koh et al., 2013). Recent researches have promoted the potential of these phytochemicals as potential QSI.

One of the QSI is phenolic products or polyphenols which constitute one of the most abundant and omnipresent as plant secondary metabolites (phytochemicals) (Nazzaro, Fratianni, & Coppola, 2013). Phenolics are considered as potential QSI because it could be used to treat ailments such as diabetes, cancer or inflammatory diseases besides having antimicrobial properties. Jagani and colleagues (2009) proved that natural occurring phenolics could act against biofouling of *P. aeruginosa* (Jagani, Chelikani, & Kim, 2009*)*. Another study by Vandeputte and colleague (2010) showed that catechin extracted from *Combretam albiflorum* reduces elastase, pyocyanin and biofilm formation in *P. aeruginosa* PAO1 (Vandeputte et al., 2010). They selected 8 types of phenolics, anarcadic acid, polyanarcadic acid, salicyclic acid, polysalicyclic acid, polyphenol, catechin, epigallocatechin and tannic acid; all 8 compounds showed significant reduction towards *P. aeruginosa* biofilm formation. Flavonoids extracted from citrus species such as quercetin and naringenin were found to hinder the biofilm formation of *E. coli* O157:H7 and *V. harveyi* BB120 (J.-H. Lee et al., 2011; Vikram, Jayaprakasha, Jesudhasan, Pillai, & Patil, 2010). Another subclass of phenolics, furocoumarins show QSI abilities in which purified furocoumarins – dihydroxybergamottin and berggamottin inhibits autoinducer activities in *V. harveyi* (Rasamiravaka, Labtani, Duez, & El Jaziri, 2015). Girennavar and colleagues (2008) further substantiated that furocoumarins from grapefruit juice inhibited more than 95% of autoinducer-1 and autoinducer-2 activities in *V. harveyi* (Girennavar et al., 2008).

Other than that, ferulic acid and gallic acid (grouped under subclass of phenolic acids) were also found to block bacterial motility, adhesion and biofilm formation of *E. coli*, *P. aeruginosa*, *S. aureus*, and *Listeria monocytogens* (Wojdyło, Oszmiański, & Czemerys, 2007). Study by Plyuta and colleagues (2013) showed that the usage of 200 µg/mL of gallic acid could reduce the biofilm formation of *P. aeruginosa* PAO1 to 30% (Plyuta et al., 2013). Gallic acid also proven to be a potential QSI where in the concentration of 1mM, 80% reduction of biofilm formation by *Eikenella corrodens* was observed (Matsunaga et al., 2010). Application of ferulic acid at a concentration lower than 8 µg/mL was found to forbid the biofilm formation of *S. aureus* (Borges, Saavedra, & Simões, 2012).

There are also some other groups of phytochemicals such as isothiocyanates and essential oils that serve as potential QSI. Isothiocyanates are products formed during glucosinolate hydrolysis and it is considered the most important biological active product in plant (Fenwick, Heaney, Mullin, & VanEtten, 1983). One of the aliphatic isothiocyanates, allylisothiocyanate interfered the adhesion-related genes in *S. aureus* in a work done by Lee and colleagues (2012) (H.-Y. Lee, Zou, & Ahn, 2013). This compounds was also demonstrated to reduce the *Pseudomonas sp.* planktonic cell growth and number of cells adhered to the *Brassica nigra*. Essential oils, on the other hand is also proven to be potential QSIs where they are complex mixtures of volatile compounds that can be synthesized from several plant organs (Sadekuzzaman, Yang, Mizan, & Ha, 2015). The QS activities of *P. aeruginosa*, *Proteus mirabilis* and *S. marcescens* – swarming, production of extracellular polymeric substances and biofilm formation were inhibited upon exposing to methanolic extracts of *Cuminum cynimum*, where one of the component is methyl eugenol, an essential oil with an aromatic ring (Packiavathy, Agilandeswari, Musthafa, Pandian, & Ravi, 2012). The plant-based QSIs will not kill the bacteria but the

bacterial infecting process could be interrupted by interfering QS in which the pathogen will be then eliminated by host immune system.

Table 2.2: Examples of QS inhibitors (QSIs) from plant origin and its effect on QS activity.

Phytochemical Group	QSIs (Phytochemicals)	QS Activity	References
Phenolics	Ascorbic acids	Reduction in autoinducer-2 activities, spore production and enterotoxin production in *Clostridium perfringens*	(Baird-Parker & Freame, 1967)
	Baicalein, Hamamelitannin	Inhibition of biofilm formation, increased permeability of vancomycin and reduced production of staphylococcal enterotoxin in *S. aureus*	(Brackman et al., 2016; Chen et al., 2016)
	Curcumin	Attenuation of virulence in *P. aeruginosa*	(Rudrappa & Bais, 2008)
	Ellagic acids	Inhibit biofilm production in *E. corrodens*; reduction of AHLs production in *E. carotovora*.	(Truchado, Larrosa, Castro-Ibáñez, & Allende, 2015)
	Epigallocatechin gallate, Catechin	Interference with biofilm formation of *E. coli* and *P. putida*. Reduction in extracellular polymeric substance of *Staphylococcus* sp.	(K.-M. Lee et al., 2009; Vandeputte et al., 2010; X. Xu, Zhou, & Wu, 2012)
	Ferulic acids	Inhibition of biofilm in *P. aeruginosa*, interference to motility of *P. fluorescens* and *B. cereus*	(Lemos et al., 2014; Zeng et al., 2008)
	Gallic acids	Inhibition of biofilm in *S. mutans*	(Kang, Oh, Kang, Hong, & Choi, 2008)
	Giganteone A	Reduction of QS-related activity in *E. coli* biosensors	(Sivasothy et al., 2016)

Table 2.2: continued.

Phytochemical Group	QSIs (Phytochemicals)	QS Activity	References
Phenolics	Gingerone	Reduction in swarming and biofilm forming capacity in *P. aeruginosa* PAO1	(Kumar, Chhibber, Kumar, Kumar, & Harjai, 2015)
	Glycyrrhiza glabra flavonoids	Interference of motility and reduction in biofilm formation in *Acinetobacter baumannii*	(N. Bhargava, Singh, Sharma, Sharma, & Capalash, 2015)
	Malabaricone C	Reduction in pyocyanin production and biofilm formation in *P. aeruginosa*	(Chong et al., 2011)
	Rosamarinic acid	Influence the protease and elastase production, biofilm formation and virulence factors of *P. aeruginosa*	(Marwat et al., 2011)
	Salicylic acids	Reduction of AHL production, interference towards twitching and swimming motility of *P. aeruginosa*	(Bandara, Zhu, Sankaridurg, & Willcox, 2006)
	Tea polyphenols (*Camellia sinensis* L.)	Reduction of proteolytic activity, elastase, swarming motility and biofilm formation in *P. aeruginosa*	(H. Yin et al., 2015)
	Pyrizine-2-carboxylic acid	Inhibition of biofilm formation in multi-drug resistant *V. cholerae*	(Hema et al., 2016)
	Proanthocyanidins	Reduction in production of QS-regulated virulence determinants in *P. aeruginosa*	(Maisuria, Lopez-de Los Santos, Tufenkji, & Déziel, 2016)

Table 2.2: continued.

Phytochemical Group	QSIs (Phytochemicals)	QS Activity	References
Essential Oils	Cinnamon oil, Ferula oil, Dorema oil	Interference of QS related phenotypes; production of pyocyanin, alginate and rhamnolipid in *P. aeruginosa*	(M. Kalia et al., 2015; Sepahi, Tarighi, Ahmadi, & Bagheri, 2015)
	Cinnamon bark oil	Modification of permeability of outer membrane and inhibition of bacterial QS-activity in *E. coli*	(Yap, Krishnan, Chan, & Lim, 2015)
	Clove oil	Reduction of violacein production in *C. violaceum* and interference of swarming ability of *P. aeruginosa*	(M. S. A. Khan, Zahin, Hasan, Husain, & Ahmad, 2009)
	Coriander oil	Inhibition of biofilm formation and lipid peroxidation in *Campylobacter coli* and *C. jejuni*	(Duarte, Luís, Oleastro, & Domingues, 2016)
	Linalool	Inhibition of biofilm formation and alteration of the adhesion of *A. baumannii*	(Alves, Duarte, Sousa, & Domingues, 2016)
	Oregano oil	Inhibition of violacein production by *C. violaceum*	(Rodriguez-Garcia et al., 2016)
	Rose oil, Geranium oil, lavender oil, Rosemary oil	Reduction in violacein pigmentation in *C. violaceum* and AHLs production in *E. coli*	(Szabó et al., 2010)
	Thyme oil	Reduction of flagella gene expression in *C. violaceum* and interference of biofilm formation in *P. fluorescens* KM121	(Kamila Myszka et al., 2016; Vattem, Mihalik, Crixell, & McLean, 2007)

Table 2.2: continued.

Phytochemical Group	QSIs (Phytochemicals)	QS Activity	References
Isothiocyanates	Allicin, Ajoene	Renders *P. aeruginosa* sensitive towards tobramycin; inhibition of biofilm	(Bjarnsholt et al., 2005; Jakobsen et al., 2012)
	Allyisothiocyanate	Interference of adhesion and motility, inhibition of biofilm formation in *E. coli*, *S. aureus*, *L. monocytogenes* and *P. aeruginosa*	(Borges, Simões, Saavedra, & Simões, 2014; de Saravia & Gaylarde, 1998; Guiamet & Gómez de Saravia, 2005; H.-Y. Lee et al., 2013)
	Iberin	Interference of rhamnolipid production and gene expression of *lasB* and *rhlA* in *P. aeruginosa*	(Galloway, Hodgkinson, Bowden, Welch, & Spring, 2012; Jakobsen et al., 2014)
	Sulforaphane, Erucin	Antagonists of transcriptional activator of LasR and inhibition of biofilm formation in *P. aeruginosa*	(Ganin et al., 2013)
Stilbenoids	Resveratrol, Piceatannol, Oxyresveratrol	Reduction of violacein in *C. violaceum* CV026; Decreased in production of pyocyanin and swarming motility in *P. aeruginosa* PAO1	(Sheng, Chen, Tan, Chen, & Jia, 2015)

2.5 RNA-Sequencing (RNA-seq)

Fredrick Sanger developed robust DNA sequencing technologies that escorted to a new era of molecular biology, in which the exact base pair composition of a gene could be viewed. With advancement of powerful new applications like RNA-seq, this breakthrough allows the advancement into understanding and discover the profile and quantification of RNA transcripts across the entire prokaryotic or eukaryotic transcriptome (Ozsolak & Milos, 2011). In brief, RNA-seq is a new approach developed to profile transcriptome with deep-sequencing technologies; in which transcriptome is a complete set of transcript in a cell and their quantity for a specific developmental stage or physiological condition (Ozsolak & Milos, 2011; Wit et al., 2012). RNA-seq offers several advantages such as (i) detection of transcripts that have not been determined by genomic sequencing (ii) reveal single nucleotide polymorphism (SNP) – nucleotide variation in transcribed regions (iii) do not have any limit of RNA quantity for quantification (iv) low RNA quantity is required (v) high specificity (vi) reproducible and (vii) high-throughput. The application of RNA-seq will quickly supersede microarray technologies by detecting desired differential splicing activity, antisense transcription and discovery of novel region of transcription (Wilhelm & Landry, 2009). In 2013, scientist developed a new RNA-seq, known as Designed Primer-based RNA-seq as a new tool to analyze RNA transcripts from samples of 50-100 cells (V. Bhargava, Ko, Willems, Mercola, & Subramaniam, 2013). This method enables the scientist to diagnose cancers in early stages in a rapid manner. Low expressed novel transcripts could be detected through this method and they hope to bring this for routine diagnosis of pathologies and into discovery of therapeutic interventions.

The RNA-seq technology had introduced into studying the transcriptomic profiling in QS related field. One of the study in *Burkholderia glumae*, QS-dependent gene expression was studied through transcriptomic profiling of two QS mutants (*tofI*- and *qsmR*- mutants) at two given time points (S. Kim et al., 2013). Through RNA-seq, the researchers found out that QS-regulated genes are involved in regulated several metabolic pathways and the results showed consistency with QS-regulated phenotypes such as motility. RNA-seq also revealed the gene expression of infection-linked *V. cholerae* (Mandlik et al., 2011). In this study, transcripts consist of majorly virulence factors of *V. cholera* and from genes and small RNAs not previously linked to virulence were found inclined in infected mice and rabbits. Besides that, four rare sRNAs (grouped in regulatory RNAs) that govern *V. cholerae* QS was also detected (Mandlik et al., 2011). From RNA-seq, CsrA/B system also found to be another player in regulating the *hapR* mRNA and QS in *Vibrio* species and this was also found generally to control the QS circuits of many bacterial species (Papenfort & Vogel, 2010). Furthermore, RNA-seq was also applied in studying the signalling pathway in *Trypanosoma brucei* in which the role of overexpressed putative RNA-binding protein RBP7 was found to be essential in normal QS and promoting cell-cycle arrest and transmission competence (Mony et al., 2014). This study reveals that QS signalling in trypasomes correspondence to fundamental quiescence pathway in eukaryotic cells, this further allow the opportunity in targeting QS interference-based therapeutics. QS-dependent RNA-seq allows the understanding of cellular, molecular and metabolic of phenomena during a time-point selected before or after onset of QS (S. Kim et al., 2013). RNA-seq based transcriptome analysis is very much appreciated that it could study the link between QS and pathogenicity of pathogens during infection where the output from RNA-seq could be robust, sensitive and reliable for evaluation of regulatory processes that drive the pathogenesis.

2.6 Mutagenesis

The development of mutagenesis is essential in identify the genetic determinants required for each metabolic regulation and for functional characterization of proteins (P. E. Hartman, 1980). There are different mutagenesis approaches had been used such as chemical mutagenesis, transposons mutagenesis and recombineering-based mutagenesis (X. Wang, 2007). Chemical mutagenesis is the interaction of certain chemical compounds and cell metabolism intermediates that may results in random genetic changes in DNA structure, altering one or more genes (Heidelberger et al., 1983). Several chemical compounds used in this approach are alkylating agent (ethyl methyl sulphonate), base analogues (5-bromouracil and 2-aminopurine), intercalating agents (ethidium bromide and daunorubicin), and metals (arsenic, cadmium and nickel) to induce random mutation. Chemical mutagenesis generate mutant in a fast and easy manner but it is difficult to elucidate their locations in the genome and occasionally it does not have any observable phenotypes (X. Wang, 2007). Another method is to use transposon to create random mutagenesis; in which transposon is a mobile genetic element containing addition transposition-unrelated genes that function to enable the foreign DNA to move to different positions within the genome of the cell (Hayes, 2003). Through this, they can create mutations and alter the amount of DNA in the genome. Until now, the transposon mutagenesis libraries have been successfully created in *Francisella* (Qin & Mann, 2006), *Pseudomonas* (Jacobs et al., 2003) and *Vibrio* (Graf, Dunlap, & Ruby, 1994).

Various methods developed to analyze mutation at distinct chromosomal locations such as site-directed mutagenesis, insertion of foreign sequences or frame-shift deletion, have become fast growing passion since complete bacterial genome sequences became easily available (Katashkina et al., 2009; Trehan et al., 2016). Over the last decade, the most dynamic and accurate method for generating a vast diverse of genetically alteration

of DNA in *E.coli* has been termed as "recombineering" (Ellis, Yu, & DiTizio, 2001; Katashkina et al., 2009). This term is coiled to describe *in vivo* genetic engineering with DNA fragments carrying short homologies with a bacterial chromosome, using the proteins of a homologous recombination system of bacteriophage λ such as λ Red system. This method has been initially developed for *E. coli* K12 genome modification (Murphy & Campellone, 2003), was later extended to other *E. coli* strains, and to *Salmonella*, *Shigella*, *Yersinia*, *Pseudomonas* and other Gram-negative bacteria (Gerlach, Blank, & Wille, 2013). Both single-stranded DNA (ssDNA) oligonucleotides and double-stranded DNA (dsDNA) have been used as the targeting construct and the Red recombination system of the bacteriophage λ leads to accurate and brisk approach to create mutant (Sharan, Thomason, Kuznetsov, & Court, 2009). The λ red operon consists of three genes encoding the Exo, Beta and Gam proteins; in which Exo is a 5'-3' specific exonuclease that degrades linear dsDNA from each end to create 3'-ssDNA tails and required for dsDNA recombination, Beta binds ssDNA greater than 35 nucleotides in length and mediates the pairing with complementary target, and Gam inhibits the host nucleases and protect the dsDNA substrate for recombination (Sawitzke et al., 2013). However, Gam is not necessary for the recombineering but it could increase the frequency of dsDNA recombination up to 20-fold. The linear DNAs, either double stranded (PCR products) or single-stranded synthetic oligonucleotides are introduce via electroporation and provide the substrates (produced by PCR amplification of a cassette encoding a drug-resistance gene using bi-partite primers) to induce genetic change adjacent to the region of homologous interaction (Sawitzke et al., 2013; Sharan et al., 2009).

This method expanded widely into studying the bacterial gene functions which include the study the QS-related gene functions. The QS-system of *Sodalis glossinidius* was found to regulate a large number of genes involved in the bacterial response to oxidative stress via the optimized lambda Red-mediated recombineering (Pontes & Dale, 2011). Another study on autoinducer-2 (AI-2) of *Y. pestis* strain YP21 in which the *luxS* mutant suggested that AI-2 QS system is involved in the metabolic activities and oxidative stress genes that help *Y. pestis* to adapt to different abiotic factors such as host temperature (Yu et al., 2013). In this study, the luxS mutant was obtained via lambda Red-mediated recombination by replacing *luxS* with Cam^r antibiotic resistant cassette amplified from pKD3. On the other hand, *sdiA* (a *luxR* homologue) of *E. coli* strain EHEC O157:H7 proven that it act as strong repressor of genes encoding flagella and curli fimbriae and its mutant, Δ*sdiA* enhanced bacterial motility (Sharma, Bearson, & Bearson, 2010). The *sdiA* mutant was generated using the lambda-red mediated recombineering method (Murphy & Campellone, 2003) by knocking out the *sdiA* with a Kan^r antibiotic resistant cassette amplified by PCR. From these studies, the λ Red-mediated recombineering can be manipulated for rapid and efficient construction of QS-related mutants of various bacteria hence enabling QS pathway to be elucidated in detail.

2.7 Biotechnology Implications of Studying QS

As the number of bacteria that employ QS systems continues to bloom, the research into QS could span a wide variety of potential applications in which mostly involved the controlling of bacteria by interfering the signalling pathways (de Kievit & Iglewski, 2000). QS cross talk is also another interesting implications as in nature bacteria always exist in mixed species population such as biofilms. This could cause outbreak of diseases or ironically, bring benefits to other higher organism (Di Cagno, De Angelis, Calasso, & Gobbetti, 2011). Looking into QS of bacterial pool from freshwater environment could aid in identify the pathogenic or beneficial bacteria that occur, and as natural water flows from upper to lower (i.e. waterfall to river), the bacteria present in the water could be transported from one region to another region (Postel & Richter, 2012). Natural occurring freshwater is also an important source of drinking water in living organisms (Nikolaou, Meric, & Fatta, 2007). Study into QS paved the way into discovery of various QSIs that is feasible as treatments for bacterial infections in all living organisms especially to human being. The rational strategy in controlling the outbreak of bacteria could be manipulating the QS properties as in the number of multi-drug resistant strain are growing. Other than that, scientists nowadays creating more possible benefits from QS and of course there will be lots of potential development for advancement ranging from freshwater to human disorder (Goldenfeld & Woese, 2007). In fact, much work remain to be done in order to fully characterize the function and pathway related with QS as in this could be the silver bullet in the context of pharmaceutical and agricultural practices.

CHAPTER 3: MATERIALS AND METHODS

3.1 Chemicals and Solvents

All chemicals and solvents used in this work were of analytic grade. Chemicals purchased from Sigma, USA; Merck, Germany; Amresco, USA; BDH Ltd. UK; Thermo Fischer Scientific, USA; Promega Ltd, USA; Bio-Rad Laboratories Ltd., USA; and BD Difco™ Laboratories, USA were used in this work. Solvents used in this work were supplied by Fisher Scientific, UK.

3.2 Equipment and Instruments

Equipment and instruments used in this study were as follows; incubators and ovens (Memmert, Germany), Tecan microplate reader (Infinite M200®, Mannerdorf, Switzerland), Agilent Cary-60 UV.Vis Spectrophotometer (Agilent, USA), OmniLog PM Systems Phenotype MicroArrays (BIOLOG, Inc., USA), Milli-Q® water purification system (Merck Millipore, Germany), UV transilluminator (UV Products, Inc.), PCR Thermal Cycler (Bio-Rad; ABI), Analytical Table-Top Microscope SEM TM3030 (Hitachi), Libra S4 spectrophotometer (Biochrom, UK), Qubit 2.0 fluorometer (Life Technologies), CFX96™ Real-Time Polymerase Chain Reaction (PCR) Detection System (Bio-Rad Laboratories Ltd., USA), weighing machine (Sartorius), shaking incubator (N-biotek), centrifuge machine (Eppendorf), thermomixer (Eppendorf), autoclave machine (Hirayama), NanoDrop spectrophotometer (Thermo Fisher Scientific, USA), 2100 Bioanalyzer (Agilent Technologies Inc., USA), Miseq Personal Sequencer (Illumina, USA), Photon Camera (Hamamatsu, Japan), Microflex MALDI-TOF MS (Bruker Daltonik GmbH, Leipzig, Germany), LCMS/MS (Agilent Technologies Inc., USA), Gene Pulser Xcell™ Electroporation System (Bio-Rad Laboratories Ltd., USA),

ice maker (Rinnai) and pipettes (Eppendorf). Other equipment include; tips, tubes, Schott's bottles, conical flask, dryer, cuvettes, petri dishes, polypropylene tubes and inoculating loop.

3.3 Growth Media, Stock Solutions and Buffers

Culture media were prepared and autoclaved for sterilization at 121 °C, 15 psi for 15 min. The pH of each buffer and medium were adjusted with 1 N of HCl and 1 N of NaOH. Antibiotics were aseptically added into indicated medium after cooling. All heat labile items were sterile syringe filtered (0.22 μm pore size) (Sartorius Minisart; unless stated otherwise).

3.3.1 Luria-Bertani (LB) Medium

LB medium contains 0.5 g of yeast extract, 1.0 g of tryptone, 1.0 g of NaCl were dissolved in 100 mL of distilled water (dH_2O). Bacterial strains cultured for AHL extraction were grown with LB medium buffered with 50 mM 3-[N-morpholino] propanesulfonic acid (MOPS) at pH6.5 to inhibit the degradation of AHLs by alkali (Yates et al., 2002).

3.3.2 Reasoner's 2A (R2A) Medium

One liter of R2A medium contains typical composition (in g/mL): proteose peptone, 0.05; casamino acids, 0.05; yeast extract, 0.05; dextrose, 0.05; soluble starch, 0.05; dipotassium phosphate, 0.03; magnesium sulfate•$7H_2O$, 0.005; sodium pyruvate, 0.03; agar, 1.5; and solution was adjusted to pH 7.

3.3.3 JNFb- Medium

This medium was developed from NFb medium by J. Dobereiner (Cassán, Okon, & Creus, 2015) where its composition (in g/mL) was followed: malic acid, 0.5; K_2HPO_4, 0.06; KH_2PO_4, 0.18; $MgSO_4 \cdot 7H_2O$, 0.02; NaCl, 0.01; $CaCl_2 \cdot 2H_2O$, 0.002. This medium also supplemented with 2 mL of micronutrient solution (contains in milligram per milliliter of $CuSO_4 \cdot 5H_2O$, 4.0; $ZnSO_4 \cdot 7H_2O$, 12.0; H_3BO_3, 140.0; $Na_2MoO_4 \cdot 2H_2O$, 10.0; $MnSO_4 \cdot H_2O$, 11.8), 2 mL of bromothymol blue (0.5 % in 0.2 N KOH), 4 mL of 1.64 % of FeEDTA, and 1 mL of vitamin solution (contains in gram per 100 mL of biotin, 10mg and pyridoxal-HCL, 20mg). Solid medium was prepared by adding 17 g/L agar and the medium was adjusted to pH 5.8.

3.3.4 Phosphate Buffered Saline, 1× PBS

PBS solution was prepared by mixing 23 mg of NaH_2PO_4, 115 mg of Na_2HPO_4 and 900 mg of NaCl in 100 mL of distilled H_2O; and adjusted to pH 6.5 prior to autoclave sterilization.

3.3.5 Synthetic *N*-acyl Homoserine Lactones (AHLs)

Synthetic AHLs used as standards in this study were obtained from Sigma-Aldrich© and Cayman Chemicals. The AHLs were dissolved in HPLC graded acetonitrile (ACN) to the required concentrations and kept at -20°C.

3.3.6 Antibiotics Stock Solution

Four antibiotics (Table 3.1) were used in this study and all stocks were filter sterilized followed by storage of aliquots at -20°C until further usage.

Table 3.1: Antibiotics with its solvent and stock concentration.

Antibiotics	Solvent	Stock Concentration
Ampicillin	dH_2O	100 mg/mL
Chloramphenicol	absolute ethanol	34 mg/mL
Kanamycin	dH_2O	50 mg/mL
Spectinomycin	dH_2O	50 mg/mL

3.3.7 5-bromo-4-chloro-3-indoyl-beta-D-galacto-pyranoside (X-gal) Stock Solution

Blue/white colonies screening for transformants involved the synthetic sugar analogous to lactone, known as X-gal. The X-gal stock (20 mg/mL) was prepared by dissolving in dimethyformamide (DMF) and stored at -20°C after filter sterilization (0.22 µm pore size).

3.3.8 Anti-QS Compound, Gallic Acid

Gallic acid or 3,4,5,-trihydroxybenzoic acid is a type of phenolic acid found usually in plant sources such as tea leaves and oak bark. The chemical formula of gallic acid is $C_6H_2(OH)_3COOH$ and the pure gallic acid compound was obtained from Sigma-Aldrich. The stock solution of gallic acid (10 mg/mL) was prepared by dissolving it in dH_2O followed by syringe filter sterilization (0.22 µm pore size) and kept in room temperature for further usage.

3.3.9 Agarose Gel electrophoresis (AGE)

3.3.9.1 Tris Borate EDTA (TBE) Buffer (10× concentrated)

Generally, 10× TBE stock solutions consisted of 10.8 g Tris base, 5.5 g boric acid and 7.44 g $Na_2EDTA●2H_2O$ was dissolved in 100 mL of distilled water with pH set to 8.0 before it was autoclaved.

3.3.9.2 Agarose Gel (1% w/v)

To prepare agarose gel with 1% w/v, agarose powder (0.5 g) was weighed and added into 50mL of 1× TBE and boiled using microwave until agarose powder was fully dissolved. After cooling down, 1 µL of GelStar™ Nucleic Acid Gel Stain (Lonza, Basel) was added prior to gel casting.

3.3.9.3 DNA Ladder Marker

GeneRuler™ 100 bp DNA ladder and GeneRuler™ 1 kb DNA ladder used in this study were obtained from Fermentas International, Inc., Canada.

3.4 Commercial Kits

The commercial kits used in the entire study are listed in Table 3.2.

Table 3.2: Usage of kits in this study with manufacturer and application details.

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

Table 3.2: continued.

Kit	Manufacturer	Application
Nextera™ DNA Sample Preparation Kit; Nextera Index Kit	Illumina, Inc., CA	DNA sample preparation for whole genome sequencing
Illumina Library Quantification Kit	KAPA Biosystems, Woburn MA	Validation of Libraries prepared for Illumina platform
Agilent High Sensitivity DNA Kit	Agilent Technologies Inc., USA	For separation, sizing and quantification of low concentrated dsDNA (50-7000 bp)
Agilent RNA 6000 Nano Kit	Agilent Technologies Inc., USA	For analysis and quantification of total and mRNA samples of 25-500 ng/µL in concentration
Agilent RNA 6000 Pico Kit	Agilent Technologies Inc., USA	For analysis and quantification of total mRNA samples of 500-5000 pg/µL
Ribo-Zero rRNA Removal Kit	Epicentre Biotechnologies, USA	Removal of ribosomal RNA in a single pass
Scriptseq v2 RNA-Seq Library Prep Kit	Illumina, Inc., CA	Requires a very little RNA: 500 pg+ of rRNA-depleted RNA or poly(A)+ RNA to create a diverse sequencing libraries

3.5 Bacterial Strains, Plasmids, Oligonucleotides

The required bacterial strains, plasmids and oligonucleotides used were listed as shown (Table 3.3, 3.4 and 3.5).

Table 3.3: List of bacterial strains used in this study.

Bacterial strain	Description	Source/Reference
C. violaceum CV026	Biosensor (mini-Tn*5* mutant derived from *C. violaceum* ATCC 3153) that forms purple violacein pigmentation	(McClean et al., 1997)
E. coli [pSB401]	Biosensor with bioluminescence production and respond to short chain AHL, *luxR luxI*' (*Photobacterium fischeri* [ATCC 7744])::*luxCDABE* (*Photorhabdus luminescens* [ATCC 29999]) fusion; pACYC184-derived, Tet^R	(Winson et al., 1996)
E. carotovora Attn	Capable in producing AHLs detectable by *C. violaceum* CV026 that enable it to be positive control for bacterial biosensor test	Dr. Chan Kok Gan, Department of Genetics, University of Malaya
E. carotovora A20	Act as negative control in bacterial biosensor tests due to incapable of producing AHLs	Dr. Chan Kok Gan, Department of Genetics, University of Malaya

Table 3.3: continued.

Bacterial strain	Description	Source/Reference
E. coli DH5α	Developed for cloning purposes. The mutations that the DH5-α strain has are: *Φ80dlacZ*Δ*M15* Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17*(rK-mK+) *supE4 gyrA96 relA1*. ***lac*Z Delta M15 mutation**: Recombinant cells can be selected via blue-white screening ***end*A1 mutation**: Higher plasmid transfer rates by lowering endonucleases degradation. ***rec*A1 mutation:** Induction of more stable insert through reduction of homologous recombination	(Sambrook, Fritsch, & Maniatis, 1989)
E. coli BL21(DE3)pLysS	High-efficiency of targeted protein expression under the control of T7 promoter in competent cells and has a ribosome binding site. T7 RNA polymerase controlled by lac UV5 promoter is encoded by T7 bacteriophage gene I. The presence of pLysS encodes T7 lysozyme that lowers that background expression level of target genes under control of T7 promoter without interfering the level of expression by IPTG induction	Novagen, Inc., Germany
TKC strain	*tetA, cat, kan* (Serves as drug cassette amplification)	Court Lab, USA

LB medium was used to culture *E. carotovora* and *C. violaceum* CV026 grown at 28°C while all *E. coli* strains grown at 37°C with shaking at 220 rpm.

Table 3.4: List of plasmids used.

Plasmid	Description	Source/Reference
pGEM®-T Easy Vector	F1 ori, Amp^R, used as cloning vector	Promega, USA
pET-28a	F1 ori, Kan^R, used as expression vector	Novagen, Inc., Gemany
pCDF-1b	CDF ori, $Spect^R$, used as expression vector	Novagen, Inc., Germany
pSIM 7	pBBR1 ori, Cm^R, Plasmids Containing the Red System Under cI857 Control	Court Lab, USA

Table 3.5: Oligonucleotides used in this study (NA = not available).

Primer	Sequence	Length (-mer), Reference
	16S rRNA gene amplification	
16S rDNA forward primer 27F	AGA GTT TGA TCM TGG CTC AG	20, (Ott, Musfeldt, Ullmann, Hampe, & Schreiber, 2004)
16S rDNA reverse primer 1525F	AAG GAG GTG WTC CAR CC	17, (Dewhirst et al., 1999)
	Gene Cloning of QS-related genes	
T7	TAA TAC GAC TCA CTA TAG GG	20, Universal Primer
SP6	TTC TAT AGT GTC ACC TAA AT	19, Universal Primer
EcnI-1-F	CAT TAG GAA TGC TGG AAC TTT TTG ACG TTG AGC	33, NA
EcnI-1-R	GGA TCC ATC AGA CCG GCA GCT TCA	24, NA
EncI-2-F	GGA TCC GAT GAT GAA AGT AAT TCA AAC ACA GC	32, NA
EcnI-2-R	CTC GAG TTC AAC TGT GCG ACT GCC A	24, NA

Table 3.5: continued.

Primer	Sequence	Length (-mer), Reference
	Colony PCR Verification for Gene Cloning	
EcnI-1-CP-F	GTG GTC TGC AGT CAG GGA AT	20, NA
EcnI-1-CP-R	TCA GCA GGT AAA TCC GCT CT	20, NA
EcnI-2-CP-F	GAA GGT TGG TCT ATC CCG CC	20, NA
EcnI-2-CP-R	CCC GGG TGA TAA AGG TTC GT	20, NA
	Knock-out Mutation of Functional QS-related gene	
Chimeric_F	TGT AAA ATC GAC GTA TTG CGG CAA TCA GCC GCG GTT TTA GGG GAT AGG GAT ATG GAC AGC AAG CGA ACC G	70, NA
Chimeric_R	AGT CAG CAA CGC CCG GCA GGC CAT CCG CCT GGG CGT TGA GCT GGA TCT GAT CAG AAG AAC TCG TCA AGA AG	71, NA
	Colony PCR for verification of Knock-out Mutation	
EcnI-1-Flanked-F	GCA ATC AGC CGC GGT TTT AG	20, NA
EcnI-1-Flanked-R	GTC GTT GTG AAG CTC GGA GT	20, NA
Kan-F	TAT GGA CAG CAA GCG AAC CG	20, NA
Kan-R	TCA GAA GAA CTC GTC AAG AAG	21, NA

Table 3.5: continued.

Primer	Sequence	Length (-mer), Reference
Housekeeping Genes for Normalization of RT-PCR Expression Data		
GyrA_F	CTG GTG AAA GAG AAG CGT GTG	21, NA
GyrA_R	CTT TCA GCG TCA TGA TCT TCG	21, NA
RecA_F	TAA AGA GGG CGA TGA AGT GGT	21, NA
ecA_R	AGT TCG CTT TAC CTT GAC CAA	21, NA
RpoS_F	GGA GAA GTT TGA CCC AGA ACG	21, NA
RpoS_R	CAT CAT CAA CCG GCT TGT CTA	21, NA
RT-PCR for RNA-Seq with Anti-QS Compound		
Lpp_F	GCG AAT CTG CGT CAG TGG AT	20, NA
Lpp_R	TGA TAC GCT GAC CAC CTT CTG	21, NA
FliN_F	ATG ATA TTT CTG CGG ACG AC	20, NA

Table 3.5: continued.

Primer	Sequence	Length (-mer), Reference
	RT-PCR for RNA-Seq with Anti-QS Compound	
FliN_R	CCC AAT TCC ACG GTA AGT TTG	21, NA
FhuC_F	GCA TCA CGA AGA AAC CAC CTT	21, NA
Fhuc_R	ACC GCA TCG CTA TTT AAC AG	20, NA
HOA_F	TAC TGC ACG GAA CCA ATC AAC	21, NA
HOA_R	TGC TGT CAC CAT ATC GTC GT	20, NA
ChD_F	AAG AGT TTA CCT GGC CGA ACA	21, NA
ChD_R	GCG CCA TGT AAT TGA ACA GAA	21, NA
MetC_F	TCA ATG GAA GAG CTG ACT GG	20, NA
MetC_R	GCG AGG ATA TTG GTC TGG AAG	21, NA
	RT-PCR for RNA-Seq of Knock-out Mutation	
FliT_F	ACT CGA TCT CAG CCA TGG AAT	21, NA
FliT_R	CCA TAC GCA ATT TCA ACA GAC	21, NA
FlxA_F	CAC AAA TTG CCA GCC TGA ATA	21, NA

Table 3.5: continued.

Primer	Sequence	Length (-mer), Reference
	RT-PCR for RNA-Seq of Knock-out Mutation	
FlxA_R	TGT GCA ATT TGA GCT TCA ACC	21, NA
FlgA_F	TAT TTA GTT GCC AGC CGT CAG	21, NA
FlgA_R	CCA CCA TCA CAT TTT GTC CTG	21, NA
SulA_ii_F	CAA CTA CAA CGG CCA GAT CAA	21, NA
SulA_ii_R	TTG CAG ATC AAT ACG GTC GTC	21, NA
SulA_i_F	ACC GAG AAG CGC AAA TTT ATC	21, NA
SulA_i_F	TGA TAC CCA GCA GCG TAG AGA	21, NA
PilP_F	AAC AAT GAC TTC CGC CAG AC	20, NA
PliP_R	GTA AAG CAC GTC AAA CTT CC	20, NA

3.6 Environmental Sampling and Isolation of Bacteria

Water sampling was conducted in 2013 at tropical rainforest waterfall, Sungai Tua Falls which is located 10km from Selayang and Ulu Yam (GPS: N 03 19.91′ E 101 42.15′). Collection of water sample was conducted at the water surface level (to the depth of 12cm) and stored in sterile plastic bottles. The collected sample was kept at 4°C until further analysis. Saline buffer (0.9 % w/v NaCl) was used for ten-fold serial dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) of the collected water sample and spread onto Reasoner's 2A agar. Overnight incubation (24h, 28°C) was conducted and isolation procedure was proceeded by selection of single bacterial colonies displaying distinctive morphologies. Pure colonies were obtained with a few repeated streaking on LB medium.

3.7 Detection of AHLs Production

3.7.1 Employment of Bacterial Biosensors in Preliminary Detection of AHLs

Cross streaking of isolates against bacterial biosensors (*C. violaceum* CV026 and *E. coli* [pSB401]) allow the preliminary screening for AHLs production. The exogenous short chain AHLs will induce purple violacein pigmentation in the biosensor *C. violaceum* CV026 while bioluminescence in *E. coli* [pSB401]. The pure isolates obtained were screened for AHL production on LB media (24 h, 28°C). After incubation, purple pigmentation was observed while luminescence was observed with a photon camera (60 s exposure) (Hamamatsu, Japan). Isolates with positive results indicated AHLs production were subjected for further investigation. *Erwinia carotovora* PNP22 and *E. carotovora* GS101 was served as negative and positive controls respectively.

3.7.2 AHLs Extraction

First, 100 mL of LB medium added with 50 mM MOPS adjusted to pH 5.5 were used to grow the isolates overnight (24 h, 28°C, 200 rpm). Spent culture was added with equal volume of acidified (0.1% v/v glacial acetic acid added) ethyl acetate and vortexed vigorously for 1 min. The upper layer formed were extracted and the procedure was repeated twice. The extract was then dried in a fume hood and 1 ml of acidified ethyl acetate was used to resuspend the extracts which were then transferred to a new 1.5 mL microcentrifuge tube and let dry. After drying, the concentrated extract was then re-suspended with 200 µL of acetonitrile and vortexed for 3 min followed by maximum speed of centrifugation for 10 min. A total of 75 µL of the top layer mixture were taken and placed in sample vial before further analysis via mass spectrometry (Agilent, USA).

3.7.3 AHLs' Profiling using High-resolution Tandem Liquid Chromatography Quadrupole Mass Spectrometry, LC-MS/MS

Agilent 1290 Infinity Liquid Chromatography (LC) system was employed for AHLs extract separation. The column used was Agilent Zorbax Rapid Resolution High Definition SB-C18 Threaded Column (2.1 mm × 50 mm, 1.8 µm particle size) and the parameters set is given in Table 3.6.

Table 3.6: Parameters set for the separation of AHLs in LC.

Flow Rate	0.5 mL/min
Temperature	37°C
Injection Volume	1 µL
Mobile Phase	A: 0.1 % v/v formic acid in HPLC graded water B: 0.1 % v/v formic acid in ACN
Gradient Profiles (time: mobile phase A, %: mobile phase B, %)	0 min: 80: 20; 7 min: 50: 50; 12 min: 20: 80; and 14 min: 80: 20

Compounds chromatographed from LC were detected via mass spectrometry (MS) with parameter given in Table 3.7. The Agilent MassHunter software was subsequently used to analyze the MS data where the analysis relied on the mass spectra comparison of retention index of extracted ion and synthetic AHL compounds.

Table 3.7: Parameters set for MS in this study.

Probe Capillary Voltage	3 kV
Sheath Gas	11 mL/h
Nebulizer Pressure	20 psi
Desolvation Temperature	200°C
Precursor Ion Scanning	Positive mode
Q3 (third stage of MS)	*m/z* 102 (represent the highly conserved lactone ring present in AHLs) ((Chan et al., 2014)
Q1 (first stage of MS)	*m/z* range: 150 - 400

3.8 Bacterial Identification

3.8.1 Bacterial Identification with Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

MALDI-TOF MS analysis was performed according to the reported work by Mellmann et al. (2008). First, the smeared fresh culture of selected isolates on MSP 96 target polished steel BC plate was overlaid with 1 µL of MALDI matrix, α-cyano-4-hydroxycinnamic (10mg/mL in 50 % acetonitrile/ 2.5 % trifluoroacetic acid) followed by air dried. The plate was analyzed in Microflex MALDI-TOF bench-top MS (Bruker, Germany) with UV laser at wavelength 337 nm. Analysis software equipped is the Bruker FlexControl version 3.3 and spectra were recorded in linear positive ion mode with mass range of analysis between 2 to 20 kDa. Various random position of laser were shots on the targeted sample plate well and Bruker MALDI Biotyper Real Time Classification Software (version 3.1) was used to analyze the MS spectra produced. Bacteria identification based on the spectra analyses were based on dedicated scoring system evaluation that was to the best match in Bruker database (Table 3.8).

Table 3.8: Score values of MADI-TOF MS (Schubert et al., 2011).

Range	Interpretation	Color
2.00-3.00	High Confidence Identification (accuracy secured until species level)	
1.70-1.99	Low Confidence Identification (accuracy secured at genus level)	
< 1.70	Non Reliable Identification	

3.8.2 Molecular Identification of Bacterial Isolates

3.8.2.1 Genomic DNA Extraction

Overnight incubation of isolates was conducted in LB broth (28°C, 220 rpm). Overnight grown cells were harvested by centrifugation (13,000 rpm, 10 min) and pelleted cells were washed once with 1× PBS and twice with sterile dH_2O twice. For each wash, the cells were harvested by centrifugation (13,000 rpm, 10 min). Bacterial genomic DNA was extracted from the pelleted cells with MasterPure™ DNA Purification Kit (Epicentre) in accordance to the manufacturer's instructions.

3.8.2.2 Polymerase Chain Reaction (PCR) Amplification of 16S rRNA gene

To amplify the bacteria 16S rRNA gene, forward primer 27F and reverse primer 1525R that yield a product fragment with expected size of 1.5 kb were used using i*Taq*™ DNA polymerase kit (iNtRON, Korea).

The PCR mixture (25 µL reaction) consisted of 10 × PCR reaction buffer, 10 mM dNTPs, 10 µM of forward and reverse primers each, 5 U/µl of *iTaq*™ DNA polymerase, template DNA (< 1,000 ng) and nuclease-free water. The PCR cycles set for this amplification involved initial denaturation (94°C, 5 min), followed by 30 cycles of denaturation (94°C, 30 s); annealing (63°C, 30 s); extension (72°C, 90 s) and the process ended with a cycle of final extension step (72°C, 5 min). Nuclease-free water replaced template DNA acted as negative control in each PCR run.

3.8.2.3 Agarose Gel Electrophoresis (AGE)

The horizontal 1.0 % w/v agarose gel submerged in 1× TBE was used to analyze the DNA samples and PCR products in electrophoretic manner. The mixture of 6× loading dye (Fermentas, Canada) added to each DNA sample at 1/5 dilution was transferred to the well of casted gel. The voltage of 80V was set to run the electrophoresis for 40 min. The resulting gel was viewed in a 302 nm UV transiluminator and image was captured with gel documentary image analyzer. The GeneRuler^{TM} 1kb DNA ladder and 100bp DNA ladder (Fermentas, Canada) were used as reference to DNA samples and PCR products respectively.

3.8.2.4 PCR Product Purification

Agarose gel slice containing the target DNA size was excised with a clean and sharp scalpel under UV transilluminator (UV Product) and a new 1.5 mL microcentrifuge tube was used for transferring of the excised gel and weighed. The excised gel was purified using QIAquick® Gel Extraction Kit (Qiagen).

3.8.2.5 Sequencing Analysis

Purified PCR products (amplification of 16S rRNA gene) were sent to 1st base (Malaysia) for Sanger sequencing analysis. Gene sequences obtained were visualized using the Chromas, a DNA sequencing software (Technelysium, Australia) followed with trimming to obtain good quality sequences. The sequences were aligned and compared with GenBank databases using the BLASTN program via National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov). Nearest identity of genus and species were evaluated based on the deposition in the databases.

3.8.2.6 Phylogenetic Analysis

FASTA file type containing the 16S rDNA sequences was proceeded with phylogenetic analysis. Molecular Evolutionary Genetic Analysis (MEGA; version 6.0) software was used to conduct the phylogenetic analysis. This software is downloadable from http://www.megasoftware.net (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The construction of phylogenetic tree was based on maximum likelihood statistical method with bootstrap replication of 1000. Each in-group taxa was added with an appropriate outgroup which is a taxon that is distantly related but nevertheless sufficient in its conserved homologues to produce a rooted tree.

3.8.3 Scanning Electron Microscopy (SEM)

SEM sample preparation was conducted based on the protocol established by Fischer and colleagues (2012) with slight modification (Fischer, Hansen, Nair, Hoyt, & Dorward, 2012). Fresh culture of bacteria was fixed by immersion in 5 % v/v glutaraldehyde with 0.1 M of PBS (pH 7.2) for 24 h. The fixed cells were washed twice with 0.1 M PBS (pH 7.2) followed by post-fixing of cells in 1% v/v osmium (OsO_4) for 1 hr and washed twice with sterile dH_2O. The post-fixed sample was then subjected to gradient dehydration with 50 %, 75 %, 95 % and 100 % of ethanol. This was then followed by treatment of hexamethydisilazane (HMDS) for 10 min with drying in desiccator for 24 h. Upon drying, the sample was mounted on aluminum stubs with gold coating in SC7620 mini sputter coater (Quorum Technologies) and viewed using the Analytical TableTop Microscope SEM TM3030 (Hitachi, Japan).

3.9 Whole Genome Sequencing

3.9.1 MiSeq Platform Sequencing

Genomic DNA was performed as according to section 3.8.2.1. Extracted DNA was quality checked by using agarose gel electrophoresis, Nanodrop (Thermo Scientific) and Qubit dsDNA High Sensitivity (HS) Assay Kit (Life Technologies). Subsequently, the DNA samples were subjected to preparation using Nextera^{TM} DNA Sample Preparation Kit and Nextera Index Kit (Illumina) based on manufacturer's instruction. The quality of prepared library was analyzed with Agilent 2100 Bioanalyzer system using High Sensitivity DNA Analysis Kit (Agilent) and the concentration was quantified using Illumina Eco qPCR machine with KAPA Library Quantification Kits (KAPA). Based on the qPCR quantification, the library templates were diluted to 2nM followed with denaturation. The denatured DNA library (further diluted to final concentration of 10 pM) were sent for sequencing in the MiSeq personal sequencer (Illumina) following the MiSeq Control Software (MSC) interface. The libraries were sequenced with a paired-end read length configuration at 2×250 bp.

3.9.2 Bioinformatics Analysis

FastQC analyzer (Andrews, 2010) was first used to evaluate the quality of the generated raw reads by MiSeq. Next, CLC Genomics Workbench 7.0 (CLC Bio, Denmark) was used to trim, filter and de novo assembly the raw data obtained from the sequencing. Prokaryote gene prediction algorithm in Prodigal (version 2.60) was used for bacterial gene prediction (Hyatt et al., 2010). The number of rRNA and tRNA present were predicted using RNAmmer (Lagesen et al., 2007) and tRNAscan SE (version 1.2.1) (Lowe & Eddy, 1997) respectively. Integrated Microbial Genomes & Microbiome software (IMG-ER)(Markowitz et al., 2009), automated SEED-based annotation with Rapid Annotation using Subsystem Technology (RAST) (Aziz et al., 2008) and NCBI-

database annotation with Prokaryotic Genome Annotation Pipeline (PGAP) were used to annotate the assembled data. One strain of interest, strain M004 was selected for further analyses. The QS-related homologues, LuxI were searched against the genome and the genes were aligned with homologues from closely-related species obtained from GenBank BLASTP program. MEGA (version 6.0) was then used to build the phylogenetic tree as accordingly to section 3.8.2.5.

3.10 Functionality Study of Gene of Interest by Cloning

3.10.1 Construction of Recombinant LuxI Expression Plasmids

There are two LuxI homologues found in strain M004. Both LuxI were named as EcnI-1 and EcnI-2 upon this study. Amplification of both the *lux*I genes from the purified genomic DNA of strain M004 were done by using PCR where two sets of primer sequences were used, EcnI-1-F/R and EcnI-2-F/R (Table 3.5) for *ecnI*-1 and *ecnI*-2 amplification respectively. For EncI-1 primers, the forward primer was added with two non-specific bases and an NdeI (underlined) restriction site while reverse primer was added with one non-specific base and BamHI (underlined) restriction site (Table 3.5). On the other hand, the NdeI and XhoI (underlined) restriction sites were added to the forward and reverse primers of EcnI-2 respectively (Table 3.5) and both primers were added with one non-specific base.

The PCR amplification process was carried out as follow: initial denaturation at 95°C (5 min); 30 cycles of denaturation at 95°C (30 s), annealing at 57 °C (30 s) and extension at 72°C (1 min); final extension at 72°C (5 min). Sterile dH_2O replaced the template DNA was served as negative control. AGE was performed to verify the size of amplicons. QIAquick Gel Extraction Kit (Qiagen, Germany) was used to purify targeted PCR products and pGEM-T Easy cloning vector (Promega, USA) was employed to ligate the

purified PCR product. The ligation process was completed by following the manufacturer's instructions. Transformation of resulting recombinant plasmids (named as pGEM-T-*ecnI*-1 and pGEM-T-*ecnI*-2) into *E. coli* DH5α were conducted separately based on steps introduced by Sambrook and Russell (2001) (Sambrook & Russell, 2001). Then, the plasmids harbored were extracted with QIAquick Spin Miniprep Kit (Qiagen) and both the *ecnI* genes were excised with required restriction enzymes (Promega) followed by gel purification. Then, the purified *ecnI*-1 and *ecnI*-2 were ligated into linearized pET28a and pCDF1b (Novagen, Germany) respectively. This step produced two recombinant plasmids designated as pET28a-*ecnI*-1 and pCDF1b-*ecnI*-2 and both these plasmids were separately transformed into the host, *E. coli* BL21(DE3)pLysS.

3.10.2 Verification of Transformants

Colony PCR was carried out to screen the desired transformants with the parameters of PCR set as according to section 3.8.2.2. The purified PCR products were sent to 1st base for Sanger sequencing (section 3.8.2.4). The AHL synthase Ecn-1 and EcnI-2 were characterized using biosensor *C. violaceum* CV026. The *E. coli* BL21(DE3)pLysS harboring the recombinant *ecnI*-1 and *ecnI*-2 were cross-streaked against CV026 to screen for AHL production. The *E. carotovora* PNP22 and BL21(DE3)pLysS harboring pET28a and pCDF1b alone were served as negative control, while the *E. carotovora* GS101 served as positive control.

Other than that, AHLs extraction and profiling with LS-MS/MS was also conducted to verify the AHL production of the transformants. One milliliter of overnight cultures of *E. coli* BL21(DE3)pLysS harboring the *ecnI*-1 and *ecnI*-2 were inoculated into a fresh 50 mL LB medium. The culture of transformant with *ecnI*-1 was added with kanamycin (50 µg/mL) and transformant with *ecnI*-2 was supplemented with spectinomycin (50 µg/mL);

chloramphenicol (34 µg/mL) was also added into both the cultures. The cultures were grown at 37°C until they reached OD_{600nm} of 0.4 – 0.5 and isopropyl-D-thiogalactopyranoside (IPTG, Sigma) was added at a final concentration of 1.0 mM for induction of both the *ecnI* expression in the recombinant *E. coli* BL21(DE3)pLysS. The cultures were induced with shaking for 8 h at 25°C. The *E. coli* BL21(DE3)pLysS harboring pET28a and pCDf1b plasmids, separately were served as negative controls. The induced *E. coli* BL21(DE3)pLysS cells (harboring pET28a-*ecnI*-1 and pCDF1b-*ecn*I-2) were subjected to AHL extraction according to method in section 3.7.2. The extracted samples were then subjected to AHL profiling with LC-MS/MS by referring to section 3.7.3.

3.11 Recombineering: Using Drug Cassette to Knock-out Genes *in vivo*

3.11.1 Construction of an *ecnI* Mutant using λ Red System

The *ecnI*-1 of strain M004 was knocked out and replaced by a kanamycin gene cassette using a recombineering method as described previously (Thomason, Sawitzke, Li, Costantino, & Court, 2007). The kanamycin cassette was amplified from TKC strain (Court Lab) using chimeric primer pair; chimeric_F/R (refer to Table 3.5) where the underlined 50 bases refers to the homology of the *ecnI*-1 site. The final amplicons consist of kanamycin cassette flanking 50 bp upstream and downstream of *ecnI*-1. After the amplification, the amplified linear DNA was gel purified using QIAquick Gel Extraction Kit (Qiagen) referring to manufacturer's instructions.

The strain M004 harboring pSIM7 (Court Lab) was cultured overnight in 5 mL LB medium with 34 µg/mL chloramphenicol (28°C, 220 rpm). Next, 0.5 mL of overnight culture was inoculated into a 30 mL fresh LB medium supplemented with 34 µg/mL chloramphenicol and let to grow until OD_{600nm} was reached at 0.4 – 0.5. Subsequently, 15 mL of the seed culture was transferred into another sterile Erlenmeyer flask and placed at 42°C water bath for induction; while the remaining 15 mL grown culture was kept at 28°C to serve as non-induced control. Both flasks were grown for 15 min at 220 rpm followed by preparation for electro-transformation procedures as previously described (Sawitzke et al., 2013). Briefly, DNA (100 ng) was mixed with the electro-competent cells prepared from both induced and non-induced cultures. The mixtures then transferred to a pre-chilled 1 mm electroporation cuvette and placed in an electroporation that was set to 1.8kV. The optimal time constants for the electro-transformation should be greater than 5 msec. After electro-transformation, 1mL of fresh LB medium was immediately added to the cuvette. Then, the transformed cells were subjected to 2 hr outgrowth period before plating onto LB plate with 50 µg/mL kanamycin. The non-induced cells served as negative control.

3.11.2 Verification of Mutant

Transformant formed (after 24 h incubation at 28°C) were confirmed by colony PCR. The PCR primers used for the verification included: EcnI-1-Flanked-F/R and Kan-F/R (Table 3.5). Two reactions were done using nearby locus specific primers with EcnI-1-Flanked-F, EcnI-1-Flanked-R, Kan-F or Kan-R to test for both new junctions. Then, a third PCR with EcnI-1-Flanked-F and EcnI-1-Flanked-R was conducted to verify the parental fragment loss and replaced by the kanamycin fragment. This purified PCR product was then sent for Sanger sequencing for verification (section 3.8.2.4). The desired colony with *ecnI*-1 mutant was routinely cultured in LB agar.

Cross streaking with AHL biosensor was conducted following steps in section 3.7.1 to verify the knock-out mutant created. The wildtype M004 was used as positive controls. To further confirm the loss of AHL production by resulting *ecnI*-1 mutant (*ΔecnI-1::Kan*^{r}), LC-MS/MS was used to check AHL production in mutant where the AHL extraction and profiling were conducted based on section 3.7.2 and 3.7.3. The AHL of wildtype M004 was also extracted and analysed at the same time to serve as positive control.

3.12 Transcriptome Profiling

Two transcriptomic studies were conducted in this study namely:

(i) Strain M004 treated with a known anti-QS compound, gallic acid

(ii) Strain M004 wildtype with its mutant (Δ*ecnI-1::Kan*^{r})

3.12.1 Total RNA Isolation and cDNA Synthesis

Overnight cultures of (i) strain M004 in two different conditions; with and without gallic acid (ii) strain M004 wildtype and mutant were sub-cultured in 100 mL fresh LB medium till OD_{600nm} reached 0.3 – 0.5 and 2.3 – 2.5 respectively. The gallic acid concentration used in this study was 1 mg/mL. All four conditions were done with triplicates. The total RNA of all conditions were extracted using a Macherey-Nagel Nucleopin RNA Kit (Macherey-Nagel) as according to the manufacturer's instructions. Extracted RNAs were analysed with NanoDrop 1000 spectrophotometer (Thermo Fischer, USA) to monitor its 260-nm/280-nm ratio and the integrity were assessed using an Agilent 2100 bioanalyzer with Agilent RNA 6000 Nano Kit (Agilent). The integrity of all extracted RNA should fall within the range of 9.0 – 10.0. Aliquots of the purified RNA samples were reverse transcribed to cDNA with QuantiTect® Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions. However, for gallic acid study, the

cDNA was synthesized from total RNA re-extracted from a new set of biological triplicates.

3.12.2 Transcriptome Sequencing

The total RNA extracted as mentioned above was followed by rRNA depletion, cDNA library preparation and sequencing on an Illumina MiSeq personal sequencer with a 2×75 bp paired end read length configuration. The rRNA molecules of each sample were removed from total RNA using Ribo-zero rRNA Removal Kit (Epicentre) and the quality was assessed using Agilent 2100 bioanalyzer with its RNA 6000 Pico Kit (Agilent). The cDNA libraries were then prepared with ScriptSeq v2 RNA-Seq Library Preparation Kit (Illumina) following the manufacturer's instructions. The libraries prepared were validated for its quality using the same bioanalyzer with Agilent High Sensitivity DNA Kit (Agilent) and concentration was quantified by Illumina Eco qPCR machine with Library Quantification Kits (KAPA Biosystems). The validated library templates were diluted to 2 nM and denatured by 0.2 N NaOH followed by further dilution to 10 pM before loading into the selected MiSeq cartridge for sequencing.

3.12.3 Transcriptome Analysis

FASTQ format containing the raw sequence reads were aligned to the reference genome of strain M004 (Deposition in NCBI database: accession number JRUP0000000). Next, CLC Genomic Workbench 7.0 (CLC Bio) was used to pre-align the end-trimming sequences reads based on read-quality scores. Normalization and statistical analyses were conducted with Partek Genomics Suite 6.6 (Saint Louis, MO) by loading the output generated in BAM file format. Briefly, the Reads per Kilobase of transcript per Million mapped (RPKM)-normalized reads were calculated. The gene expression level was estimated with ANOVA to determine the differential expression, according to manufacturer's manual. The false discovery rate (FDR)-corrected cut off P-value (P) ≤ 0.05 with fold change of ≤-2.0 and ≥ 2.0 were applied to the differential expressed gene lists generated. The free web server, WebMGA (http://weizhong-lab.ucsd.edu/metagenomic-analysis/) was used for further bioinformatics analyses. Venn diagram was generated with Venn diagram generator from Bioinformatics.lu (http://www.bioinformatics.lu/index.html).

3.12.4 Validation of RNA-seq with Quantitative Real-time PCR assay (qRT-PCR)

Selected QS-regulated genes were verified by conducting qRT-PCR. The cDNA generated as mentioned in section 3.12.1 was used as the template in the qRT-PCR. Gene-specific primers used for this PCR were as shown in Table 3.5. The qRT-PCRs were set up based on the manufacturer's protocols using the SolGent^{TM} qPCR assay (SolGent) and a Bio-Rad CFX-96 real-time detection system (Bio-Rad). Standard curves of 10-fold serial dilution of cDNA were generated for each gene to evaluate the primer efficiency. The qRT-PCRs reactions were performed in triplicates with 2X SolGent master mix, 10 µM of forward and reverse primers and 10 ng of cDNA template in a final volume of 15 µL. Three housekeeping genes (*gyrA*, *recA* and *rpoS*) were selected as internal control

genes and were used to normalize variability in expression levels. The housekeeping genes' expressions were checked by SolGent^{TM}-qPCR using cDNA synthesized from each condition as mentioned in section 3.12.1. Cycling conditions set were as follow: 95°C for 15 min; 40× of 95°C for 20 s, 55°C for 40 s and 72°C for 1 min; followed by 72°C for 5 min; and a melt curve step (from 75°C, gradually increasing 0.5°C/s to 95°C, with acquisition data every 5 s). Melting peaks were generated through melt curves by plotting the negative derivatives versus temperature. CFX Manager^{TM} Software version 1.6 (Bio-Rad. USA) was used to determine the efficiency, slope and correlation coefficient of generated data.

3.13 Phenotypical Microarray (PM)

3.13.1 OmniLog PM Analysis

Twenty 96-well PMs (metabolic panels PM1 to PM8 and sensitivity panels PM9-20) (Biolog, Inc., USA) were used in this study. Patented redox chemistry was employed by PMs for cell phenotypes monitoring where if the bacterial growth is supported by the assay well medium, reduction of tetrazolium dye by actively metabolizing cells will occurred. The "positive" results were indicated by the color formation (purple) in the well. The intensity of the color formed showed how active the cells are metabolizing. OmniLog Instrument was used to perform plate incubation and phenotype data recording. Digital images of microarray were captured and qualitative color change / turbidity values kept in a computer file (output) and kinetic graphs could be created. In this study, the phenotypic comparison between wildtype M004 and its mutant was carried out where 1,920 phenotypes for each strain were recorded four times simultaneously at each hour by OmniLog (Biolog, Inc., USA). The phenotypic changes between wildtype and mutant were recorded and tabulated; after filtering in OmniLog-PM software, the area of kinetic

curves was used as the analysis parameters (significant results were filtered at a cut-off value of 5000).

The PM assay was performed by employing Biolog's Manual (Hayward), in which PM procedures for *E. coli* and GN bacteria were selected. The isolates were plated out on LB agar at 28°C prior to conducting the sample preparation. A Biolog turbidimeter was used to allow a uniform suspension to be adjusted in IF-0a (PM1-8) and IF-10 (PM9-20) until a turbidity of 85 % transmittance (T) was achieved. Next, 1 % of dye A was added into the mixture. Then, multichannel pipette was used to transfer 100 µL of cell suspension into each well of the PM plates. The plates were then incubated for 48 h (28°C).

To assess the altered phenotypes the *ecnI*-1 mutant was compared to its wildtype, M004 (the wildtype was recorded as green tracing and the mutant was recorded as blue tracing). The differences of overlaid kinetic graphs were visualized and quantified after generation using OmniLog-PM software. The overlapped region showed purple color and the phenotypes that showed significant values were tabulated.

3.13.2 Antimicrobial Susceptibility Test

Antibiotics-related phenotypes that showed significant results were selected to substantiate a subset of the PM data. Oxoid^{TM} antimicrobial susceptibility test disks (Thermo Scientific) were used to test the selected drug sensitivity phenotypes with the agar disk diffusion test procedure recommended by the manufacturer. The disk diffusion procedure was carried out on LB agar and conducted in triplicates.

3.14 Biofilm Formation Assay

Two different biofilm assays (i) untreated and treated with anti-QS compound, gallic acid (ii) wildtype with its knock-out mutant were carried out. The biofilm assay was conducted as previously reported with slight modifications (Vandeputte et al., 2010). Briefly, fresh LB medium was used to dilute the overnight culture of the bacterial strain to OD_{600nm} of 0.1. A 96-well microtiter plate was used in the assay. The next step involved addition of 5 µL of diluted culture into 95 µL of fresh LB medium; in which for treated condition the wells were supplemented with 1 mg/mL gallic acid. The plates with different culture conditions were incubated statically (72 h, 28°C). Sterile dH_2O was used to wash three times in order to remove the planktonic bacteria allowed to be air-dried for 15 min before straining with 200 µL/per well of 0,1 % (w/v) crystal violet for 30 min. Then, the excess crystal violet was removed and washed with sterile dH_2O twice. The qualitative analysis of biofilm production was done re-suspending each well with 200 µL of 95 % (v/v) ethanol and 100 µL of the mixture was transferred to a new microtiter plate. Tecan microplate reader (Infinite M200®) was used to read the absorbance of the mixture at OD590. All experiments were performed in triplicates.

3.15 *In-situ* Plate Assay for Indole-3-acetic Acid (IAA) Production

Plant assay for indole-3-acetic acid (IAA) production assay was conducted based on protocol proposed by Shrivastava and colleague with slight modifications (Shrivastava & Kumar, 2011). The JFNb- agar plate containing 100 µg/mL of tryptophan was prepared and a cavity of approximately 1.5-2.0 cm diameter with 0.5 cm depth was made in the middle of agar by a sterile cork borer (Shrivastava & Kumar, 2011). The overnight culture of strain M004 wildtype and *ecnI*-1 mutant were transferred into the cavity separately and incubated at 30°C. After 24 h incubation, the culture was removed gently and 200 µL of indole-acetic acid (IAA) reagent, Kovac's reagent was added into the cavity. A halo zone formation was visualized and measured. The IAA production index was calculated based on the formula proposed (Figure 3.1).

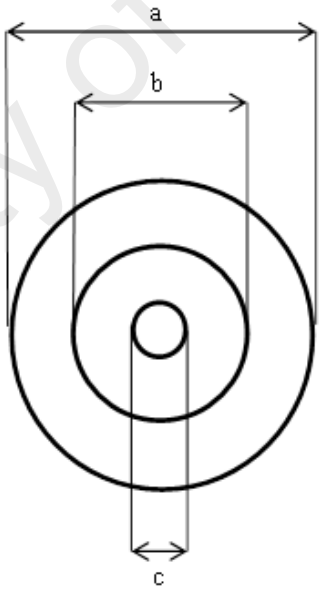


Figure 3.1: The IAA production plate assay: a – culture plate diameter; b – total halozone diameter; c – cavity diameter.

CHAPTER 4: RESULTS

4.1 Water Properties

The water sample was collected at the highest source of waterfall and the water was crystal clear. The water temperature on collection day was 25°C at 11.00 am and the pH value of the water sample was 7.

4.2 Bacterial Isolation and Screening of AHL Producing Bacteria

A total of 90 isolates were isolated from the water sample. However, 10 of them did not grow in sub-culture. The isolates were labeled starting from M001 to M080. After pure colonies were obtained, 80 isolates were screened for AHL molecules production. The bacterial biosensors CV026 and *E. coli* [pSB401] were used for preliminary screening of AHL producing bacteria (Supplementary Appendix D). There were 13 out of 80 isolates observed to induce purple pigmentation of CV026 and bioluminescence in *E. coli* [pSB401] suggesting AHL production by these isolates.

4.3 Bacterial Identification

The 13 isolates were selected and subjected for identification via MALDI-TOF MS (Table 4.1).

Table 4.1: Identification of QS positive isolates. List of 13 isolates showing positive QS results in bacterial biosensor tests as identified by MALDI-TOF MS.

Bacterial Isolates	Organism (Best Match)	Score Value[a]
M004	Not reliable ID	1.592
M005	*Dickeya chrysanthemi*	1.909
M006	*Cedecea neteri*	2.145
M009	*Pantoea stewartii*	1.814
M013	*Aeromonas hydrophila*	2.226
M022	*Pectobacterium carotovorum*	1.833
M023	*Aeromonas hydrophila*	2.103
M043b	Not reliable ID	1.583
M052	*Aeromonas hydrophila*	2.259
M053	*Aeromonas hydrophila*	2.187
M062	*Aeromonas hydrophila*	2.231
M073a	*Pantoea stewartii*	1.934
M074	*Dickeya chrysanthemi*	1.863

[a] Score values of MALDI-TOF MS (2.00-3.00 = high confidence identification; 1.70-1.99 = low confidence identification; <1.70 = non-reliable identification).

Five isolates (M005, M009, M022, M073a and M074) showed score values less than 2.0 and two isolates M004 and M043b were obtained as not-reliable identification. These seven isolates were followed up with molecular identification using PCR amplification of 16S rDNA sequence. EzTaxon database (O.-S. Kim et al., 2012) was used for 16S rRNA gene sequence-based identification in which the results were indicated in Table 4.2.

Table 4.2: EzTaxon database identification of isolates by PCR amplification of 16S rDNA sequence.

Isolates	Closest-related identity	Sequence similarity[a] (%)
M004	*Enterobacter cancerogenus* LMG 2693 (T) (GenBank accession = Z96078.1	98.29
M005	*Dickeya solani* IPO 2222 (T) (GenBank accession = KF639914.1)	98.78
M009	*Pantoea stewartii* subsp. *stewartii* LMG 2715 (T) (GenBank accession = Z96080.1)	99.51
M022	*Pectobacterium aeroidearum* SCRI 109 (T) (GenBank Accession = JN600322.1)	97.93
M043b	*Erwinia typographi* DSM 22678 (T) (GenBank accession = GU166291)	97.51
M073a	*Pantoea stewartii* subsp. *stewartii* LMG 2715 (T) (GenBank accession = Z96080.1)	99.50
M074	*Dickeya chrysanthemi* LMG 26270 (T) (GenBank accession = Z96093.1)	97.01

[a] Pairwise alignment to calculate the sequence similarity values between the query sequence and the sequence potentially closely related using the BLASTN program.

4.4 LC-MS/MS Analysis on AHL Profiles

AHL extracts from overnight cultures of the 13 isolates were subjected to LC-MS/MS analysis. The analysis was conducted with Agilent MassHunter software by comparing the retention times of extraction ion chromatogram (EIC) between standards (synthetic AHL compounds) and AHL extracts of isolate. The EIC showed similar peak patterns between the synthetic AHL and extracted AHL (Ortori et al., 2011). The list of AHL extracted from spent culture supernatant of the 13 isolates were tabulated in Table 4.3 and the mass spectra were supplied in Supplementary Appendix E. However, there were no AHL molecules detected from extracts of *E. typographi* strain M043b and *D.*

chrysanthemi M074 although these isolates were observed to trigger violacein production of CV026 and bioluminescence of *E. coli* [pSB401].

Table 4.3: AHL profiling via LC-MS/MS. The list of AHL produced by the 13 isolates.

Bacterial Strains	**AHL produced**
E. cancerogenus M004	3-oxo-C6-HSL, 3-oxo-C8-HSL
D. solani M005	3-oxo-C6-HSL, C8-HSL, C10-HSL
C. neteri M006	C6-HSL, 3-oxo-C8-HSL, C10-HSL, 3-oxo-C10-HSL
P. stewartii M009	3-oxo-C6-HSL
A. hydrophila M013	C4-HSL
P. aeroidearum M022	3-oxo-C8-HSL
A. hydrophila M023	C4-HSL
E. typographi M043b	-
A. hydrophila M052	C6-HSL
A. hydrophila M053	C6-HSL, 3-oxo-C6-HSL
A. hydrophila M062	C6-HSL
P. stewartii M073a	3-oxo-C6-HSL, 3-oxo-C8-HSL
D. chrysanthemi M074	-

4.5 Scanning Electron Microscopy (SEM)

From the bacterial identification, *E. cancerogenus* strain M004 was selected for further analyses. Its appearance and morphology was observed using Analytical TableTop Microscope SEM TM 3030. The result showed that this isolate is non-motile, aerobic rods with a length of 1.18 µm (Figure 4.1).

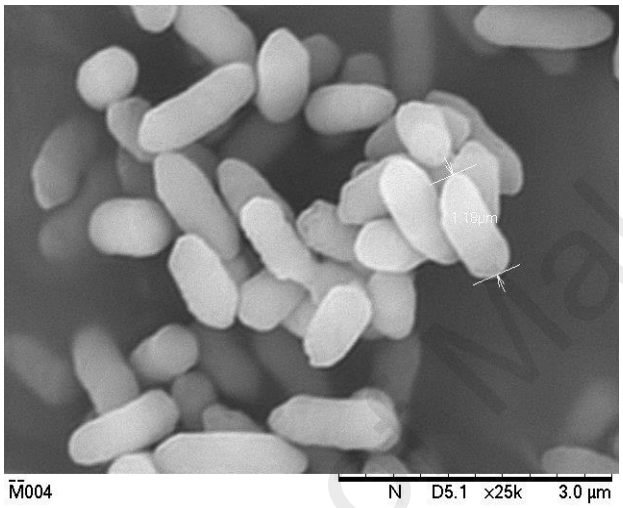


Figure 4.1: Electron micrograph of *E. cancerogenus* strain M004. This image was viewed under the microscope at 25000 × magnification size. Bar (left bottom): 3.0 µm.

4.6 Data Analyses on Genomic Features of *E. cancerogenus* Strain M004

4.6.1 Genome Project History

E. cancerogenus strain M004 was sent for whole genome sequencing using MiSeq platform. The genome sequence for this isolate was deposited in GenBank (Appendix A). The project and the Minimum Information about the Genome Sequence (MIGS) were shown in Table 4.4.

Table 4.4: Genome sequencing project information.

MIGS ID	Property	Term
MIGS 31	Finishing quality	Complete
MIGS-28	Libraries used	MiSeq
MIGS 29	Sequencing platforms	MiSeq
MIGS 31.2	Fold coverage	45.83 ×
MIGS 30	Assemblers	CLCbio CLC Genomics Workbench, version 6.5.1
MIGS 32	Gene calling method	IMG-ER/RAST
	Locus Tag	NH00
	Genbank ID	JRUP00000000
	Genbank Date of Release	2014/11/10
	GOLD ID	Gp0108990
MIGS 13	BIOPROJECT	PRJNA263657
	Project relevance	Environmental

4.6.2 Genome Properties

The genome of *E. cancerogenus* strain M004 comprised of approximately 5.67 Mbp and 53.75% G + C content. Genome sequencing resulted in 101 contigs and of the 5,286 predicted genes, 5,205 were protein-coding genes. The properties of and the statistics for the genome are summarized in Table 4.5.

Table 4.5: Genome statistics. The genome properties of *E. cancerogenus* strain M004 after analysis.

Attribute	Value	% of total
Genome size (bp)	5,670,247	100.00
DNA coding (bp)	4,938,456	87.09
DNA G+C (bp)	3,047,864	53.75
DNA scaffolds	101	100.00
Total genes	5,286	100.00
Protein coding genes	5,205	98.47
RNA genes	81	1.53
rRNA genes	7	0.13
tRNA	71	1.34
Pseudo genes	141	2.67
Genes in paralog clusters	3,999	75.65
Genes with function prediction	4,598	86.98
Genes assigned to COGs	4,012	75.90
Genes with Pfam peptides	4,614	87.29
Genes with signal peptides	499	9.44
Genes with transmembrane helices	1,239	23.44

4.6.3 Classification and Features

Based on PCR amplification of 16S rDNA sequence, strain M004 was identified closest to *E. cancerogenus*. Due to availability of whole genome sequence, the 16S rRNA gene was searched out and identified using the EzTaxon database. Similar result was obtained indicating that strain M004 was most closely related to *E. cancerogenus* LMG 2693(T) (GenBank accession = Z96078.1; sequence similarity: 99.12%). Next, 16S rDNA sequence of strain M004 and its related species was analyzed phylogenetically (Figure 4.2). Maximum-likelihood method in MEGA version 6 was employed in building the phylogenetic tree (Tamura & Nei, 1993; Tamura et al., 2013). The *E. cancerogenus* strain M004 was also classified according to MIGS recommendations (Table 4.6).

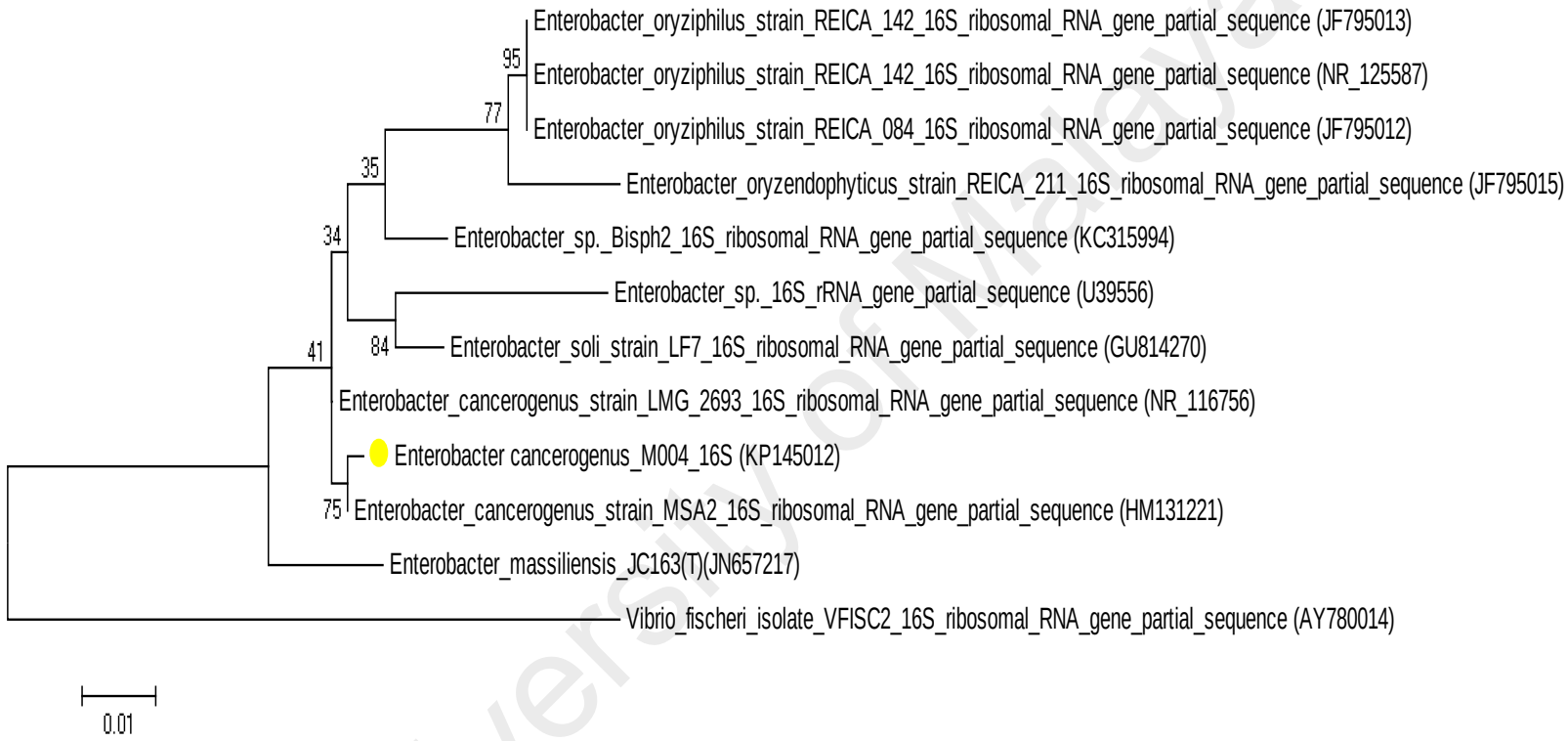


Figure 4.2: Phylogenetic tree of *E. cancerogenus* strain M004 and its related species. This study of evolutionary history of strain M004 with its closest species employed the Maximum-Likelihood method algorithm based on Tamura-Nei model (Tamura & Nei, 1993; Tamura et al., 2013) with bootstrap value of 1,000 replicates. *E. cancerogenus* strain MSA2 was found to be closest to strain M004 (yellow circle).

Table 4.6: Classification and general features of *E. cancerogenus* strain M004 according to MIGS recommendations (Field et al., 2008).

MIGS ID	Property	Term	Evidence code[a]
	Classification	Domain Bacteria	TAS (Woese, Kandler, & Wheelis, 1990)
		Phylum *Proteobacteria*	TAS (G. Garrity et al., 2006; Oren & Garrity, 2015)
		Class *Gammaproteobacteria*	TAS (A. Garrity, 2005; G. M. Garrity, Bell, & Lilburn, 2005; K. P. Williams & Kelly, 2013)
		Order *Enterobacteriales*	TAS (G. Garrity et al., 2006)
		Family *Enterobacteriaceae*	TAS (Commission, 1958; Rahn, 1937; SKERMAN, McGowan, & Sneath, 1980)
		Genus *Enterobacter*	TAS (Commission, 1958; Hormaeche & Edwards, 1960; SKERMAN et al., 1980)
		Species *cancerogenus*	IDA
	Gram stain	negative	TAS (DICKEY & ZUMOFF, 1988)
	Cell shape	rod	TAS (DICKEY & ZUMOFF, 1988)
	Motility	motile	TAS (DICKEY & ZUMOFF, 1988)

Table 4.6: continued.

MIGS ID	Property	Term	Evidence code[a]
	Sporulation	Not reported	NAS
	Temperature range	4-30°C	IDA
	Optimum temperature	28°C	IDA
	pH range; Optimum	e.g., 5.0-8.0; 7	IDA
	Carbon source	Acetate, citrate, glutamate, DL-lactate, malate, succinate, L-alanine, DL-α-alanine, L-serine	TAS (DICKEY & ZUMOFF, 1988; Garazzino et al., 2005)
MIGS-6	Habitat	waterfall	IDA
MIGS-22	Oxygen requirement	Facultative anaerobic	TAS (DICKEY & ZUMOFF, 1988)
MIGS-15	Biotic relationship	Free-living	IDA
MIGS-14	Pathogenicity	Non-pathogen	IDA
MIGS-4	Geographic location	Sungai Tua Waterfall, Malaysia	IDA
MIGS-5	Sample collection	2013	IDA

Table 4.6: continued.

MIGS ID	Property	Term	Evidence code[a]
MIGS-4.1	Latitude	N 03 19.91′	IDA
MIGS-4.2	Longitude	E 101 42.15′	IDA

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

4.6.4 Functional Annotation and Molecular Cloning of Gene of Interest

RAST annotation allowed the insight of subsystem category distribution of *E. cancerogenus* strain M004. This category enabled the understanding of metabolic pathways, multi-subunit complexes or protein classes that make up the functional roles of *E. cancerogenus* strain M004 (How et al., 2015). There is a total of 553 subsystems and the most abundant subsystem feature belonged to carbohydrates metablism (n=662; out of the a total of 4,014 subsystem feature counts), followed by feature responsible for amino acids and derivaties (n=533); cofactors, vitamins, prosthetic groups, pigments (n=281) and protein metabolism (n=281). The fatty acids, lipids and isoprenoids share the same counts as regulation and cell signaling features (n=158). One subsystem feature grouped as regulation and cell signaling system was being focused to search for functional genes related to QS activity (Figure 4.3).

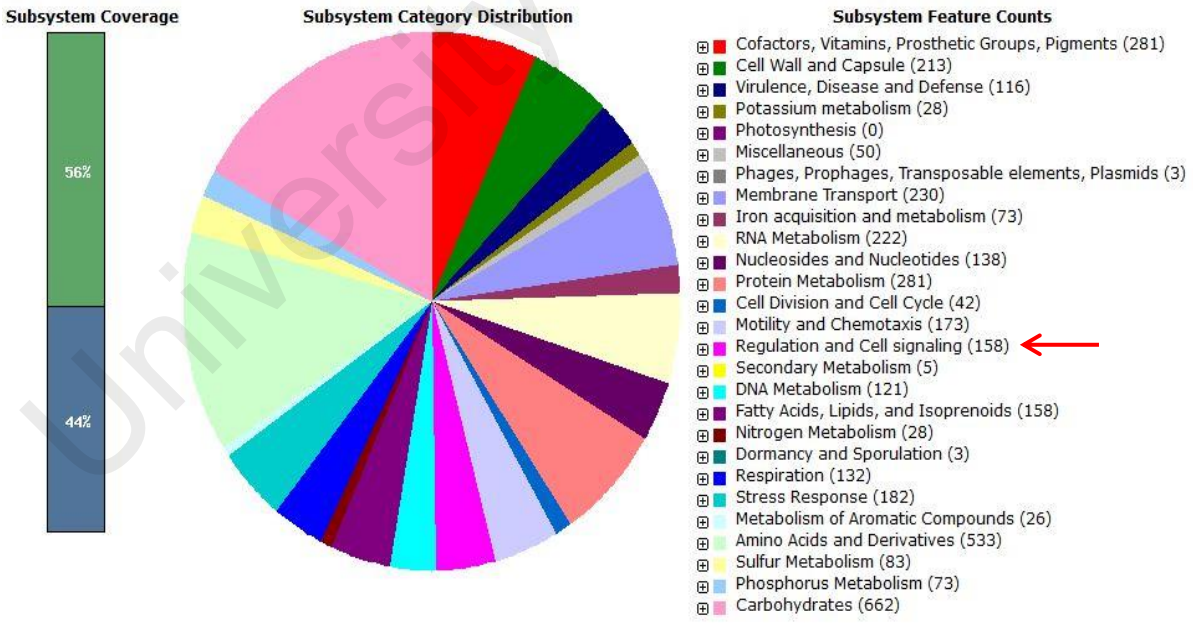
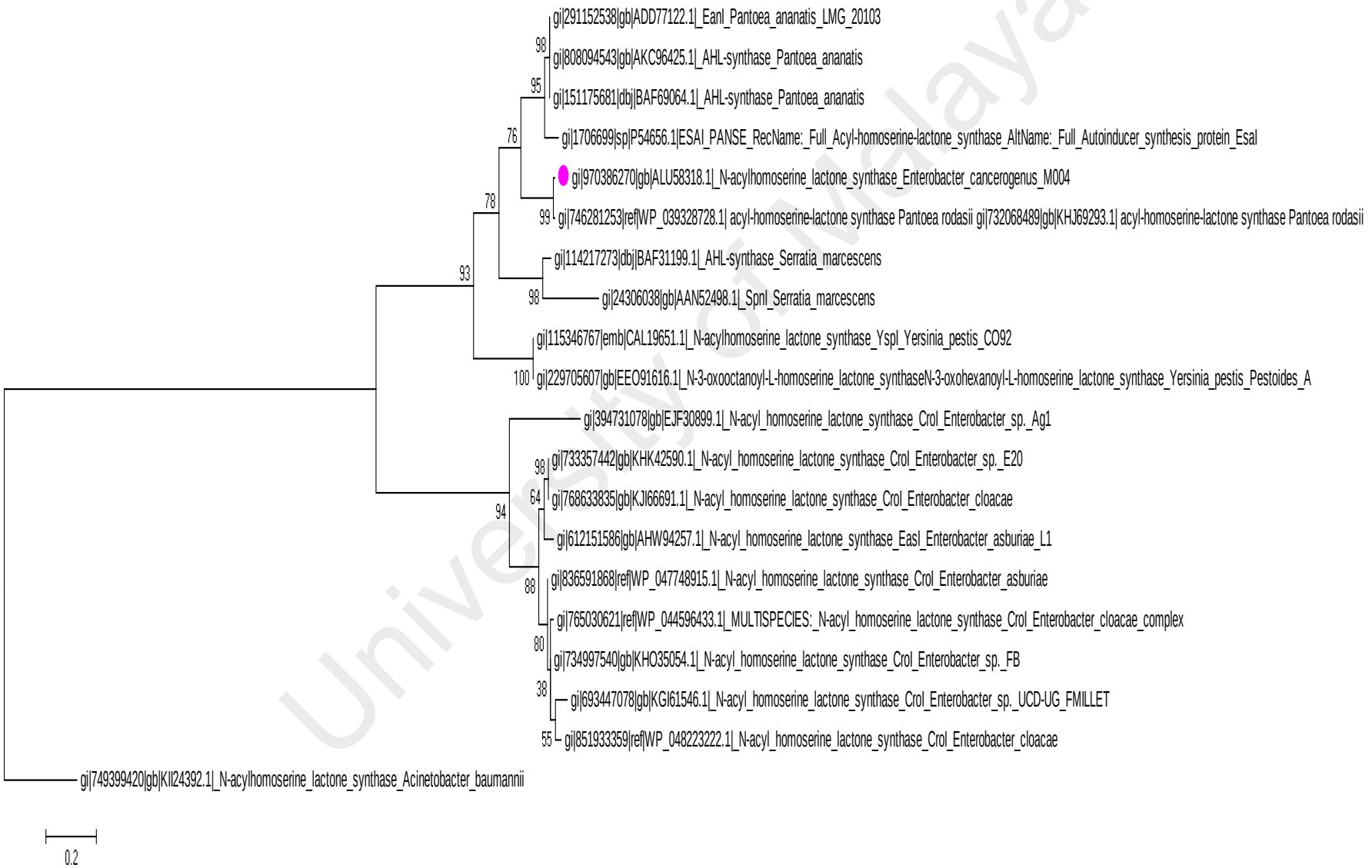


Figure 4.3: RAST annotation provided a view of subsystem category distribution of *E. cancerogenus* strain M004. Genes responsible for QS activity in strain M004 can be found in regulation and cell signaling subsystem (red arrow).

Through RAST analysis, a *luxI* homologue with size of 633 bp was found and hitherto, named as *ecnI*-1 (Appendix B). This homologue can also be found in the data generated by NCBI PGAP and from BLAST analysis, *ecnI*-1 shared a 97% base pair similarity with *luxI* homologue of *Pantoea rodasii*. From InterPro Scan analysis of EcnI-1 amino acid sequence, there was presence of three domains, known as autoinducer synthesis protein, acyl-coA-*N*-acyltransferase and autoinducer synthesis, a conserved site which is the structural domain of AHL synthase. These identified domains further postulates that the gene is a *luxI* homologue. On the other hand, another 615 bp *luxI* homologue, hitherto named as *ecnI*-2 (Appendix C). However, unlike EcnI-1, there are only two domains found in the amino acid sequence of EcnI-2 via InterPro Scan analysis, known as the acyl-CoA *N*-acyltransferase and autoinducer synthase. EcnI-2 shared the highest similarity with *Pantoea* sp. YR343 (97%). The phylogenetic trees were then constructed based on the amino acid sequence alignment illustrating independently on the EcnI-1 and EcnI-2 that clustered respective with other LuxI homologue of closely-related genus (Figure 4.4). Furthermore, EMBOSS Water software was used for pairwise protein sequence alignment of EcnI-1 and Ecn-2 and both sequences scored a similarity value of 63.5 (Figure 4.5).This indicated that both the protein sequences were not similar.

(a)



(b)

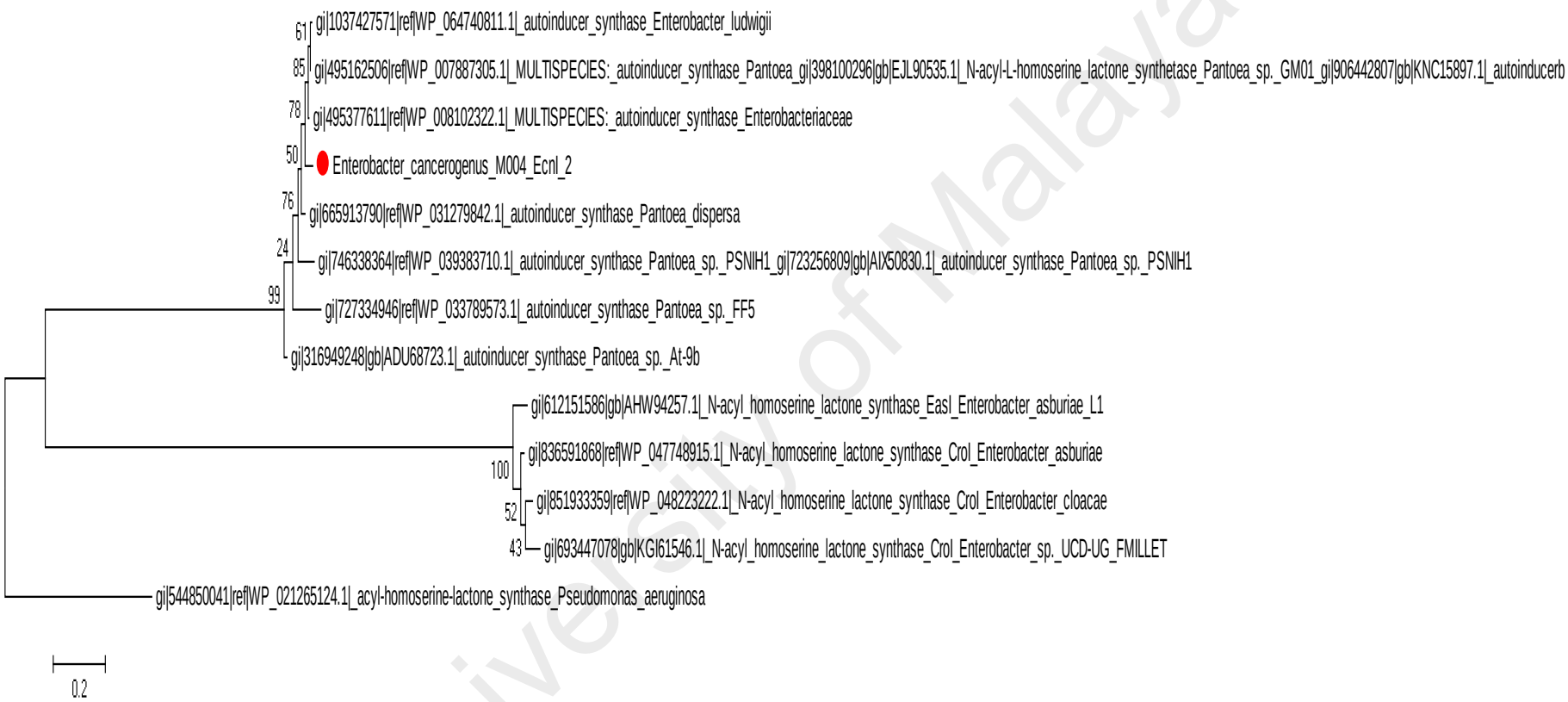


Figure 4.4: Phylogenetic trees of (a) EcnI-1 and (b) EcnI-2. This study of evolutionary history of EcnI with its closest species by using Maximum-Likelihood method algorithm based on Tamura-Nei model (Tamura & Nei, 1993; Tamura et al., 2013) with bootstrap value set with 1,000 replicates. EcnI-1 (purple circle) was found to be related closest with LuxI homologue of *P. rodasii* while autoinducer synthase of *P. dispersa* was found to be closest with EcnI-2 (red circle).

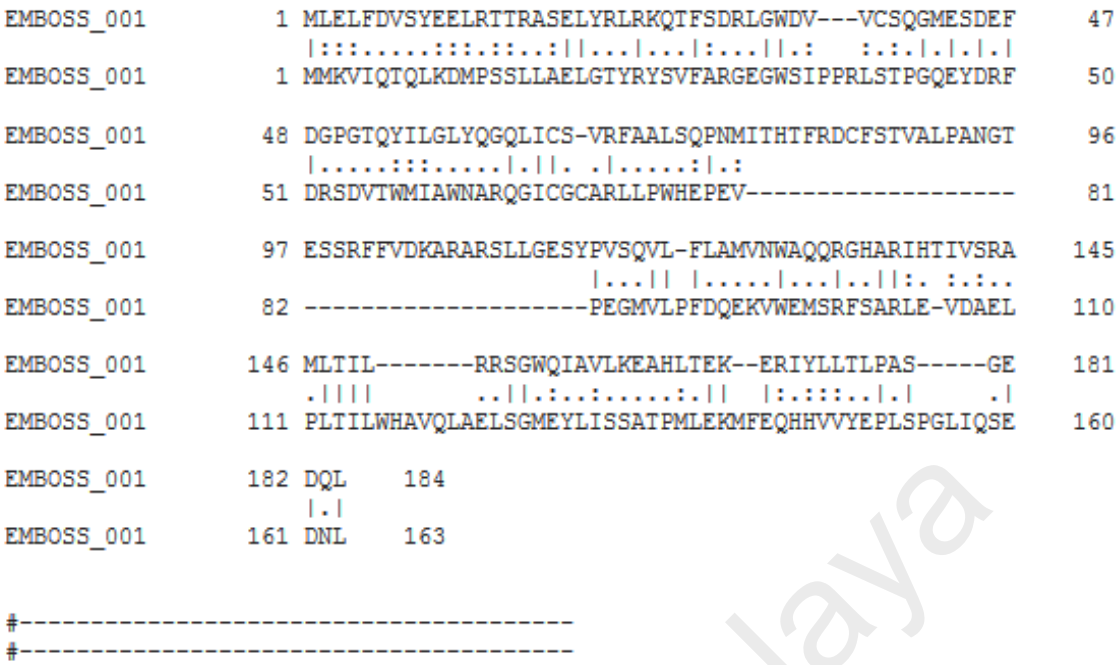


Figure 4.5: The pairwise alignment of EcnI-1 and EcnI-2 amino acid sequences using EMBOSS Water software. Identical residues are show by '|'; residues which are members of the same physiochemical group are indicated by ':'; and residues aligned at the coresponding positions are indicated by ".".

Owing to the availability of both *ecnI*-1 and *ecnI*-2 sequences of *E. cancerogenus* strain M004, characterization of functionality of both these gene were conducted by molecular cloning. Both *ecnI*-1 and *ecnI*-2 were cloned in pET28a and pCDF1b respectively and overexpressed in *E.coli* BL21(DE3)pLysS individually. Colony PCR was performed to verify the presence of recombinant plasmids harboring *ecnI*-1 and *ecnI*-2 respectively in *E. coli*. For preliminary screening of *E. coli* harboring *ecnI*-1 using CV026 biosensor, induced purple pigmentation was observed indicating the production of AHL molecules by the recombinant strain (Figure 4.6). On the other hand, preliminary screening of *E. coli* harboring *ecnI*-2 with CV026 showed no production of purple pigmentation (Figure 4.7). This confirmed the prediction of *ecnI*-2 might be a non-fucntional *luxI* homologue of *E. cancerogenus* strain M004.

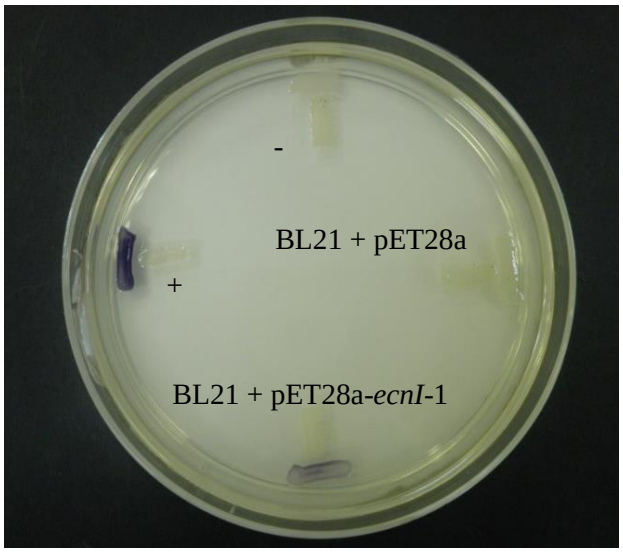


Figure 4.6: Cross-streaking bioassay to preliminary screen the production of AHL by *E. coli* BL21(DE3)pLysS with pET28a-*ecnI*-1. *E. carotovora* PNP22 (-) and BL21(DE3)pLysS harboring pET28a plasmid alone served as negative controls, while *E. carotovora* GS101 (+)served as positive control.

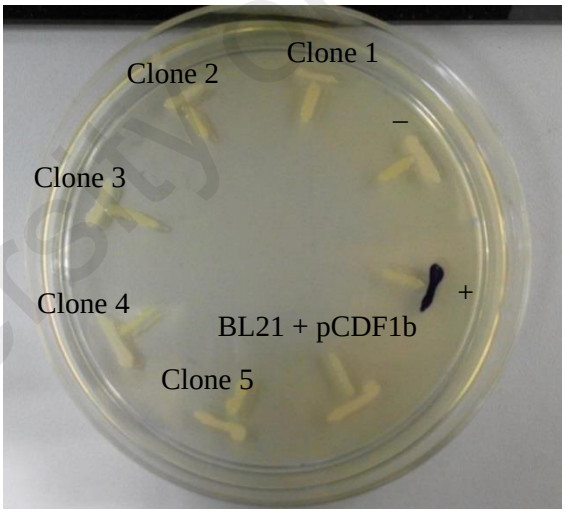


Figure 4.7: Cross-streaking bioassay to preliminary screen the production of AHL by *E. coli* BL21(DE3)pLysS with pCDF1b-*ecnI*-2. *E. carotovora* PNP22 (-) and BL21(DE3)pLysS harboring pCDF1b plasmid alone served as negative controls, while *E. carotovora* GS101 (+) served as positive control. (Clones 1-5: *E. coli* BL21+ pCDF1b-*ecnI*-2.

Next, AHLs were extracted from both the IPTG-induced spent culture supernatant of the *E. coli* BL21(DE3)pLysS harboring pET28a-*ecnI*-1 and *E. coli* BL21(DE3)pLysS harboring pCDF1b-*ecnI*-2. The AHLs extracted were analyzed and profiled via Agilent 6490 Triple-Quad LC-MS/MS system. From mass spectra analysis of induced *E. coli* BL21(DE3)pLysS harboring pET28a-*ecnI*-1, two AHLs were identified; 3-oxo-C6-HSL and 3-oxo-C8-HSL (Figure 4.8) and these AHLs found were similar with the AHLs produced by *E. cancerogenus* strain M004. This finding suggested that both the AHLs were produced by Ecn-1. The mass spectra were indistinguishable to the corresponding synthetic compounds at their specific retention times. Both AHLs were not found in negative control (*E. coli* BL21(DE3)pLysS harboring pET28a alone). No AHL was detected from analysis by high-resolution tandem mass spectrometry of induced *E. coli* BL21(DE3)pLysS harboring pCDF1b-*ecnI*-2 (Figure 4.9). Coupling of the verifications through CV026 bioassay and LC-MS/MS, *ecnI*-2 was proven to be a non-fucntional *luxI* homologue of strain M004.

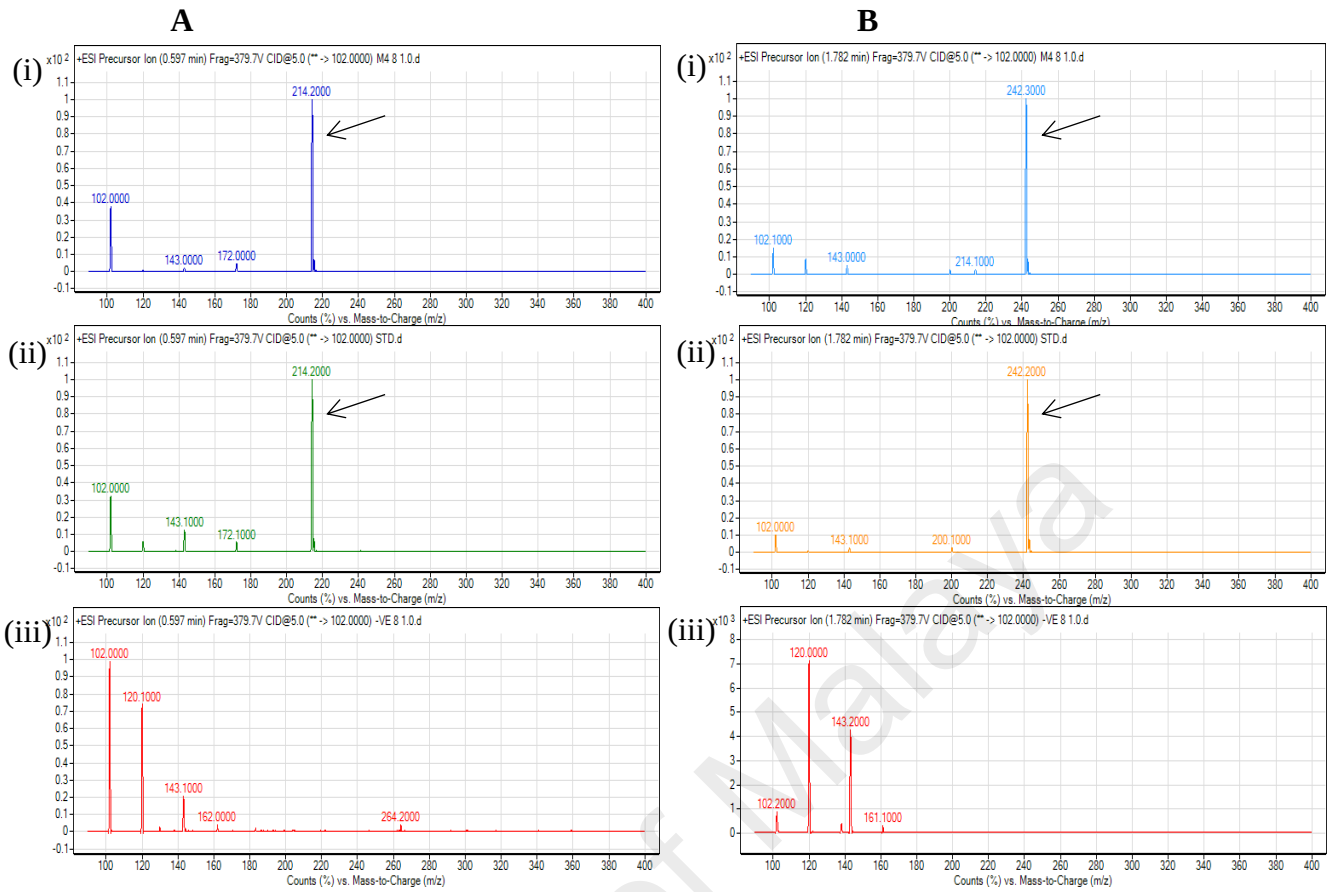


Figure 4.8: Mass spectrometry (MS) analyses of the extract of spent culture supernatant of induced *E. coli* BL21 cells harboring pET28a-*ecnI*-1. The mass spectra of AHL extracted exhibited the presence of (A) 3-oxo-C6-HSL at *m/z* 214.2000 (B) 3-oxo-C8-HSL at *m/z* 242.3000 (i) Mass spectra of *E. coli* BL21 harboring pET28a-*ecnI*-1 (ii) Mass spectra of synthetic AHL (iii) Mass spectra of *E. coli* BL21 harboring pET28a alone (negative control). The arrows indicating the presence of the AHL molecules at specific *m/z* value.

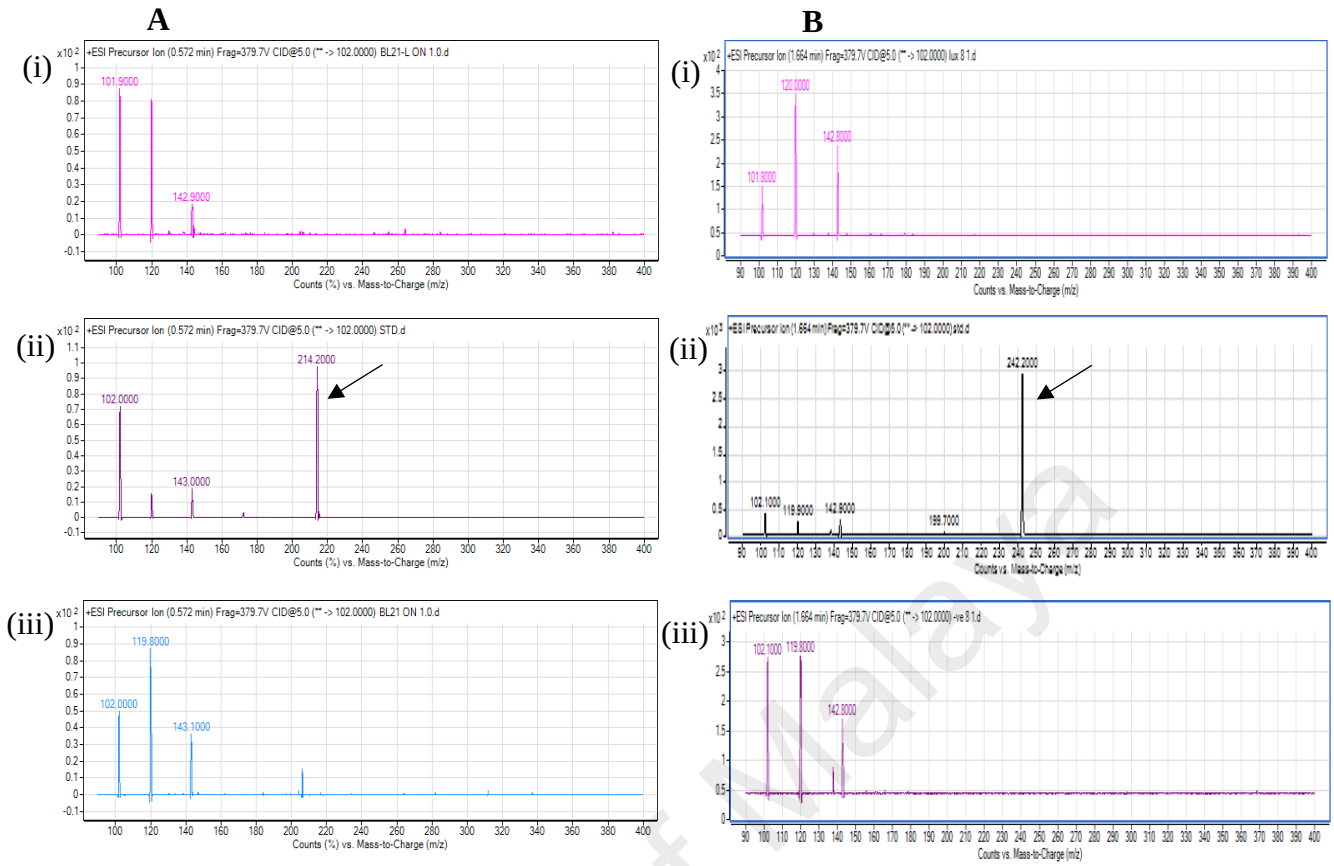


Figure 4.9: Mass spectrometry (MS) analyses of the extract of spent culture supernatant of induced *E. coli* BL21 cells harboring pCDF1b-*ecnI*-2. The mass spectra of AHL extracted exhibited the presence of (A) 3-oxo-C6-HSL at *m/z* 214.2000 (B) 3-oxo-C8-HSL at *m/z* 242.3000 (i) Mass spectra of *E. coli* BL21 harboring pCDF1b-*ecnI*-2 (ii) Mass spectra of synthetic AHL (iii) Mass spectra of *E. coli* BL21 harboring pCDF1b alone (negative control). The arrows indicating the presence of the AHL molecules at specific *m/z* value.

4.7 Verification of A *E. cancerogenus* strain M004 Mutant (Δ*ecnI-1::Kan*^{r}) Produced by *λ* Red System

By using λ Red system, *ecnI*-1 was knocked out from *E. cancerogenus* strain M004 and was replaced with kanamycin cassette. AHL biosensor CV026 and high-resolution tandem mass spectrometry were used for verification of the knock-out mutant's AHL production; meanwhile M004 wildtype served as positive control in both verification tests. There was no production of purple pigmentation observed when mutant cross-streaked against the CV026 and allowed the preliminary indication that the mutant produced was successful (Figure 4.10). The mutant was further verified by mass spectra anaysis whereby no AHL was profiled (Figure 4.11).

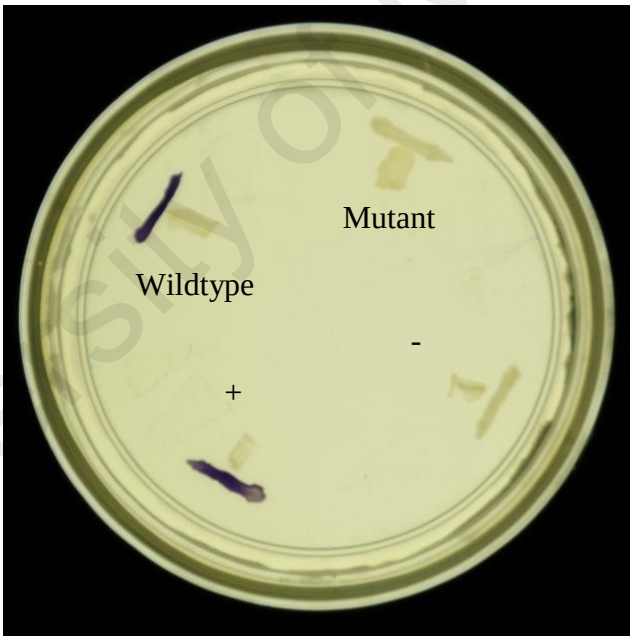


Figure 4.10: Preliminary verification of *E. cancerogenus* strain M004 mutant by cross-streaking against CV026. In this bioassay, *E. cancerogenus* strain M004 (Wildtype) and *E. carotovora* GS101 (+) served as positive control while *E. carotovora* PNP22 served as negative control (-). "Mutant" represented the *ecnI*-1 knock-out mutant.

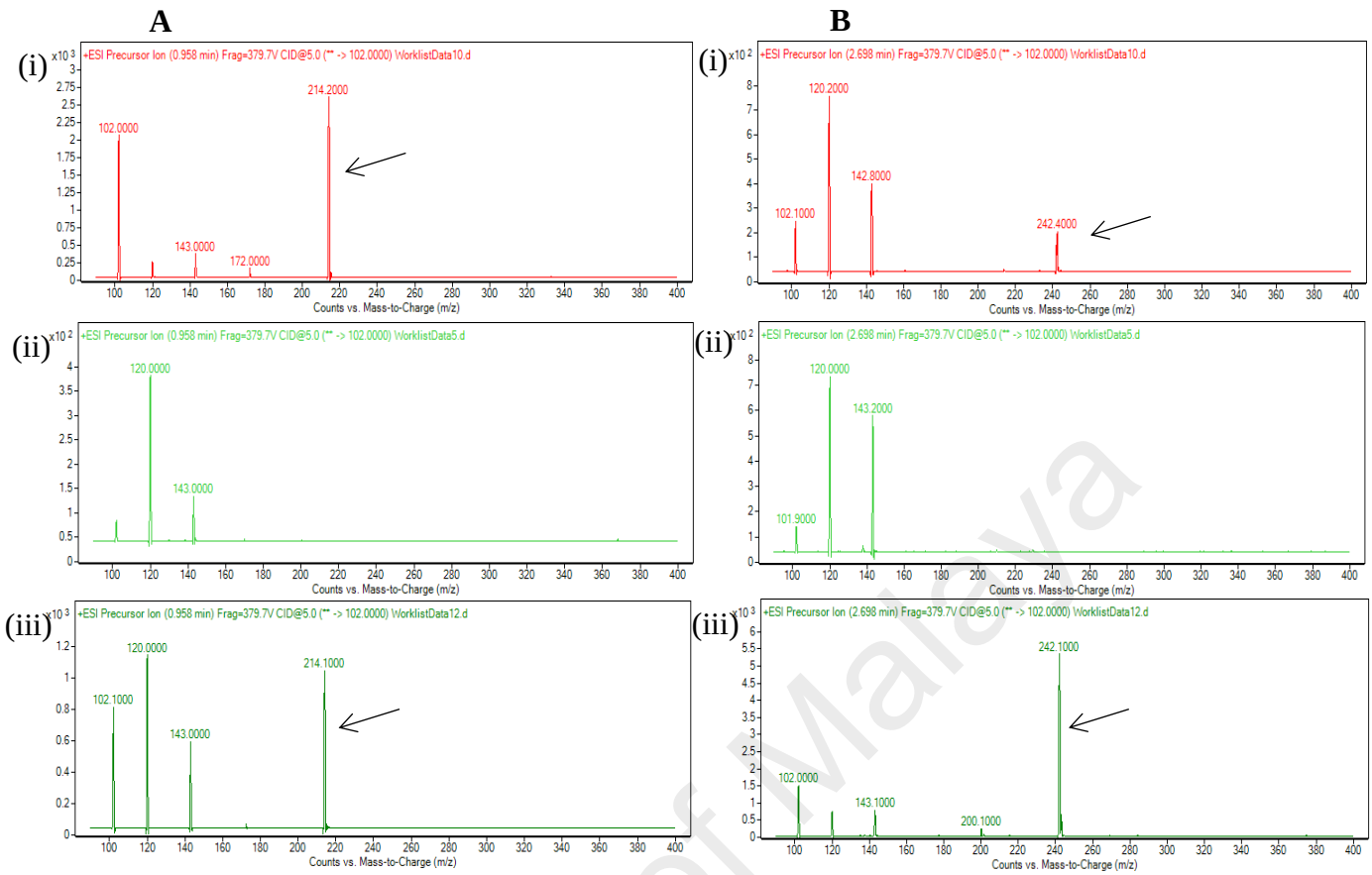


Figure 4.11: LC-MS/MS analyses on the extract of spent culture supernatant *E. cancerogenus* strain M004 wildtype (control) and its *ecnI*-1 mutant. The mass spectra of AHL extracted exhibiting the presence of (A) 3-oxo-C6-HSL at *m/z* 214.0000 (B) 3-oxo-C8-HSL at *m/z* 242.0000; mass spectra of i) wildtype (ii) *ecnI*-1 mutant and (iii) synthetic AHL. The arrows indicating the presence of the AHL molecules at specific *m/z* value.

4.8 Phenotypic Microrray (PM) Comparing Wildtype and *ecnI*-1 Mutant

Investigation into phenotypic processes such as carbon and nitrogen metabolism, pH control, antibiotics' resistance and others could be recorded and analyzed via Omnilog system (Sandle, Skinner, Sandle, Gebala, & Kothandaraman, 2013). PMs were adapted in this study to compare the phenotypic characteristics of wildtype and its respective mutant (Δ*ecnI-1::Kan*^{r}) in which revealing particular nutrients utilization influenced by the *ecnI*-1 knock-out mutation (Figure 4.12; other details are provided in Supplementary Appendix F).

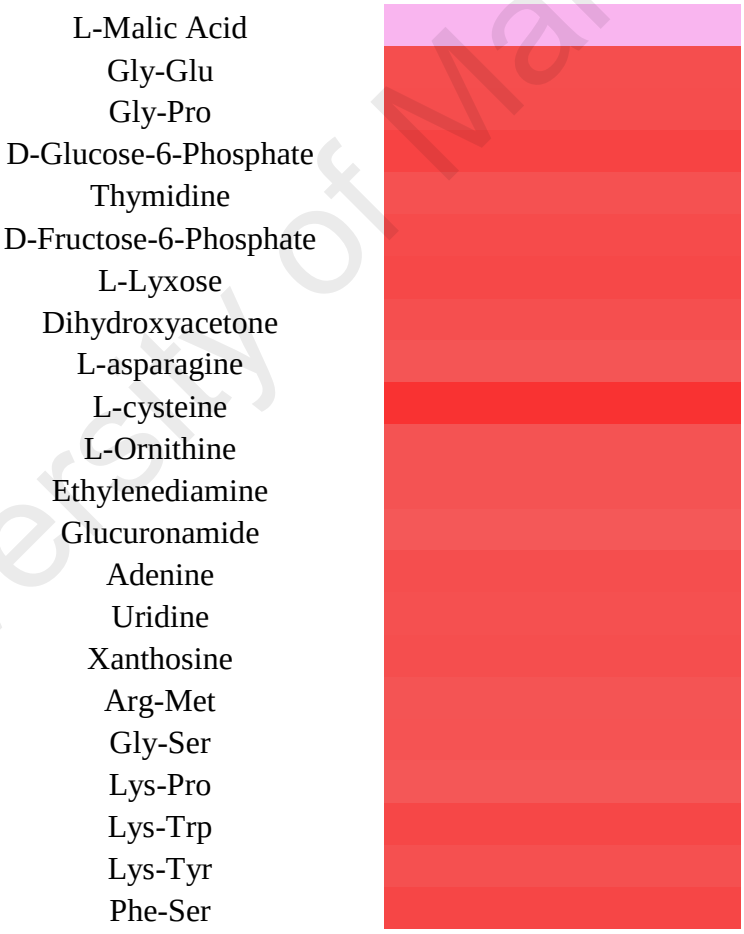


Figure 4.12: Differences of area of kinetic curves between *E. cancerogenus* strain M004 wildtype and mutant. The phenotypes downregulation and upregulation were illustrated by the intensity of red and purple coloration respectively.

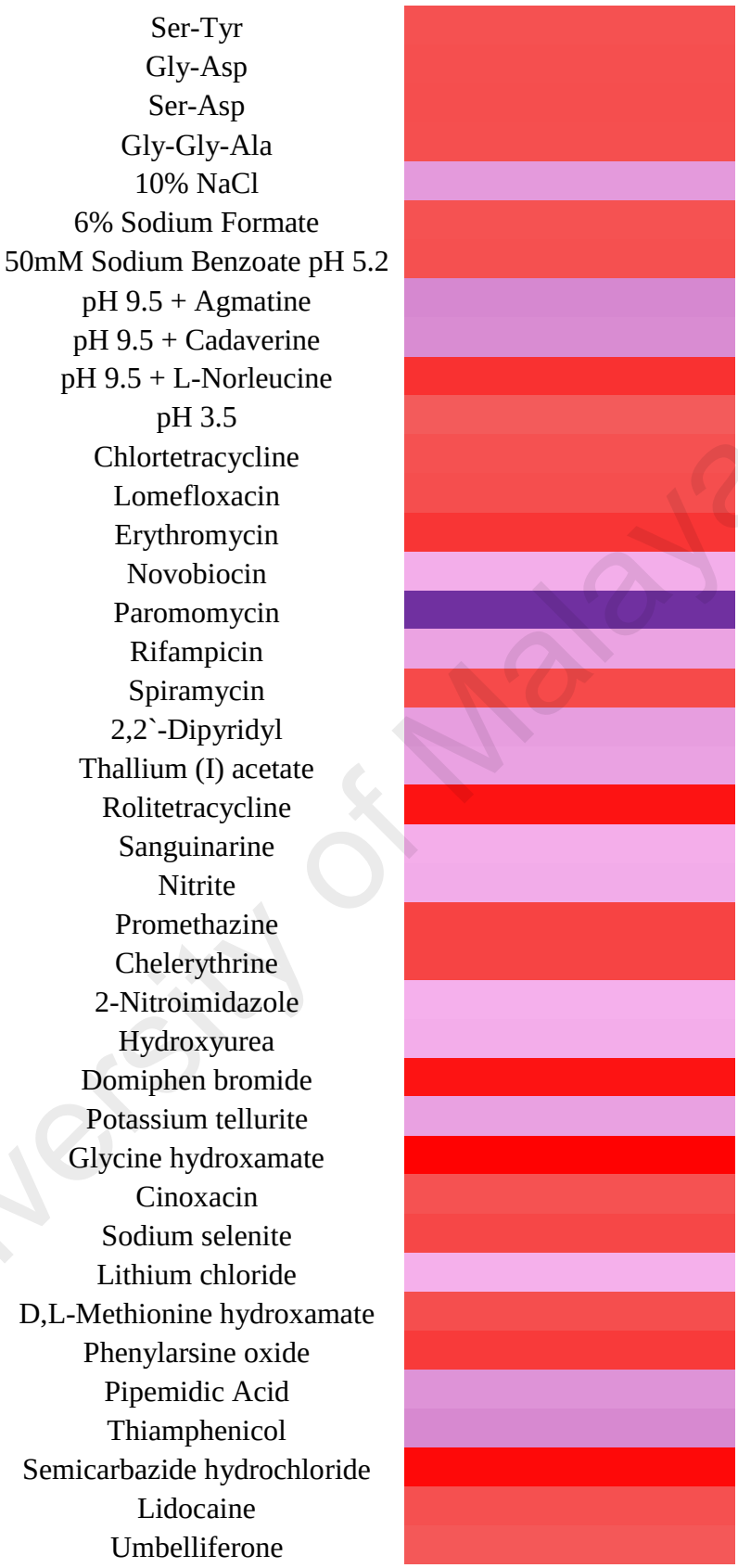


Figure 4.12: continued.

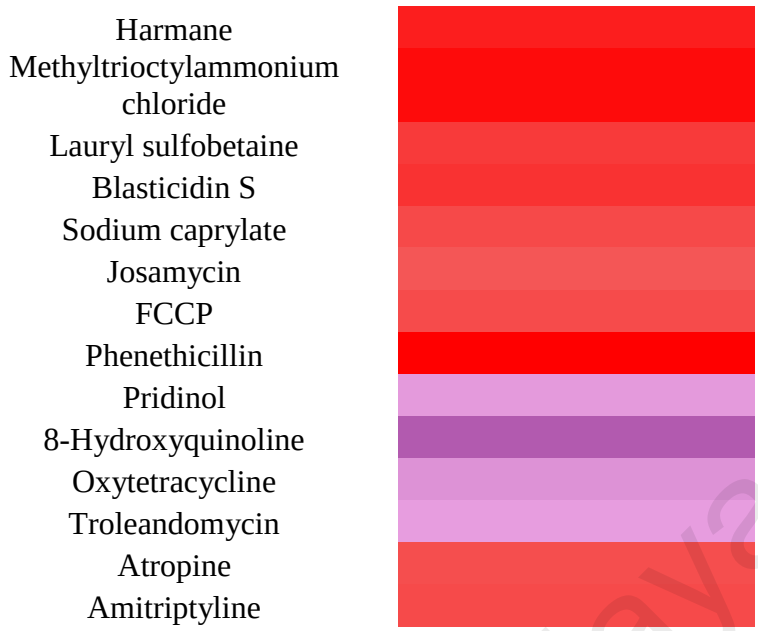
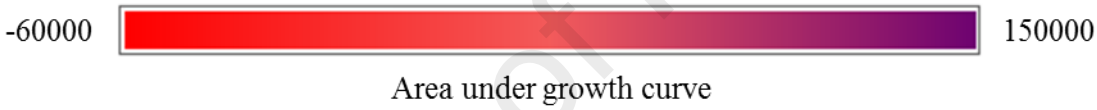


Figure 4.12: continued.

Legend:



Several phenotypes were then selected for further confirmation studies. Four phenotypes which belonged to antimicrobial agents, namely lomefloxacin, erythromycin, rifampicin and pipemidic acid were selected and subsequently independent studies were conducted. All assays were biological replicates and assayed in triplicates. In the agar disk diffustion tests, the differences between wildtype and mutant observed were in good accordance with the results of PM analysis (Figure 4.13). The figures of the antimicrobial susceptibility disk tests were supplied in Supplementary Appendix G.

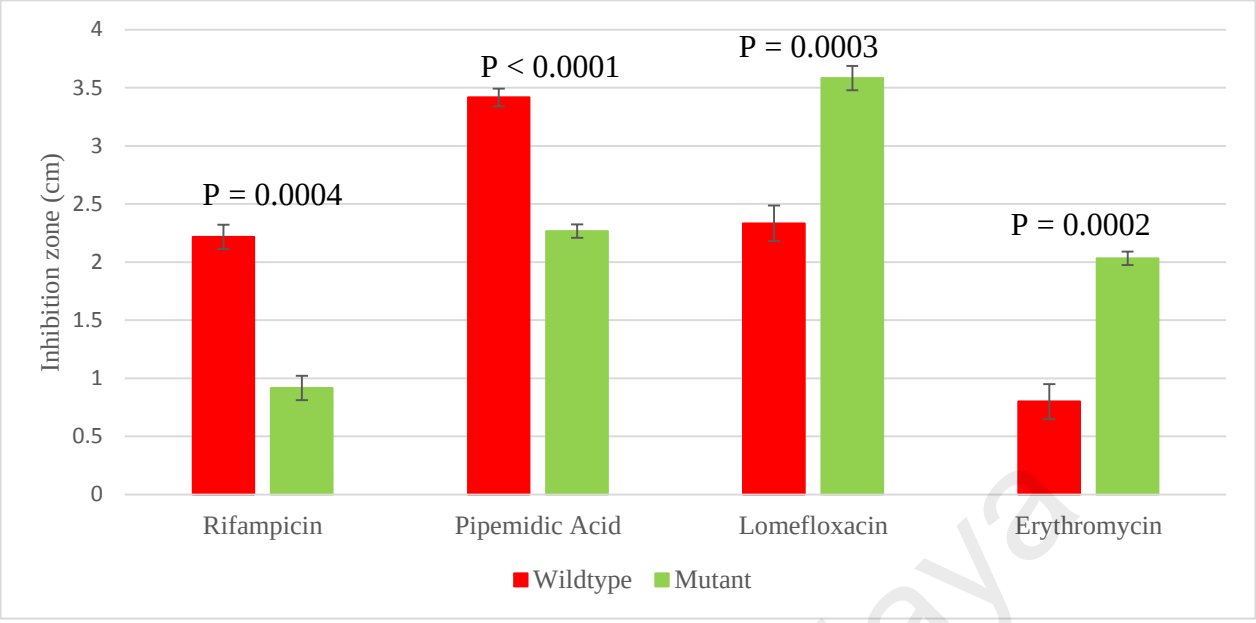


Figure 4.13: The diameter of clear inhibition zone in the *E. cancerogenus* strain M004 wildtype and mutant independent studies. All assays were carried out in triplicates. Bars: standard errors of the mean; unpaired t-test, $p < 0.05$.

4.9 Transcriptomic Profiling

Transcriptome studies were conducted on (i) *E. cancerogenus* strain M004 treated with a known anti-QS compound, gallic acid while non-treated act as control; and (ii) *E. cancerogenus* strain M004 wildtype with its mutant (Δ*ecnI-1::Kan*^{r}). In both the independent studies, Partek Genomic Suite was used to analyze the distribution of genes expression level in treated against treated samples and mutant against wildtype samples. Both untreated condition and wildtype served as control. The relative quantification of sequenced RNAs were measured by reads per kilobase of transcripts per million mapped reads (RPKM). The filtering and selection criteria for the differential expressed genes was two-fold changes with $\log_2$ ratio of RPKM values (S. Kim et al., 2013). In the study of gallic acid (treated vs. untreated), a total of 221 genes were up-regulated and 165 genes were down-regulated. On the other hand, a total of 68 genes were up-regulated and 115 genes were down-regulated in the study as mutant was compared against wildtype. Tables 4.7 and 4.8 showed the differential expressed genes from the RNA-seq analysis of gallic acid and mutation studies respectively.

Table 4.7: Expression profiling of genes involved in the gallic acid treatment.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
Up-regulated		
HTH-type transcriptional regulator BetI	0.00094	5.89603
NAD/NADP-dependent betaine aldehyde dehydrogenase	0.00039	4.29164
Oxygen-dependent choline dehydrogenase	0.00046	4.13615
hypothetical protein	0.01093	3.03965
hypothetical protein	0.00121	2.90762
Ribonuclease	0.00208	2.87452
Glucose--fructose oxidoreductase precursor	0.03388	2.65612
hypothetical protein	0.01039	2.44867
Homoserine O-acetyltransferase	0.00020	2.44780
L-arabinose-binding periplasmic protein precursor	0.00559	2.42281
Fimbrial protein precursor	0.01767	2.33041
hypothetical protein	0.04257	2.32403
Ribose transport system permease protein RbsC	0.00919	2.26083
F pilus assembly Type-IV secretion system for plasmid transfer	0.03755	2.13564
Fructose dehydrogenase small subunit precursor	0.01007	2.09086
Methyl-accepting chemotaxis protein III	0.01675	2.05668
Exopolysaccharide production protein YjbE	0.01081	2.03082
L-glyceraldehyde 3-phosphate reductase	0.00165	1.96969
hypothetical protein	0.01412	1.93719
Putative osmoprotectant uptake system substrate-binding protein OsmF precursor	0.00697	1.92083

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
	Up-regulated	
hypothetical protein	0.03548	1.90065
Gluconate 2-dehydrogenase subunit 3 precursor	0.00437	1.88639
HTH-type transcriptional regulator GntR	0.02463	1.87145
Hemolysin transporter protein ShlB precursor	0.00034	1.78527
D-ribose pyranase	0.00231	1.77893
molybdate ABC transporter periplasmic substrate-binding protein	0.00371	1.79898
Stress-induced bacterial acidophilic repeat motif protein	0.04397	1.77047
hypothetical protein	0.01352	1.76375
Malonyl-S-ACP:biotin-protein carboxyltransferase MADD	0.01114	1.76160
hypothetical protein	0.00970	1.75322
Glycerol dehydrogenase	0.00919	1.74167
hypothetical protein	0.00058	1.71941
hypothetical protein	0.00332	1.71642
1,5-anhydro-D-fructose reductase	0.00450	1.70275
hypothetical protein	0.00210	1.68717
Arginine transport system permease protein ArtQ	0.01703	1.68465
Flavohemoprotein	0.00540	1.68294
Streptogramin A acetyltransferase	0.00127	1.67555
Gluconate 2-dehydrogenase flavoprotein precursor	0.00099	1.67442
hypothetical protein	0.03402	1.65635

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
Up-regulated		
Catecholate siderophore receptor Fiu precursor	0.00051	1.65619
Methyl-accepting chemotaxis protein I	0.00236	1.65185
Aerobactin synthase	0.03963	1.64525
Multiple antibiotic resistance protein MarA	0.00387	1.64308
ATP-dependent dethiobiotin synthetase BioD 1	0.02322	1.63248
hypothetical protein	0.00496	1.62789
hypothetical protein	0.00675	1.59940
hypothetical protein	0.00243	1.59918
Pertactin autotransporter precursor	0.00062	1.57041
Cyclic di-GMP phosphodiesterase response regulator RpfG	0.01542	1.56823
HTH-type transcriptional repressor NicR	0.01659	1.55857
Xylose isomerase-like TIM barrel	0.03174	1.55283
Ribose import ATP-binding protein RbsA	0.00012	1.54202
Inner membrane protein YohK	0.00732	1.54129
Homoserine O-succinyltransferase	0.00406	1.53930
Cupin domain protein	0.03520	1.53533
2,6-dihydropseudooxynicotine hydrolase	0.00166	1.53152
dTDP-3-amino-3,6-dideoxy-alpha-D-galactopyranose 3-N-acetyltransferase	0.01774	1.49992
Aspartate aminotransferase	0.04485	1.49541
hypothetical protein	0.00016	1.49155

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
	Up-regulated	
hypothetical protein	0.00947	1.49044
Protein TolB	0.00588	1.48357
Flavin reductase like domain protein	0.03337	1.47518
Major exported protein	0.01101	1.47358
HTH-type transcriptional regulator DmlR	0.04939	1.47305
Putative 2-aminoethylphosphonate transport system permease protein PhnV	0.02408	1.47158
Putative osmoprotectant uptake system permease protein YehW	0.00481	1.46858
Ribose transport system permease protein RbsC	0.02857	1.46197
hypothetical protein	0.01780	1.45345
Serine/threonine-protein kinase pkn1	0.00568	1.45242
Peroxiredoxin OsmC	0.03855	1.44377
Proline/betaine transporter	0.02163	1.44170
Chaperone protein ClpB	0.00024	1.43656
putative glucarate transporter	0.03010	1.43032
Chemotaxis protein CheY	0.00191	1.42855
Aldose 1-epimerase	0.00816	1.42805
hypothetical protein	0.00266	1.41859
UV DNA damage endonuclease	0.00927	1.41800
hypothetical protein	0.01627	1.41654
ADP-ribose pyrophosphatase	0.00284	1.41622

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
	Up-regulated	
Lysine-arginine-ornithine-binding periplasmic protein precursor	0.00209	1.41557
Phenolic acid decarboxylase subunit C	0.01157	1.41456
D-alanyl-D-alanine carboxypeptidase DacD precursor	0.00066	1.41053
putative HTH-type transcriptional regulator YdfH	0.00807	1.40916
hypothetical protein	0.00023	1.40527
Multidrug resistance protein stp	0.00513	1.40210
Proline/betaine transporter	0.01702	1.39954
hypothetical protein	0.00142	1.39342
Ribosomal protein S12 methylthiotransferase RimO	0.01045	1.37906
Outer membrane porin F precursor	0.00217	1.37695
Gluconate 2-dehydrogenase flavoprotein precursor	0.04647	1.37172
hypothetical protein	0.00039	1.37168
hypothetical protein	0.00132	1.36766
Ribonuclease T	0.00451	1.35552
Fructokinase	0.00084	1.35280
hypothetical protein	0.01726	1.35167
Inner membrane protein YqjF	0.00233	1.34896
hypothetical protein	0.00537	1.34520
hypothetical protein	0.02587	1.34452
5-keto-4-deoxy-D-glucarate aldolase	0.00017	1.34338
Flagellar hook-associated protein 1	0.00043	1.34117

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
	Up-regulated	
hypothetical protein	0.01912	1.33984
Stage V sporulation protein D	0.00199	1.33769
ribosomal-protein-L7/L12-serine acetyltransferase	0.01091	1.32598
hypothetical protein	0.01100	1.32490
hypothetical protein	0.01990	1.32203
Guanidinobutyrase	0.00569	1.31865
hypothetical protein	0.00022	1.31739
Peptidase M15	0.00019	1.30661
hypothetical protein	0.01932	1.30660
Glucarate dehydratase	0.01659	1.30415
Proline/betaine transporter	0.02964	1.29909
Bacterial regulatory proteins, gntR family	0.01081	1.29335
Arabinose import ATP-binding protein AraG	0.00323	1.28717
Tyrosine recombinase XerC	0.03540	1.28268
Multiple stress resistance protein BhsA precursor	0.04575	1.27244
Inner membrane protein YfeZ	0.01698	1.27034
Phosphocarrier protein HPr	0.00380	1.26659
hypothetical protein	0.02356	1.26048
Plasmid SOS inhibition protein (PsiB)	0.04078	1.25708
putative RNA polymerase sigma factor FecI	0.02814	1.24804
hypothetical protein	0.00202	1.24377
hypothetical protein	0.04878	1.24341

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
	Up-regulated	
Nitrate regulatory protein	0.04356	1.23932
hypothetical protein	0.00131	1.23584
Acyl-CoA thioesterase 2	0.02607	1.23019
putative HTH-type transcriptional regulator YdcR	0.01688	1.22714
Proline/betaine transporter	0.00012	1.22546
5-keto-4-deoxy-D-glucarate aldolase	0.00938	1.22460
Type VI secretion lipoprotein	0.00665	1.21546
hypothetical protein	0.01296	1.20975
4-oxalmesaconate hydratase	0.00251	1.20851
putative diguanylate cyclase YedQ	0.02001	1.20376
hypothetical protein	0.00608	1.20370
Prophage CP4-57 regulatory protein (AlpA)	0.03349	1.20239
hypothetical protein	0.00780	1.19995
Galactose/methyl galactoside import ATP-binding protein MglA	0.01242	1.19885
putative diguanylate cyclase YdaM	0.01504	1.19368
FhuE receptor precursor	0.00024	1.19289
Cell division protein ZapB	0.01040	1.18393
Chemotaxis protein methyltransferase	0.00196	1.18108
hypothetical protein	0.04226	1.17345
hypothetical protein	0.02949	1.17027
Cytochrome c-type biogenesis protein CcmH precursor	0.00471	1.16959

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
	Up-regulated	
hypothetical protein	0.00145	1.16933
6-phosphogluconate phosphatase	0.04240	1.16888
hypothetical protein	0.01341	1.16885
D-ribose-binding periplasmic protein precursor	0.04491	1.16351
Alpha-D-glucose-1-phosphate phosphatase YihX	0.00080	1.15832
hypothetical protein	0.02585	1.15773
CsbD-like protein	0.02352	1.15311
50S ribosomal protein L14	0.01314	1.14474
hypothetical protein	0.00122	1.14352
hypothetical protein	0.04457	1.14296
Methyl-accepting chemotaxis protein I	0.01202	1.14252
Glycine zipper 2TM domain protein	0.00336	1.13852
hypothetical protein	0.02226	1.13506
hypothetical protein	0.00623	1.13327
hypothetical protein	0.00956	1.13148
putative HTH-type transcriptional regulator YbbH	0.01862	1.12700
Putative aliphatic sulfonates transport permease protein SsuC	0.01734	1.12650
HTH-type transcriptional regulator GntR	0.04051	1.12617
hypothetical protein	0.00480	1.12369
putative two-component-system connector protein YcgZ	0.02874	1.12182

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
Up-regulated		
Ascorbate-specific phosphotransferase enzyme IIA component	0.01788	1.11930
hypothetical protein	0.01795	1.11900
Hca operon transcriptional activator	0.00708	1.11750
Chemotaxis response regulator protein-glutamate methylesterase	0.00366	1.10998
hypothetical protein	0.01319	1.10994
putative two-component-system connector protein YcgZ	0.00813	1.10679
Inositol 2-dehydrogenase/D-chiro-inositol 3-dehydrogenase	0.00344	1.10591
Fosmidomycin resistance protein	0.00944	1.10437
hypothetical protein	0.00969	1.09528
hypothetical protein	0.00836	1.08674
putative D,D-dipeptide-binding periplasmic protein DdpA precursor	0.00118	1.08494
OPT oligopeptide transporter protein	0.00018	1.07955
hypothetical protein	0.04387	1.07802
hypothetical protein	0.00248	1.07523
hypothetical protein	0.00048	1.07220
Putrescine importer PuuP	0.01797	1.07015
hypothetical protein	0.00585	1.06788
D-amino acid dehydrogenase small subunit	0.00070	1.06587

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
	Up-regulated	
hypothetical protein	0.00031	1.06301
Formylglycine-generating sulfatase enzyme	0.00113	1.06240
hypothetical protein	0.00283	1.06227
hypothetical protein	0.01052	1.06044
hypothetical protein	0.00056	1.05976
hypothetical protein	0.01624	1.05830
DNA-directed RNA polymerase subunit omega	0.00667	1.05419
hypothetical protein	0.01165	1.05392
Phage lysozyme	0.03660	1.05156
Motility protein A	0.00418	1.05096
ECF RNA polymerase sigma factor SigK	0.02785	1.05091
Phage shock protein D	0.02284	1.04925
hypothetical protein	0.03419	1.04720
Indole-3-acetyl-aspartic acid hydrolase	0.02806	1.04351
hypothetical protein	0.01227	1.04308
Cyclic di-GMP phosphodiesterase YhjH	0.03859	1.03812
Sensor protein QseC	0.00893	1.03551
HTH-type transcriptional regulator MurR	0.01591	1.03142
Acylamidase	0.03668	1.03075
hypothetical protein	0.01502	1.03057
hypothetical protein	0.00170	1.02421
hypothetical protein	0.00246	1.02348

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
	Down-regulated	
Antitoxin HigA	0.04533	1.02106
N-acetylmuramoyl-L-alanine amidase AmiA precursor	0.01138	1.01834
Gluconate 2-dehydrogenase subunit 3 precursor	0.00052	1.01675
Methyl-accepting chemotaxis protein III	0.03737	1.01348
putative lipoprotein YgdR precursor	0.00779	1.01150
hypothetical protein	0.02930	1.00352
HMP-PP phosphatase	0.03040	1.00319
Dihydrolipoyl dehydrogenase	0.00373	1.00286
Formylglycine-generating sulfatase enzyme	0.00429	1.00220
hypothetical protein	0.00084	-3.14889
Gluconolactonase precursor	0.00379	-2.60022
Inner membrane protein YebE	0.00734	-2.59671
PAAR motif protein	0.00097	-2.50618
Iron(3+)-hydroxamate import ATP-binding protein FhuC	0.02865	-2.44729
Octopine permease ATP-binding protein P	0.02016	-2.42011
Cold shock-like protein CspE	0.02739	-2.41404
50S ribosomal protein L34	0.03178	-2.40630
hypothetical protein	0.03356	-2.38007
Spermidine export protein MdtJ	0.00892	-2.37497
Iron(3+)-hydroxamate import ATP-binding protein FhuC	0.02337	-2.35658
50S ribosomal protein L32	0.00791	-2.32937
hypothetical protein	0.02371	-2.14916

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
	Down-regulated	
Cold shock-like protein CspC	0.01020	-2.07789
Major outer membrane lipoprotein Lpp precursor	0.02041	-1.98941
Transcriptional regulatory protein RcsB	0.00075	-1.95801
hypothetical protein	0.03877	-1.94095
Inner membrane protein YqjE	0.02858	-1.85768
Periplasmic protein CpxP precursor	0.00020	-1.83109
3',5'-cyclic adenosine monophosphate phosphodiesterase CpdA	0.01839	-1.82650
Methionine import ATP-binding protein MetN	0.03749	-1.82241
N-formylglutamate amidohydrolase	0.03106	-1.80333
hypothetical protein	0.01650	-1.76868
50S ribosomal protein L33	0.02570	-1.74608
putative MFS-type transporter YhjX	0.00038	-1.73947
putative D,D-dipeptide transport ATP-binding protein DdpF	0.03296	-1.70438
Crossover junction endodeoxyribonuclease RuvC	0.01664	-1.68636
Cytochrome bo(3) ubiquinol oxidase subunit 3	0.03183	-1.67932
Putative acid--amine ligase YgiC	0.00671	-1.67088
Thiazole synthase	0.00491	-1.66708
HTH-type transcriptional regulator cbl	0.00068	-1.66231
Pyridoxal phosphate phosphatase YigL	0.02974	-1.65452
Prolipoprotein diacylglyceryl transferase	0.01634	-1.63009

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
	Down-regulated	
30S ribosomal protein S5	0.04846	-1.60926
8-oxoguanine deaminase	0.01498	-1.56704
Protoheme IX farnesyltransferase	0.04505	-1.56590
hypothetical protein	0.01813	-1.54602
NADH dehydrogenase	0.00470	-1.53948
2-dehydro-3-deoxy-6-phosphogalactonate aldolase	0.01807	-1.53397
TraU protein	0.02483	-1.52635
Protein YceI	0.00478	-1.51673
Hydroxymethylpyrimidine/phosphomethylpyrimidine kinase	0.00248	-1.50626
50S ribosomal protein L20	0.00842	-1.49603
Flagellar assembly protein FliH	0.04092	-1.46942
Zinc import ATP-binding protein ZnuC	0.01232	-1.46795
50S ribosomal protein L25	0.00367	-1.46055
putative amino-acid-binding protein YxeM precursor	0.01138	-1.44471
Outer membrane protein F precursor	0.02663	-1.43072
Cys-tRNA(Pro)/Cys-tRNA(Cys) deacylase YbaK	0.00341	-1.42265
50S ribosomal protein L36 2	0.00848	-1.40317
Flagellar motor switch protein FliN	0.02564	-1.39781
Ribosome-binding factor A	0.03537	-1.38110
ATP synthase subunit delta	0.01330	-1.37979
Flagellar motor switch protein FliM	0.00360	-1.35957

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
	Down-regulated	
Phospholipase YtpA	0.00690	-1.35929
tRNA-specific adenosine deaminase	0.03976	-1.35745
hypothetical protein	0.01269	-1.35464
Ribosomal protein L11 methyltransferase	0.02479	-1.34001
ATP synthase protein I	0.00827	-1.33380
DNA-binding protein Fis	0.04233	-1.33082
Phosphatidate cytidylyltransferase	0.01927	-1.32785
hypothetical protein	0.02996	-1.31679
hypothetical protein	0.01384	-1.31678
Ribosome modulation factor	0.00915	-1.31598
Inososе isomerase	0.00907	-1.31422
hypothetical protein	0.02500	-1.31391
Multiple antibiotic resistance protein MarA	0.02808	-1.31286
Purine nucleoside phosphorylase DeoD-type	0.01357	-1.31058
Fimbria A protein precursor	0.00076	-1.30218
hypothetical protein	0.01101	-1.29071
Inner membrane protein YeiU	0.00698	-1.28984
D-allose-binding periplasmic protein precursor	0.01282	-1.28026
Iron(3+)-hydroxamate import ATP-binding protein FhuC	0.03994	-1.27897
Flagellar basal-body rod protein FlgF	0.04013	-1.27712
hypothetical protein	0.00397	-1.26928
HTH-type transcriptional regulator IscR	0.04494	-1.26581

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
	Down-regulated	
hypothetical protein	0.01715	-1.26475
tRNA-dihydrouridine synthase C	0.02556	-1.26458
Biopolymer transport protein ExbB	0.04196	-1.25966
hypothetical protein	0.03389	-1.25842
Inner membrane protein YnbA	0.00234	-1.24917
Aspartate-semialdehyde dehydrogenase	0.02253	-1.24103
Succinate dehydrogenase hydrophobic membrane anchor subunit	0.01669	-1.24019
Ribosomal RNA small subunit methyltransferase J	0.02475	-1.23234
D-alanyl-D-alanine carboxypeptidase DacC precursor	0.00268	-1.23146
Protein-export membrane protein SecF	0.03445	-1.23128
putative periplasmic iron-binding protein precursor	0.01795	-1.22546
23S rRNA (guanosine-2'-O-)-methyltransferase RlmB	0.01478	-1.21322
Putative peroxiredoxin bcp	0.00028	-1.21114
Cell division protein FtsP precursor	0.02994	-1.21040
HTH-type transcriptional regulator CdhR	0.00213	-1.20173
Methionine import system permease protein MetP	0.03996	-1.20008
Phosphinothricin N-acetyltransferase	0.02623	-1.18535
Protein Ves	0.02194	-1.17887
hypothetical protein	0.00439	-1.17598
hypothetical protein	0.00347	-1.16842
Putative metal chaperone YciC	0.00658	-1.16724

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
	Down-regulated	
hypothetical protein	0.02948	-1.16579
inner membrane protein	0.00715	-1.16570
hypothetical protein	0.02066	-1.15734
Stringent starvation protein B	0.02804	-1.15580
Molybdopterin synthase catalytic subunit	0.04393	-1.15484
Biopolymer transport protein ExbD	0.02910	-1.14390
ATP synthase subunit b	0.00978	-1.14004
50S ribosomal protein L31	0.01362	-1.13456
Barstar (barnase inhibitor)	0.01190	-1.12720
3-oxoacyl-[acyl-carrier-protein] reductase FabG	0.02466	-1.12364
Pyrimidine-specific ribonucleoside hydrolase RihA	0.03241	-1.12287
30S ribosomal protein S20	0.00806	-1.12208
Octopine transport system permease protein OccM	0.04670	-1.12048
Phospho-2-dehydro-3-deoxyheptonate aldolase, Phe-sensitive	0.02568	-1.11971
Inositol 2-dehydrogenase/D-chiro-inositol 3-dehydrogenase	0.02662	-1.11508
HTH-type transcriptional regulator CynR	0.02122	-1.11257
Lipopolysaccharide export system ATP-binding protein LptB	0.03487	-1.11247
Sulfurtransferase TusD	0.04117	-1.10341
Flagellar basal-body rod protein FlgG	0.04129	-1.10277

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
	Down-regulated	
Ribosomal large subunit pseudouridine synthase A	0.02177	-1.09934
putative lipoprotein YiaD precursor	0.03669	-1.09930
Ferric enterobactin receptor precursor	0.01320	-1.09474
Inner membrane amino-acid ABC transporter permease protein YecS	0.01330	-1.09240
Transcriptional regulatory protein OmpR	0.01596	-1.09182
hypothetical protein	0.01120	-1.08894
Flagellar P-ring protein precursor	0.03094	-1.08667
HTH-type transcriptional regulator PuuR	0.00700	-1.08520
Gamma-glutamylcyclotransferase family protein YtfP	0.00266	-1.08513
2-deoxyglucose-6-phosphate phosphatase	0.01321	-1.08418
Putative metal chaperone YciC	0.00564	-1.08068
Putative uroporphyrinogen-III C-methyltransferase	0.03229	-1.07986
Persistence and stress-resistance toxin PasT	0.00132	-1.07929
Acylphosphatase	0.04604	-1.07770
Flagellar hook protein FlgE	0.01571	-1.07581
Peptidoglycan hydrolase FlgJ	0.02373	-1.07444
HTH-type transcriptional repressor of iron proteins A	0.01205	-1.07121
30S ribosomal protein S18	0.00563	-1.07041
Aldehyde reductase Ahr	0.01751	-1.06437
Integration host factor subunit alpha	0.03225	-1.06426

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
	Down-regulated	
Membrane-bound lytic murein transglycosylase D precursor	0.00397	-1.06407
Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha	0.01111	-1.06387
Inositol-1-monophosphatase	0.02997	-1.06301
hypothetical protein	0.01899	-1.06244
Flagellum site-determining protein YlxH	0.03878	-1.05995
Phosphatidate cytidylyltransferase	0.02698	-1.05828
30S ribosomal protein S6	0.00221	-1.05196
Inner membrane protein YohC	0.00239	-1.05127
3-ketoacyl-CoA thiolase	0.01369	-1.04735
hypothetical protein	0.03939	-1.04700
D-methionine transport system permease protein MetI	0.00133	-1.04578
Manganese ABC transporter substrate-binding lipoprotein precursor	0.00169	-1.04481
hypothetical protein	0.04869	-1.03906
30S ribosomal protein S17	0.03367	-1.03488
Low molecular weight protein-tyrosine-phosphatase wzb	0.03882	-1.03383
Aminodeoxyfutalosine deaminase	0.01589	-1.02971
Riboflavin synthase	0.03507	-1.02889
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	0.01192	-1.02295

Table 4.7: continued.

Protein	p-value (Treated vs. Untreated)	Log_2 (Treated vs. Untreated)
	Down-regulated	
3-methyl-2-oxobutanoate hydroxymethyltransferase	0.03573	-1.02280
Ribosomal RNA large subunit methyltransferase G	0.00785	-1.01325
Putative bacterial virulence factor	0.02543	-1.00774
Arginine transport ATP-binding protein ArtM	0.02504	-1.00749
ATP synthase subunit beta	0.01495	-1.00383
Low-affinity putrescine importer PlaP	0.01241	-1.00048

Note: Log_2 (fold change) value is statistically significant ($p < 0.05$) in treated versus untreated conditions.

Table 4.8: Expression profiling of genes involved in the *ecnI*-1 knock-out mutation.

Protein	p-value (Mutant vs. Wildtype)	Log_2 (Mutant vs. Wildtype)
Up-regulated		
hypothetical protein	0.00405	3.88425
Exopolysaccharide production protein YjbE	0.00160	2.51774
hypothetical protein	0.00410	1.96924
hypothetical protein	0.00336	1.87710
Outer membrane porin F precursor	0.00647	1.81982
Flagella basal body P-ring formation protein FlgA precursor	0.00044	1.81384
hypothetical protein	0.01331	1.69559
hypothetical protein	0.02035	1.67854
Stress-induced bacterial acidophilic repeat motif protein	0.01913	1.53154
ATP-dependent Clp protease adapter protein ClpS	0.01181	1.52043
putative 6-phospho-beta-glucosidase	0.01514	1.46934
N,N'-diacetylchitobiose-specific phosphotransferase enzyme IIA component	0.00131	1.43454
Xylose isomerase-like TIM barrel	0.00441	1.42867
30S ribosomal protein S10	0.00119	1.42257
Tyrosine recombinase XerC	0.00109	1.39313
hypothetical protein	0.00725	1.36414
Bacterioferritin	0.01497	1.36051
hypothetical protein	0.00088	1.35428
Oligo-beta-mannoside-specific phosphotransferase enzyme IIB component	0.00026	1.33773

Table 4.8: continued.

Protein	p-value (Mutant vs. Wildtype)	Log_2 (Mutant vs. Wildtype)
Up-regulated		
ABC transporter arginine-binding protein 1 precursor	0.00213	1.33699
putative HTH-type transcriptional regulator YdcR	0.00098	1.32959
6-phospho-beta-glucosidase BglA	0.00292	1.31546
HIT-like protein	0.01389	1.30969
Lichenan permease IIC component	0.00125	1.30210
Proteolipid membrane potential modulator	0.00782	1.28299
Regulator of RpoS	0.01150	1.27387
Flagellar hook-basal body complex protein FliE	0.00188	1.25149
DNA-binding protein H-NS	0.00017	1.23182
hypothetical protein	0.00452	1.22995
Flavin reductase like domain protein	0.00174	1.22913
Pyruvate dehydrogenase complex repressor	0.00281	1.22818
Dipeptide transport system permease protein DppB	0.00739	1.21575
Flagellar protein FliT	0.00085	1.20272
3-deoxy-D-manno-octulosonate 8-phosphate phosphatase KdsC	0.00147	1.19440
hypothetical protein	0.02800	1.19122
Basal-body rod modification protein FlgD	0.00000	1.17485
Flagellar basal-body rod protein FlgC	0.00593	1.17333
Flagellar transcriptional regulator FlhD	0.02795	1.17147
hypothetical protein	0.00542	1.17144
10 kDa chaperonin	0.00909	1.17102

Table 4.8: continued.

Protein	p-value (Mutant vs. Wildtype)	Log_2 (Mutant vs. Wildtype)
	Up-regulated	
HTH-type transcriptional regulator DmlR	0.00310	1.16842
hypothetical protein	0.04275	1.16745
hypothetical protein	0.02314	1.16470
hypothetical protein	0.00935	1.13254
hypothetical protein	0.01450	1.13115
Flagellar protein FliS	0.00480	1.12876
Erythritol kinase	0.00076	1.11732
Isochorismate synthase EntC	0.01957	1.10700
hypothetical protein	0.00819	1.10552
Multiple antibiotic resistance protein MarA	0.00451	1.10355
Inner membrane protein YfeZ	0.02097	1.10224
hypothetical protein	0.00359	1.09443
Lysine-arginine-ornithine-binding periplasmic protein precursor	0.00133	1.09348
Flagellar hook-length control protein	0.00102	1.07858
Antibiotic biosynthesis monooxygenase	0.01986	1.06106
PAP fimbrial minor pilin protein precursor	0.04635	1.05538
Ribose-phosphate pyrophosphokinase	0.03944	1.05163
Acetyltransferase (GNAT) family protein	0.01438	1.04600
Very short patch repair protein	0.04737	1.04574
Peroxiredoxin OsmC	0.02315	1.03780
hypothetical protein	0.01751	1.03401

Table 4.8: continued**.**

Protein	p-value (Mutant vs. Wildtype)	Log_2 (Mutant vs. Wildtype)
Up-regulated		
hypothetical protein	0.00162	1.03077
Negative regulator of flagellin synthesis	0.01552	1.02191
Putative O-methyltransferase/MSMEI_4947	0.00834	1.00573
Glycerol-3-phosphate transporter	0.01617	1.00388
Glucarate dehydratase-related protein	0.00454	1.00247
hypothetical protein	0.01753	1.00228
DNA-binding protein HU-beta	0.00146	1.00179
Down-regulated		
hypothetical protein	0.01448	-3.34578
hypothetical protein	0.02156	-2.80736
Type-F conjugative transfer system pilin assembly protein	0.03217	-2.39855
Sulfate adenylyltransferase subunit 2	0.03070	-2.03623
Glutamine-binding periplasmic protein precursor	0.02282	-2.03477
Sulfate/thiosulfate import ATP-binding protein CysA	0.00417	-1.97054
Sulfate adenylyltransferase subunit 1	0.00926	-1.96159
DNA adenine methyltransferase YhdJ	0.02071	-1.91146
hypothetical protein	0.00156	-1.88042
Adenylyl-sulfate kinase	0.02099	-1.87824
Sulfate transport system permease protein CysW	0.02568	-1.83138
hypothetical protein	0.00237	-1.81497
Maltose/maltodextrin import ATP-binding protein MalK	0.00281	-1.80736
Phosphoadenosine phosphosulfate reductase	0.01210	-1.79680

Table 4.8: continued**.**

Protein	p-value (Mutant vs. Wildtype)	Log_2 (Mutant vs. Wildtype)
	Down-regulated	
Sulfite reductase [NADPH] hemoprotein beta-component	0.03741	-1.73904
Integration host factor subunit beta	0.00750	-1.71213
3-alpha-hydroxysteroid dehydrogenase/carbonyl reductase	0.02671	-1.68147
hypothetical protein	0.02476	-1.64386
Dipeptide transport system permease protein DppC	0.01085	-1.57469
hypothetical protein	0.01508	-1.56635
sulfur carrier protein ThiS	0.02770	-1.55020
L-cystine uptake protein TcyP	0.00349	-1.52863
Flagellar biosynthetic protein FliP precursor	0.02306	-1.51457
hypothetical protein	0.00248	-1.51434
Sulfate transport system permease protein CysT	0.02745	-1.51245
TraK protein	0.00184	-1.50524
Inner membrane protein YqjA	0.00022	-1.49260
hypothetical protein	0.03364	-1.49185
hypothetical protein	0.00227	-1.48543
Gluconate 2-dehydrogenase subunit 3 precursor	0.00963	-1.47999
hypothetical protein	0.02268	-1.47805
50S ribosomal protein L33	0.03912	-1.46896
Iron(3+)-hydroxamate import ATP-binding protein FhuC	0.02105	-1.46713
Arginine transport ATP-binding protein ArtM	0.00417	-1.45066
Putative aliphatic sulfonates transport permease protein SsuC	0.03208	-1.43858

Table 4.8: continued**.**

Protein	p-value (Mutant vs. Wildtype)	Log_2 (Mutant vs. Wildtype)
	Down-regulated	
lipoprotein	0.02276	-1.42450
Nitrilotriacetate monooxygenase component A	0.02790	-1.41913
Nitrate transport protein NrtA precursor	0.02153	-1.37439
leucine/isoleucine/valine transporter permease subunit	0.03074	-1.37049
Transcription antitermination protein RfaH	0.03675	-1.36923
Type IV conjugative transfer system lipoprotein (TraV)	0.00370	-1.36678
50S ribosomal protein L30	0.00805	-1.36558
multidrug transporter membrane component/ATP-binding component	0.00020	-1.36257
Glutathione transport system permease protein GsiC	0.03206	-1.35908
hypothetical protein	0.02623	-1.34792
Biofilm development protein YmgB/AriR	0.00003	-1.34514
50S ribosomal protein L17	0.01089	-1.34215
Methanesulfonate monooxygenase	0.03089	-1.34211
Chaperone protein EcpD precursor	0.03302	-1.33631
hypothetical protein	0.00277	-1.33184
Bacterial conjugation TrbI-like protein	0.03098	-1.33184
Putative 2-aminoethylphosphonate import ATP-binding protein PhnT	0.00276	-1.32616
Inner membrane amino-acid ABC transporter permease protein YecS	0.00226	-1.32611
putative lipoprotein GfcB precursor	0.03024	-1.32567

Table 4.8: continued.

Protein	p-value (Mutant vs. Wildtype)	Log_2 (Mutant vs. Wildtype)
	Down-regulated	
Succinate dehydrogenase iron-sulfur subunit	0.00496	-1.32537
Sensor protein QseC	0.01697	-1.32193
Chemotaxis response regulator protein-glutamate methylesterase	0.03260	-1.32193
Cysteine synthase B	0.00523	-1.31276
Flagellar motor switch protein FliN	0.01139	-1.30550
hypothetical protein	0.03727	-1.30257
30S ribosomal protein S5	0.00442	-1.30021
(R)-stereoselective amidase	0.01973	-1.29123
Aspartate aminotransferase	0.01085	-1.27552
Major outer membrane lipoprotein Lpp precursor	0.00902	-1.19459
Glutathione transport system permease protein GsiC	0.00421	-1.17218
Putative aliphatic sulfonates-binding protein precursor	0.02462	-1.17176
Glyoxylate/hydroxypyruvate reductase A	0.03207	-1.16784
putative hydrolase YxeP	0.02529	-1.16601
Flagellar basal-body rod protein FlgF	0.04232	-1.16294
hypothetical protein	0.02636	-1.16227
TraE protein	0.03892	-1.15966
L-Ala-D/L-Glu epimerase	0.00349	-1.15948
Flagellar biosynthetic protein FlhB	0.04197	-1.15891
Putative 2-aminoethylphosphonate-binding periplasmic protein precursor	0.03882	-1.15807

Table 4.8: continued.

Protein	p-value (Mutant vs. Wildtype)	Log_2 (Mutant vs. Wildtype)
Down-regulated		
Type-F conjugative transfer system protein (TrbI_Ftype)	0.00528	-1.15754
Putative monooxygenase MoxC	0.03436	-1.15381
TraU protein	0.04618	-1.14776
hypothetical protein	0.02700	-1.14303
Putative aliphatic sulfonates transport permease protein SsuC	0.04092	-1.13883
Flagellar hook-basal body complex protein FliE	0.04310	-1.11330
FMN reductase (NADH) RutF	0.00177	-1.11321
Glutathione import ATP-binding protein GsiA	0.02233	-1.11171
Putrescine transport system permease protein PotH	0.04293	-1.10383
1-deoxy-D-xylulose-5-phosphate synthase	0.02965	-1.09882
Putative gamma-glutamyltransferase YwrD	0.02866	-1.09558
Succinate dehydrogenase hydrophobic membrane anchor subunit	0.02361	-1.08125
Spermidine/putrescine import ATP-binding protein PotA	0.02868	-1.07276
Inner membrane protein YqjF	0.03401	-1.07215
Flagellar M-ring protein	0.03416	-1.07088
Inner membrane amino-acid ABC transporter permease protein YecS	0.00300	-1.06926
Inositol 2-dehydrogenase	0.02788	-1.06343
NADH-quinone oxidoreductase subunit K	0.01227	-1.06159
50S ribosomal protein L31	0.04746	-1.04978

Table 4.8: continued.

Protein	p-value (Mutant vs. Wildtype)	Log_2 (Mutant vs. Wildtype)
	Down-regulated	
Protein-export membrane protein SecF	0.01988	-1.03431
NMT1/THI5 like protein	0.02673	-1.03157
Sulfate transport system permease protein CysT	0.00450	-1.02928
hypothetical protein	0.04583	-1.02106
Putative aminoacrylate peracid reductase RutC	0.02188	-1.01707
Gluconate 2-dehydrogenase flavoprotein precursor	0.03402	-1.01443
Glutamine transport system permease protein GlnP	0.00042	-1.01372
Putative 2-aminoethylphosphonate-binding periplasmic protein precursor	0.00251	-1.01355
High-affinity branched-chain amino acid transport system permease protein LivH	0.03480	-1.01290
Flagellar hook protein FlgE	0.03784	-1.01168
Spermidine export protein MdtJ	0.04776	-1.01089
UDP-glucose 6-dehydrogenase	0.00788	-1.00616
Allophanate hydrolase	0.00387	-1.00595
Branched-chain amino acid transport system / permease component	0.03505	-1.00572
putative glycosyltransferase EpsJ	0.04059	-1.00313
hypothetical protein	0.01705	-1.00000
hypothetical protein	0.01912	-1.00000
Bicarbonate transport ATP-binding protein CmpD	0.02966	-1.00000
Putative acyl-CoA dehydrogenase YdbM	0.00350	-1.00000

Table 4.8: continued.

Protein	p-value (Mutant vs. Wildtype)	Log_2 (Mutant vs. Wildtype)
	Down-regulated	
Acylamidase	0.03550	-1.00000
L-cystine import ATP-binding protein TcyC	0.03854	-1.00000

Note: Log_2 (fold change) value is statistically significant ($p < 0.05$) in mutant versus wildtype conditions.

Functional annotation (Clusters of Orthologous Groups, COGs) provides the insight of funtional category distribution of differential expressed genes in the RNA-seq study. This category enabled the understanding of metabolic pathways, multi-subunit complexes or protein classes that make up the functional gene roles involved upon treated with gallic acid or in *ecnI*-1 knock out mutation in *E. cancerogenus* strain M004. RPSBLAST program on COGs database was employed in the server of WebMGA to conduct the functional annotation (Altschul, Gish, Miller, Myers, & Lipman, 1990). In the RNA-seq study where *E. cancerogenus* strain M004 was treated with gallic acid, there is a total of 386 genes expressed differentially. Out of the 386 genes, only 287 genes were assigned (Figure 4.14) and the most abundant COG feature belonged to function unknown (n=35 out of 287 differential expressed genes), followed by transcription (n=29) and then multiple classes (n=28).

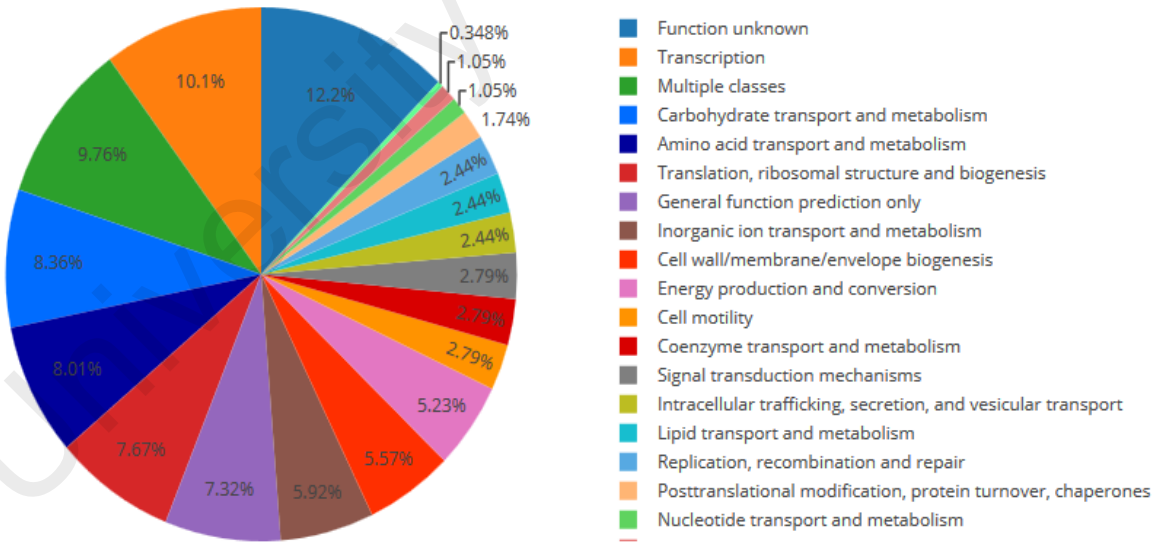


Figure 4.14: Genes associated with general COG functional categories. The RNA-seq of gallic acid-treated study in *E. cancerogenus* strain M004.

On the other hand, RNA-seq data analysis showed a total of 183 differential expressed genes when mutant was compared against wildtype. From the functional annotation (COGs), only a total of 139 genes were assigned to its COG (Figure 4.15) and most abundant COG feature belonged to multiple classes (n=29 out of entire 183 differentially expressed genes), followed by inorganic ion transport and metabolism (n=16) and amino acids transport and metabolism (n=16).

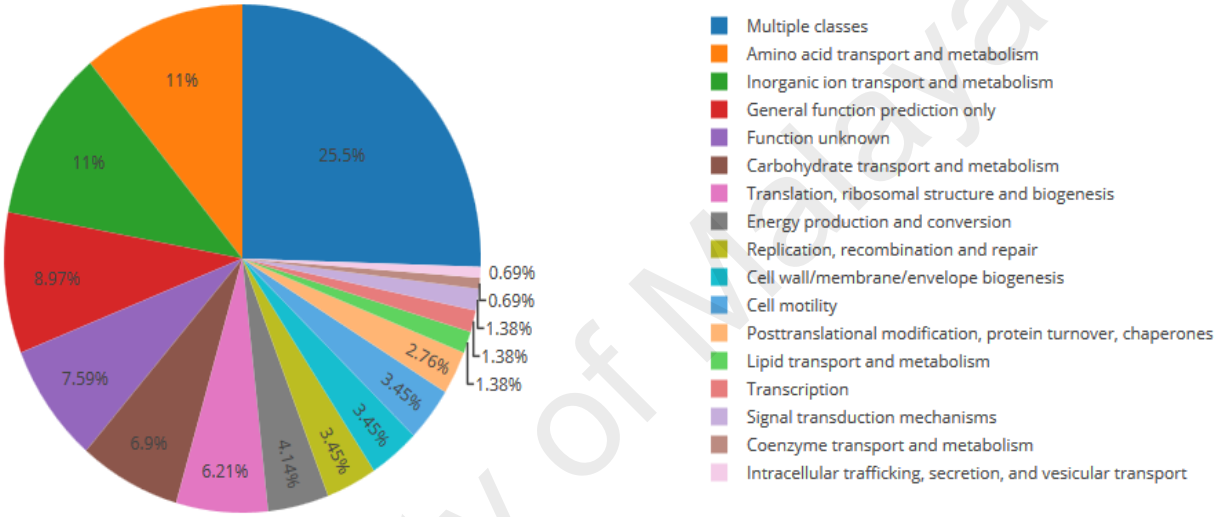


Figure 4.15: Genes associated with general COG functional categories for *E. cancerogenus* strain M004. The RNA-seq of mutational study in *E. cancerogenus* strain M004.

Venn diagram was generated by Venn diagram generator from Bioinformatics.lu to identify the relationships of differential genes expression of RNA-seq between the gallic acid and mutation study. The overlapping circles indicates the 27 genes were similar and influenced by both gallic acid and mutation (Figure 4.16). Amino acid sequences of the similar genes involved were functional annotated by WebMGA server (Altschul, Gish, Miller, Myers, & Lipman, 1990). From the analysis, 18 out of 27 genes associated with general COG functional categories is shown in Figure 4.17.

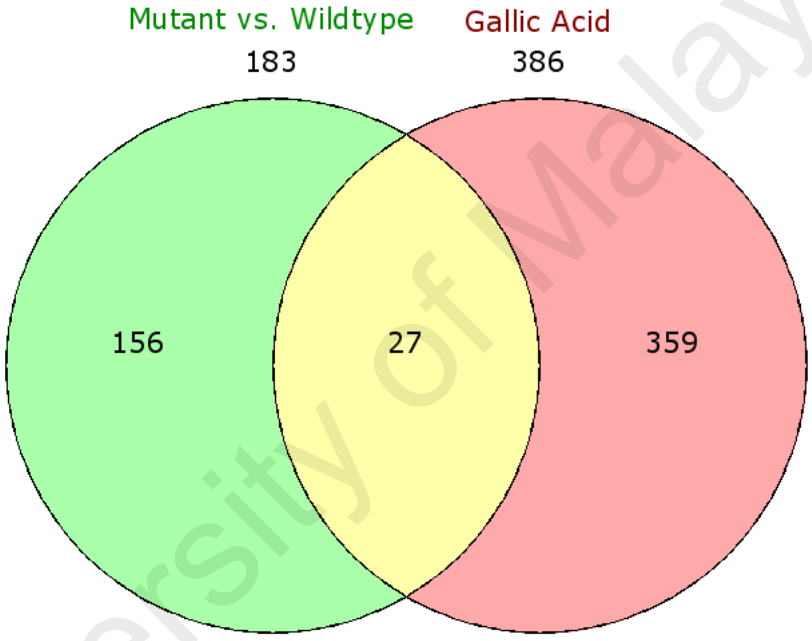


Figure 4.16: Venn diagram indicating the similarities and differences of differential *E. cancerogenus* strain M004 genes expression in RNA-seq between gallic acid and mutation study. The overlapped region (n = 27) is statistically significant, $p < 0.05$ (R's phyper test, $p = 0.000184$).

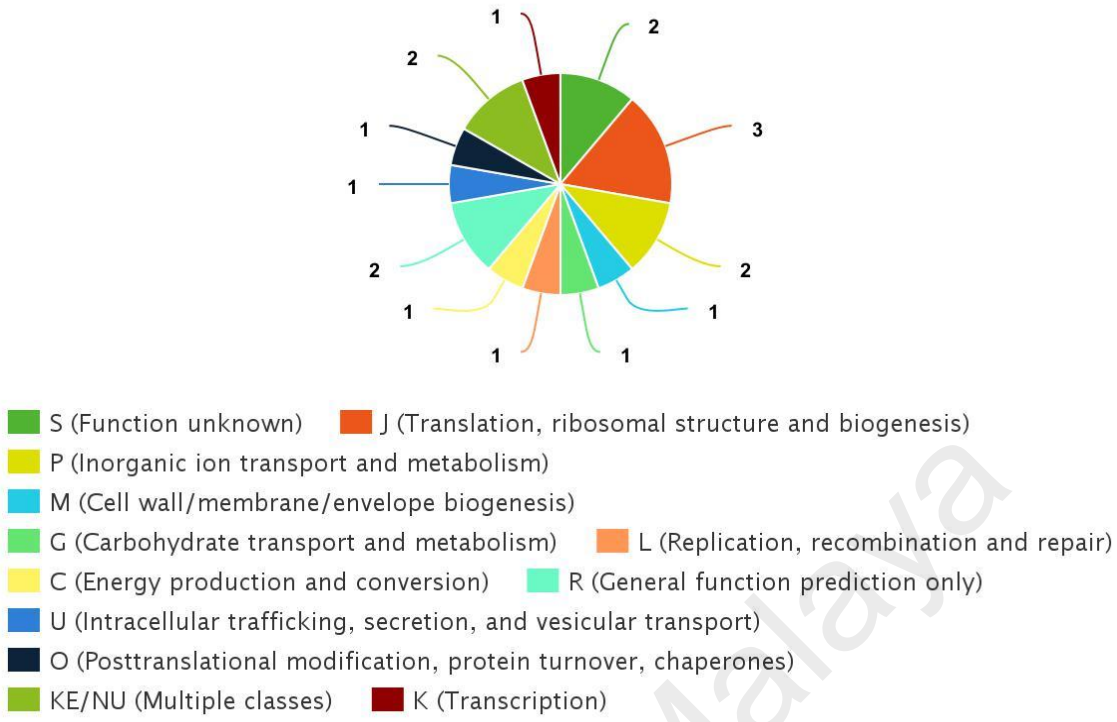


Figure 4.17: Count of genes associated with general COG functional categories.

4.9.1 Quantitative Real-time Polymerase Chain Reaction (qRT-PCR) Validation of RNA-seq Experiments

The qRT-PCR validation to validate RNA-seq results for both studies were conducted independently. Six of the QS-regulated genes from each RNA-seq study were randomly selected for validation with gene-specific primers (listed in Table 3.5). The values obtained were of average of biological triplicates. According to the results from qRT-PCR inluding the reference genes (*gyrA*, *recA* and *rpoS*), the trends of QS-regulated genes expression patterns were in good accordance with the expression levels obtained through qRT-PCR; Figure 4.18 showing the qRT-PCR validation of gallic acid-treated RNA-seq study while qRT-PCR of *ecnI*-1 knock-out mutation RNA-seq study is shown in Figure 4.19. In both studies, all genes selected for validation were in good accordance with respective RNA-seq analysis.

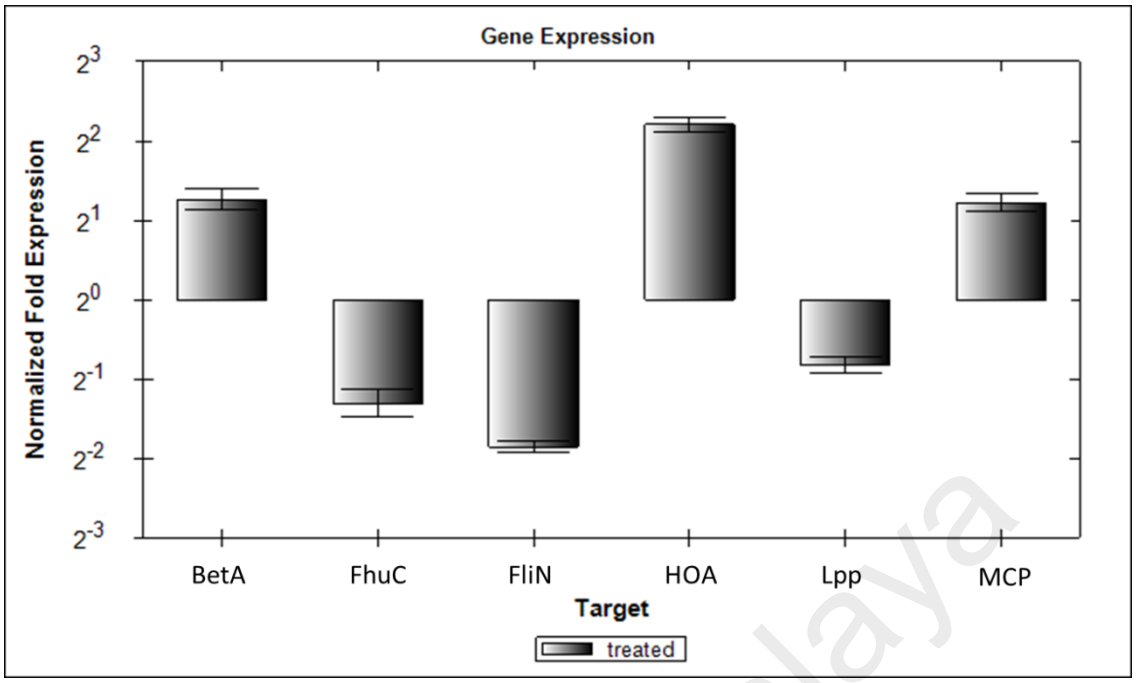


Figure 4.18: qRT-PCR validation on RNA-seq-based expression pattern in gallic acid-treated study. The up-regulated *E. cancerogenus* strain M004 genes were oxygen-dependent choline dehydrogenase (BetA), homoserine O-acetyltransferase (HOA) and methyl-accepting chemotaxis protein III (MCP). The down-regulated *E. cancerogenus* strain M004 genes were iron (3+)-hydroxamate import ATP-binding protein (FhuC), flagellar motor switch protein (FliN) and major outer membrane lipoprotein Lpp precursor (Lpp). The values of the gene expression were normalized to the housekeeping gene expression. CFX Manager™ Software version 1.6 (Bio-Rad. USA) were used for analysis.

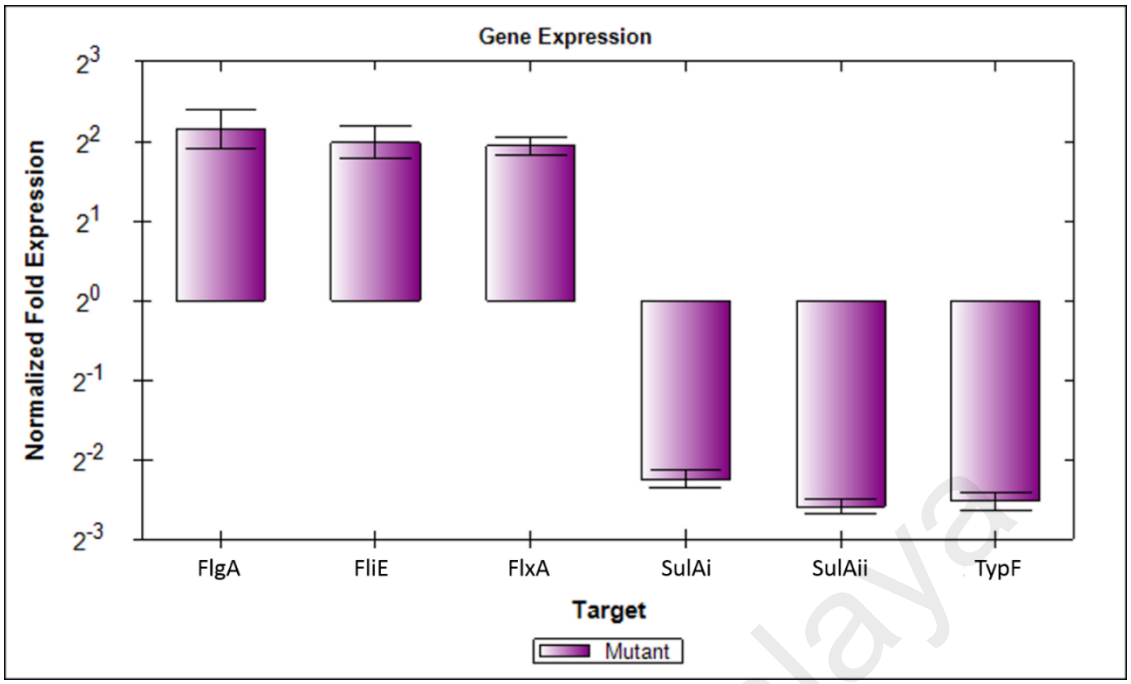


Figure 4.19: qRT-PCR validation on RNA-seq-based expression pattern in *E. cancerogenus* strain M004 *ecnI*-1 knock-out study. The up-regulated *E. cancerogenus* strain M004 genes were flagella basal body P-ring formation protein FlgA precursor (FlgA), flagellar hook-basal body complex protein (FliE) and flagellar class 3 operons (FlxA). The down-regulated *E. cancerogenus* strain M004 genes were sulfate adenylyltransferase subunit 1 (SulAi) and subunit 2 (SulAii) and type-F conjugative transfer system pilin assembly protein (TypF). The values of the gene expression were normalized to the housekeeping gene expression. CFX Manager™ Software version 1.6 (Bio-Rad. USA) were used for analysis.

4.10 Biofilm Formation Assay

4.10.1 Biofilm Formation of *E. cancerogenus* Strain M004 Treated with Gallic Acid

The RNA-seq with the use of a known QS inhibitor (QSI) - gallic acid focused on QS-dependent genes and the link of phenotypic characteristic which is tightly regulated by QS, the biofilm assay (Rutherford & Bassler, 2012) was selected. The QSI, gallic acid (Dusane, O'May, & Tufenkji, 2015; Myszka et al., 2016) was used and it showed reduction in biofilm formation of *E. cancerogenus* strain M004 (Figure 4.20). The concentration of the gallic acid used in this study is 1 mg/mL. There was noticeable difference in structure biofilm formed by *E. cancerogenus* strain M004 treated with QSI from the untreated. The biofilm matrix in gallic acid treated condition was not firmly attached to the microtiter plate and there was a noticeable large gap while wild type biofilms were uniform and cover completely on the attached surface.

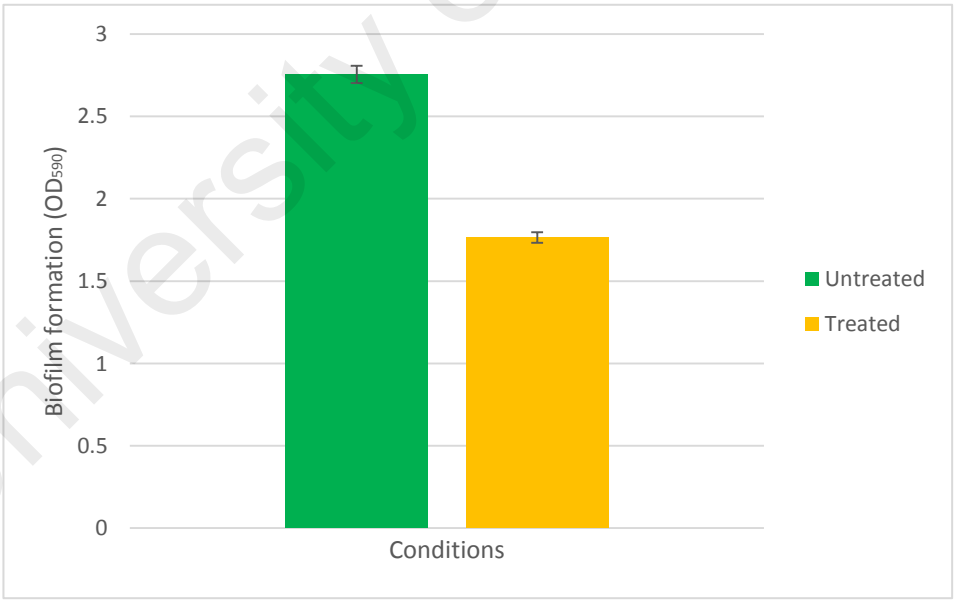


Figure 4.20: Qualitative analysis of inhibition of *E. cancerogenus* strain M004 biofilm by QSI, gallic acid. Bars: standard errors of the mean. (Unpaired t-test, $p < 0.0001$).

4.10.2 Biofilm Formation of *E. cancerogenus* Strain M004 Wildtype and Mutant (Δ*ecnI-1::Kan*^{r})

The *E. cancerogenus* strain M004 mutant had a significant reduced capacity in biofilm formation as compared with the wildtype (Figure 4.21). During the biofilm staining process, noticeable difference in the formed biofilm structure between wildtype and mutant can be observed with naked eyes. The matrix in wildtype was thick, uniform and firmly attached to the microtiter plate but the mutant biofilm matrix was with noticeable large gap and fragile.

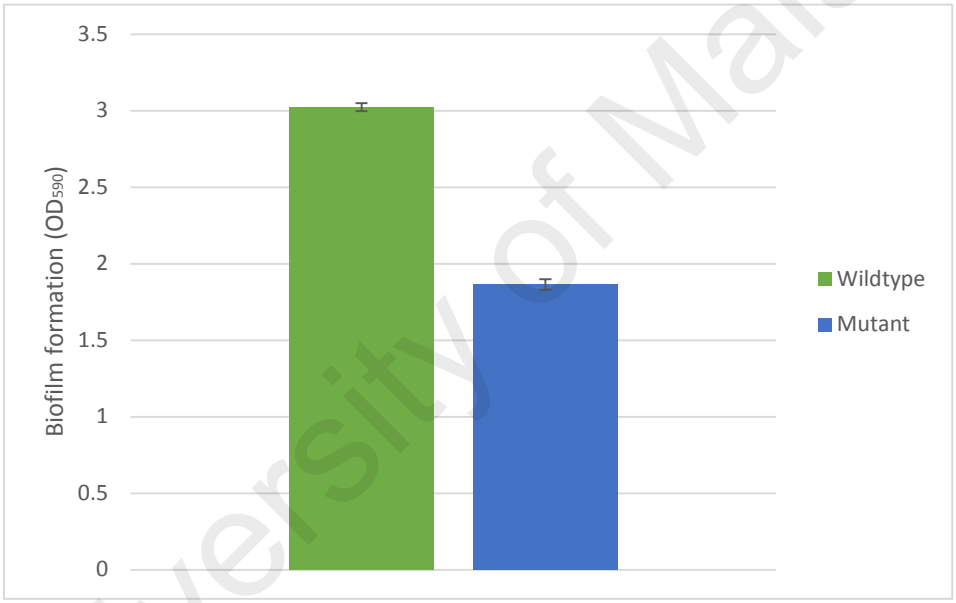


Figure 4.21: Qualitative analysis of *E. cancerogenus* strain M004 biofilm reduction of mutant compared with the wildtype. Bars: standard errors of the mean. (Unpaired t-test, $p < 0.0001$).

4.11 Auxin Biosynthesis of *E. cancerogenus* Strain M004 Wildtype and *ecnI*-1 Mutant

In the rapid plate assay established by Srivastava & Kumar (2011), it allows the screening of auxin production by *E. cancerogenus* strain M004. The reddish-pink halo zone formation preliminary indicating that *E. cancerogenus* strain M004 produced auxin which made it a potential plant growth promoting bacteria. Besides that, no reddish-pink zone formation in *E. cancerogenus* strain M004 mutant provide us the preliminary insights that QS might be the role player in auxin biosynthesis in this strain (Figure 4.22).

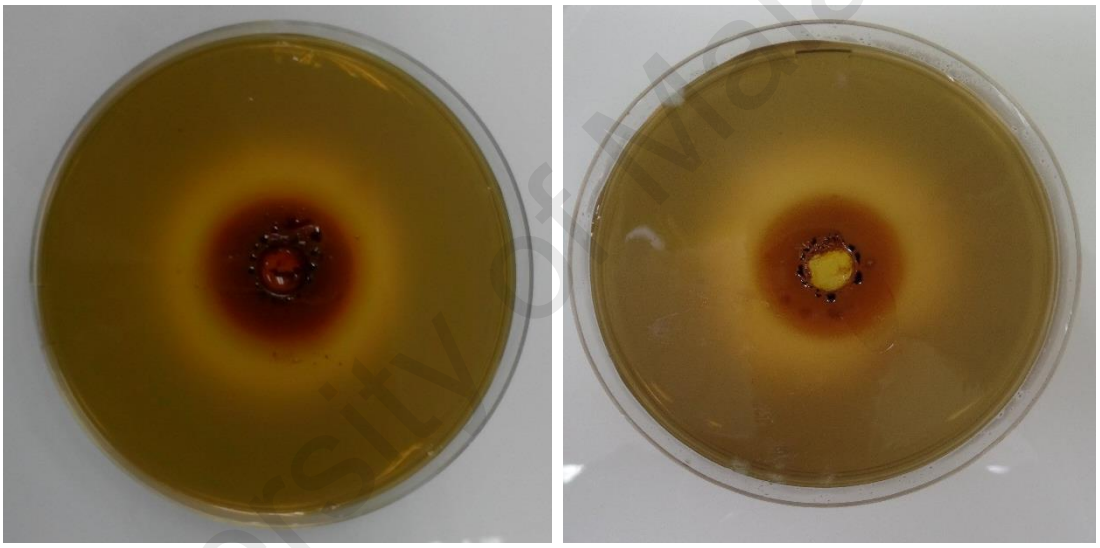


Figure 4.22: The reddish-pink halo formation of *E. cancerogenus* strain M004 wildtype (left) and mutant (right).

From RAST analysis of *E. cancerogenus* strain M004, there are genes encoded for the biosynthesis of plant hormones such as tryptophan synthase and indole-3-glycerol phosphate synthase. Figure 4.23 shows the details of the pathway for tryptophan synthesis and tryptophan-dependent auxin biosynthesis with the highlighted (yellow) illustrated the genes involved that present in genome of *E. cancerogenus* strain M004.

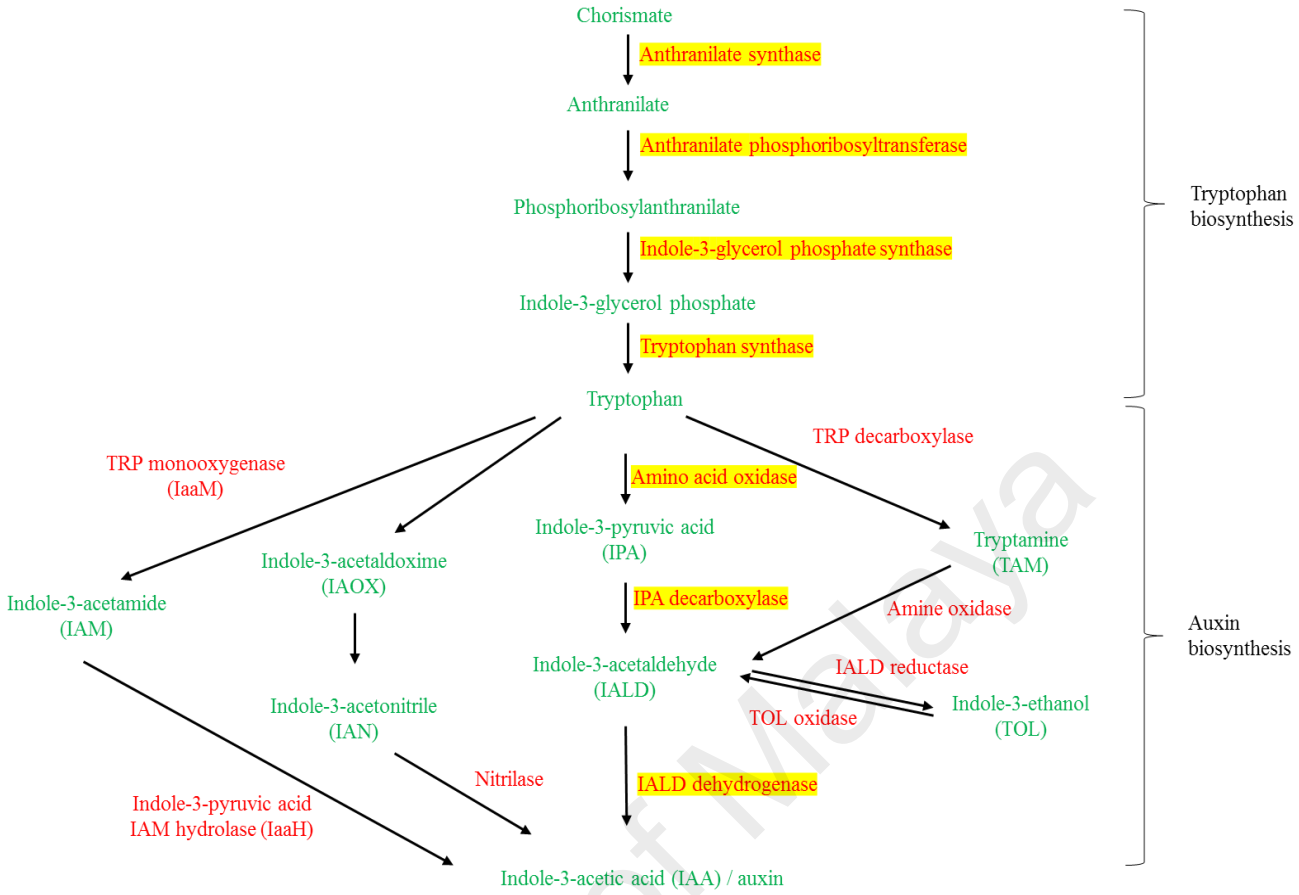


Figure 4.23: Metabolism of tryptophan in bacteria in which anthranilate synthase catalyzes the first reaction branching from the aromatic amino acid pathway and tryptophan-dependent pathways of auxin biosynthesis in bacteria. The yellow highlighted indicated the genes involved in the pathways that are present in the genome of *E. cancerogenus* strain M004. Enzymes are indicated in red while substrates, intermediates and final product of the pathways are indicated in green.

CHAPTER 5: DISCUSSION

5.1 Water Properties

The understanding into microbial composition in tropical freshwater is still remain understudied and the aim of this study is to study AHL-producing bacteria from tropical waterfall. The water samples were collected at the highest source with less human activities to reduce the fecal contamination during water collection (Madrid & Zayas, 2007).

5.2 Bacterial Isolation and Screening of AHL Producing Bacteria and Their Identification

Aeromonads are common bacteria isolated from water environment (Chan et al., 2011) and from this study, 5 out of 13 isolates were aeromonads. Other isolates commonly found in this habitat include plant-associated bacteria such as *Dickeya* sp., *Pantoea* sp. and *Pectobacterium* sp. This is common as the water source is collected from the highest source with flora surrounding the water source (Morris & Monier, 2003). To identify the isolates that are able to produce AHLs, bacterial biosensors were employed. There are many types of bacterial biosensors available for screening of AHL production (McClean et al., 1997). Both CV026 and [pSB401] were used in this study to preliminary detect the AHL production. Out of the 13 isolates, strain M004 was further selected for analyses as it showed AHL production activity as this isolate has not been shown to exhibit QS activity.

5.3 *Enterobacter cancerogenus* Strain M004

Enterobacter, is a group of rod-shaped bacteria of the family Enterobacteriaceae and this morphology is shown in the SEM image of *E. cancerogenus* strain M004. The *Enterobacter* spp. are found ubiquitously in the natural environment such as water, sewage, vegetables and soil and they could exhibit different roles ranging from disease-causing, as food spoilage agent, and plant pathogens (Sanders & Sanders, 1997). Although strains of *E. cancerogenus* have been reported to be isolated predominantly from human blood and spinal fluid, but there are other reports showing that this bacterium were isolated from environmental sources such as soil (Madhaiyan et al., 2010; Manter, Hunter, & Vivanco, 2011) and plant rhizosphere (Jha, Patel, & Saraf, 2012). This study is one of the very few findings that isolated *E. cancerogenus* from environmental water sources. *E. cancerogenus* has been reported as human pathogen (Abbott & Janda, 1997; Stock & Wiedemann, 2002) and plant growth-promoting endophyte (Rezzonico, Smits, & Duffy, 2012). Rezzonico and colleagues hypothesized that QS might be the key player behind two different functioning of *E. cancerogenus* and therefore strain M004 was selected as bacterium of interest in this study. Hence, the study expanded to understand the genetic makeup and to further decipher the genome for the presence of communicated-encoded genes.

Several environmental *E. cancerogenus* isolates have also been reported to be plant growth stimulant. For example, *Enterobacter* sp. strain 638 was found to be potential plant growth promoting endophytic bacterium in which it enhanced poplar growth up to 40% (Taghavi et al., 2010). In this study, strain 638 was identified to be closest to *E. cancerogenus* instead of the clinical *Enterobacter* spp.. In 2011, a study showed that *E. cancerogenus* is as plant growth promoting proteobacterium by enhancing seed germination of Pigeon peas (Rani & Reddy, 2011). *E. cancerogenus* again showed the

potential as plant growth promoting bacterium in which a novel strain MSA2 promote the root growth of an important biofuel feed stock plant, *Jatropha curcas* (Jha et al., 2012). This led to the speculation that *E. cancerogenus* can be beneficial to plants by production of auxin.

Ironically, *E. cancerogenus* was also reported to be opportunistic pathogen. The knowledge regarding to the epidemiology and clinical significance of *E. cancerogenus* was still remain insignificant, but sporadic cases of causing bacteremia, osteomyelitis, urinary tract infection, pneumonia and wound infection have been reported. In one of the earliest studies, *E. cancerogenus* has been reported to be associated with severe trauma or crush injuries as it caused wound infections and septicemia in persons exposed to it environmentally, during traumatic events (Abbott & Janda, 1997). In 2005, traumatic injury that led to osteomyelitis was again reported to be caused by *E. cancerogenus*. Following this incident, 16 documented cases related to *E. cancerogenus* were reported and these were majorly due to environmental exposure such as crush injury or laceration (Bowles, Truesdale, Levi, & Trotter, 2006). Until last year, a case study on surgical site infection caused by *E. cancerogenus* was first to be reported (Tena et al., 2015). Most reports showed that infection caused by *E. cancerogenus* was secondary to trauma and injuries and it is assumed that this infection is introduced to exposed wounds. As this bacterium was commonly found and isolated from environmental sources, supporting that potential pathogenic bacteria can be inhabitants of the environment. More research should be conducted in order to provide insights of transmission routes of *E. cancerogenus* to human.

5.4 AHL Detection and Profiling with LC-MS/MS in *E. cancerogenus* Strain M004

The synthetic AHL compounds served as standard during LC-MS/MS analysis (Ortori et al., 2011). From LC chromatogram, AHL extracts of strain M004 had the same retention time as the synthetic compounds; 3-oxo-C6-HSL and 3-oxo-C8-HSL. Under the same parameters set, 3-oxo-C6-HSL is a more polar compound as compared to 3-oxo-C8-HSL, hence the elution time was shorter; 3-oxo-C6-HSL retention time at 0.5-1.0 min and 3-oxo-C8-HSL retention time at 2.2-2.7 min. The electrospray ionization mode was set as positive as the masses of possible AHLs in protonated form, demonstrated $[M+H]^+$ species (Cataldi, Bianco, Fonseca, & Schmitt-Kopplin, 2013). Furthermore, *m/z* 102 was set as common product ion fragments across all AHL analytes that allow search of the signaling molecules during MS analyses (Gould, Herman, Krank, Murphy, & Churchill, 2006).

Some members of *Enterobacter* genera displayed QS properties such as *E. sakazakii* produces 3-oxo-C6-HSL and 3-oxo-C8-HSL and its QS is associated with food-borne diseases; meningitis and enteritis (Lehner et al., 2005). Other work done by Yin and colleagues (2012) showed that member of *Enterobacter* genus isolated from human tongue surface exhibited long chain AHL production; C12-HSL (W.-F. Yin, Purmal, Chin, Chan, & Chan, 2012). *E. asburiae* was found to produce C4-HSL and C6-HSL and this strain was isolated from lettuce leaves (Lau, Sulaiman, Chen, Yin, & Chan, 2013). *Enterobacter* sp. strain SST3 was also observed to have AHL-positive phenotypes (Gan et al., 2012). The production of two *N*-3-oxo-acylhomoserine lactone by *E. cancerogenus* is firstly reported in this study. It is curious to understand AHL production of *E. cancerogenus* strain M004 and by expanding the work to whole genome sequencing (WGS) would gain insights on the QS gene homologue system in this strain.

5.5 Functional Annotation and Molecular Cloning of Gene of Interest

E. cancerogenus strain M004 genome was sequenced by Illumina MiSeq platform with deposition in GenBank database. The availability of whole-genome of this isolate could provide appreciative foundation in functional study of QS in strain M004. The annotated genome of strain M004 led to finding and characterize the two *luxI* homologues, hereafter designated as *ecnI*-1 and *ecnI*-2. Verification of synthases activities which are responsible for AHL production could pave a way into understanding the regulatory roles of AHLs on physiological traits of this bacterium (How et al., 2015).

Mass spectrometry analysis showed the presence of equal amount of 3-oxo-C6-HSL and 3-oxo-C8-HSL and this finding is parallel with the AHL profiles detected in the wildtype strain M004. Both AHLs are produced equally indicates that they might play essential roles in regulating physiological behavior of strain M004. Besides that, they may work synergistically or complementary to each other in influencing the expression of targeted genes. Through BLAST analysis, the EcnI-1 showed highest similarity with LuxI homologue of *P. rodasii*. Apart from this, EcnI-1 was also observed to cluster between EsaI of *Pantoea ananatis*, YspI of *Yersinia pestis* and SpnI of *Serratia marcescens*. The four highest similarity LuxI homologue with EcnI-1, EsaI (Morohoshi, Nakamura, et al., 2007), SpnI (Ryu et al., 2013), YspI (Kirwan et al., 2006) and LuxI homologue of *P. rodasii* (Yunos et al., 2014) were reported to produce one of the same AHL as EcnI-1, 3-oxo-C6-HSL. The production of 3-oxo-C8-HSL by EcnI-1 was also reported in YspI but not found in LuxI homologue of *P. rodasii*, *S. marcescens* and *P. ananatis*. Production of 3-oxo-C6-HSL by EsaI in *P. ananatis* regulates biofilm formation and infection towards onion leaves (Morohoshi, Nakamura, et al., 2007). Study demonstrated that rhizobacterium *S. marcescens* strain 90-166 producing *N*-3-oxo-AHL is essential to induced systemic resistance in plants (Ryu et al., 2013). Other than that, production of 3-

oxo-C6-HSL by *S. marcescens* strain AS-1 was found to be responsible for formation of biofilm and swarming motility. This is evident by inhibition of swarming motility and biofilm formation were observed upon introduction of synthetic QS inhibitor, C_9-CPA in the strain AS-1 (Morohoshi, Shiono, et al., 2007). Kirwan and colleagues showed that 3-oxo-C6-HSL and 3-oxo-C8-HSL produced by YspI of *Y. pestis* can caused bubonic and pneumonic plagues in the mammalian host (Kirwan et al., 2006).

The production of 3-oxo-C6-HSL and 3-oxo-C8-HSL are also found across other species of *Enterobacter* such as *E. aerogenes* (H. Wang et al., 2006) and *E. sakazakii* (Lehner et al., 2005). In *E. aerogenes*, both these AHLs encapsulate polysaccharide production in order to prevent phagocytosis (Olaitan, Morand, & Rolain, 2014; H. Wang et al., 2006). Production of both 3-oxo-C6-HSL and 3-oxo-C8-HSL by *E. sakazakii* mediate its biofilm formation and putative production of cellulose as one of the components in extracellular matrix (Lehner et al., 2005). These AHLs-orientated behaviors could enhance the survival and persistence of *E. sakazakii* towards various abiotic and biotic factors. Steindler and colleagues (2009) proven that QS can be involved in regulation of beneficial traits, particularly playing a role in the roots of rice plants. The study reveals the potential roles of 3-oxo-C6-HSL and 3-oxo-C8-HSL produced by *P. aeruginosa* PUPa3 involving in plant growth-promoting properties and expressing the antifungal activities (Steindler et al., 2009). Despite of that, *P. aeruginosa* PUPa3 exhibited pathogenicity characteristics in two non-mammalian infection models. These findings provide the insight where environmental strain able to behave divergently in different hosts. Interestingly, *P. aeruginosa* PUPa3 was found to harbor two QS systems that are highly homologous to the LasI/R and RhlI/R systems of the model strain PAO1 (Latifi et al., 1995; Steindler et al., 2009).

Furthermore, Wei and Zhang (2006) showed that wheat root colonization properties in *P. fluorescens* 2P24 regulated by QS can be highly exploited for biological control (Wei & Zhang, 2006). They researched the PcoI/PcoR QS-system in strain 2P24 plays a significant role in suppressing wheat take-all disease by producing the antibiotic 2,4-DAPG that acts against the plant pathogen. These studies have further elucidated the role of QS in regulating beneficiary traits of *P. aeruginosa*, but clinical significant regulated by QS in *P. aeruginosa* is well-documented. Nevertheless, information on the roles and mechanism of QS in *Enterobacter* sp. is still limited. This study could spiked the research interest to understand how QS could regulate either pathogenicity or the plant stimulant properties in strain M004.

Many studies show intimate relationship between clinical and environmental isolates suggest that the environment (especially rhizosphere) could serve as a reservoir for opportunistic human pathogens. The ironic is that despite of being a plant symbiont, the same bacterial strain from the exact environment could be a threatening human opportunistic pathogen. The information of bacterial QS-related genes demonstrates the initial step in elucidating the roles and molecular mechanisms of the signaling system portrayed by the bacterium. Efforts are should be focused on the link of QS with physiological traits to understand its mechanisms in establishing the niche in different environment. Strong indication from analyses of mass spectrometry that AHLs production of strain M004 were directed by recombinant EcnI-1. This study first reported two *luxI* homologue in *E. cancerogenus* strain M004. In addition, non-functional *ecnI*-2 further supported that QS-mediated phenotypes in strain M004 depends on one *luxI* gene.

5.6 Mutant (Δ*ecnI-1::Kan*^{r}) Produced by λ Red System

Development of efficient methods in functional analysis of newly identified genes is in need to grow according with the increasing number of availability of sequenced genomes. The traditional way to construct knockout mutants by gene replacement is time consuming due to involvement of several sub-cloning steps (Guan, Ye, Yang, & Gao, 2010; Ji, 2002). Thus, creation of λ Red based methodology ease mutagenesis through enhancing time efficacy that abandoned cloning process and have been successfully employed across various bacteria and fungi (Lesic & Rahme, 2008; Tobin et al., 2013). This methods had been first described success in *E. coli* (Datsenko & Wanner, 2000) and *Aspergillus nidulans* (Chaveroche, Ghigo, & d'Enfert, 2000); followed by application to *Yersinia* (Derbise, Lesic, Dacheux, Ghigo, & Carniel, 2003; Kaniga, Delor, & Cornelis, 1991), *Salmonella* (Husseiny & Hensel, 2005), *Shigella flexneri* (Beloin, Deighan, Doyle, & Dorman, 2003), *Serratia* (Rossi, Paquelin, Ghigo, & Wandersman, 2003; Serra-Moreno, Acosta, Hernalsteens, Jofre, & Muniesa, 2006), *Sodalis glossinidus* (Pontes & Dale, 2011) and *Vibrio* (Yamamoto, Izumiya, Morita, Arakawa, & Watanabe, 2009). Through all these studies, λ Red system procedure was proved to be widely useful across all other bacteria because the produce can be done in wildtype cells. λ Red system recombineering involved a homologous recombination between the chromosomal region of interest and a PCR-product that contains an antibiotic cassette flanked by a region of homology with the target DNA was involved in deleting the chromosomal genes (Sawitzke et al., 2013).

This technique proved useful in functional study on *luxI* homologue of strain M004. Because the cloning process of the *luxI* homologue was a success, the *ecnI*-1 was identified to be the only functional *luxI* homologue in strain M004. Therefore, the knock-out mutation of *ecnI*-1 was made possible. The λ Red recombineering system was adapted

from protocol established by Court Lab (Sawitzke et al., 2013). Prior of mutation process, the strain M004 was tested for its antibiotic susceptibility test. Any antibiotics which is naturally resistance by bacterium of interest shall not be selected; as to avoid any false positive results during the verification process (Heermann, Zeppenfeld, & Jung, 2008). Besides that, making sure the bacterium of interest can incorporate with the λ Red system is also important to ensure successful mutation. In Court Lab database, there are 11 different types of curable and lower copy number Red plasmids which are able to synthesize phage λ Red recombinase under the control of an inducible promoter that allow researcher to select (Lesic & Rahme, 2008; "RECOMBINEERING - 2016," 2016). The Red plasmids have a temperature-sensitive origin of replication and after desired genetic modifications have been engineered, the plasmid is cured by growing the selected cells in the absence of plasmid selection at non-permissive temperature (> 32°C) (Pontes & Dale, 2011). But strain M004 replicates at lower temperature, curing was achieved by growing cells at 28°C in the absence of plasmid selection (Tan, Yunos, et al., 2014). After sixth passages, the loss of pSIM7 was confirmed when over 95% of the cells were chloramphenicol sensitive. Other than that, customized primer pairs also play vital part in this technique. Tailor-made chimeric primer pair is needed to amplify the selected antibiotic cassette. The λ Red-system recombination allow the precise and rapid knock-out mutation of *ecnI*-1 in this study. All gene disruption mutants were verified by PCR strategy which tested for the presence of new locus and junction-specific fragments of predicted sizes; in this study, predicted size of kanamycin insert was approximately 950 bp (Murphy & Campellone, 2003). Through bacterial biosensor assay and AHLs detection via LC-MS/MS, successful mutant was selected for further analyses (refer to Chapter 4; section 4.6). The knock-out of *ecnI*-1 gene and replaced by a kanamycin cassette ease the mutant selection by looking at kanamycin resistant colonies formed.

5.7 Phenotypic Comparison between *E. cancerogenus* Strain M004 Wildtype and Mutant (Δ*ecnI-1::Kan*^{r})

The study into understands bacterial phenotype is the large major area of analysis that are amenable to efficient global analysis (B. R. Bochner, 2003). Traditionally, analysis of cellular phenotypes was time consuming as one phenotype was analyzed at a time. It has not been obvious that one could devise an efficient method with adequate scope and sensitivity for global analysis and phenotypes were often vaguely defined. Recently, Bochner developed a technology that is able to detect cellular respiration by measuring in a colorimetric means. As respiration is obligatory to cell growth, many can understand and explore into biological pathways and physiological functions that are linked to cell growth (B. R. Bochner, 2009). The OmniLog instrument was developed to relieve the bottleneck of conventional phenotypic testing. The developed phenotypic microarray (PM) would allow the detection of phenotypes in a rapid and easy manner and allow broad phenotypic testing to become a simple standard practice. To date, there are many successful adaptation of PMs in *P. aeruginosa* (Starkey et al., 2009), *Burkholderia cepacia* (Morgan, Boyette, Goforth, Sperry, & Greene, 2009), *Vibrio fluvialis* (Su & Liu, 2007) and *Aspergillus nidulans* (B. Bochner, 2003).

Phenotypic studies in *Enterobacter* spp. still remain understudied and the biochemical profile comparison on *E. cancerogenus* strain M004 *ecnI*-1 mutant and wildtype was firstly studied and reported here. The survey of mutant phenotypes was conducted using PM1 to PM20 because this study aimed to reveal the general phenotypes that were affected when *ecnI*-1 was knock-out from strain M004. PM1 to 8 serve to confirm phenotypes detected with the metabolic arrays while PM9-20 allow the phenotypes detection with the inhibitor sensitivity arrays. From 1,920 phenotypes tested by comparing mutant to wildtype of *E. cancerogenus* strain M004, 58 phenotypes showed

highly significant difference (kinetic curve value of difference > 5000). And from these 58 phenotypes, 36.2 % were up-regulated and 63.8% were down-regulated.

In PM 1-8, there were 7 loss-of-function phenotypes and mostly belonged to amino acid / carbohydrate biosynthesis and metabolism. This findings have yet reported in other *Enterobacter*, but Sperandio and colleagues (2001) where they acknowledged that amino acid biosynthesis and metabolism was regulated by QS (Sperandio, Torres, Girón, & Kaper, 2001). Besides that, further finding in *E. coli* substantiated that uptake, synthesis or degradation of amino acids are regulated by QS (Baca-DeLancey, South, Ding, & Rather, 1999). Besides, carbohydrate utilization influences the changes in amino acids of bacteria (Jones, 1920), this further indicated that observation of down-regulation of carbohydrate phenotypes relates with the down-regulation of the amino acid biosynthesis in *E. cancerogenus* strain M004. Meanwhile, in PM 9-20, there was a loss-of-function in pH 3.5 in mutant as compared to wildtype indicating that QS up-regulated bacterial growth in acidic condition. This suggested that QS allows the adaptation in environmental pH shift at a population density. Besides, the PM result also suggested that QS involved in regulation of cell division and growth in *E. cancerogenus* strain M004. Some phenotypes involved in tRNA synthetase, DNA topoisomerase and respiration-related were significantly loss in mutant as compared with wildtype. Baca-DeLancey and colleagues (1999) reported that QS indeed influenced the cell division and growth in *E. coli*.

Phenotypes involving higher NaCl and more alkaline conditions were gained in mutant when compared to wildtype. It is hypothesized that there is another cellular mechanism in *E. cancerogenus* strain M004 in order to allow planktonic cells to adapt in certain harsh environment (Ryall, Eydallin, & Ferenci, 2012). Hogan and Kolter (2002) stated that signals that control gene regulation, such as QS molecules present in biofilm to regulate

expression of antibiotic-related genes. However, antibiotic-related phenotypes were observed in both gain (pipemedic acid and rifampicin) and loss (lomefloxacin and erythromycin) patterns. Although there is yet clear explanation on this finding, it is suggested that there is other metabolic pathway that synchronize with QS system to regulate antibiotic-related genes in *E. cancerogenus* strain M004.

5.8 Treatment of Gallic Acid on *E. cancerogenus* Strain M004

QS allow bacteria to monitor changes of population density that lead to regulation of variety of physiological processes. Hence, QS become attractive target in developing anti-virulence therapies which led to identification of compounds that quench QS, known as QS inhibitors (QSI). To date, increasing of documented discoveries on QSI from medicinal and dietary plant sources, including phenolic compounds from extracts of *Acacia nilotica* L. (Singh et al., 2009), methanolic extracts from *Phyllanthus amarus* (Priya, Yin, & Chan, 2013), malabaricone C from *Myristica cinnamomea* (Chong et al., 2011), and polyphenolic extract from *Rosa rugose* tea (Zhang et al., 2014). Plants have co-evolved and build up symbiotic or syntrophic relationship with bacteria and led to discovery of QSI that established effective alternative anti-infective agents to replace antibiotics.

Phenolic products or polyphenols are QSI compounds that are most abundant and omnipresent as plant secondary metabolites. One of the polyphenols, gallic acid showed potential as QSI as discussed in multiple reports. Gallic acids which is also known as 3,4,5-trihydroxybenzoic acid can be extracted from various plant origins ranging from oak barks, tea leaves, grape seeds to witch hazel (Daglia, Di Lorenzo, Nabavi, Talas, & Nabavi, 2014). Porat and colleagues (2006) documented that gallic acid can be important in treating Alzheimer's and Parkinson's disease by inhibiting the formation of amyloid

fibrils, a protein associated with the onset of neurodegenerative diseases (Porat, Abramowitz, & Gazit, 2006). Similar findings were also reported by Daglia and colleagues (2014). Besides that, this compound also portray anti-fungal, anti-bacterial and anti-viral properties (Kratz et al., 2008). Several previous reports nominated gallic acid as QSI candidate.

One study showed that application of 200 µg/mL of gallic acid reduced the biofilm formation of *P. aeruginosa* PAO1 by 30% (Plyuta et al., 2013). Other than that, 1 mM of gallic acid reduced 80% of biofilm formed by *Eikenella corrodens* (Matsunaga et al., 2010). Gallic acid can be potential QSI, Borges and colleagues observed a 59% reduction of violacein production upon introduction of 1 mg/mL gallic acid to treat *C. violaceum* CV12472 (Borges et al., 2012). Furthermore, high amount of gallic acid present in extracts of green pod of *Acacia nilotica* L. that was reported to inhibit QS-regulated violacein pigmentation in *C. violaceum* CV 12472 without interference of the bacterial growth (Singh et al., 2009). In this study, it is observed approximately 40 % reduction in biofilm formation of *E. cancerogenus* M004 when treated by 1 mg/mL gallic acid. This study used 1 mg/mL gallic acid by referencing the concentration used by Borges and colleagues (2012) and this concentration selected has showed no growth inhibition against *E. cancerogenus* strain M004.

5.9 Transcriptome Study

The expression analyses by qRT-PCR technology was used in this work to confirm the RNA-seq data (Figure 4.20 and 4.21). RNA-seq approach was used to investigate the transcriptomic profiles in strain M004 and to further understand the potential of gallic acid as an anti-QS therapeutic agent. From the functional annotation of COGs, the differential expressed genes could be categorized into their functional groups. This analysis also provides the understanding into metabolic pathways, multi-subunit complexes or protein classes that make up the genes' functions that were involved upon treated with gallic acid.

From the RNA-seq analysis, genes such as oxygen-dependent choline dehydrogenase (*betA*), homoserine O-acetyltransferase (*metX*) and methyl-accepting chemotaxis protein III (*MCP-III*) were up-regulated in gallic acid treatment culture. Synthesis of betaine is important for bacterial osmoprotectant, the *betA* gene is a part of *bet* regulon that encodes an enzyme that synthesized betaine (Lamark, Røkenes, McDougall, & Strøm, 1996). This gene was assigned to the COG of amino acid transport and metabolism (class E). Bacterial stress responses is known to be widely recognize by cytoplasmic accumulation of certain organic solutes such as betaine. These organic solutes are main players in osmoregulation and oxidative stress tolerance in bacteria (Wood et al., 2001). With presence of gallic acid interfering the QS system in strain M004, up-regulated of osmoprotectant-related genes helps to increase bacterial sensitivity towards osmotic stresses. Thus, this benefits the bacterial survivability in diverse environments (Cameron, Frirdich, Huynh, Parker, & Gaynor, 2012).

Another interesting finding would be the up-regulation of methyl-accepting chemotaxis protein III (*MCP-III*). This MCP-III belonged to multiple classes in functional category, class NT for cell motility/signal transduction mechanism respectively. This MCP-III is a transmembrane receptor protein that is responsible in aiding bacteria to alter their swimming behavior by detecting the changes of chemical molecules in extracellular matrix (de Kievit & Iglewski, 1999). When QS is interrupted, up-regulation of chemotaxis-related protein such as MCP-III would induce bacteria to tumble and re-orient itself in order to move away from toxic environment. Most of the pathogenic bacterial species acquired motility and chemotaxis in order to colonize and invade the host of interest. The sensing to a wider range of signals enable the bacteria to generate pathogenic responses. In fact, biofilm formation and development was shown to relate with chemotaxis and to allow bacteria interaction with various chemical signals (Wadhams & Armitage, 2004). Upon introduction of gallic acid in culture of M004, chemotaxis-related genes up-regulated to allow the bacteria to detect and sense the presence of unfavorable molecules. Homoserine-*O*-acetyltransferase, MetX is assigned to COG class E, amino acid transport and metabolism, is an enzyme that responsible in methionine biosynthesis (Hwang, Yeom, Kim, & Lee, 2002; H.-S. Lee & Hwang, 2003). The up-regulation of this gene could correlates with up-regulation of MCP-III in this work, as methionine biosynthesis and its metabolites are associated with chemotaxis, thus enhancing the tumbling and twitching movement of bacteria (Springer et al., 1975). Furthermore, methionine is reported essential for methylation of MCP-III and in sensory adaptation (Goy, Springer, & Adler, 1977). Hence, certain genes upregulated in QS-interfered mode could give preliminary insights that bacteria could increase their physiological mechanisms for adaptability and survivability in the presence of QSI.

Several cell attachment-related proteins expressed caught the attention, namely major outer membrane lipoprotein Lpp precursor (Lpp), flagellar motor switch (FliN) and iron (3+)-hydroxamate import ATP-binding protein (FhuC) were found to be down-regulated in the gallic acid-treated culture. QS is well-known to be essential in regulating various phenotypes especially the bacterial swarming activity and biofilm formation, the introduction of gallic acid would reduce the expression of genes involved in regulation of both these phenotypes. The Lpp interacts with peptidoglycan layer which is vital to the maintenance of the structural and functional integrity of bacterial cell envelope (Yakushi, Masuda, Narita, Matsuyama, & Tokuda, 2000) and this protein is assigned in class M, responsible for cell wall/membrane/envelope biogenesis. Chang and colleagues (2012) reported that outer membrane lipoprotein Lpp of Enterobacteriaceae interacts with antimicrobial peptides and inhibit the growth of bacteria (Chang, Lin, Wang, & Liao, 2012). Hence, the observation of reduction in strain M004 biofilm formation may highly suggest that gallic acid caused Lpp to be down-regulated, thus led to disruption of the cell surface integrity, eventually causing bacterial cell density to decrease.

Correspondingly, the down-regulation of *fliN* (class NU; N=cell motility, U=Intracellular trafficking, secretion, and vesicular transport) encodes for flagellar motor switch will lead to reduction of cell attachments and motility. One of the three protein components of the flagellar motor switch, FliN regulates the direction of flagellar rotation, thereby controlling the bacterial swimming pattern (Roman, Frantz, & Matsumura, 1993). This protein is predicted to act as a protein exporter to transport other proteins for building the exterior structures of the flagellum (rod, hook and filament). This may explain the morphology observed in cultures treated with gallic acid. In addition, the *fhuC* gene, which is a component of *fhu* cassette operons, encodes iron (3+)-hydroxamate import ATP-binding protein and its expression was downregulated in strain M004 upon

treated with gallic acid. Movement of iron (3+)-hydroxamate in the cells depends on necessary energy produced by this protein as it serves as a cognate ATPase (Holland & Blight, 1999). As reported previously, reduction of bacteria pathogenicity and virulence expression occurred when synthesis of FhuC protein reduced (Crossley, Jefferson, Archer, & Fowler, 2009; Lechowicz & Krawczyk-Balska, 2015). Iron (3+)-hydroxamate is one of the protein that play a key role in siderophores uptake system. Siderophores allow pathogenic bacteria to acquire iron and in pathogenic *E. cloacae*, there are two types of siderophores produced, identified as enterochelin and aerobactin for iron scavenging. Several reports showed that bacteria that are deficient in siderophore production exhibited lower virulence than the parent strain (Lamont, Beare, Ochsner, Vasil, & Vasil, 2002; Mathew, Tan, Rao, Lim, & Leung, 2001; Wertheimer et al., 1999). Dhaenens and colleagues (1999) stated that siderophore-mediated iron acquisition system is common to all species of Enterobacteriaceae (Dhaenens, Szczebara, Van Nieuwenhuyse, & Husson, 1999). These findings showed parallel observation in this study in which gallic acid potentially act as an anti-QS agent in reducing pathogenicity of *E. cancerogenus* such as reduction of biofilm formation.

On the other hand, RNA-seq approach was used in this study to understand the changes in transcriptome profiles of *ecnI*-1 knock-out mutant when compared with its wildtype. As gram-negative bacterium, *E. cancerogenus* requires nutrients pass through inner and outer membranes (Yu et al., 2013). From Figure 4.17, COG functional category with highest gene count is the inorganic ion transport and metabolism (n=16). Most of the genes involved were related to sulfur metabolism, such as sulfate/thiosulfate import ATP-binding protein *cysA*, putative aliphatic sulfonates transport permease protein *ssuC*, sulfate adenylyltransferase *cysD*, adenylyl-sulfate kinase and sulfate transport system permease protein *cysW* and were down-regulated in Δ*ecnI-1::kan*^{r} mutant. Sulfur is vital

for all living organisms in which it is involved in the metabolism of amino acids such as methionine and cysteine that connected to energy supply in bacteria (Kiene, Linn, González, Moran, & Bruton, 1999). Sulfur plays vital roles in various cellular processes, such as redox cycle, enzyme reaction and metabolism of secondary products. Cellular requirements for sulfur can be fulfilled by the uptake of sulfur-containing amino acid such as cysteine. Thus, this led to correlation of down-regulation in expression of genes grouped in amino acid transport and metabolism (Figure 4.17) especially cysteine synthase gene. This finding is parallel with the phenotype microarray analysis, where in the PM plate 3, well A11, containing the L-cysteine showed phenotype lost when kinetic curves of mutant and wildtype were compared. Sulfur was reported to be essential in biofilm forming-cells by Pysz and colleagues (2004). They also further reported that gene encoded for cysteine synthase was up-regulated in biofilm forming-cells (Pysz et al., 2004). These patterns of differential expressed genes may explain the reduction of biofilm formation in mutant of strain M004. Furthermore, down-regulation of sulfur-related genes also could relate with down-regulation of genes involved in the COG group of energy production and conversion. Reduced sulfur compounds could be oxidized by most organisms and some conserved energy from oxidizing sulfur. In some bacteria, sulfur could be the sole energy source (Schulte & Bonas, 1992).

Another interesting finding involved genes grouped in COG of cell wall/membrane/envelope biogenesis. Genes such as glycosyltransferase EpsJ, UDP-glucose 6-dehydrogenase, major outer membrane lipoprotein Lpp precursor involved in cell wall/membrane/envelope biogenesis were down-regulated in *ecnI*-1 knock-out mutant. This result is parallel with the finding of differential gene expressed in gallic acid-treated culture. Biofilm formation is one of the phenotypes highly regulated by QS and down-regulation of these genes led to biofilm formed in mutant of strain M004 to be

easily disrupted and lack of attachment ability to the surface. Ironically, two of the genes belonged to this COG group, namely outer membrane porin F precursor and membrane fusion protein were up-regulated in the mutant. Although there is yet clear explanation on this finding, but it is suggested that there is other mechanism pathway that synchronize with QS system in cell wall/membrane/envelope biogenesis.

In the category of carbohydrate transport and metabolism (6.9 %), genes involved such as erythritol kinase (*eryA*), 6-phospho-β-glucosidase (*chbF*) and glycerol-3-phosphate transporter (*glpT*) were up-regulated in the Δ*ecnI-1::kan*^{r} mutant. This finding was similar in the study reported on QS system of *Sinorhizobium meliloti* by Hoang and colleagues (2004) that genes involved in carbohydrate transport and metabolism (group G) exhibited an up-regulated level of expression when *sinI* was disrupted (Hoang, Becker, & González, 2004). These results were further substantiated by a study in QS mutant (*tofI* mutant) of *B. glumae*, carbohydrate-related genes such as glucose-6-phosphate, phosphoenolpyruvate and phosphoglycerate kinase were elevated in mutant than in wildtype strain (An, Goo, Kim, Seo, & Hwang, 2014). They supported that QS mechanism can regulate nutrient uptake in individual cells as a form of cooperative activity. In this study, the result suggested that the QS mechanism involved in repression of carbohydrate/sugar acquisition of individual cells in crowded conditions. Thus, in bacterial communication-deficient condition, up-regulation of carbohydrate-related genes could help planktonic bacteria to overcome the environmental factors which are abiotic-related such as pH changes, presence of toxic compounds and osmotic pressure (Brown, Hammerschmidt, & Orihuela, 2015). However, in phenotypic microarray observation, carbohydrate-related phenotypes were lost in mutant when compared with wild-type. In PM plate 1, D-glucose-6-phosphate, thymidine, D-fructose-6-phosphate and L-lyxose were up-regulated in wildtype. This suggested that the up-regulated carbohydrate-related genes in mutant

obtained from RNA-seq data were not directly linked to carbohydrate-related phenotypes in the PM analysis. This indicated that QS could play different role in carbohydrate transport and metabolism in *E. cancerogenus* strain M004.

Two individual sets of QS-regulated genes were identified in strain M004 culture treated with gallic acid and AHL synthase mutants using RNA-seq. Among them were the genes linked to flagella, motility, nutrient transport and metabolism and stress-related gene. There are a total of 27 differential expressed genes similar between RNA-seq of gallic acid-treated and AHL synthase mutant and these similar genes were assigned to their own functional categories. Although the similarity of genes expressed between the two conditions is not high, this might due to the selection of culture OD_{600nm} was different for the two conditions, 0.3-0.5 for culture treated with gallic acid and 2.3-2.5 for AHL synthase mutant culture. Hence, it is suggested that the OD_{600nm} selection affects the variation of expression of genes captured at the selected time point. However, these gene expression similarities could indicate that gallic acid can be a potential QSI. In short, sensing and responding to the environmental stimuli to modulate gene expression occurred consistently in bacteria to adapt to external changes and QS is one of the mechanisms used by bacteria to regulate gene expression for adaptation.

5.10 Biofilm Formation of *E. cancerogenus* Strain M004

Biofilm formation has long been demonstrated to be a QS-regulated phenotype which associates with high cell density and close proximity of microorganisms. Biofilm serves as a "house" where microbial communities can interact symbiotically in it (Bhinu, 2006). The driving force into biofilm development could be due to biotic stresses such as competition of nutrients, drought and other growth parameters. *E. cancerogenus* strain M004 forms biofilm (Tan et al., 2014). There are also other *Enterobacter* reported with the ability in forming biofilms such as *E. sakazakii*, a food-borne pathogen (Lehner et al., 2005). Potential vehicles for outbreak of *E. sakazakii* infections are usually through food sources, such as powdered infant formula, vegetables and cheese. When bacterial cells attached to a food surfaces, the density of bacteria increases, followed by exopolysaccharides production which eventually lead to biofilm formation (Vu, Chen, Crawford, & Ivanova, 2009). Upon consumption of food containing the pathogen often results in the attachment and biofilm formation in human body (Costerton, Stewart, & Greenberg, 1999). The study by Kim and colleagues (2006) reported that *E. sakazakii* was able to attach and form biofilm onto inert surfaces (H. Kim, Ryu, & Beuchat, 2006).

Besides that, *E. cloacae* was reported to attach on medical equipment and form biofilm (Revdiwala, Rajdev, & Mulla, 2012), and *E. aerogenes* is able to form electroactive biofilm and this biofilm type is generated by electrochemically active microbes that could have potential applications in bioenergy and chemicals production (Borole et al., 2011; Zhuang et al., 2011). In this study, when culture of strain M004 was treated with gallic acid, the biofilm formed reduced significantly compared to the untreated culture. This finding supports that QS-associated genes are responsible in mediating biofilm formation in strain M004. By coupling with the whole transcriptomic study on gallic acid facilitates the initial step in elucidating its effect on signaling system in the bacterium and provide

significant evident that gallic acid could be a potential QSI. Besides that, significant reduction in biofilm formation was observed and quantitated in strain M004 *ecnI*-1 knock-out mutant. This further suggested that biofilm formation in strain M004 indeed was QS-regulated phenotype.

5.11 Auxin Biosynthesis of *E. cancerogenus* Strain M004 Wildtype and *ecnI*-1 Mutant

The rapid plate assay proposed by Shrivastava and colleague is to determine bacterial IAA production in a fast and reproducible manner (Shrivastava & Kumar, 2011). The principle of this rapid plate assay relies on the rate of diffusion of IAA on agar plate which is directly proportional to the concentration of the IAA produced by bacteria. Hence, this study extends the understanding of *E. cancerogenus* strain M004 which was preliminary indicated to be able in production of IAA thus making *E. cancerogenus* strain M004 a potential candidate as PGPB. First attempt was made to link QS and plant growth stimulation characteristic in strain M004. Establishment of bacterial-plant relationship often achieved through colonization process and by understanding whether PGPB is regulated by QS could be a stepping stone into enhancing various field applications such as in agriculture (Compant, Clément, & Sessitsch, 2010).

Observation from the assay further provides a preliminary indication that *ecnI*-1 could be responsible for the bacterial IAA production. Besides, there are also several reports substantiate the ability of *E. cancerogenus* in producing IAA that enhance various plant growth such as poplar plant (Taghavi et al., 2010), Pigeon peas (Rani & Reddy, 2011) and *J. curcas* (Jha et al., 2012). From the RNA-seq analysis by comparing *ecnI*-1 knock-out mutant and its wildtype, several genes involved in auxin biosynthesis were down-regulated, such as branched-chain amino acid transport system/permease component

(*livM*), anthranilate synthase (*trpE*), transcriptional regulatory factor (*tyrR*) and aldehyde dehydrogenase (ALDH). This finding is further supported by the reduction of halo reddish-pink formation of mutant in the rapid plate assay. From RAST annotation, genes involved in plant hormone biosynthesis in *E. cancerogenus* strain M004 suggested that this strain produces auxin via tryptophan-dependent indolepyruvate pathway. The soil bacterium *E. cloacae* UW5 also produced auxin via indolepyruvate pathway (Parsons, Harris, & Patten, 2015). Parsons and colleagues (2015) further showed that IPA decarboxylase was regulated by TyrR with response to aromatic amino acids and branched-chain amino acids.

Bacteria are able to synthesize, conjugate and degrade IAA, regulating the IAA homeostasis in their cells. Bacterial IAA production can be influenced by different plant and environmental cues and IAA can be effector molecules in plant-microbe interactions that serves in both pathogenesis and phytostimulations (Maheshwari, 2015; Spaepen & Vanderleyden, 2011). IAA can also be used by bacteria as signal molecule to steer microbial behavior, hence more efforts should be continued to characterize the microbial biosynthetic pathways of IAA (Spaepen et al., 2007). Further studies will hence allow deeper insights on the role of IAA in microbial physiology.

5.12 Future Work

As genomic sequencing approaches are improving, a complete genome sequencing of *E. cancerogenus* strain M004 by using sequencing platform such as PacBio SMRT sequencing is one of the future work that is interesting to be explored. A complete genome could provide a full overview of the genes encoded in the bacterial system and hence, allow deeper exploitation on gene studies (Chin et al., 2013). Because the RAST analysis of *E. cancerogenus* strain M004 genome show 2 copies of LuxR homologues, hence it will be important to confirm the functionality of LuxR homologues in *E. cancerogenus* strain M004 via development of LuxR knock-out mutant by using recombineering approach, followed by transcriptome analysis. In addition to this, double mutant by knocking out *luxI* and *luxR* could be conducted and genes regulated by *luxI* and/or *luxR* can be elucidated. *E. cancerogenus* strain M004 showed plant growth stimulant properties and it is linked with QS. More work should be conducted (genotypic and phenotypic) in order to reveal the mechanisms involved which will lead to design of strategy for improving efficacy of PGPB agents.

CHAPTER 6: CONCLUSION

Bacterial isolation from Malaysia tropical rainforest waterfall has yielded 13 isolates with QS activity. In this study, *E. cancerogenus* strain M004 portray QS activity and produced two types of AHLs. This is the first discovery of *E. cancerogenus* strain M004 from tropical rainforest waterfall and this bacterium appears to be a plant stimulant via production of bacterial auxin that is QS-dependent. Transcriptome analysis showed that several metabolic processes such as (i) amino acid transport and metabolism (ii) inorganic ion transport and metabolism (iii) carbohydrate transport and metabolism (iv) cell wall / membrane / envelope biogenesis (v) cell motility of *E. cancerogenus* were significantly altered in both *ecnI*-1 knock-out mutant and treatment of gallic acid, as confirmed by RNA-seq.

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SUPPLEMENTARY

LIST OF PUBLICATIONS AND PAPER PRESENTED

A: List of Publications

Tan, W.S., Yunos, N. Y. M., Tan, P. W., Mohamad, N. I., Adrian, T. G. S., Yin, W. F., & Chan, K. G. (2014). Freshwater-borne bacteria isolated from a Malaysian rainforest waterfall exhibiting quorum sensing properties. *Sensors, 14*(6), 10527-10537.

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B: List of Presentations

Tan, W. S., Yunos, N. Y. M., Tan, P. W., Mohamad, N. I., Adrian, T. G. S., Yin, W. F., & Chan, K. G. (2014, June). *Freshwater-borne Bacteria "Talk" With N-acyl homoserine Lactone as Their "Language".* Poster presented at Monash Science Symposium – Monash University (International).

Tan, W. S., Yunos, N. Y. M., Tan, P. W., Mohamad, N. I., Adrian, T. G. S., Yin, W. F., & Chan, K. G. (2014, December). *N-acylhomoserine Lactone: Communication Bridge for Enterobacter sp., a Freshwater-borne Bacterium*. Poster presented at 19th Annual Biological Sciences Graduate Congress – National University of Singapore (International). *sponsored student