CHARACTERIZATION OF AHL-TYPE QUORUM SENSING IN *Cedecea neteri* SSMD04

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ABSTRACT

Bacteria demonstrate a form of cell-to-cell signalling for the regulation of their gene expression according to the change of population density. This is called quorum sensing. Different bacterial species utilizes different signalling molecules for quorum sensing and the most well studied quorum sensing system is the *N*-acyl homoserine lactone type quorum sensing found commonly in Gram-negative Proteobacteria. *Cedecea neteri* is an uncommonly studied bacteria in the Enterobacteriaceae family. It is a known human pathogen with unknown etiology. In a previous study to investigate the presence of bacteria that exhibit *N*-acyl homoserine lactone type quorum sensing isolated from food sources, a strain of *C. neteri* SSMD04 was found to exhibit quorum sensing activity, which was the first in this genus. By using triple quadrupole liquid chromatography mass spectrometry, it was identified that *C. neteri* SSMD04 produces C4-HSL as its signalling molecule. The gene responsible for C4-HSL production and the gene for the receptor that binds to C4-HSL, named *cneI* and *cneR*, were later found from its genome. These genes were found to be most closely related to a new species in the *Klebsiella* genus, *Klebsiella michiganensis*. However, *K. michiganensis* has never been reported to exhibit quorum sensing activity. A quorum sensing deficient mutant of *C. neteri* SSMD04 was later created by λ Red recombineering. Through global comparative transcriptomics, it was shown that *N*-acyl homoserine lactone type quorum sensing is responsible for the modulation of its metabolism.

Keywords: quorum sensing, AHL, *Cedecea neteri*, transcriptomics, metabolism

PENCIRIAN PENDERIAAN KUORUM JENIS AHL DI *Cedecea neteri* SSMD04

ABSTRAK

Bakteria menggunakan sejenis komunikasi antara sel untuk mengawal ekspresi gen mengikut pertukaran kepadatan populasi bakteria tersebut. Ini dikenali sebagai penderiaan kuorum. Spesies bakteria yang berbeza menggunakan jenis molekul isyarat yang berbeza, di mana molekul isyarat yang paling banyak dikaji ialah *N*-acyl homoserine lactone. Jenis molekul isyarat ini paling umum dijumpai di Proteobakteria Gram negatif. *Cedecea neteri* merupakan salah satu ahli *Enterobacteriaceae* yang jarang dikaji. Ia merupakan patogen manusia tetapi etiologinya tidak diketahui. Dalam satu kajian sebelum yang bertujuan untuk menemui bakteria yang menunjukkan aktiviti penderiaan kuorum jenis *N*-acyl homoserine lactone dari sumber makanan, *C. neteri* strain SSMD04 telah didapati menunjukkan aktiviti penderiaan kuorum jenis *N*-acyl homoserine lactone. Penemuan ini merupakan kes pertama dalam genus ini. Dengan menggunakan spektroskopi jisim selaras resolusi tinggi, *C. neteri* SSMD04 didapati menghasilkan C4-HSL sebagai molekul isyaratnya. Gen yang bertanggungjawab untuk penghasilan C4-HSL dengan gen reseptor C4-HSL tersebut, dinamakan *cneI* dan *cneR* masing-masing, telah dikenal pastikan dari genomnya. Kedua-dua gen ini mempunyai kaitan warisan yang paling dekat dengan spesies baru dalam genus *Klebsiella*, iaitu *Klebsiella michiganensis*. Walau bagaimanapun, *K. michiganensis* tidak pernah dilaporkan menunjukkan aktiviti penderiaan kuorum. Mutan kekurangan penderiaan kuorum *C. neteri* SSMD04 telah dihasilkan melalui λ Red recombineering. Dengan menggunakan perbandingan transkriptomik berskala global, penderiaan kuorum jenis *N*-acyl homoserine lactone di *C. neteri* SSMD04 didapati mengawal metabolisme.

Kata kunci: penderiaan kuorum, AHL, *Cedecea neteri*, transkriptomik, metabolisme

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SYMBOLS

^{0}C	Degree Celsius
× *g*	Gravity
µm	Micrometre
%	Percent
×	Times

ABBREVIATIONS

A	Adenine
ACN	Acetonitrile
ACP	Acyl carrier protein
AEA	Acidified ethyl acetate
AGE	Agarose gel electrophoresis
AHL	*N*-acylhomoserine lactone
AI-2	Autoinducer-2
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	Base pair
C	Cytosine
C4-HSL	*N*-butyrylhomoserine lactone
CBB	Coomassie Brilliant Blue R-250
CDC	Centers for Disease Control
cDNA	Complementary DNA
CDS	Coding sequences
CHD	Cyclohexanedione
cm	Centimetre
DAR	Diakylresorcinols
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DPD	4,5-dihydroxy-2,3 pentadione
EDTA	Ethylenediaminetetraacetic acid
EIC	Extracted-ion chromatogram
et al.	*et alii*
G	Guanosine
g	Gram
H_2O	water
hrs	Hours
HS	High sensitivity
IF	Inoculating fluid
kb	Kilo bases
kDa	Kilo dalton
LB	Luria-Bertani
LCMS/MS	Triple quadrupole liquid chromatography mass spectometry
M	Molarity
m/z	Mass to charge ratio
MALDI-TOF	Matrix-assisted laser desorption ionization time-of-flight
MS	Mass spectometry
MEGA	Molecular Evolutionary Genetics Analysis
mg	Milligram

min	Minute
mL	Millilitre
mM	Millimolar
MOPS	3-(*N*-morpholino)propanesulfonic acid
mRNA	Messenger RNA
N	Normality
NaCl	Sodium chloride
NADH	Nicotinamide adenine dinucleotide
NCBI	National Center for Biotechnology Information
NGS	Next generation sequencing
OC12-HSL	*N*-(3-oxododecanoyl)-homoserine lactone
OC6-HSL	*N*-(3-oxohexanoyl)homoserine lactone
PacBio SMRT	Pacific Biosciences single-molecule real-time
PATRIC	Pathosystems Resources Integration Center
PCR	Polymerase chain reaction
PM	Phenotype microarray
PPY	Photopyrone
PQS	*Pseudomonas* quinolone signal
psi	Pounds per square inch
qRT-PCR	Quantitative reverse-transcriptase PCR
QS	Quorum sensing
RAST	Rapid Annotation using Subsystem Technology
RIN	RNA Integrity Numbers
RLU	Relative light units
RNA	Ribonucleic acid
RNA-seq	RNA sequencing
rpm	Revolutions per minute
rRNA	Ribosomal RNA
s	Seconds
SAM	S-adenosylmethionine
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide electrophoresis
SLE	Systemic lupus erythematosus
SNP	Single nucleotide polymorphism
sp.	Species
spp.	*Species pluralis*
ssDNA	Single stranded DNA
subsp.	Subspecies
T	Thymine
TBE	Tris borate EDTA
tRNA	Transfer RNA
TSA	Tryptic soy agar
UV	Ultraviolet
v/v	Volume over volume
w/v	Weight over volume
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galacto-pyranoside

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Research Background

Bacteria are able to communicate among themselves through a chemical signaling system called quorum sensing (QS) which enables responses to be made accordingly to the changes of the bacterial population density. The term QS was first coined by Fuqua et al. (Fuqua et al., 1994). QS involves the synthesis, release, detection, and response to small, diffusible signaling molecules. Many different types of signaling molecules have been identified so far, and the most commonly studied signaling molecules are the ones deployed by Gram-negative bacteria, the *N*-acylhomoserine lactone (Eberhard et al., 1981).

Cedecea neteri is a Gram-negative bacterium that is known to be a human pathogen. It was named in 1982 (Farmer et al., 1982) but later isolation cases remain rare and very little studies has been done on this bacterium. However, it has been known to be resistant to multiple antimicrobial compounds, and this phenotype is common among the members of the same genus (Farmer et al., 1982; Aguilera et al., 1995). A strain of *C. neteri* has been isolated from pickled mackerel sashimi (*Shime saba*) and it has been found to demonstrate AHL-type QS activity (Tan, 2014, unpublished data). Therefore, it is interesting to study its QS activity as well as its genomic components involved in its QS circuit. Besides that, due to the wide range of phenotypes controlled by QS in other bacterial species, it will be fascinating to look into the regulatory role played by QS in this bacterium.

1.2 Hypothesis

C. neteri SSMD04, a rare bacterium demonstrates AHL-type QS activity, which has never been reported before. Therefore, the study of its QS system can lead to a better understanding of this bacterium and possibly unearth unknown regulatory role played by QS.

1.3 Objectives

1. To identify *C. neteri* SSMD04 using molecular and phenotypic approaches.
2. To characterize the AHL-type QS in *C. neteri* SSMD04.
3. To identify the *luxI/R* homologues from *C. neteri* SSMD04 and to characterize the *luxI* homologue by gene cloning and its heterologous expression in *E. coli*.
4. To construct a QS-deficient mutant strain of *C. neteri* SSMD04 through site-targeted mutagenesis and subsequently investigate the genes regulated by the AHL-type QS system in *C. neteri* SSMD04 through comparative transcriptomics between the wild type and QS-deficient mutant strain.

CHAPTER 2: LITERATURE REVIEW

2.1 Quorum Sensing

Bacteria demonstrate cell-to-cell communication for the regulation of gene expression in response to fluctuations in cell population density. This process is termed quorum sensing (QS) (Miller & Bassler, 2001). This involves the synthesis, release, detection and response to a type of small diffusible signaling molecules, called the autoinducers. The detection of the autoinducers allows the bacteria to distinguish the variation between low and high cell population density so that they can alter gene expression accordingly (Schauder & Bassler, 2001). In order to switch on QS circuits, the bacterial population needs to reach a threshold level of population density, as a high density of cells would lead to high extracellular concentration of the autoinducers. If such condition is met, the autoinducer molecule would bind to the cognate receptor and the signaling molecule-receptor complex drives the downstream gene regulation. As it is a synchronized response, QS enables bacteria to exhibit a phenotype in unison, similar to chemical signaling in multicellular organism.

The concentration of autoinducer molecules as a function of population density is, despite a popular one, not the only existing theory to explain cell-to-cell signalling. Another interesting explanation was also proposed to explain the function of bacterial cell-to-cell signaling as an opposition to QS (Redfield, 2002). This hypothesis, called diffusion sensing, states that the bacterial cells use autoinducer concentration to monitor the rate of diffusion of extracellular molecules instead of population density. The higher the concentration of autoinducer in the extracellular environment, the lower the rate of diffusion. This is because high rate of diffusion would lead to autoinducer molecules diffusing away from the cells. A threshold level would signify a favorable conditions for

the production of exo-products, as they remain within the proximity. This theory, apparently, fits the observation of exo-products production controlled by QS, but not some other phenotypes. Apart from that, another hypothesis, the efficiency sensing (Hense et al., 2007) had also been proposed after the introduction of diffusion sensing to better explain the function of this form of cell-to-cell signaling. The author explained that this behavior of cell-to-cell signaling is not a simple function of bacterial cell density, but rather the action of other factors such as diffusion rate and spatial distribution acting in conjunction. Nevertheless, QS has been the most fitted term for its explanation (Lee & Zhang, 2015) despite other attempts as described. In fact, population density, population size, as well as the convective rate of solution surrounding a microcolony has been shown to affect cell-to-cell signaling, and it is generally described in broad sense as QS. (Connell et al., 2010).

QS was first reported in *Aliivibrio fischeri* (formerly known as *Vibrio fischeri* (Urbanczyk et al., 2007)), a bioluminescent marine bacterium that demonstrates symbiotic relationship with marine animal hosts (Hastings & Nealson, 1977; Nealson & Hastings, 1979). The most stereotypical model in the explanation of QS is the partnership between *A. fischeri* and *Euprymna scolopes*, a species of Hawaiian squid. This squid harbours *A. fischeri* within its light organ in the center of its body. When it is feeding at night, it emits light downward to counteract the shadow imposed by the moonlight, thus concealing its presence. This phenomenon is called counterillumination. This helps the squid in the evasion of predator lurking at the bottom of the sea. The bioluminescence of *A. fischeri* is now known to be controlled by QS, where light is emitted only when the population density of *A. fischeri* is high such as in the environment provided in the light organ of *E. scolopes*. Interestingly, the light organ of *E. scolopes* does not illuminate all the time. Apparently, *E. scolopes* expulses *A. fischeri* each morning following sunlight, which

signals the end of nocturnal activity. The population of *A. fischeri* then builds up over the day until the population is sufficiently dense to activate its bioluminescence again (Ruby & Lee, 1998). It has been found that *A. fischeri* can reach a density of 10^{11} cells per cm^3 within the said organ (Visick & McFall-Ngai, 2000). This exploitation of *A. fischeri*'s bioluminescence is an exhibition of a symbiotic relationship as *E. scolopes*, in return, provides *A. fischeri* with nutrients. Although bacterial co-operation through cell-to-cell signaling has previously been shown, such as the formation of fruiting body in myxobacteria (Kiskowski et al., 2004), this study of *A. fischeri* was the first instance where the autoinducer molecule was identified.

QS has later been found to not be an exclusive behavior exhibited by *A. fischeri*, but a norm in all bacterial communities (Bassler & Losick, 2006). In fact, we have slowly come to an understanding to the mechanisms of QS in many other bacteria such as *Agrobacterium tumefaciens* (Swiderska et al., 2001), *Pectobacterium carotovora* (Koiv & Mae, 2001), *Burkholderia cepacia* (Huber et al., 2001), *Pseudomonas aeruginosa* (Smith et al., 2002), *Bacillus subtilis* (Lazazzera & Grossman, 1998), *Staphylococcus aureus* (Tegmark et al., 1998), *Enterococcus faecalis* (Haas et al., 2002), and many others. Interestingly, with regard to bioluminescence in *A. fischeri*, phenotypes regulated by QS are extremely diverse. Hitherto, QS has been found to be responsible for the regulation of biofilm formation, plasmid conjugation, virulence, swarming motility, antibiotics production, and genetic competence (Okada et al., 2005; Williams et al., 2007).

2.2 Types of Autoinducer

The autoinducer responsible for the light production in *A. fischeri* was later identified as an *N*-acylhomoserine lactone (AHL) (Eberhard et al., 1981). Different bacterial species deploy AHLs of varying structure in terms of degrees of saturation (none, one or two

double bonds) and length of acyl side chain (C4 to C18), as well as the nature of the C3 substituent (3-oxo, 3-hydroxy or unsubstituted) (Ortori et al., 2007). Variations in the AHL molecules give rise to signal specificity of different quorum sensing circuits. It is now established that Gram-negative Proteobacteria typically employs AHL as the signaling molecules, which is detected by its cognate receptor, the LuxR type proteins. This AHL-LuxR complex binds to DNA and activates transcription of targeted QS genes.

However, as the study of QS encapsulates a broader range in taxonomical sense, more classes of autoinducer have been unveiled. Even though there has been fewer focus on Gram-positive bacteria in terms of QS study, we now know that Gram-positive bacteria such as *Bacillus* and *Streptococcus* mostly communicate with short peptides as they do not harbour *luxI* or *luxR* homologues. The autoinducers used by *B. subtilis*, for example, is a short peptide (6 amino acids) with modification on the tryptophan residue (Okada et al., 2005). In this system, the peptides are bound by two-component sensor histidine kinases found on the cell membrane. To be specific, the peptide binds to the membrane bound histidine kinase as the population density is high, resulting in an ATP mediated autophosphorylation. The phosphate group is added to a highly conserved histidine kinase residue that faces the cytoplasm. This phosphate group is used to activate a cytoplasmic response regulator which acts as a transcriptional regulator. Therefore, in Gram-positive bacteria, the histidine kinase (autoinducer recognition) and the response regulator (transcriptional regulator) are not part of the same protein, and thus 'two-component'. Interestingly, this form of signal transduction proceeded by phosphorylation kinases is reminiscent to the mechanism of hormone signaling in humans. Expectedly, this peptide QS system, like AHL type QS system, exhibits signal specificity. These two signaling systems are by far the most well-known QS systems.

Another type of signaling molecule is derived from 4,5-dihydroxy-2,3 pentadione (DPD), termed autoinducer-2 (AI-2). This molecule, first found and identified in *Vibrio harveyi* (Cao & Meighen, 1989), is synthesized by LuxS type protein. Unlike the aforementioned AHLs and short peptides, AI-2 has been found to be employed by a wide range of bacterial species, suggestive of the use of AI-2 in interspecies communication (Xavier & Bassler, 2005). However, this role is yet to be studied in detail. There are many other less studied signaling molecules such as γ-butyrolactones used by streptomycetes (Khokhlov et al., 1967; Onaka et al., 1995); *Pseudomonas* quinolone signal (PQS) found in *Pseudomonas aeruginosa* (Bredenbruch et al., 2006); mixture of amino acids by *Myxococcus xanthus* (Kuspa et al., 1992), 3-OH palmitic acid methy ester, cyclic dipeptides (Waters & Bassler, 2005). Studies have continuously been discovering new signaling molecules synthesized by bacteria as well as the complexity in the cell-to-cell signaling system. For instance, LuxR solo, LuxR that has no cognate LuxI, were first thought to sense AHLs produced by other LuxIs, but it has recently been found to sense photopyrones (PPYs) in *Photorhabdus luminescens* (Brachmann et al., 2013); dialkylresorcinols (DARs) and cyclohexanediones (CHDs) in *P. asymbiotica* respectively (Brameyer et al., 2015), despite their sequence similarity to LuxR type proteins. Evidently, bacterial cell-to-cell signaling remains a largely elusive field to be explored.

2.3 Genetic basis of AHL type QS

AHL type QS is dependent on two central components, the autoinducer synthase which mediates the final synthesis step of autoinducer, and the cognate autoinducer receptor protein. The QS systems in the model organisms, *A. fischeri* and *P. aeruginosa* provides very good examples for the understanding of AHL type QS systems. The QS system in *A. fischeri* provides a direct insight into the genetic basis of QS. In *A. fischeri*, the autoinducer synthase gene and the receptor gene were named *luxI* and *luxR* respectively

(Engebrecht & Silverman, 1984). LuxI protein synthesizes the autoinducer, *N*-(3-oxohexanoyl)homoserine lactone (OC6-HSL) of which when accumulated to a threshold level favorably binds to LuxR due to the shift of dynamic equilibrium. LuxI type protein catalyzes the synthesis of AHLs by forming an amide bond between S-adenosylmethionine (SAM) and an acylated acyl carrier protein (ACP) (Schaefer et al., 1996). The use of SAM is invariant in homologs of LuxI protein, but the selectivity of variable length of acyl chain from acylated ACP, which is dependent on the LuxI type protein, results in different types of AHL molecule (Parsek et al., 1999; Watson et al., 2002; Gould et al., 2004). Upon the binding of OC6-HSL to LuxR molecule, the autoinducer-receptor complex subsequently binds to the upstream of the *lux* operon to initiate transcription of genes required for the enzymatic production of light (Engebrecht et al., 1983). Gene cloning experiments allowed identification of genes responsible for this light production as well as their chromosomal arrangements. The experiments have shown that the *luxI* and *luxR* genes are arranged divergently where the functional genes required for light production are arranged tandemly downstream of *luxI* in the order of *luxCDABEG*. The *luxA* and *luxB* encode the α and β subunits of luciferase enzyme which catalyzes the oxidation of aldehyde and reduced flavin mononucleotide, while releasing light energy (Dunn et al., 1973). On the other hand, *luxCDE* are translated into components of a fatty acid reductase system with reductase, synthetase and transferase activities which are involved in the synthesis of aldehyde substrate required for the reaction catalyzed by luciferase (Boylan et al., 1989). Lastly, *luxG* encodes a flavin reductase that supplies luciferase with reduced flavin mononucleotide (Zenno & Saigo, 1994).

A. fischeri's QS system provides a simplified insight into its regulatory roles. However, an AHL-type QS system can be very complicated. Fortuitously, the advancement in

molecular biology technologies provided evidence of the complexity of QS system. *P. aeruginosa*, for example, possesses two AHL type QS systems, the *las* and *rhl* systems, that work in hierarchy, and they regulate approximately six percent of *P. aeruginosa*'s total number of genes (Schuster et al., 2003) as revealed by genome wide global study. This number, in comparison to what was known of the QS system in *A. fishceri* in the 80s, is shocking. However, this is also how scientists have come to the realization that QS system does not govern only one operon, but a master regulator of multiple of them. In *P. aeruginosa*, the *las* system consists of a LasI that is involved in the synthesis of *N*-(3-oxododecanoyl)-homoserine lactone (OC12-HSL), which is detected by LasR (Pearson et al., 1994), similar to a canonical QS system; the *rhl* system, on the other hand, consists of a RhlI that is involved in the synthesis of *N*-butyryl-homoserine lactone (C4-HSL), which is detected by RhlR (Pearson et al., 1995). Both of the LuxR homologues bind to their cognate AHLs to form complexes that bind to conserved *las* and *rhl* boxes found in the promoter of the target genes. Surprisingly, transcriptomic studies have shown that these two systems do not operate in a mutually exclusive manner. While some genes are regulated by either one of the systems, some others can be regulated by both of the system, including some of the key virulence genes (Schuster & Greenberg, 2006). To complicate things further, *rhlI/R* were found to be within the regulon of LasI/R system, such that the activation of LasI/R system activates the RhlI/R system as well (Latifi et al., 1996).

Besides that, LasR also upregulates a transcriptional repressor of *lasI*, RsaL, which forms a negative feedback loop to counteract its own autoinduction, thereby controlling the QS circuit upon activation (Rampioni et al., 2007). This network of regulation have shown how QS system is finely tuned to tightly and carefully regulate gene expressions. Furthermore, in recent years, a study have shown that the two QS circuits in *P. aeruginosa*

acts combinatorially to assess the social and physical environment. The social environment refers to the cellular density and the physical environment refers to the diffusion rate of AHL molecules away from the producing cells. Due to different decay rates of the AHLs, the cell can differentiate the differences of the aforementioned environment in the presence of either one of the AHLs, or both, or none (Cornforth et al., 2014). More studies are required to know if this phenomenon is widespread in the prokaryotic kingdom but it nonetheless have demonstrated the intricate complexity of bacterial QS systems. More intriguingly, QS control of biofilm formation in *P. aeruginosa* (Davies et al., 1998) has been found to be influenced by nutritional environment (Shrout et al., 2006), showing multiple factors to the regulation of QS regulate genes.

2.4 QS inhibition

Prolong abuse of antimicrobial compounds have created a critical phenomenon in which pathogenic bacteria have attained the resistance to traditional antimicrobial compounds, rendering the once miraculous treatment ineffective. Such abuse is commonly seen in animal rearing where sub-therapeutic doses of antibiotics are used for promoting growth or preventing diseases. This leads to the development of resistant microorganisms which can eventually spread to humans. This phenomenon happens because conventional antibiotics acts either by killing the bacterial cells or inhibiting growth by interfering with cellular functions, therefore imposing a selection pressure. Eventually, this results in the emergence of antibiotic resistant strains (Prestinaci et al., 2015). To make matters worse, the plasticity of bacterial genome coupled with horizontal gene transfer promote accumulation of multiple antibiotic resistance genes within a bacterial species, and this phenomenon is becoming common. As multidrug resistant strain of pathogenic bacteria is on the rise, we are running out of antibiotics to combat these lethal infections (Hentzer

& Givskov, 2003). Recently, there was a report on the emergence of plasmid-mediated colistin resistance in veterinary *E. coli* in China, the antibiotic considered to be the last resort in the treatment of multidrug resistance infections (Liu et al., 2016). Despite being thought to be confined within China, multiple findings in other countries such as United States and United Kingdom have shown that colistin resistance is more widespread than speculated (Gallagher, 2015; Smith, 2016). This calls for the need for an alternate disease control strategy.

Many bacterial species employ QS to control virulence as well as the formation of biofilm, thus making it an ideal target for novel therapeutic treatment. This form of treatment offers an advantage as it does not inhibit growth but merely silencing microbial activity, and thus in theory will not exert selective pressure that would lead to the development of resistance mechanism. A study carried out by Dong and his colleagues, for example, has shown some encouraging results of QS inhibition in combating plant pathogens. When an AHL degrading enzyme, AiiA, was expressed in a line of transgenic potato and tobacco plants, strong resistance to *P. carotovora* infection was observed (Dong et al., 2001). Besides that, virulence of the human pathogen, *P. aeruginosa* was significantly attenuated in mice when treated with synthetic furanones, a compound found to destabilize LuxR type proteins (Hentzer et al., 2003; Wu et al., 2004). Multiple lines of approach have been attempted to attenuate pathogenic bacteria through action on their QS systems similar to these two experiments, but QS inhibition generally employs three strategies: inhibition of AHL signal generation, inhibition of AHL signal dissemination, and inhibition of AHL signal reception. Inhibition of AHL signal generation typically involves the use of SAM analogs such as *S*-adenosylcycsteine, *S*-adenosylhomocysteine, sinefungin to interfere with AHL synthesis; inhibition of AHL signal dissemination can be achieved by degrading AHL molecules through the action of AHL degrading enzymes;

inhibition of AHL signal reception, on the other hand, involves the use of antagonist molecules to compete with AHL in the binding towards LuxR-type proteins (Hentzer & Givskov, 2003). Therefore, it has become even more important than before to study the molecular mechanism of the underlying regulatory role played by QS for the execution of such strategies.

2.5 *Cedecea neteri*

Cedecea spp. are uncommonly isolated Gram-negative bacteria that belong to the Enterobacteriaceae family (Berman, 2012). The representative of the genus is lipase-positive and resistant to colistin and cephalothin. The name *Cedecea* was coined by Grimont and Grimont, from the abbreviation of the Centers for Disease Control (CDC) (Grimont et al., 1981). Originally recognized as Enteric group 15, this genus is comprised of five species, out of which only three are now validly published, *C. neteri*, *C. lapagei*, *C. davisae*, while the other two were not validly published and are known as *Cedecea* species 3 and *Cedecea* species 5 (Brenner, 2005).

This genus started as a group of 17 unidentified lipase positive Enterobacteriaceae strains discovered in Centers for Disease Control (CDC) in 1977. These strains were later grouped into a new genus *Cedecea* in 1981, with the name derived from the abbreviation of CDC. Originally, only two valid species were published, *C davisae* and *C lapagei*. As they were newly identified, *Cedecea* spp. were not known for their clinical significance, as none of the collected strains was from blood or spinal fluid (Grimont et al., 1981). In 1982, *C. neteri* (pronunciation: suh dee' see ah knee' ter eye) was first reported for its clinical significance when a case of bacteremia caused by *C. neteri* was discovered in a patient of 62 years old (Farmer et al., 1982). *C. neteri* differs from other members of the genus *Cedecea* in that *C. neteri* is negative for ornithine decarboxylase (Moeller's),

fermentation of raffinose and melibiose, but positive for fermentation of sucrose, D-sorbitol, D-xylose, malonate utilization and it grows in media without thiamine (Farmer et al., 1982). Nevertheless, subsequent isolation of *C. neteri* remains rare. In 1995, another case of *C. neteri* infection was reported that seemingly led to the patient's death. The 27 years old patient was treated with immunosuppressive drugs for systemic lupus erythematosus (SLE), which resulted in predisposition to severe bacterial infection, despite treatment with multiple antibiotics including vancomycin, ceftazidime and gentamicin (Aguilera et al., 1995). Due to the fact that *C. neteri* has been found to infect old and immunocompromised patients, the authors of this report have also suggested that *C. neteri* be viewed as an opportunistic pathogen, which seems fit to the first clinical case as well. However, the role of *C. neteri* in human disease is still largely unknown. In fact, there is no other publication on *C. neteri* infection in the PubMed database during the writing of this thesis (7th Feb 2017).

Other members of *Cedecea* spp. have later been shown to be clinically important as well. The first documentation of *C. davisae* bacteremia was in 1986 (Perkins et al., 1986). Multiple cases of *C. davisae* infections were reported since then together with *C. lapagei*. They appear to be emerging pathogens and they were found to be causal agents of pneumonia. Table 2.1 shows the previously reported cases of bacterial infections in humans caused by *Cedecea* spp.

Table 2.1: Previous reports of *Cedecea* spp. infections.

Species	Diagnosis	Resistant to	Susceptible to	Reference
neteri	bacteremia	colistin, cephalothin, ampicillin	cefamandole, chloramphenicol, tetracycline, gentamicin, tobramycin, amikacin	(Farmer et al., 1982)
neteri	bacteremia	amoxicillin, cephalosporins, amoxicillin/clavulanic acid, aminoglycosides	vancomycin	(Aguilera et al., 1995)
lapagei	peritonitis	-	-	(Davis & Wall, 2006)
lapagei	bacteremia	gentamicin, tobramycin, cefalothin, cefuroxime sodium, cefoxitin, ampicillin, piperacillin, nitrofurantoin, tetracycline	amikacin, meropenem, ceftazidime, cefotaxime, cefepime, aztreonam, amoxicillin/clavulanate, piperacillin/tazobactam, trimethoprim/sulfamethoxazole, ciprofloxacin, levoflaxacin	(Dalamaga et al., 2008)

Table 2.1, continued.

Species	Diagnosis	Resistant to	Susceptible to	Reference
lapagei	pneumonia	amoxicillin, amoxicillin/clavulonic acid, cefuroxime, ceftazidime, ceftriaxone, imipenem, ciprofloxacin, gentamicin, amikacin	sulbactam/cefoperazone	(Yetkin et al., 2008)
lapagei	pneumonia	amikacin, ampicillin, aztreonam, cefazolin, cefepime, ceftriaxone, cirpofloxacin, ertapenem, imipenem, meropenem, moxifloxacin, nitrofurantoin, piperacillin/tazobactam, trimethoprim/sulfamethoxazole	ampicillin/sulbactam, gentamicin, tobramycin, tigecycline	(Lopez et al., 2013)

Table 2.1, continued.

Species	Diagnosis	Resistant to	Susceptible to	References
lapagei	traumatic wound infection	ampicillin, ampicillin/sulbactam	cefuroxime, amikacin, cotrimoxazole, ciprofloxacin, cefotaxime, carbapenems	(Salazar et al., 2013)
lapagei	malignant oral ulcer	ampicillin/sulbactam, tetracycline, tigecycline	amikacin, gentamicin, ceftazidime, ceftriaxone, cefepime, ciprofloxacin, meropenem, trimethoprim/sulfamethoxazole	(Biswal et al., 2015)
lapagei	pneumonia	amoxicillin-clavulanic acid, cefoxitin	piperacillin, cefotaxime, ceftazidime, cefepime, imipenem, amikacin, ciprofloxacin, tetracycline, trimethoprim-sulfamethoxazole	(Hong et al., 2015)
davisae	bacteremia	cefoxitin, ampicillin, cephalothin	amikacin, cabenicillin, gentamicin, tobramycin, cefotaxime, cefoperazone, piperacillin, tetracyline	(Perkins et al., 1986)

Table 2.1, continued.

Species	Diagnosis	Resistant to	Susceptible to	Reference
davisae	leg ulcer and bacteremia	cefalothin, cefuroxime sodium, cefoxitin, ampicillin, peperacillin, nitrofurantoin, tetracycline	amikacin, gentamicin, tobramycin, meropenem, ceftazidime, cefotaxime, cefepime, aztreonam, amoxicillin/clavulanate, piperacillin/tazobactam, trimethoprim/sulfamethoxazole, ciprofloxacin, levofloxacin	(Dalamaga et al., 2008)
davisae	oral ulcer	cefazolin	fluoroquinolones, trimethoprim/sulfamethoxazole, cefepime, ceftazidime, ceftriaxone, gentamicin, cefotetan, ampicillin	(Mawardi et al., 2010)
davisae	bacteremia	-	aminoglycosides, cefepime	(Abate et al., 2011)
davisae	polymicrobial pulmonary infection	beta-lactams, aminoglycosides, fluoroquinolones, tigecycline	trimethoprim/sulfamethoxazole	(Ismaael et al., 2012)

Table 2.1, continued.

Species	Diagnosis	Resistant to	Susceptible to	Reference
davisae	bacteremia	-	cefoxitin, ciprofloxacin, ceftriaxone, ceftazidime, imipenem, gentamicin, aztreonam	(Akinosoglou et al., 2012)
davisae	bacteremia	ampicillin, ampicillin/sulbactam, cefazolin	amikacin, ceftazidim, ciprofloxacin, gentamicin, meropenem, trimethoprim/sulfa, levofloxacin	(Peretz et al., 2013)
davisae	retroperitoneal bleeding	third generation cephalosporins and carbapenems	-	(Ammenouche et al., 2014)
davisae	atrophic rhinitis	-	levoflaxacin	(Bayir et al., 2015)

2.6 Global Transcriptomics in the study of QS

The study of QS was initiated by the observation on the bioluminescence in *A. fischeri* in liquid culture that was absent until the bacterium reaches its logarithmic growth phase (Nealson et al., 1970). A typical forward genetics procedure to seek the genetic basis of a known phenotype generally involves creating mutants through random mutagenesis, followed by identification of the genetic locus responsible for the loss of the selected phenotype. Once identified, complementation test is carried out to verify the function of the gene. The genetic circuit of QS in *A. fischeri* was discovered in such manner (Engebrecht et al., 1983). However, scientists have come to the realization that QS does not control merely one operon but multiple of them. In fact, this has been shown in *A. fischeri* where 30 genes are under the *luxI/R* regulation through DNA microarray study, a substantial contrast to the number of genes when *luxI/R* were first discovered. Apart from the genes related to light production, genes that code for protease and peptidase functions, an ABC-type transporter, and a mechanosensitive ion channel were found to be regulated by *luxI/R*, distributed among 4 operons and 9 single-gene units (Antunes et al., 2007). This has shown that typical forward genetics is a limited measure in the understanding of QS regulation. The instigation of reverse genetics, where the genotype is known but the phenotype is being searched for, helps providing more information on QS in its entirety. Global transcriptome study has also therefore been more widely adapted for QS studies as it provides a holistic view on the regulatory role of QS.

Transcriptomics have been extremely helpful in the understanding of gene structures and gene regulation at the transcript level as it helps in the interpretation of functional sequences of a gene as well as quantification of its transcripts. However, whole transcriptome studies in prokaryotes have not been a major focus due to several reasons. Firstly, the prokaryotic transcript structures were long regarded as simplistic compared to

eukaryote's, as they typically lack introns and therefore alternative splicing is non-existent. Each gene is generally transcribed into one type of transcript with minimal modifications. Secondly, the majority of the transcripts recovered from a prokaryotic cell is made up of ribosomal RNA and tRNA (>95%). The sequencing of such content would yield mostly non-informative sequences, by which whole transcriptome study is therefore implausible. Besides that, enrichment or purification of mRNA is an important step in the success of a transcriptomic experiment. However, they were technically challenging in prokaryotes as prokaryotic mRNAs lack the 3'-end poly(A) tail, a typical signature of mature mRNA in eukaryotes (Sorek & Cossart, 2010), which is typically used for RNA capturing in eukaryotes. However, enrichment of mRNA through procedures such as rRNA capture using magnetic beads or degradation of processed RNA by the action of exonucleases (Wang et al., 2009) have been proven successfully on prokaryotic RNA. Successful mRNA enrichment also bypasses the problem imposed by the presence of rRNA and tRNA. It is therefore a growing phenomenon that prokaryotic transcriptomic study is expanding quickly.

Evidently, in recent years, QS studies have been conducted in a more systematic and thorough method. This is critically important in the study of QS systems as the transcriptional regulator typically regulates more than one gene/operon in a given organism. A whole genome scrutiny for QS regulated genes gives a more detailed and in-depth picture as the number and types of genes controlled can be found. For example, a recent whole genome transcriptomics were conducted on *Burkholderia thailandensis*, a bacterium that possesses three AHL type QS systems. By comparing the transcriptomic profiles of different mutants, that is, mutant deficient in one of the QS systems each, different regulons can be identified and categorized into their respective regulator. Furthermore, since transcriptomics of QS are growth phase dependent due to its

dynamics, the effect of QS systems at different growth phase can be investigated. However, due to the complexity of the QS systems present in *B. thailandensis* coupled with the challenges in the interpretation of transcriptomic data, it was difficult to group the genes under specific transcriptional regulator, or the possibility of the presence of other regulatory factors, as well as the interactions between the QS systems, as pointed out by the authors (Majerczyk et al., 2014). Clearly, stronger resolution in terms of bioinformatics power is still required for the resolution of such large amount of data from this type of transcriptomic study. Meanwhile, similar experimental procedures were performed on a common plant pathogen, *Pantoea stewartii* subsp. *stewartii*, which is known to cause Stewart's wilt disease in corn plants. Even though its QS system has been extensively studied and multiple genes regulated by its QS system identified, a whole genome transcriptomic study managed to identify additional QS regulated targets that had been overlooked from previous investigations (Ramachandran et al., 2014). Such reports have demonstrated clearly the superiority of whole genome comparative transcriptomics in the study of QS regulatory system.

2.7 *C. neteri* strain SSMD04

A non-clinical strain of *C. neteri* namely SSMD04 was isolated from *Shime saba*, a traditional Japanese cuisine of *saba* (mackerel) marinated with salt and rice vinegar, enabling the usually perishable saba to be preserved and consumed in the form of *sashimi* (raw fish). The isolation stemmed from an unpublished study (JY Tan, 2014, unpublished data) that aimed to profile and characterize the bacteria that demonstrate AHL-type QS as well as to investigate the role of AHL-type QS in terms of food spoilage and food safety. We believe that this was the first isolation of *C. neteri* from a food source, despite *C. neteri* not being a known microbial flora in food. Due to the novelty of this bacterium as well as the absence of publicly available genome sequence of *C. neteri*, the genome of

C. neteri SSMD04 was sequenced and published (Chan et al., 2014). The whole genome of strain SSMD04 was sequenced with a Pacific Biosciences single-molecule real-time (PacBio SMRT) sequencer with 20-kb SMRTbell library. De novo assembly of the reads resulted in a single contig of 4.88 MB in size and has a GC content of 55.1 %. Figure 2.1 shows the illustration of the genome features of *C. neteri* SSMD04 retrieved from the PATRIC (Pathosystems Resources Integration Center) (Wattam et al., 2014).

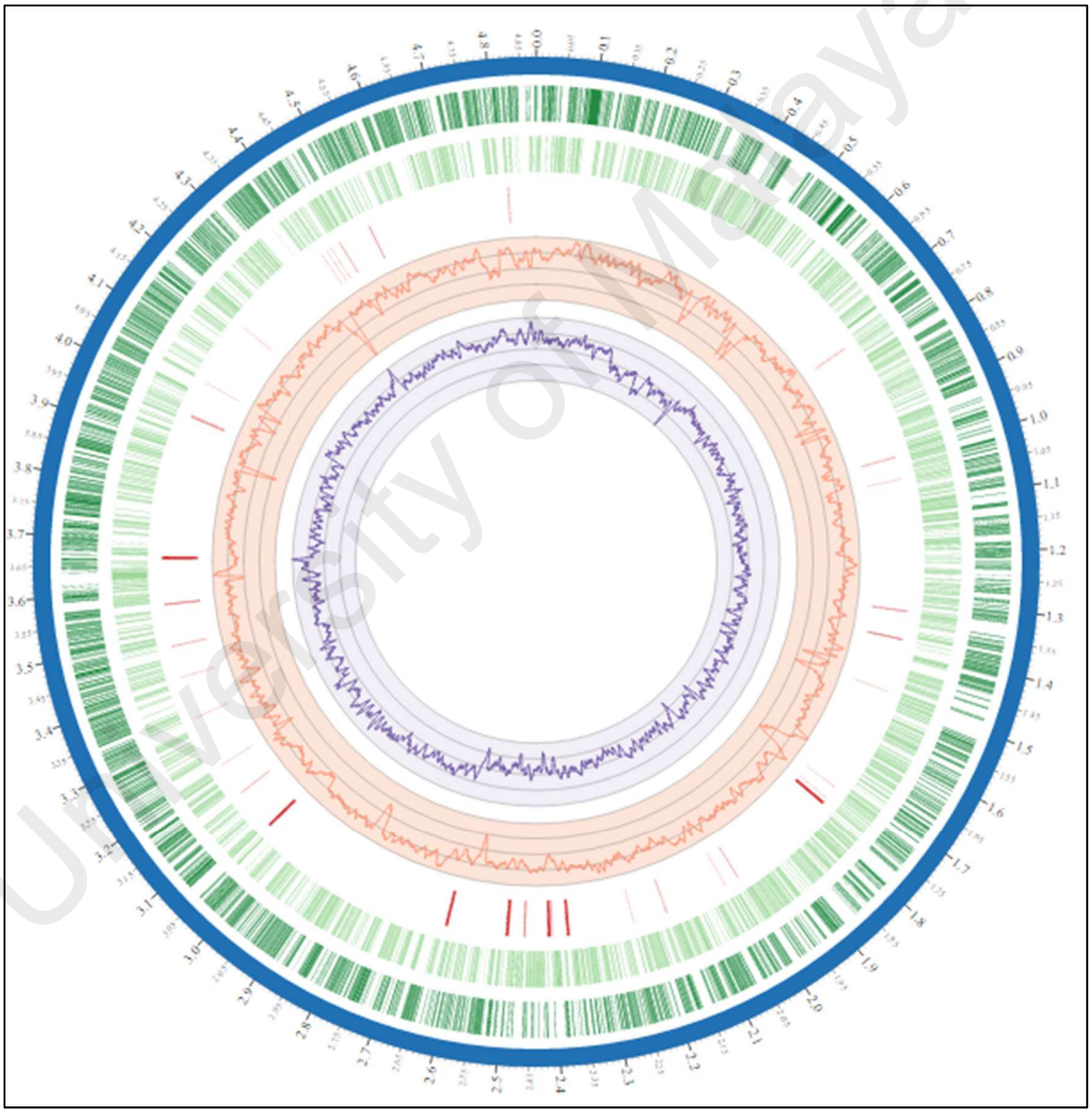

Figure 2.1: Illustration of *C. neteri* SSMD04 genome. The list of tracks from outside to inside: Contig, CDS (forward strand), CDS (reverse strand), RNAs, GC content, GC skew.

The annotation using RAST (Rapid Annotation using Subsystem Technology) (Aziz et al., 2012) have identified 4472 coding sequences as well as 103 RNA sequences. Only a total of 56.64 % of the annotated coding sequences were characterized into 539 subsystems available in the RAST database. Besides the genes common to the survival requirements of the bacterium itself, strain SSMD04 harbours 101 genes that were categorized into the virulence, disease and defense subsystem. However, the majority of these genes belongs to resistance to antibiotics and toxic compounds, suggesting the high resistance of this bacterium towards antibiotics and bactericidal compounds (Figure 2.2).

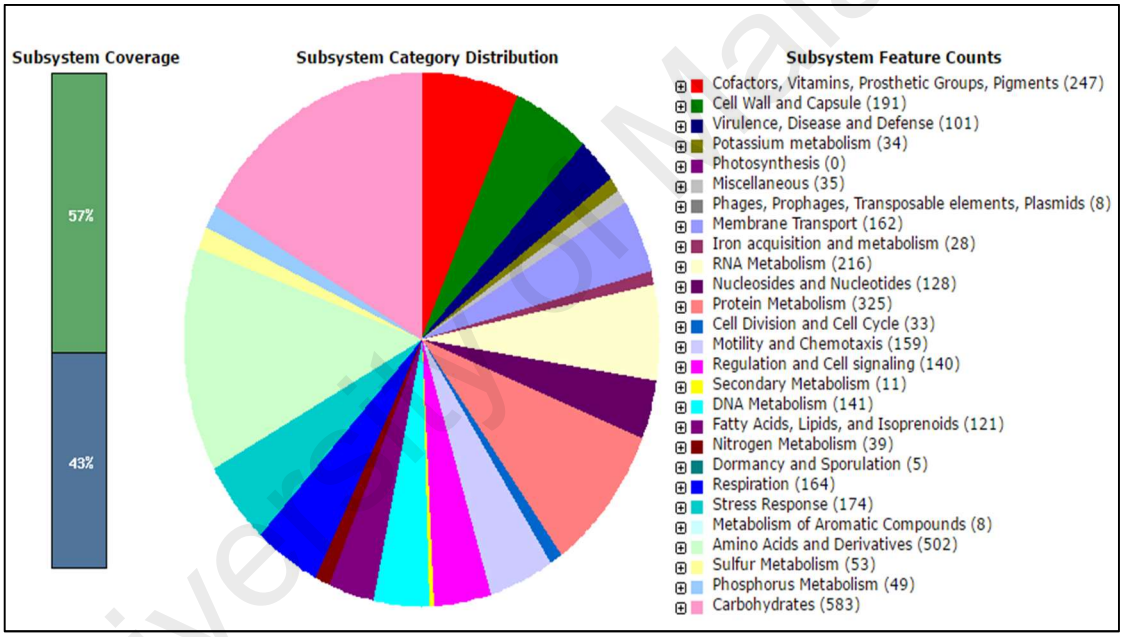

Figure 2.2: Subsystem distribution of coding sequences of *C. neteri* SSMD04. The bar on the right shows the percentage of coding sequences categorized in the subsystem (green = in subsystem, blue = not in subsystem). The pie chart represents the distribution of coding sequences into each of the subsystem.

2.8 Phenotype Microarray

The central dogma of molecular biology states that the genetic information stored in the DNA molecule is transcribed to RNA of which is then translated into proteins (Crick, 1970). The advent of next generation sequencing technologies allowed us to obtain the complete genome sequence of *C. neteri* SSMD04. This provides insight into the

physiology of this bacterium at great depth but this information does not allow a conclusive biological conclusion to be drawn. The presence of lipase gene, for example, is not necessarily conclusive of the lipid degrading ability of the strain. Therefore, phenotypic information becomes indispensable in the understanding of the physiology of an organism. The technology developed by Bochner and colleagues (Bochner, 2009) for the global analysis of phenotypes was therefore applied on *C. neteri* SSMD04. This technology is aptly called the "Phenotype Microarray".

This technology runs on 96-wells microtitre plates pre-loaded with lyophilized chemicals at the bottom of the wells that act as the substrates. The cell suspension of the tested organism, prepared by inoculating bacterial colony on agar plate to the commercial cell suspension solution to a standardized cell density, is then dispensed into the wells. Tetrazolium redox dye added to the cell suspension acts as the reporter for the respiration of the cells. As the cells grow in the presence of the tested chemical, NADH produced reduces tetrazolium dye through redox chemistry, thus producing purple coloration. The intensity of the purple color is the indication of the degree of cellular respiration, which is captured and recorded by the Omnilog instrument, an incubator equipped with a camera. This technology was designed to run a large array of tests in a single run. It can accommodate up to nearly 2000 tests to run simultaneously. It also examines a variety of phenotypes such as carbon, nitrogen, phosphorus, and sulphur metabolism, biosynthetic pathways, nitrogen pathways, osmotic and ion effects, pH effects, and sensitivity to toxic chemicals (Bochner, 2003, 2009).

2.9 Aim of this Study

The isolation of *C. neteri* SSMD04 from *Shime saba* is the first report of isolation of *C. neteri* from a food source, providing more clues to the physiology of this bacterium. The

adaptability of this bacterium to the harsh environment of *Shime saba* as well as its ability to survive in a human body make it an interesting subject to be studied. Furthermore, due to the original objective of the study of the isolation, *C. neteri* SSMD04 was subjected to screening for AHL production and it exhibits AHL-type QS activity. It has long been known that QS can be involved in the regulation of virulence (pathogenic bacteria) and food spoilage traits (food spoilage agent) (Passador et al., 1993; Brint & Ohman, 1995; Bruhn et al., 2004; Skandamis & Nychas, 2012), which led to the speculation that QS is involved in the regulation of virulence and food spoilage traits in *C. neteri* SSMD04. The genome sequence mentioned previously enabled the search of AHL-type QS related genes in this study. Detailed characterization of QS properties of *C. neteri* SSMD04 was also reported. This led to comparative transcriptomics study to investigate the differentially expressed genes caused by the activation of QS circuit, therefore allowing us to understand the roles played by QS in *C. neteri* SSMD04.

CHAPTER 3: MATERIALS AND METHODS

3.1 Equipment and Instruments

Equiment used for this study were: Infinite M200 luminometer-spectrophotometer (Tecan, Switzerland), incubators and ovens (Memmert, Germany), NanoDrop spectrophotometer (ThermoScientific, USA), Qubit 2.0 fluorometer (Life Technologies, USA), Milli-Q® water purification system (Merck Millipore, Germany), Veriti® Thermal cycler (ThermoScientific, USA), CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories Ltd., USA), Omnilog Phenotype Microarray Systems (BIOLOG Inc., USA), turbidimeter (BIOLOG Inc., USA), MicroPlate reader (BIOLOG Inc., USA), Bioanalyzer (Agilent Technologies, USA), Microflex MALDI-TOF bench-top mass spectrometer (Bruker Daltonik GmbH, Germany), Gene Pulser Xcell™ Electroporation System (Bio-Rad Laboratories Ltd., USA), LCMS/MS (Agilent Technologies, USA), autoclave machine (Hirayama, Japan), weighing machine (Sartorius, Germany), shaking incubators (N-biotek, Korea), centrifuge machines (Eppendorf, Germany), heat blocks (Eppendorf, Germany), pipettes (Eppendorf, Germany), and tips, polypropylene tubes, Schott's bottles, conical flasks, beakers, cuvettes, petri dishes, inoculating loops, hockey sticks, cotton swabs.

3.2 Growth media and chemical preparations

Luria-Bertani (LB) agar was prepared by first mixing 20 g of Difco™ LB agar powder, Miller (Difco Laboratories Inc., USA) (5.0 g tryptone, 2.5 g yeast extract, 5.0 g sodium chloride, 7.5 g agar) in 500 mL distilled water followed by autoclaving at 121 ^{0}C, 15 psi for 15 min .

Tryptic Soy Agar (TSA) was prepared by first mixing 20 g of Difco™ Tryptic Soy Agar powder (Difco Laboratories Inc., USA) (7.5 g pancreatic digest of casein, 2.5 g papaic digest of soybean, 2.5 g sodium chloride, 7.5 g agar) in 500 mL distilled water. Then, the mixtures were sterilized by autoclaving at 121 ^{0}C, 15 psi for 15 min. The agar was cooled before being poured into petri dishes. LB broth was prepared by dissolving 12.5 g of Difco™ LB broth, Miller (Difco Laboratories Inc., USA) (5.0 g tryptone, 2.5 g yeast extract, 5.0 g sodium chloride) in 500 mL distilled water, whereas low salt LB broth were prepared by dissolving 5.0 g of tryptone (Difco Laboratories Inc., USA), 2.5 g of yeast extract (Difco Laboratories Inc., USA), and 5.0 g of sodium chloride (Merck Millipore, Germany) in 500 mL distilled water, followed by autoclaving at 121 ^{0}C, 15 psi for 15 min. All growth media had their pH adjusted to 7.0 using 1 N of HCL and 1 N of NaOH before autoclaving. For every 500 mL of LB media, 5.23 g of 3-(*N*-morpholino)propanesulfonic acid (MOPS) (Sigma-Aldrich, USA) (50 mM) powder, where necessary, were added to the media powder before dissolving in distilled water. LB media supplemented with MOPS have had their pH adjusted to 5.5 instead of 7.0.

Antibiotics and synthetic AHLs used in this study were purchased from Sigma-Aldrich®. Antibiotics, where necessary, were prepared at 100 mg/mL, filtered sterilized (0.22 µm pore size filter) and kept as stocks. They were supplemented to cooled growth media aseptically. Synthetic AHLs were dissolved in acetonitrile (ACN) to 25 mg/mL. 5-bromo-4-chloro-3-indoyl-β-D-galacto-pyranoside (X-gal) were prepared by dissolving it in dimethylformamide (DMF) to 20 mg/mL, followed by filter sterilization (0.22 µm pore size filter). Antibiotics, synthetic AHLs and X-gal were stored at -20 ^{0}C for long term storage.

3.3 Agarose gel electrophoresis (AGE)

Tris borate EDTA (TBE) buffer with 10× stock concentration was prepared by dissolving 10.8 g Tris base, 5.5 g boric acid, and 7.44 g $Na_2EDTA•2H_2O$ in 100 mL distilled water. The pH was adjusted to 8.0 before autoclaving. The agarose gel was prepared at 1 % (w/v) by mixing 0.4 g agarose powder (Bio-Rad Laboratories Ltd., USA) with 40 mL 1× TBE buffer, followed by microwaving for 1.5 min. The gel was cooled and 1 µL of GelStar™ Nucleic Acid Gel Stain (Lonza, Basel, Switzerland) was added. After mixing by gentle swirling, the gel was poured into a gel cast before solidifying. GeneRuler™ 1kb DNA ladder (Fermentas, Canada) was used.

3.4 Bacterial strains, media and culture conditions

C. neteri SSMD04, *Chromobacterium violaceum* CV026, *P. carotovora* GS101, *P. carotovora* PNP22, were maintained in LB medium at 28 ºC. *E. coli* DH5α (Invitrogen, USA), *E. coli* BL21(DE3)pLysS (Novagen, Germany), and *lux*-based AHL biosensor *E. coli* (pSB401) were grown in LB medium at 37 ºC, unless otherwise stated. Ampicillin (Sigma-Aldrich, USA), kanamycin (Sigma-Aldrich, USA), tetracycline (Sigma-Aldrich, USA) or chloramphenicol (Sigma-Aldrich, USA) were supplemented at 100 µg/mL, 30 µg/mL, 20 µg/mL or 34 µg/mL, respectively, when necessary. The description of the bacterial strains used is shown in Table 3.1.

Table 3.1: Description of bacterial strains used in this study.

Bacterial strain	Description	Source/Reference
C. neteri SSMD04	Subject of this study, isolated from pickled mackerel sashimi	(Chan et al., 2014)
C. violaceum CV026	Short chain AHL biosensor (mini-Tn*5* mutant derived from *C. violaceum* ATCC 3152)	(McClean et al., 1997)
P. carotovora GS101	Short chain AHL producer strain	Dr. Chan Kok Gan Department of Genetics and Molecular Biology, University of Malaya
P. carotovora PNP22	AHL synthase deficient mutant of *P. carotovora*	Dr. Chan Kok Gan Department of Genetics and Molecular Biology, University of Malaya

Table 3.1, continued.

Bacterial strain	Description	Source/Reference
E. coli DH5α	Host for gene cloning. Genotype: *F– Φ80lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (rK–, mK+) phoA supE44 λ– thi-1 gyrA96 relA1*	(Sambrook, 1989)
E. coli BL21(DE3)pLysS	Commercial strain used for gene expression study. Genotype: *F- ompT hsdSB(rB- mB-) gal dcm (DE3) pLysS (CamR)*	Novagen Inc., Germany
E. coli (pSB401)	*lux*-based short chain AHL biosensor, *luxR luxI* (*Photobacterium fischeri* [ATCC 7744])::*luxCDABE* (*Photorhabdus luminescens* [ATCC 2999]) fusion; pACYC184-derived, Tet^R	(Winson et al., 1998)
TKC strain	*tetA*, *cat*, *kan* (used for drug cassette amplification)	(Sawitzke, 2011)

3.5 Species identification of isolate SSMD04

3.5.1 Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS)

Sample preparation was carried out according to Mellmann and his colleagues (Mellmann et al., 2008). In brief, isolate SSMD04 was streaked on LB agar (Section 3.2) and incubated at 28 ^{0}C overnight (16 hrs). A single colony of SSMD04 was picked with a sterile toothpick and smeared evenly onto a MSP 96 target polished steel BC plate, followed by overlaying the target with 1 µL of matrix solution (10 mg/mL α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile/2.5 % trifluoroacetic acid, in v/v) and air dried. The sample was then analyzed using Microflex MALDI-TOF (Bruker Daltonik GmbH, Germany) bench-top mass spectrometer equipped with UV laser at wavelength 337 nm. Bruker FlexControl software version 3.3 (Build 108) was used for subsequent analysis and the spectra were recorded in linear positive ion mode with the analysis conducted in the mass range of 2 to 20 kDa. The identity of the sample was evaluated by comparison to the Bruker database. The matches were given score values based on the similarity of the queried sample's spectrum to the spectra of known organisms in the database. Score values ranging from 2.3000 to 3.000 were considered highly probable species identification; 2.000 to 2.299 were considered secure genus identification, probable species identification; 1.700 to 1.999 were considered probable genus identification; 0.000 to 1.699 were considered not reliable identification.

3.5.2 Phylogenetic analysis of 16S rRNA gene sequences

Whole genome sequencing, sequence assembly and genome annotation were performed as previously described (Chan et al., 2014). The genome sequence of *C. neteri* SSMD04 was uploaded to Rapid Annotation using Subsystem Technology (RAST) online server (Aziz et al., 2012) for automated annotation. The 16S rRNA gene sequence of *C. neteri*

SSMD04 was then searched using "Genome Browser" function in RAST. 16S rRNA gene sequences of other *Cedecea* spp. were obtained from GenBank through text search. MEGA version 6.0 was used in sequence alignment (ClustalW) and construction of phylogenetic tree. The Maximum likelihood tree was constructed using 1000 bootstrap replications.

3.5.3 BIOLOG GEN III microbial identification system

The identity of strain SSMD04 was also checked using the BIOLOG GEN III microbial identification system (BIOLOG Inc., USA) according to manufacturer's protocol. Briefly, overnight plate culture (16 hrs) of strain SSMD04 grown on TSA (Section 3.2) was used to inoculate inoculating fluid (IF) A to a cellular density of 90-98 % transmittance, read using a turbidimeter (BIOLOG Inc., USA). The inoculum was subsequently loaded into each well in BIOLOG GEN III MicroPlate (100 µL per well), followed by incubation at 28 ºC for 24 hrs. Each well were pre-loaded with different metabolite in which if the bacterium can utilize a particular metabolite, tetrazolium redox dyes will be reduced and thus turning purple. The wells were then read using MicroPlate reader (BIOLOG Inc., USA) where the wells will be scored with "negative" if the colour did not change to purple or "positive" if the colour changed to purple. This resulted in a unique "Phenotypic Fingerprint", which in turn was matched to the existing database to find the best match of known bacteria.

3.6 Detection of AHL production using *Chromobacterium violaceum* CV026

AHL production in *C. neteri* SSMD04, *cneI* knockout mutant of *C. neteri* SSMD04 and *E. coli* BL21(DE3)pLysS expressing *cneI* was detected using *C. violaceum* CV026 (McClean et al., 1997). The tested strain was streaked perpendicularly against *C. violaceum* CV026 on a LB agar (Section 3.2) plate and left to grow overnight (16 hrs) at

28 ºC. AHL molecules produced by the tested strain will activate the violacein production in *C. violaceum* CV026, turning the culture purple. The same was performed on *P. carotovora* GS101 and *P. carotovora* PNP22 which act as positive and negative controls, respectively (Jones et al., 1993).

3.7 AHL extraction

C. neteri SSMD04 was cultured in 100 mL of LB broth supplemented with 50 mM of MOPS (pH 5.5) (Section 3.2). Then, the culture was incubated overnight (16 hrs) with shaking (220 rpm) at 28 ^{0}C. *E. coli* BL21(DE3)pLysS harbouring pET-28a as well as *E. coli* BL21(DE3)pLysS harbouring pET-28a_*cneI* were prepared according to Section 3.14. The cultures were then centrifuged at 10,000 × *g* for 10 min to collect the supernatant. Equal volume of acidified ethyl acetate (AEA) (0.1 % v/v glacial acetic acid) was added to the spent supernatant and mixed rigorously. After separation, the organic layer (top layer) was collected into a beaker. The extraction was carried out twice and the extracts pooled together. The extracts were subsequently air dried in a fume hood. Finally, the dried extracts were dissolved in 1 mL of AEA and transferred into sterile microcentrifuge tubes and dried again. The dried AHL extracts were kept at -20 ^{0}C for storage.

3.8 AHL identification by triple quadrupole LC/MS

Dried AHL extracts were reconstituted in 1 mL acetonitrile (ACN) before being analyzed by LC/MS using Agilent Zorbax Rapid Resolution High Definition SB-C18 Threaded Column (2.1 mm × 50 mm, 1.8 µm particle size). Then, 200 µL of the reconstituted AHL extracts was pipetted into the LC vials. Mobile phase A used was water with 0.1 % formic acid and mobile phase B used was ACN with 0.1 % formic acid. The flow rate was set to 0.5 mL/min. The gradient profile was adjusted to A:B 80:20 at 0 min, 50:50 at 7 min,

50:50 at 7.10 min, 80:20 at 12 min, 80:20 at 12.10 min, 20:80 at 14 min, 20:80 at 14.10 min. Precursor ion mode scan mode was used in positive ion mode with Q1 programmed to monitor *m/z* 90 to *m/z* 400 and Q3 set to monitor *m/z* 102, which is the trademark peak for lactone ring moiety, the core structure of AHL molecules. ACN was used as a blank in all runs.

3.9 Measurement of bioluminescence

Detection of exogenous short chain AHLs present in the extracts was carried out using a *lux*-based AHL biosensor, *E. coli* (pSB401) (Winson et al., 1998). First, the biosensor strain was grown in LB broth supplemented with tetracycline (20 µg/mL) (Section 3.2) overnight (16 hrs) at 37 ^{0}C with shaking (220 rpm). Fresh LB broth with tetracycline was then used to dilute the overnight culture of *E. coli* (pSB401) to an OD_{600} reading of 0.1. The diluted culture was then supplemented with the extracted AHL by mixing the diluted cultures with the dried extract by pipetting, which was then dispensed into a 96-well optical bottom microtitre plate for the reading of bioluminescence. Cellular density and bioluminescence measurements were carried out in an Infinite M200 luminometer-spectrophotometer (Tecan, Switzerland) over a period of 24 hrs at 37 ^{0}C. Diluted *E. coli* (pSB401) cultures without the supplementation of AHL extracts was read for normalization whereas sterile broth was used as a negative control. The results were shown as the relative light units (RLU)/OD_{495} against incubation time.

3.10 Lipase activity

Agar plate with corn oil supplemented (0.5 % v/v) was used to examine the lipase activity of *C. neteri* SSMD04. The agar was prepared by mixing 0.25 g yeast extract, 0.5 g tryptone, 5g NaCl, and 7.5g bacteriological agar powder with 500 mL distilled water, followed by autoclaving at 121 ^{0}C at 15 psi for 15 min. Corn oil was emulsified by mixing

100 mL of corn oil with 400 mL of distilled water and 2.5 mL of Tween 80, which was then sterilized by autoclave at 121 ^{0}C at 15 psi for 15 min. After sterilization, the oil was added into the molten agar aseptically and mix rigorously until it was emulsified completely. Opaque suspension formed once the agar solidifies due to the presence of corn oil. *C. neteri* SSMD04 was streaked onto the corn oil supplemented agar and incubated for 48 hrs at 28 ^{0}C. The presence of lipase activity was seen as the formation of halo zone around the bacterial colonies due to the breakdown of the lipids.

3.11 *cneI* and *cneR* phylogenetic analysis

The nucleotide sequences of *cneI* and *cneR* were retrieved by searching through "Genome Browser" function on RAST. These two gene sequences were used to blast against GenBank databases using BLASTP program (http://www.ncbi.nlm.nih.gov/) using default parameters. Ten LuxI homologues and twelve LuxR homologues of the highest identity were selected from the protein database, omitting redundant or ambiguous sequences. Sequence alignment and construction of phylogenetic tree were carried out with the same method as elaborated in section 3.2.2. Sequence alignments were presented using ESPript (Robert & Gouet, 2014).

3.12 DNA extraction

C. neteri SSMD04 was grown in LB broth (Section 3.2) overnight (16 hrs) at 28 ^{0}C with shaking (220 rpm). The cell pellet was collected by centrifuging 1 mL of the overnight culture at 10,000 × *g* for 10 min. Genomic DNA was isolated from the cell pellet using a commercial kit, the MasterPure DNA Purification kit (Epicentre, Inc., USA) according to manufacturer's protocol. The purity and quantity of the extracted DNA was assessed using NanoDrop spectrophotometer (ThermoScientific, USA) and Qubit 2.0 fluorometer (Life Technologies, USA).

3.13 Functional study of *cneI* by gene cloning

3.13.1 Construction of recombinant *cneI* expression plasmids

Polymerase chain reaction (PCR) was carried out using the following primers: *cneI*-F-NcoI (5'- CCATGGCGATGTGTTCTGCAATTGAA -3') and *cneI*-R-BamHI (5'-GGATCCTTGAGGTGGTTGAGCTGTGT -3') to amplify the autoinducer synthase gene, *cneI*, from the genomic DNA of *C. neteri* SSMD04. The underlined sequences in both of the primer sequences are the NcoI and BamHI restriction sites, respectively. The stop codon of *cneI* was removed in the primer design of *cneI*-R-BamHI in order to incorporate a C-terminal His-tag into the recombinant protein. The PCR was carried out on Veriti® Thermal cycler (ThermoScientific, USA) according to the following cycling conditions: initial denaturation at 95 ^{0}C for 3 min, followed by 30 cycles of the following PCR conditions: denaturation at 95 ^{0}C for 30 s, annealing at 49 ^{0}C for 30 s, extension at 72 ^{0}C for 1 min, and a final extension at 72 ^{0}C for 7 min. Gel electrophoresis was run to view the PCR product. The colour of the gel image was inverted to produce sharper image. The desired amplicon was extracted after running agarose gel electrophoresis (Section 3.3) using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). Following manufacturer's protocol, the purified amplicon was subsequently ligated into a commercially available cloning vector, pGEMT-Easy (Promega, USA). The ligated product was later transformed into competent *E. coli* DH5α by heat shock (Sambrook, 1989). This recombinant vector, designated pGEMT-Easy_*cneI*, was purified from *E. coli* DH5α using QIAprep Spin Miniprep Kit (Qiagen, Germany) following manufacturer's protocol, followed by digestion by both NcoI and BamHI restriction enzymes (NEB, USA). An expression vector, pET28a was treated with the same restriction enzymes, followed by ligation of the released insert from pGEMT-Easy_*cneI* to the linearized pET28a vector backbone. The resulting recombinant plasmid was designated pET28a_*cneI*. The ligated products were transformed into *E. coli* BL21(DE3)pLysS.

3.13.2 Verification of transformants

The *E. coli* BL21(DE3)pLysS transformed with pET28a_*cneI* was plated on LB agar supplemented with kanamycin (30 µg/mL) and chloramphenicol (34 µg/mL) (Section 3.2) and left to grow for 16 hrs at 37 ^{0}C for selection of successful transformants. Ten potential transformants were selected and their DNA extracted by heat lysis at 99 ^{0}C for 10 min. PCR using primers *cneI*-F-NcoI and *cneI*-R-BamHI was carried out on the extracted DNA according to the cycling conditions stated at Section 3.13.1. A band corresponding to the *cneI* band signifies the presence of pET28a_*cneI*. The bands were later excised and the DNA extracted with Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The DNA samples were sent to a sequencing service provider, 1st BASE (1st BASE Laboratories, Malaysia) for automated Sanger sequencing with the same primers, *cneI*-F-NcoI and *cneI*-R-BamHI. The results were aligned with the sequence of *cneI* for verification of faithful transformant. The transformants were also subjected to AHL production screening using biosensor *C. violaceum* CV026 (Section 3.6) and triple quadrupole LC/MS (Section 3.8).

3.14 Heterologous expression of CneI and His-tagged protein purification

E. coli BL21(DE3)pLysS harbouring pET-28a_*cneI* was grown in LB broth supplemented with kanamycin (30 µg/mL), chloramphenicol (34 µg/mL) and MOPS (50 mM) (Section 3.2) at 37 ^{0}C for 16 hrs with shaking (220 rpm). It was then diluted with fresh broth to an OD_{600} reading of 0.1 (total volume of 100 mL), and then incubated further until the reading reached 0.5 – 0.6. The volume was split evenly into two flasks (50 mL each). One of the flasks was supplemented with 0.5 mM of IPTG (Section 3.2) and the other 1.0 mM of IPTG. The cultures were then incubated for another 8 hrs with shaking (220 rpm) at 25 ^{0}C. With this induction, the cultures were used for subsequent AHL extraction (Section 3.7) or protein purification. Uninduced *E. coli*

BL21(DE3)pLysS harbouring pET28a_*cneI* and *E. coli* BL21(DE3)pLysS harbouring native pET28a (both induced and uninduced) were used as controls. The cultures for protein purification were first centrifuged at 10,000 × *g* for 10 min to collect the cell pellet. The cells were then lysed using BugBuster™ Protein Extraction Reagent supplemented with protease inhibitors (Novagen, Germany). His-tagged protein was isolated from the cell lysate using Ni-NTA Fast Start Kit (Qiagen, Germany) according to manufacturer's protocol.

3.15 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

E. coli BL21(DE3)pLysS harbouring pET28a_*cneI* and *E. coli* BL21(DE3)pLysS harbouring native pET28a were prepared according to Section 3.14 and the cells harvested by centrifugation at 10,000 × *g* for 10 min. The cell pellet was subsequently resuspended in BugBuster™ Protein Extraction Reagent supplemented with protease inhibitors (Novagen, Germany). The lysate wes then collected and heated at 95 ^{0}C for 3 min to linearize the proteins before being loaded into 12.5 % (w/v) polyacrylamide gel electrophoresis system (Bio-Rad Laboratories Ltd., USA) in the presence of sodium dodecyl sulfate (SDS). The gel was stained by Coomassie Brilliant Blue R-250 (CBB) (Bio-Rad Laboratories Ltd., USA) for visualization of the protein bands.

3.16 *cneI* knockout

The AHL synthase gene, *cneI*, was knockout using the bacteriophage λ Red recombineering system according to the Court Lab protocol (Sawitzke, 2011). The linear substrate was generated by amplifying the tetracycline resistance gene cassette from *E. coli* strain TCK by PCR. The primers used were *cneI*_mut_F (5'- CTA CAG GTG GTT GAG CTG TGT AAG CGT GTC GCG CAG CCA TGC GCT GTT GTA CTC GAC -3') and *cneI*_mut_R (5'- ATG TGT TCT GCA ATT GAA TTT TTT ATA AGT CAT

CAT AAT GAT TTA CAC CCC AAG AGG GTC -3'). The cycling condition used was: initial denaturation at 98 ^{0}C for 30 s, followed by 25 cycles of denaturation at 98 ^{0}C for 10 s, annealing at 54 ^{0}C for 30 s, and extension at 72 ^{0}C for 2 min. Final denaturation was carried out at 72 ^{0}C for 2 min. *C. neteri* SSMD04 was prepared for recombineering by transforming it with the λ Red plasmid pSIM9 by electroporation using Gene Pulser Xcell™ Electroporation Systems (Bio-Rad Laboratories., USA) according to manufacturer's protocol. The transformed cells were then made competent for recombineering by first growing the cells at 32 ^{0}C to an OD_{600} reading of 0.4-0.6 in 35 mL of low salt LB broth (0.5 %) (Section 3.2) supplemented with chloramphenicol (34 µg/mL), under shaking condition (200 rpm). Half the volume of the cell culture was transferred to another flask followed by incubation at 42 ^{0}C with shaking at 200 rpm (induced) where the other half of the culture was maintained at 32 ^{0}C that served as a control (non-induced). After 15 min of incubation, the cultures were chilled immediately in an ice-water slurry with gentle swirling. After 5 min, the cells were collected by centrifugation at 6500 × *g* for 7 min at 4 ^{0}C. The cell pellet was resuspended with 1 mL of ice-cold sterile distilled water. Another 30 mL of ice-cold sterile distilled water was added to the resuspended cells for washing, followed by centrifugation at 6500 × *g* for 7 min at 4 ^{0}C again. The pellet was then resuspended in 1 mL of ice-cold sterile distilled water again and transferred to pre-chilled microcentrifuge tubes. The cells were centrifuged at 15,000 × *g* for 30 s at 4 ^{0}C and resuspended in 200 µL of ice-cold sterile distilled water. The cells were ready for transformation and kept on ice until application. The amplified tetracycline resistance gene cassette was used in electroporation of the induced *C. neteri* SSMD04 harbouring pSIM9. The transformants were selected based on tetracycline resistance on LB agar supplemented with tetracycline (20 µg/mL) (Section 3.2), and automated Sanger sequencing (1st BASE Laboratories, Malaysia) using primers *cneI*_UP294 (5'- GTG TAG CGA TCC TGA GAG ATA TAA -3') for the identification

of 5' sequence of tetracycline resistance gene and *cneI*_DOWN200 (5'- CGC GTG AGC AGC AAA TTT TG -3') for the identification of 3' sequence of tetracycline resistance gene. Selected mutant strain was screened for AHL synthesis using the AHL biosensor *C. violaceum* CV026 (Section 3.6) and triple quadrupole LC/MS (Section 3.8). The mutant strain was then subcultured on LB agar three times in consecution to remove the tetracycline resistance gene cassette, which was tested by its inability to grow in LB agar supplemented with tetracycline (20 µg/mL).

3.17 Comparison of growth rates of *C. neteri* SSMD04 and SSMD04Δ*cneI*

C. neteri SSMD04 and SSMD04Δ*cneI* were grown in LB broth overnight (16 hrs, 28 ^{0}C, 220 rpm). They were then subcultured into fresh LB broth to a starting OD_{600} of ~0.1, followed by incubation at 28 ^{0}C with shaking at 220 rpm. The growth rate of both strains were inspected by measuring their OD_{600} reading using NanoDrop spectrophotometer (ThermoScientific, USA) every 30 min until plateau phase was reached. The experiment was conducted in duplicate and the reading of each time point is the average of the two.

3.18 RNA extraction and transcriptome sequencing library preparation

C. neteri SSMD04 and SSMD04Δ*cneI* were grown in LB broth (Section 3.2) overnight (16 hrs, 28 ^{0}C, 220 rpm). They were then subcultured with fresh LB broth to a starting OD_{600} reading of 0.1. They were grown at 28 ^{0}C with shaking (220 rpm) until an OD_{600} reading of 2.5. Then, their total RNA were extracted using MasterPure^{TM} RNA Purification Kit (Epicentre, USA) according to manufacturer's instructions. Final purified RNA was resuspended in sterile RNase-free water (prepared by autoclaving Milli-Q water at 121 ^{0}C, 15 psi for 30 min). NanoDrop Spectrophotometer (ThermoScientific, USA) was used to assess the purity of the RNA samples and Qubit 2.0 fluorometer (Life Technologies, USA) was used to assess their quantity. All samples were selected based

on the threshold of $A_{260/280}$ and $A_{260/230}$ values of more than 2.0. All samples were then analyzed using Agilent Bioanalyzer-RNA 6000 Pico Kit (Agilent Technologies, USA) to determine the integrity of the samples. Only samples with RNA Integrity Numbers (RIN) of more than 8 were chosen for further work. Qualified RNA samples were subsequently used for rRNA depletion using Ribo-Zero™ rRNA Removal Kits (Bacteria) (Epicentre, USA) for the removal of rRNA before cDNA synthesis. The quality of the resulted rRNA depleted samples were assessed using Bioanalyzer (Agilent Technologies, USA). ScriptSeq™ v2 RNA-seq Library Preparation Kit (Epicentre, USA) was used to prepare the final sequencing library, according to manufacturer's protocol. The quality and quantity of the final library was assessed by Agilent Bioanalyzer-High sensitivity DNA Chip, Qubit 2.0 fluorometer, as well as qPCR using KAPA library quantification kits (KAPA Biosystems, USA), according to manufacturer's protocol.

3.19 Differential gene expression analysis

C. neteri SSMD04 genome annotated with NCBI Prokaryotic Genome Annotation Pipeline was used as the reference genome for this analysis. Processed reads from RNA sequencing were first trimmed off of ScriptSeq adapters sequences (Illumina, USA) using Trim Sequences tool under the NGS Core Tools toolbox in CLC Genomic Workbench version 7 (CLC Bio, Denmark), based on default parameters. The trimmed reads were then aligned to the reference genome using the RNA-Seq Analysis tool located in the Transcriptomics Analysis toolbox of CLC in BAM file format, and the output files were sorted with Samtools (Li et al., 2009). HTSeq-count (EMBL, Heidelberg, Germany) was used to generate the number of reads according to gene or CDS. DESeq2 package (Love, Huber, & Anders, 2014) was used to analyze differentially expressed genes between the wild type and *cneI* mutant. The genes were accepted as differentially expressed if the $\log_2$ change is more than 2 or less than -2 with *q*-value lower than 0.05.

3.20 Validation of RNA-seq using quantitative reverse transcriptase PCR (qRT-PCR)

In order to verify the reliability of RNA-seq result, qRT-PCR was conducted on selected differentially expressed genes based on RNA-seq data. The extracted total RNA samples from *C. neteri* SSMD04 and SSMD04Δ*cneI* (Section 3.18) were subjected to cDNA synthesis by QuantiTect Reverse Transcription Kit (Qiagen, USA). Three housekeeping genes were selected as reference (*recA* that encodes recombinase A, *gyrA* that encodes gyrase A, and *ybhF* that encodes an uncharacterized ABC transporter ATP-binding protein YbhF). From the differential gene expression result from RNA-seq data, three genes were selected from the upregulated genes (lipase, D-ribose transporter ATP-binding protein, inositol-2-dehydrogenase) and three from downregulated genes (type I secretion protein TolC, pilus assembly protein PapD, GNAT family acetyltransferase) for qRT-PCR. The primers were designed using Primer3 version 0.4.0 (http://bioinfo.ut.ee/primer3-0.4.0/). The cycling condition was: initial denaturation at 95 ^{0}C for 2 min, followed by 40 cycles of denaturation at 95 ^{0}C for 5 s and annealing/extension at 63 ^{0}C for 10 s. The reaction was carried out in a Bio-Rad CFX96 real-time system (Bio-Rad, USA). The fluorescent signals were read at the end of each cycle. Eventual data were analyzed by Bio-Rad CFX Manager^{TM} Software version 1.6 (Bio-Rad, USA). The genes were designated as LIP (lipase), ATP (D-ribose transporter ATP-binding protein), I2D (inositol 2-dehydrogenase), TolC (type I secretion protein TolC), PapD (pilus assembly protein PapD), GNAT (GNAT family acetyltransferase). Table 3.2 lists the primer sequences designed for qRT-PCR.

Table 3.2: Primers used for qRT-PCR.

Primer	Sequence	Length (bp)
recA forward	5'- ATT GGC GTC ATG TTC GGT AAC -3'	21
recA reverse	5'- CCG TAG AAG TTG ATG CCT TCG -3'	21
gyrA forward	5'- ACC TTG CCA GAG AAA TTA CGC -3'	21
gyrA reverse	5'- CCA GTC ATT GCC CAA TAC GTT -3'	21
ybhF forward	5'- TTC AAG ATG ATG TGC GGT TTG -3'	21
ybhF reverse	5'- AAA CCC CGG AGA AGA AAC GTA -3'	21
LIP forward	5'- CAG CGC CAT CCT TAT CAA CT -3'	20
LIP reverse	5'- CTT CGT TCT GGT CGT GGG TA -3'	20
ATP forward	5'- GGT TCT TGG CCT GTC GAT CC -3'	20
ATP reverse	5'- CGC GCA AGG ATC ACT TTT TG -3'	20
I2D forward	5'- CAA ACA CGG CAA GGC TAT TT -3'	20
I2D reverse	5'- TGC AGC ACT TCG TTT TTC AC -3'	20
TolC forward	5'- ACC AGC CTC AAC AAA ATC AG -3'	20
TolC reverse	5'- CCC GGA GTA GTT CTT GTT CA -3'	20
PapD forward	5'- GTT TTC AAC TGG CAG CAA AG -3'	20
PapD reverse	5'- GGA ATA GCG CAT CAC AAA CT -3'	20
GNAT forward	5'- ATT TCA GGG GAG AGG AAT CG -3'	20
GNAT reverse	5'- CCT ATC AAC CCC CTC CTC AC -3'	20

3.21 Phenotype microarray analysis

Global phenotypic characterization of *C. neteri* SSMD04 and the screening of carbon source utilization of *C. neteri* SSMD04 and SSMD04Δ*cneI* was carried out using Phenotype Microarray (PM) system (BIOLOG Inc., USA) according to manufacturer's instructions. Microtitre plates PM-1 to PM-20 were used for the global phenotypic

characterization of *C. neteri* SSMD04, whereas only PM-1 and PM-2 were used for the comparison of carbon source utilization between *C. neteri* SSMD04 and SSMD04Δ*cneI*. All plates were prepared with similar methods with slight modifications. *C. neteri* SSMD04 and SSMD04Δ*cneI* were first streaked on LB agar (Section 3.2) and left to grow (16 hrs) at 28 ^{0}C. Then, the cells were scraped from colonies on LB agar plates using a sterile cotton swab and suspended into IF0 inoculating fluid to a transmittance level of 85 %. BIOLOG Redox Dye Mix H was used to detect the respiration rate of the cells according to the formation of purple colour in the inoculum. Then, 100 µL of the inoculum was dispensed into each of the wells in the microtitre plates before being incubated at 28 ^{0}C in the Omnilog incubator (BIOLOG Inc., USA). For PM-3 to PM-8, 5 mM of sodium pyruvate was supplemented to the IF0 before inoculating the bacteria in IF0. For PM-9 to PM-20, IF10 was used to dilute the cell suspension in IF0 (85 % transmittance) 200 times before being loaded to the plates. The same dye was used for all plates. The experiment was carried out for 3 days. The colour changes in the well were measured at an interval of 15 min. The information of the contents of the plates can be found at (http://www.biolog.com/pdf/pm_lit/PM1-PM10.pdf) and (http://www.biolog.com/pdf/pm_lit/PM11-PM20.pdf).

CHAPTER 4: RESULTS

4.1 Identification of strain SSMD04

C. neteri SSMD04 was isolated from *Shime saba* and was preliminary identified as a member of Enterobacteriaceae. It was later identified by MALDI-TOF MS but the ID score was low (1.787) despite the fact that the resulted ID was *C. neteri*. The identification therefore fell into the category where only the genus is likely to be precise but not the species. Table 4.1 shows the top spectrum match with that of *C. neteri* SSMD04 produced by comparison to Bruker's MALDI-TOF MS database. A dendrogram was produced from the mass spectra of *C. neteri* SSMD04 and other organisms shown in Table 4.1 (Figure 4.1). Subsequently, phylogenetic analysis of 16S rRNA gene sequences was carried out for a more reliable identification. Seven copies of 16S rRNA gene sequences were found in the genome of strain SSMD04, six of which were identical and one other has two SNPs (gi|689262542|:1761486-1763036 and gi|689262542|:2500435-2501985). Therefore, both variants were included in the phylogenetic analysis with other 16S rRNA gene sequences of *Cedecea* spp. retrieved from the GenBank. As shown in Figure 4.2, both 16S rRNA gene sequences of strain SSMD04 were clustered together with others of *C. neteri*, forming a monophyletic clade, thus confirming that strain SSMD04 is a member of *C. neteri*.

Table 4.1: List of organisms with MALDI-TOF spectra best matched to that of *C. neteri* SSMD04. The list is arranged according to descending score value.

Rank	Matched Pattern	Score value
1	*Cedecea neteri* DSM 13693T HAM	1.787
2	*Xenorhabdus ehlersii* DSM HAM	1.65
3	*Proteus mirabilis* 9482_2 CHB	1.642
4	*Pantoea agglomerans* CCM 4412 CCM	1.598
5	*Pantoea agglomerans* CCM 2406 CCM	1.538
6	*Raoultella terrigena* VA5551_09 ERL	1.491
7	*Cronobacter sakazakii* DSM 4485T DSM	1.468
8	*Enterobacter asburiae* DSM 17506T DSM	1.461
9	*Pantoea agglomerans* CCM 4413 CCM	1.457
10	*Enterobacter cowanii* DSM 18146T DSM	1.436

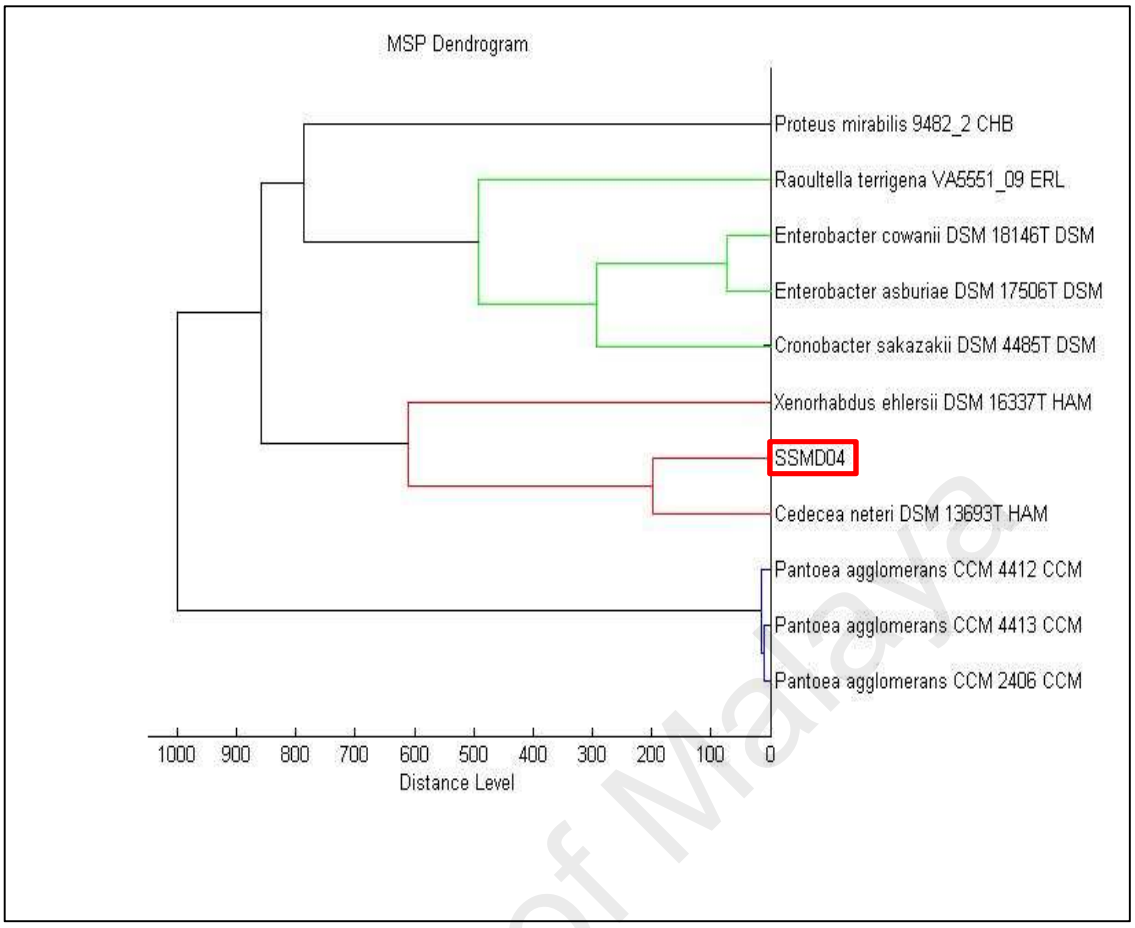

Figure 4.1: Dendrogram of relatedness among *C. neteri* SSMD04 (red box) and other microorganisms with the highest matched score value to *C. neteri* SSMD04.

The BIOLOG GEN III microbial identification system was also used to determine the identity of strain SSMD04, in complement to the 16S rRNA gene sequences identification. The system classified this strain to be *C. neteri* with a probability and similarity score of 0.697, compared to 0.182 for *Cedecea davisae*, 0.130 for *Cedecea lapagei* and 0.016 for *Yersinia intermedia*. Apart from that, the positive reaction in the sucrose well and D-sorbitol well agrees with the observation by Farmer et al. (1982).

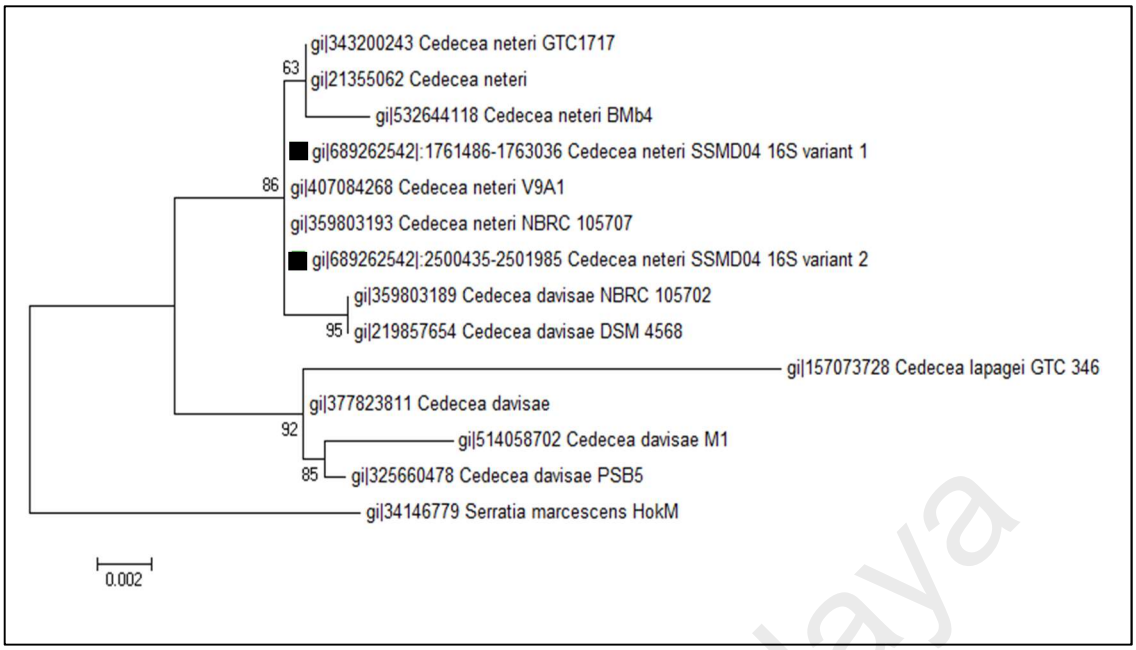

Figure 4.2: Phylogenetic analysis of 16S rRNA gene sequences of strain SSMD04 with other *Cedecea* spp. The maximum likelihood tree was inferred form 1297 aligned positions of the 16S rRNA gene sequences using Hasegawa-Kishino-Yano substitution model. The numbers shown at the node represent the bootstrap values. The positions of *C. neteri* SSMD04 16S rRNA gene variants were highlighted by black squares. Bar denotes the number of substitutions per nucleotide position. *Serratia marcescens* strain HokM was used as an outgroup.

4.2 Phenotype microarray analysis

C. neteri SSMD04 showed respiration in 85 wells out of 190 types of carbon source tested (Table 4.2). It can be observed from the cellular respiration kinetics that this strain showed some preference in the utilization of carbon sources, as it grows more optimally in the presence of some carbon sources than the others. For example, in wells containing thymidine, D-glycosaminic acid, L-threonine, γ-amino butyric acid, and D-tartaric acid, the growth has an extended log phase, and it takes more than 24 hours to reach the plateau phase. In the presence of some other non-optimal carbon sources such as dextrin and butyric acid, the growth was stunted such that the plateau phase has a lower reading than the others of optimal growth.

Table 4.2: Growth of *C. neteri* SSMD04 in wells loaded with different carbon sources. The sign "+" represents growth observed and "-" represents no growth observed.

Biolog Microplates	Compound	Growth observed
PM1	L-arabinose	+
	N-acetyl-D-glucosamine	+
	D-saccharic acid	-
	Succinic acid	+
	D-galactose	+
	L-aspartic acid	+
	L-proline	+
	D-alanine	+
	D-trehalose	+
	D-mannose	+
	Dulcitol	-
	D-serine	+
	D-sorbitol	+
	Glycerol	+
	L-fucose	+
	D-glucuronic acid	+
	D-gluconic acid	+
	D,L-α-glycerol-phosphate	+
	D-xylose	+
	L-lactic acid	+
	Formic acid	-
	D-mannitol	+
	L-glutamic acid	+
	D-glucose-6-phosphate	+
	D-galactonic acid-γ-lactone	+
	D,L-malic acid	+
	D-ribose	+
	Tween 20	+
	L-rhamnose	-

Table 4.2, continued.

Biolog Microplates	Compound	Growth observed
	D-fructose	+
	Acetic acid	+
	α-D-glucose	+
	Maltose	-
	D-melibiose	+
	Thymidine	+
	L-asparagine	+
	D-aspartic acid	-
	D-glucosaminic acid	-
	1,2-propanediol	-
	Tween 40	+
	α-keto-butyric acid	-
	α-methyl-D-galactoside	-
	A-D-lactose	+
	Lactulose	-
	Sucrose	+
	Uridine	+
	L-glutamine	+
	m-tartaric acid	+
	D-glucose-1-phosphate	+
	D-fructose-6-phosphate	+
	Tween 80	+
	α-hydroxy glutaric acid-γ-lactone	-
	α-hydroxy butyric acid	-
	β-methyl-D-glucoside	+
	Adonitol	-
	Maltotriose	+
	2-deoxy adenosine	+

Table 4.2, continued.

Biolog Microplates	Compound	Growth observed
	Adenosine	+
	Glycyl-L-aspartic acid	+
	Citric acid	+
	m-inositol	+
	D-threonine	-
	Fumaric acid	+
	Bromo succinic acid	+
	Propionic acid	-
	Mucic acid	-
	Glycolic acid	-
	Glyoxylic acid	-
	D-cellobiose	+
	Inosine	+
	Glycyl-L-glutamic acid	+
	Tricarballylic acid	-
	L-serine	+
	L-threonine	-
	L-alanine	+
	L-alanyl-glycine	+
	Acetoacetic acid	-
	N-acetyl-β-D-mannosamine	-
	Mono methyl succinate	-
	Methyl pyruvate	+
	D-malic acid	-
	L-malic acid	+
	Glycyl-L-proline	+
	p-hydroxy phenyl acetic acid	-
	m-hydroxy phenyl acetic acid	-
	Tyramine	-
	D-psicose	+
	L-lyxose	+
	Glucuronamide	-

Table 4.2, continued.

Biolog Microplates	Compound	Growth observed
	Pyruvic acid	+
	L-galactonic acid-γ-lactone	+
	D-galacturonic acid	+
	Phenylethylamine	-
	2-aminoethanol	-
PM2	Chondroitin sulfate C	-
	α-cyclodextrin	-
	β-cyclodextrin	-
	γ-cyclodextrin	-
	Dextrin	+
	Gelatin	-
	Glycogen	-
	Inulin	-
	Laminarin	-
	Mannan	-
	Pectin	-
	N-acetyl-D-galactosamine	+
	N-acetyl-neuraminic acid	-
	β-D-allose	+
	Amygdalin	-
	D-arabinose	+
	D-arabitol	+
	L-arabitol	-
	Arbutin	+
	2-deoxy-D-ribose	-
	i-erythritol	-
	D-fucose	-
	3-0-β-D-galactopyranosyl-D-arabinose	-
	Gentiobiose	+

Table 4.2, continued.

Biolog Microplates	Compound	Growth observed
	L-glucose	-
	Lactitol	-
	D-melezitose	+
	Maltitol	+
	a-methyl-D-glucoside	-
	β-methyl-D-galactoside	+
	3-methyl glucose	+
	β-methyl-D-glucuronic acid	-
	α-methyl-D-mannoside	-
	β-methyl-D-xyloside	-
	Palatinose	+
	D-raffinose	-
	Salicin	+
	Sedoheptulosan	-
	L-sorbose	-
	Stachyose	-
	D-tagatose	+
	Turanose	+
	Xylitol	-
	N-acetyl-D-glucosaminitol	-
	γ-amino butyric acid	-
	δ-amino valeric acid	-
	Butyric acid	-
	Capric acid	-
	Caproic acid	-
	Citraconic acid	-
	Citramalic acid	-
	D-glucosamine	+
	2-hydroxy benzoic acid	-
	4-hydroxy benzoic acid	-
	β-hydroxy butyric acid	-
	γ-hydroxy butyric acid	-

Table 4.2, continued.

Biolog Microplates	Compound	Growth observed
	a-keto-valeric acid	-
	Itaconic acid	-
	5-keto-D-gluconic acid	+
	D-lactic acid methyl ester	+
	Malonic acid	+
	Melibionic acid	-
	Oxalic acid	-
	Oxalomalic acid	-
	Quinic acid	-
	D-ribono-1,4-lactone	-
	Sebacic acid	-
	Sorbic acid	-
	Succinamic acid	-
	D-tartaric acid	-
	L-tartaric acid	-
	Acetamide	-
	L-alaninamide	-
	N-acetyl-L-glutamic acid	-
	L-arginine	-
	Glycine	-
	L-histidine	+
	L-homoserine	-
	Hydroxyl-L-proline	-
	L-isoleucine	-
	L-leucine	-
	L-lysine	-
	L-methionine	-
	L-ornithine	-

Table 4.2, continued.

Biolog Microplates	Compound	Growth observed
	L-phenylalanine	-
	L-pyroglutamic acid	-
	L-valine	-
	D,L-carnitine	-
	Sec-butylamine	-
	D,L-octopamine	-
	Putrescine	+
	Dihydroxy acetone	+
	2,3-butanediol	-
	2,3-butanone	-
	3-hydroxy 2-butanone	-

Despite its ability to grow on a wide range of carbon sources, this strain did not grow on any of the phosphorus (phosphate, pyrophosphate, trimetaphosphate, tripolyphosphate, triethyl phosphate, hypophosphite, adenosine-2'-monophosphate, adenosine-3'-monophosphate, adenosine-5'-monophosphate, adenosine-2',3'-cyclic monophosphate, adenosine-3',5'-cyclic monophosphate, thiophosphate, dithiophosphate, D,L-α-glycerol phosphate, β-glycerol phosphate, carbamyl phosphate, D-2-phosphoglyceric acid, D-3-phosphoglyceric acid, guanosine-2'-monophosphate, guanosine-3'-monophosphate, guanosine-5'-monophosphate, guanosine-2',3'-cyclic monophosphate, guanosine-3',5'-cyclic monophosphate, phosphoenol pyruvate, phosphoglycolic acid, D-glucose-1-phosphate, D-glucose-6-phosphate, 2-deoxy-D-glucose 6-phosphate, D-glucosamine-6-phosphate, 6-phosphogluconic acid, cytidine-2'-monophosphate, cytidine-3'-monophosphate, cytidine-5'-monophosphate, cytidine-2',3'-cyclic monophosphate, cytidine-3',5'-cyclic monophosphate, D-mannose-1-phosphate, D-mannose-6-phosphate, cysteamine-S-phosphate, phospho-L-arginine, O-phospho-D-serine, O-

phospho-L-serine, O-phospho-L-threonine, uridine-2'-monophosphate, uridine-3'-monophosphate, uridine-5'-monophosphate, uridine-2',3'-cyclic monophosphate, uridine-3',5'-cyclic monophosphate, O-phospho-D-tyrosine, O-phospho-L-tyrosine, phosphocreatine, phosphoryl choline, O-phosphoryl-ethanolamine, phosphono acetic acid, 2-aminoethyl phosphonic acid, methylene diphosphonic acid, thymidine-3'-monophosphate, thymidine-5'-monophosphate, inositol hexaphosphate, thymidine-3',5'-cyclic monophosphate) or sulphur sources (sulfate, thiosulfate, tetrathionate, thiophosphate, dithiophosphate, L-cysteine, D-cysteine, L-cysteinyl glycine, L-cysteic acid, cysteamine, L-cysteine sulfinic acid, N-acetyl-L-cysteine, S-methyl-L-cysteine, cystathionine, lanthionine, glutathione, D,L-ethionine, L-methionine, D-methionine, glycyl-L-methionine, N-aceetyl-D,L-methionine, L-methionine sulfoxide, L-methionine sulfone, L-djenkolic acid, thiourea, 1-thio-β-D-glucose, D,L-lipoamide, taurocholic acid, taurine, hypotaurine, p-amino benzene sulfonic acid, butane sulfonic acid, 2-hydroxyethane sulfonic acid, methane sulfonic acid, tetramethylene sulfone) tested. Besides that, this strain did not grow in many of the nitrogen sources tested. Table 4.3 shows the growth observed of *C. neteri* SSMD04 in the presence of the nitrogen sources tested.

Table 4.3: Growth of *C. neteri* SSMD04 in wells loaded with different nitrogen sources. The sign "+" represents growth observed and "-" represents no growth observed.

Biolog Microplate	Compound	Growth observed
PM3	Ammonia	-
	Nitrite	-
	Nitrate	-
	Urea	-
	Biuret	-
	L-alanine	-
	L-arginine	-

Table 4.3, continued.

Biolog Microplate	Compound	Growth observed
	L-asparagine	+
	L-aspartic acid	-
	L-cysteine	-
	L-glutamic acid	+
	L-glutamine	+
	Glycine	-
	L-histidine	-
	L-isoleucine	-
	L-leucine	-
	L-lysine	-
	L-methionine	-
	L-phenylalanine	-
	L-proline	+
	L-serine	-
	L-threonine	-
	L-tryptophan	-
	L-tyrosin	-
	L-valine	-
	D-alanine	-
	D-asparagine	-
	D-aspartic acid	-
	D-glutamic acid	-
	D-lysine	-
	D-serine	-
	D-valine	-
	L-citruline	-
	L-homoserine	-
	L-ornithine	-
	N-acetyl-L-glutamic acid	-
	N-phthaloyl-L-glutamic acid	-
	L-pyroglutamic acid	-
	Hydroxylamine	-

Table 4.3, continued.

Biolog Microplate	Compound	Growth observed
	Methylamine	-
	N-amylamine	-
	N-butylamine	-
	Ethylamine	-
	Ethanolamine	-
	Ethylenediamine	-
	Putrescine	-
	Agmatine	-
	Histamine	-
	β-phenylethylamine	-
	Tyramine	-
	Acetamide	-
	Formamide	-
	Glucuronamide	-
	D,L-lactamide	-
	D-glucosamine	-
	D-galactosamine	-
	D-mannosamine	-
	N-acetyl-D-glucosamine	+
	N-acetyl-D-galactosamine	+
	N-acetyl-D-mannosamine	-
	Adenine	-
	Adenosine	+
	Cytidine	+
	Cytosine	-
	Guanine	-
	Guanosine	-
	Thymine	-

Table 4.3, continued.

Biolog Microplate	Compound	Growth observed
	Thymidine	-
	Uracil	-
	Uridin	-
	Inosine	-
	Xanthine	-
	Xanthosine	+
	Uric acid	-
	Alloxan	-
	Allantoin	-
	Parabanic acid	-
	D,L-α-amino-N-butyric acid	-
	γ-amino-N-butyric acid	-
	ε-amino-N-caproic acid	-
	D,L-α-amino-caprylic acid	-
	δ-amino-N-valeric acid	-
	α-amino-N-valeric acid	-
	Ala-Asp	+
	Ala-Gln	+
	Ala-Glu	+
	Ala-Gly	+
	Ala-His	+
	Ala-Leu	+
	Ala-Thr	+
	Gly-Asn	+
	Gly-Gln	+
	Gly-Glu	+
	Gly-Met	-
	Met-Ala	+

When tested in a range of pH and varying concentrations of NaCl, *C. neteri* SSMD04 showed high level of adaptability (Figure 4.3). In the range of pH tested (3.5 to 10), *C. neteri* showed growth from 4 to 10. It grows optimally within the range of pH 5.5 to 10, and the growth is slower in lower pH. The growth in pH 4 has an extended lag phase, but it eventually reaches the same plateau level as in other wells. *C. neteri* SSMD04 can also survive in salinity ranging from 1 % to 9 % (w/v) (Figure 4.4). However, in the presence of higher concentrations of NaCl, the growth is slower (4 % to 9 % w/v), demonstrating its facultative halophilic property.

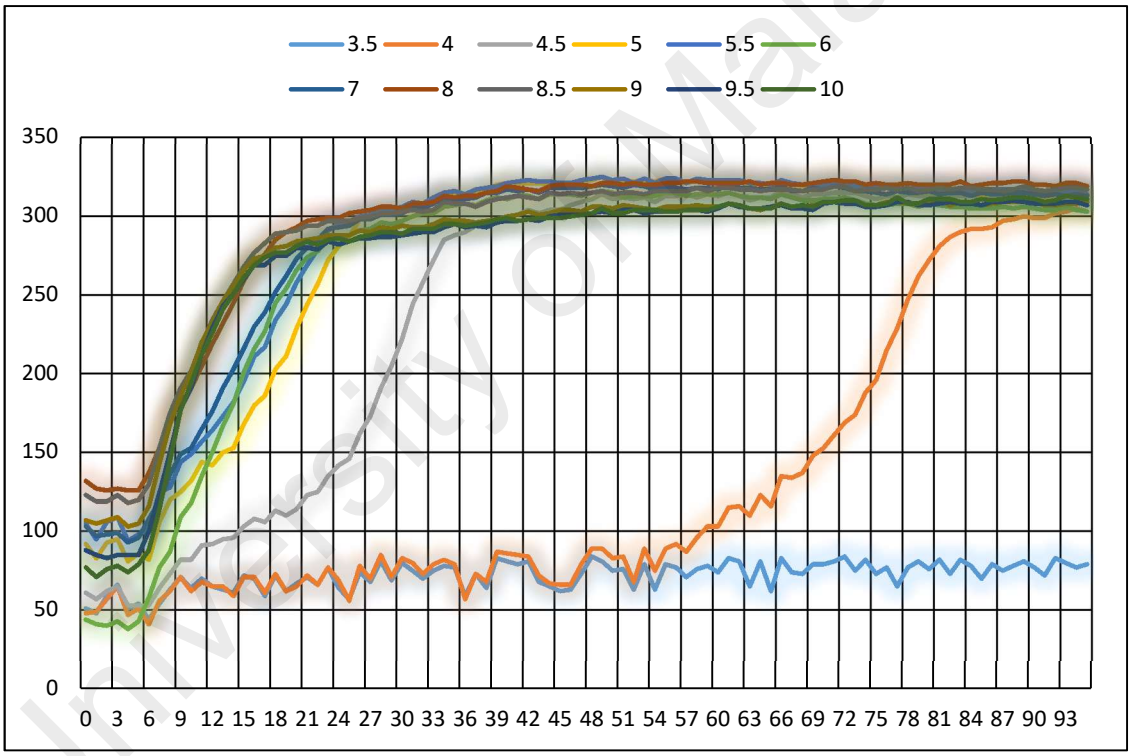

Figure 4.3: Growth kinetics of *C. neteri* SSMD04 in a range of pH values. The x-axis denotes the time in hour, and the y-axis denotes the absorbance reading of the tetrazolium dye. Different pH values were represented by different coloured lines.

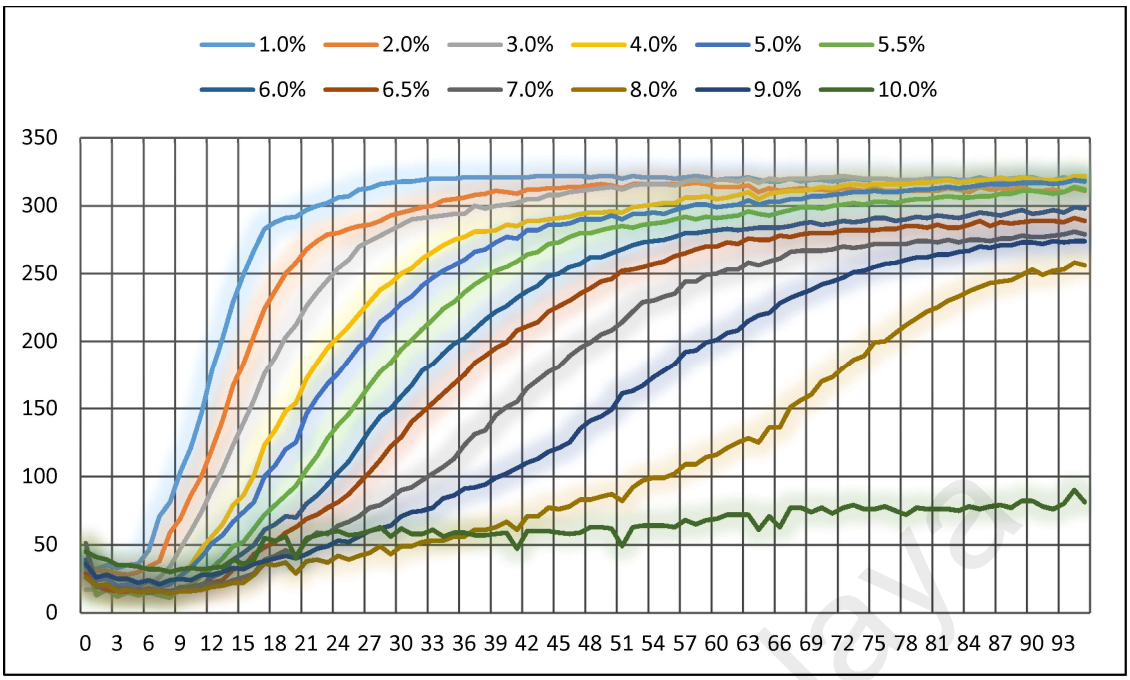

Figure 4.4: Growth kinetics of *C. neteri* in a range of NaCl concentrations (% in w/v). The x-axis denotes the time in hour, and the y-axis denotes the absorbance reading of the tetrazolium dye. Different NaCl concentrations were represented by different coloured lines.

C. neteri SSMD04 showed a certain degree of growth in all of the antibiotics tested, with growth in two lowest concentrations to growth in all four concentrations tested. It grew least well in the wells containing enoxacin, nalidixic acid, chloramphenicol, cinoxacin, thiamphenicol, pipemidic acid, and sulfisoxazole (Table 4.4).

Table 4.4: Growth of *C. neteri* SSMD04 in wells containing antibiotics. "+" represents the number of wells that *C. neteri* SSMD04 grew in.

Biolog Microplates	Antibiotics	Class	Growth Observed
PM11	amikacin	Aminoglycoside	++++
	chlortetracycline	Tetracycline	++++
	lincomycin	Lincosamide	++++
	amoxicillin	Penicillin	++++
	cloxacillin	Penicillin	++++
	lomefloxacin	Quinolone	++++
	colistin	Polymyxin	++++
	minocycline	Tetracycline	++++

Table 4.4, continued.

Biolog Microplates	Antibiotics	Class	Growth Observed
	capreomycin	Aminoglycoside	++++
	demeclocycline	Tetracycline	+++
	nafcillin	Penicillin	++++
	cefazolin	Cephalosporin	++++
	enoxacin	Quinolone	++
	nalidixic acid	Quinolone	++
	chloramphenicol	Miscellaneous	++
	erythromycin	Macrolide	++++
	neomycin	Aminoglycoside	++++
	ceftriaxone	Cephalosporin	++++
	gentamicin	Aminoglycosides	++++
	cephalothin	Cephalosporin	++++
	kanamycin	Aminoglycoside	++++
	ofloxacin	Quinolone	++++
PM12	penicillin G	Penicillin	++++
	tetracycline	Tetracycline	++++
	carbenicillin	Penicillin	++++
	oxacillin	Penicillin	++++
	penimepicycline	Tetracycline	++++
	polymyxin B	Polymixin	++++
	paromomycin	Amebicide	+++
	vancomycin	Glycopeptide	++++
	sisomicin	Aminoglycoside	++++
	sulfamethazine	Sulfanamide	++++
	novobiocin	Aminocoumarin	+++
	sulfadiazine	Sulfanamide	++++
	tobramycin	Aminoglycoside	++++
	sulfathiazole	Sulfonamide	++++
	spectinomycin	Aminocyclitol	++++
	sulfamethoxazole	Sulfonamide	++++
	spiramycin	Macrolide	++++
	rifampicin	Miscellaneous	++++

Table 4.4, continued.

Biolog Microplates	Antibiotics	Class	Growth Observed
PM13	ampicillin	Penicillin	++++
	azlocillin	Penicillin	++++
	oxolinic acid	Quinolone	++++
	doxycycline	Tetracycline	++++
	cefuroxime	Cephalosporin	++++
	rolitetracycline	Tetracycline	++++
	geneticin (G418)	Aminoglycoside	++++
	moxalactam	Oxa-beta lactam	++++
	tylosin	Macrolide	++++
PM14	fusaric acid	Miscellaneous	+++
	cefoxitin	Cephalosporin	++++
	nitrofurantoin	Miscellaneous	++++
	chloramphenicol	Miscellaneous	+++
	piperacillin	Penicillin	++++
	carbenicillin	Penicillin	++++
PM15	cefmetazole	Cephalosporin	++++
	D-cycloserine	Anti-TB agent	++++
	fusidic acid	Miscellaneous	+++
	phleomycin	Glycopeptide	++++
	oleandomycin	Macrolide	++++
	puromycin	Protein synthesis inhibitor	+++
PM16	cefotaxime	Cephalosporin	+++
	phosphomycin	Miscellaneous	++++
	trimethoprim	Antimetabolite	+++
	cinoxacin	Quinolone	++
	streptomycin	Aminoglycoside	++++
	rifamycin SV	Anti TB agent	+++
PM17	hygromycin B	Aminoglycoside	++++
	ethionamide	Anti TB agent	++++

Table 4.4, continued.

Biolog Microplates	Antibiotics	Class	Growth Observed
	cefamandole nafate	Cephalosporin	++++
	cefoperazone	Cephalosporin	++++
	cefsulodin	Cephalosporin	++++
PM18	thiamphenicol	Miscellaneous	++
	pipemidic acid	Pyridopyrimidine	++
	sulfisoxazole	Sulfonamide	++
	aztreonam	Monobactam	++++
PM19	josamycin	Macrolide	++++
	phenethicillin	Penicillin	++++
	blasticidin S	Miscellaneous	++++
	polymyxin B	Polymyxin	++++
	dihydrostreptomycin	Aminoglycoside	++++
PM20	apramycin	Aminoglycoside	++++
	ciprofloxacin	Quinolone	+++
	oxytetracycline	Tetracycline	++++
	troleandomycin	Macrolide	+++

With the interest in the antibiotic resistant phenotypes in *C. neteri* SSMD04, its genome was searched for antibiotics resistance related genes that was categorized by RAST. All genes related to antibiotics resistance were listed in Table 4.5 in comparison to that found in the genome of *C. neteri* NBRC150707, a clinical isolate of *C. neteri*. As shown, there is no difference in terms of number of genes harboured by both *C. neteri* strains except genes encoding MdtA, BLc, CmeB, CmeC/TolC_14, AcrR/TetR/Reg, MFS, MacA, MacB, and AcrB.

Table 4.5: Antibiotics resistance related genes found in *C. neteri* SSMD04 and NBRC105707. The number denotes the number of gene copy found within the genome.

Category	Protein abbreviation	NBRC105707	SSMD04	Functional role
The mdtABCD multidrug resistance cluster	BaeS	1	1	sensory histidine kinase BaeS
	BaeR	1	1	response regulator BeaR
	MdtA	2	1	probable RND efflux membrane fusion protein
	MdtB	1	1	multidrug transporter MdtB
	MdtC	1	1	multidrug transporter MdtC
	MdtD	1	1	multidrug transporter MdtD
Fosfomycin resistance	FosA	1	1	fosfomycin resistance protein FosA
Resistance to fluoroquinolones	parC	1	1	topoisomerase IV subunit A
	parE	1	1	topoisomerase IV subunit B
	gyrA	1	1	DNA gyrase subunit A
	gyrB	1	1	DNA gyrase subunit B
Beta-lactamase	BL	1	1	Beta-lactamase
	BLc	0	1	Beta-lactamase class C and other penicillin binding proteins
	BLI	1	1	Metal-dependent hydrolases of the beta-lactamase superfamily I
	BLIII	1	1	Metal-dependent hydrolases of the beta-lactamase superfamily III
Multidrug resistance efflux pumps	CmeA	1	1	RND efflux system, membrane fusion protein CmeA
	CmeB	3	2	RND efflux system, inner membrane transporter CmeB
	CmeC/TolC_14	6	2	RND efflux system, outer membrane lipoprotein CmeC/Type I secretion outer membrane protein, TolC precursor

Table 4.5, continued.

	AcrR/TetR/Reg	2	1	Transcription repressor of multidrug efflux pump acrAB operon, TetR (AcrR) family/ Transcription regulator of multidrug efflux pump operon, TetR (AcrR) family/ Probable transcription regulator protein of MDR efflux pump cluster
	MATE_all/(YdhE /NorM)	1	1	Multidrug antimicrobial extrusion protein (Na(+)/drug antiporter), MATE family of MDR efflux pumps/ Multidrug and toxin extrusion (MATE) family efflux pump YdhE/NorM
	MFS	2	1	Multidrug-efflux transporter, major facilitator superfamily (MFS)
	MacA	5	2	Macrolide-specific efflux protein MacA
	MacB	5	2	Macrolide export ATP-binding/permease protein MacB
	RND	1	1	Membrane fusion protein of RND family multidrug efflux pump
	AcrB	2	1	Acriflavin resistance protein
Lipid A-Ara4N pathway (Polymyxin resistance)	PmrM	1	1	Polymyxin resistance protein PmrM
	PmrL	1	1	Polymyxin resistance protein PmrL, sucrose-6 phosphate hydrolase
	PmrK	1	1	Polymyxin resistance protein PmrK, undecaprenyl phosphate-α-4-amino-4-deoxy-L-arabinose arabinosyl transferase
	PmrJ	1	1	Polymyxin resistance protein PmrJ, predicted deacetylase
	ArnC	1	1	Polymyxin resistance protein ArnC, glycosyl transferase

4.3 Detection of AHL-type QS activity in *C. neteri* SSMD04

In this work, *Chromobacterium violaceum* CV026 was the first AHL biosensor used to detect the presence of exogenous AHL produced by *C. neteri* SSMD04. *C. violaceum* CV026 is an AHL synthase mutant but it retains its functional copy of AHL receptor. Upon activation of its own QS circuit in the presence of AHL from external sources, mainly AHL with side chain of length of four to eight carbons, the biosynthesis of a purple pigment, violacein, is activated and thus turning the colony purple (McClean et al., 1997). Therefore, when streaked against the biosensor strain on LB agar plate, purple pigmentation in *C. violaceum* CV026 indicates the presence of exogenous AHL in the tested strain. Figure 4.5 shows that *C. neteri* produced AHL which led to the purple pigmentation in *C. violaceum* CV026. Two *P. carotovora* strains were used as control in this experiments, the AHL producing *P. carotovora* GS101, and the AHL-synthase mutant, *P. carotovora* PNP22.

Figure 4.5: Detection of AHL-type QS activity of *C. neteri* SSMD04 using the biosensor *C. violaceum* CV026. *C. violaceum* CV026 were streaked perpendicularly against the tested strains at the periphery of the agar plate. *P. carotovora* PNP22 served as negative control while *P. carotovora* GS101 served as positive control.

Another AHL biosensor, *E. coli* (pSB401) was also used to screen for AHL production in *C. neteri* SSMD04. *E. coli* (pSB401) is a *lux*-based biosensor that produces bioluminescence in the presence of short chain AHLs. Figure 4.6 shows that the AHL extract of *C. neteri* SSMD04 activated the light production in *E. coli* (pSB401), confirming that *C. neteri* produces AHL(s).

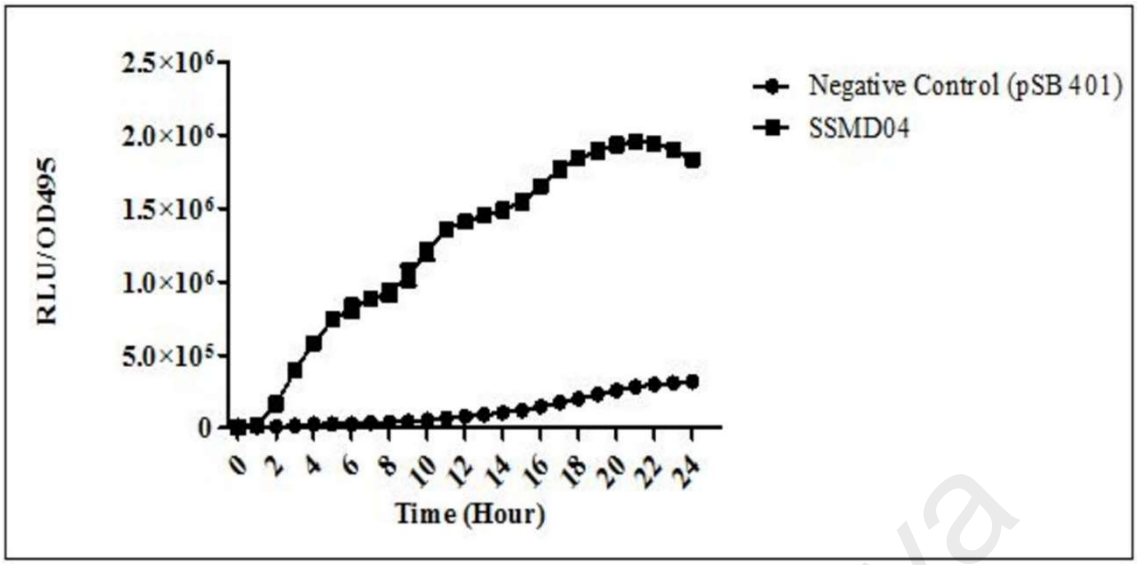

Figure 4.6: Detection of the presence of AHL in the organic extract of *C. neteri* SSMD04 using *E. coli* (pSB401). The results were shown as relative light unit (RLU)/OD_{495} against incubation time of *E. coli* (pSB401) in the presence of the AHL extract of *C. neteri* SSMD04 (square plots). The negative control (circle plots) was *E. coli* (pSB401) grown without AHL extract.

4.4 AHL identification by triple quadrupole LC/MS

Both AHL biosensors (*C. violaceum* CV026 ad *E. coli* (pSB401)) detect a range of AHLs and thus do not provide information on the specific type of AHL produced by *C. neteri* SSMD04. For the identification of AHL produced by *C. neteri*, triple quadrupole LC/MS was used where the AHL extract of *C. neteri* SSMD04 showed a prominent peak that has the same retention time with that of synthetic *N*-butyryl-homoserine lactone (C4-HSL), and it is consistently present in all three replicates, as seen in the extracted-ion chromatogram (EIC) (Figure 4.7). Detailed inspection of the peak seen have shown the presence of a product ion peak with mass-to-charge (*m/z*) ratio of 172.1000, a reported value for C4-HSL (Ortori et al., 2007) (Figure 4.8). This is confirmed by the presence of another product ion peak of *m/z* value of 102, a signature peak for AHL, which signify the presence of a homoserine lactone ring, the invariant structure of AHLs. Therefore, it was found that *C. neteri* SSMD04 produces only one type of AHL, namely C4-HSL. This is in agreement with the biosensor assay as both biosensors respond to C4-HSL.

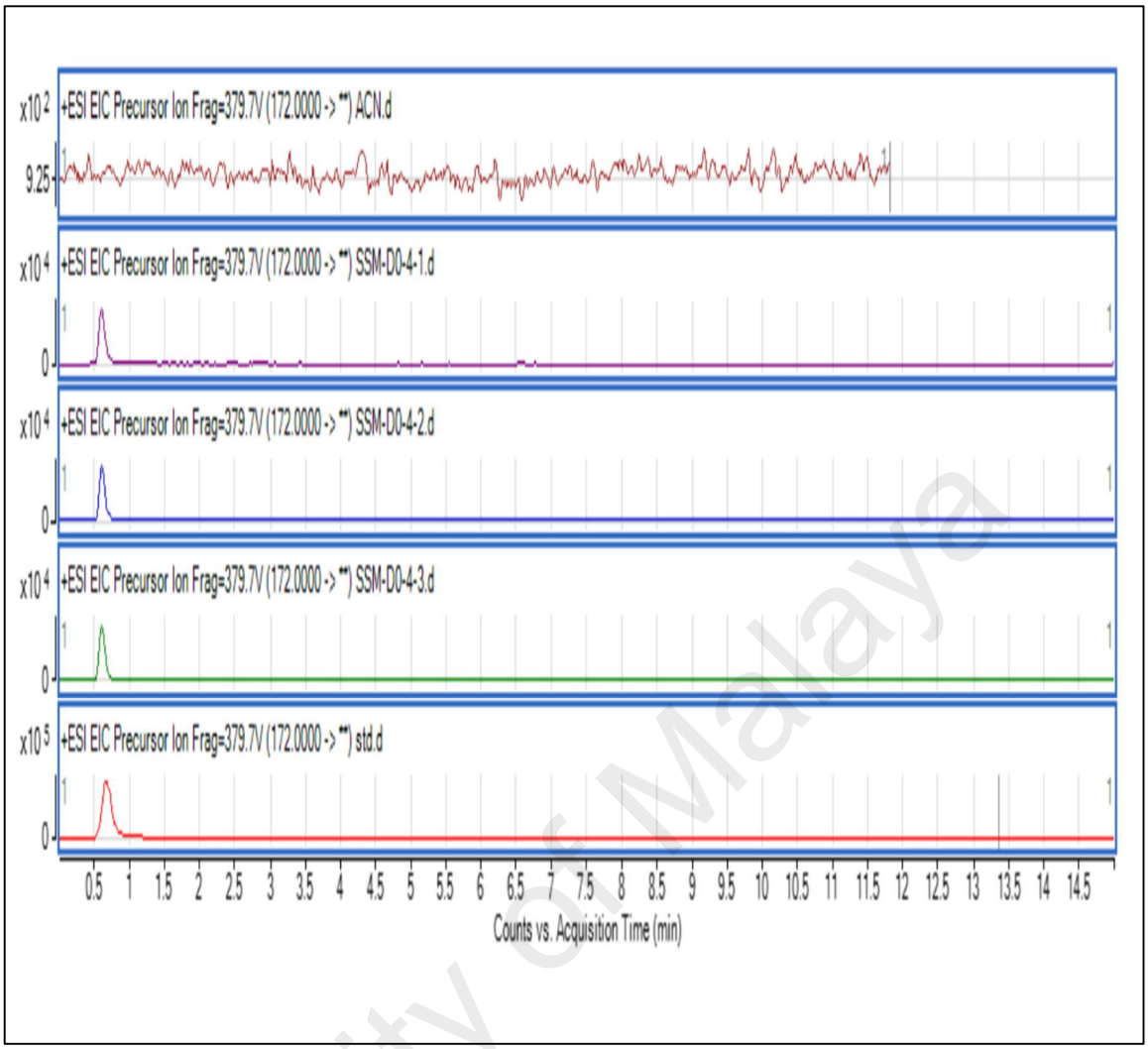

Figure 4.7: EIC of *C. neteri* SSM04 organic extract. The experiment was carried out in triplicates with a blank (ACN). Synthetic C4-HSL was used for comparison, labelled as "std".

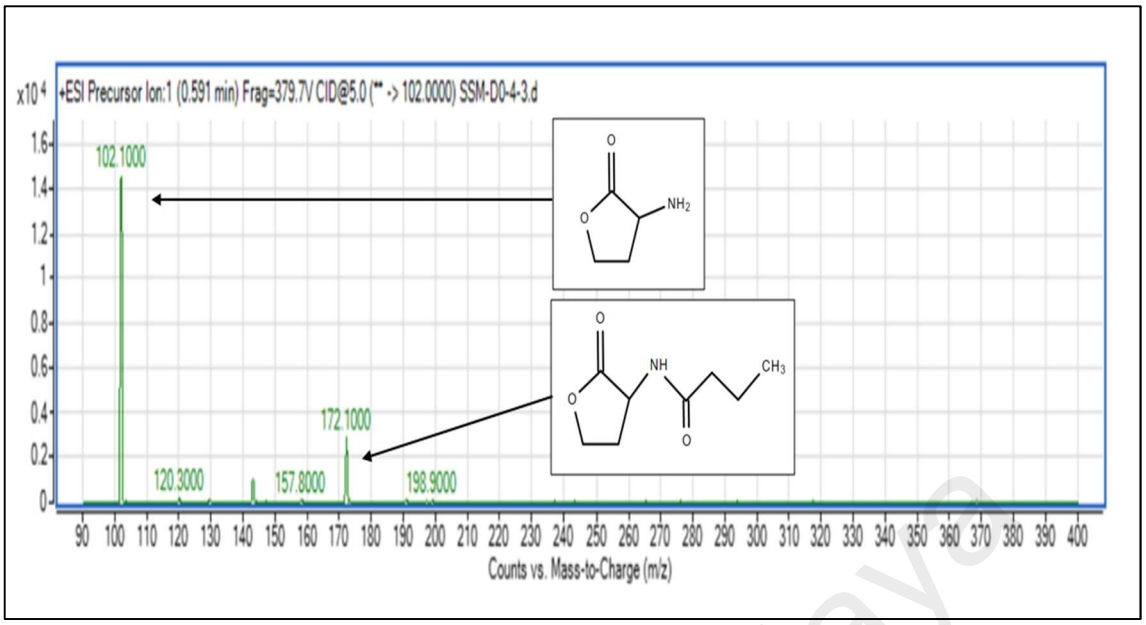

Figure 4.8: Product ion peaks of the peak seen in EIC. The peak with *m/z* value of 172.1000 represents the complete structure of C4-HSL and the peak with *m/z* value of 102.1000 represents the homoserine lactone ring. Insets: molecular structures of homoserine lactone ring and C4-HSL.

4.5 Lipase activity

C. neteri SSMD04 was cultured on an agar plate supplemented with 0.5 % (v/v) corn oil to demonstrate its lipase activity. After incubation overnight, clearance zone can be observed around the colonies, indicating the degradation activity of lipase. The activity was observable after 24 hrs incubation, but the result was more prominent after 48 hrs incubation (Figure 4.9).

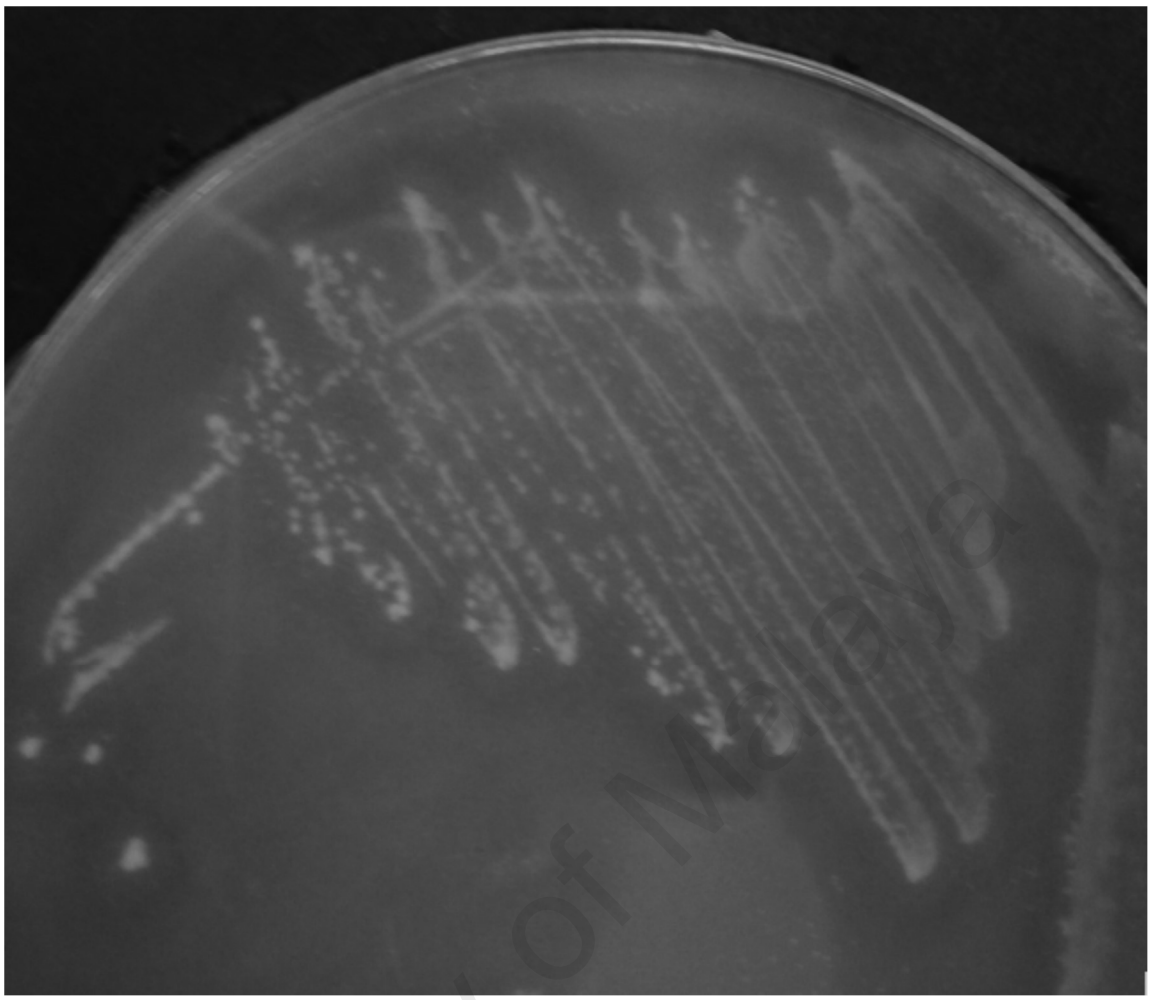

Figure 4.9: *C. neteri* SSMD04 cultured on medium supplemented with 0.5 % corn oil. The clearance zone around the colonies is a result of lipase activity.

4.6 *luxI/R* homologues search and analysis

The complete genome of *C. neteri* SSMD04 was submitted to GenBank and consequently annotated by NCBI prokaryotic genome annotation pipeline, which prompted the search for the *luxI/R* homologue responsible for QS activity. A 636 bp gene sequence homologous to *luxI* was found in the genome, hereafter named *cneI* (gi|689266443|). This gene was found to be highly similar to the *N*-acyl homoserine lactone synthase found in *Klebsiella michiganensis* RC10 (90 % base pair similarity) as well as the homologous gene in *Citrobacter rodentium* ICC168 (70 % base pair similarity). In order to strengthen the identity of this gene, protein domain analysis was carried out using InterPro (Mitchell et al., 2015). An acyl-CoA-*N*-acyltransferase domain was identified, which is the

structural domain of canonical *N*-acyl homoserine lactone synthethases (Watson et al., 2002; Gould et al., 2004).

Another gene potentially coding for the cognate receptor, the *luxR* homologue, located 8 nucleotides away from *cneI* was annotated as a hypothetical protein by NCBI prokaryotic genome annotation pipeline. This 705 bp gene was found to be in convergent order with *cneI*, sharing 89 % sequence similarity with the *luxR* homologue in *K. michiganensis* RC10 and 70 % sequence similarity with the *luxR* homologue in *C. rodentium* ICC168, *croR*. The identity of this gene was revealed by protein domain search which identified two signature domains of a typical luxR homologue, the autoinducer-binding domain and the C-terminal effector. This *luxR* homologue is hereafter named *cneR* (gi|689266442|).

Since both *cneI* and *cneR* share high sequence similarity with the homologous genes in *K. michiganensis* RC10 and *C. rodentium* ICC168, the loci of this gene pair, including the flanking regions were aligned to examine their similarity in terms of genome organization. Two other available genomes of *C. neteri*, a non-clinical strain, M006 as well as a clinical strain, NBRC105707 were included for comparison as well (Figure 4.10). As shown in the result, the gene organization of the loci of interest between the two strains SSMD04 and M006 are highly similar. However, genes found upstream of *cneI* in these two strains are not present in NBRC105707, suggesting the underlying genome structural disparity between clinical and non-clinical strains. Besides that, the genomic organizations of *C. neteri* SSMD04 and *K. michiganensis* RC10 at the *luxI/R* homologues locus are very similar too. This is not true for *C. rodentium* ICC168. Interestingly, a publicly available genome of *C. davisae* DSM4568 was examined but it was found that it does not harbor any *luxI/R* homologous pair, suggesting that AHL-type QS is not conserved in all members of the genus *Cedecea*.

Besides that, another gene sequences in *C. neteri* SSMD04 genome was found to encode an orphan *luxR* type receptor, without a cognate *luxI* homologue. This 723 bp orphan *luxR* homologue (gi|740696391|) shares 69 % sequence homology with the *luxR* homologue of *Enterobacter asburiae* L1. A multiple sequence alignment of CneR, the orphan LuxR homologue, and other canonical LuxR-type proteins were made (Figure 4.11). It was found that both LuxR homologues in *C. neteri* SSMD04 shares the conserved amino acid residues as in other known LuxR-type proteins, the residue position 57, 61, 70, 71, 85, 113, 178, 182, 188 (TraR sequence numbering used as a reference). These conserve sites are present in at least 95 % of LuxR-type proteins (Whitehead et al., 2001; Zhang et al., 2002), thus justifying the functionality of both of the LuxR homologue in *C. neteri* SSMD04.

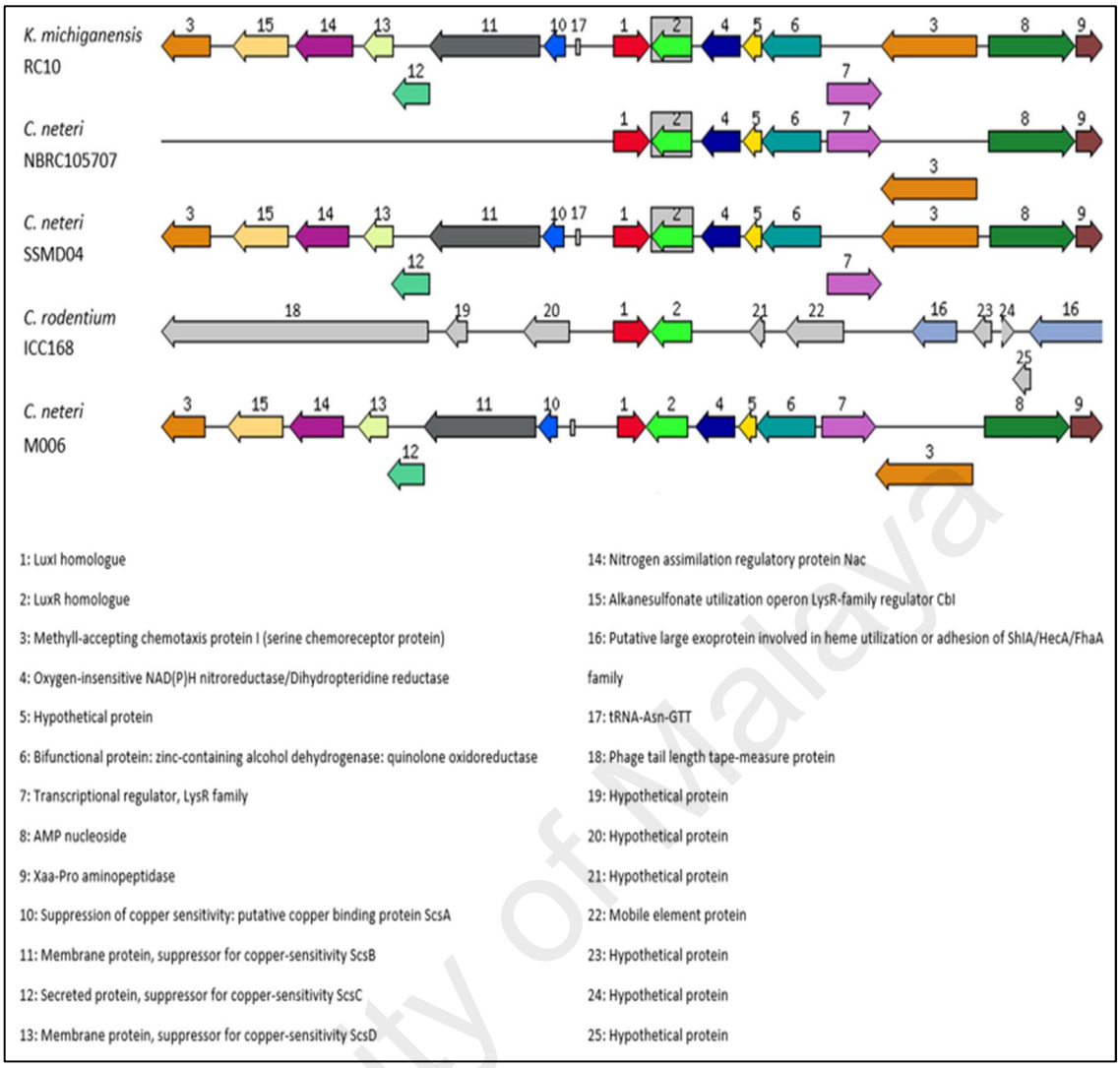

Figure 4.10: Schematic representation of *cneI/R* locus of *C. neteri* SSMD04 in comparison with *K. michiganensis* RC10, *C. neteri* NBRC105707, *C. rodentium* ICC168, and *C. neteri* M006 (GenBank ID: CP011077.1, GCA_001571265, FN543502.1, CP0009458.1, respectively). The species and strains are shown at the left of the genome loci. Each of the genes is numbered and labelled accordingly. Homologous genes are presented in similar colour and the arrows represent the orientation of the genes. Genes arranged below the line are overlapping genes.

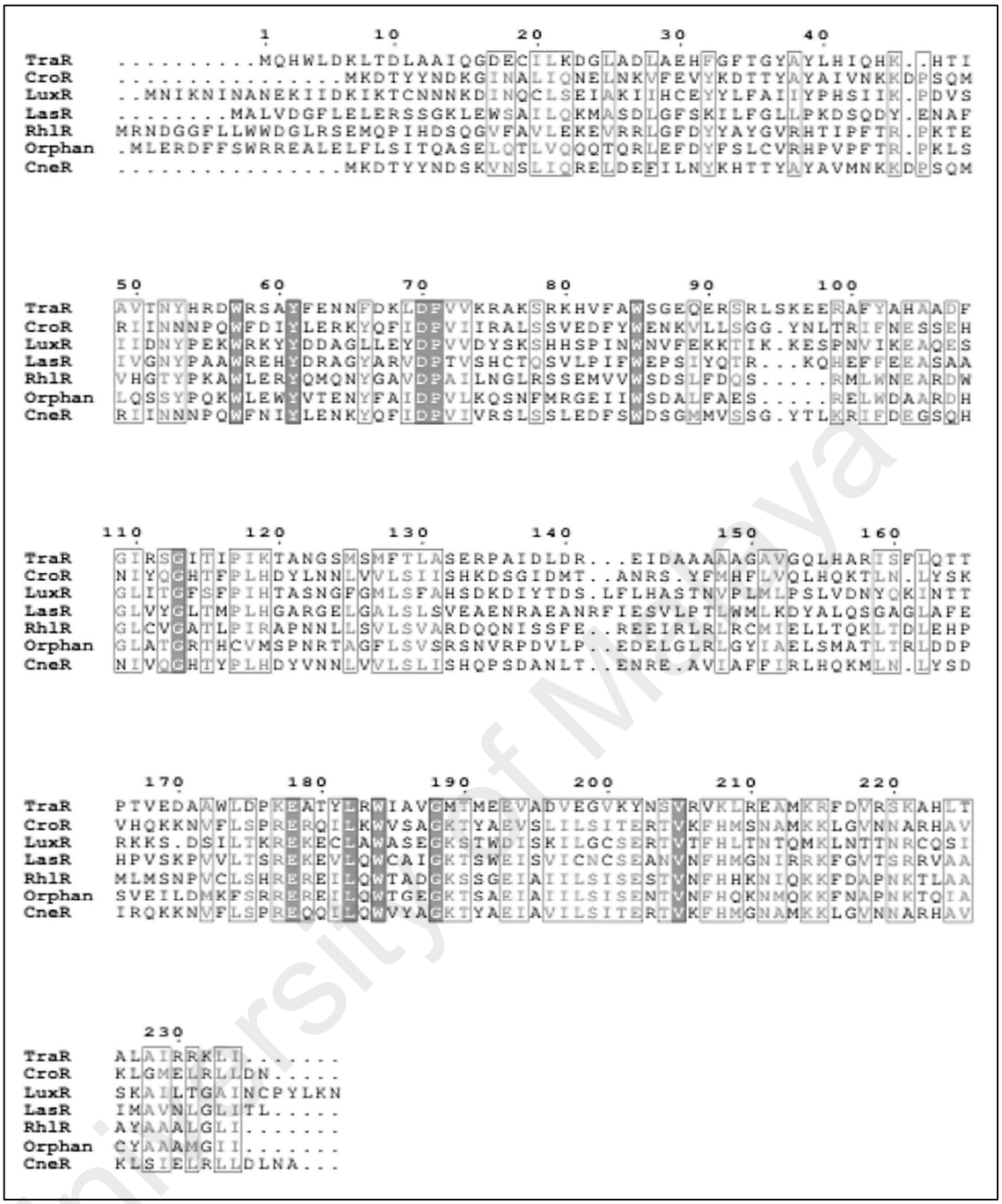

Figure 4.11: Multiple sequence alignment of CneR, *C. neteri* SSMD04 orphan LuxR (Orphan) with five other canonical QS LuxR-type proteins. TraR, CroR, LuxR, LasR, and RhlR (GenBank ID: 282950058, 59482356, 299361, 541657, 1117981, 740696391, 689266442) are known LuxR-type proteins responsible for QS from *A. tumefaciens*, *C. rodentium*, *A. fischeri*, *P. aeruginosa*, (LasR and RhlR), respectively. Identical residues are shown in vertical filled bars. Conserved but not identical residues are shown in outline bars. TraR residue numbering is shown above the alignment as a reference.

It is known that orphan LuxR is present in some bacteria that does not harbor any *luxI* gene, such as *E. coli* and *Salmonella enterica*. The presence of orphan LuxR in these bacteria allows detection of exogenous AHLs produced by other sources (Ahmer, 2004).

Nevertheless, the function of the aforementioned orphan LuxR in *C. neteri* SSMD04 is not known. However, genome comparison of such loci in *C. neteri* SSMD04 and M006, *C. davisae* DSM4568, *C. rodentium* ICC168, *S. enterica* subsp. *enterica* serovar Choleraesuis str. SC-B67, and *E. coli* K12 shows a high degree of conservation (Figure 4.12). The homology is the highest among the three strains of *Cedecea* spp. despite the absence of canonical *luxI/R* pair in *C. davisae* DSM4568 genome.

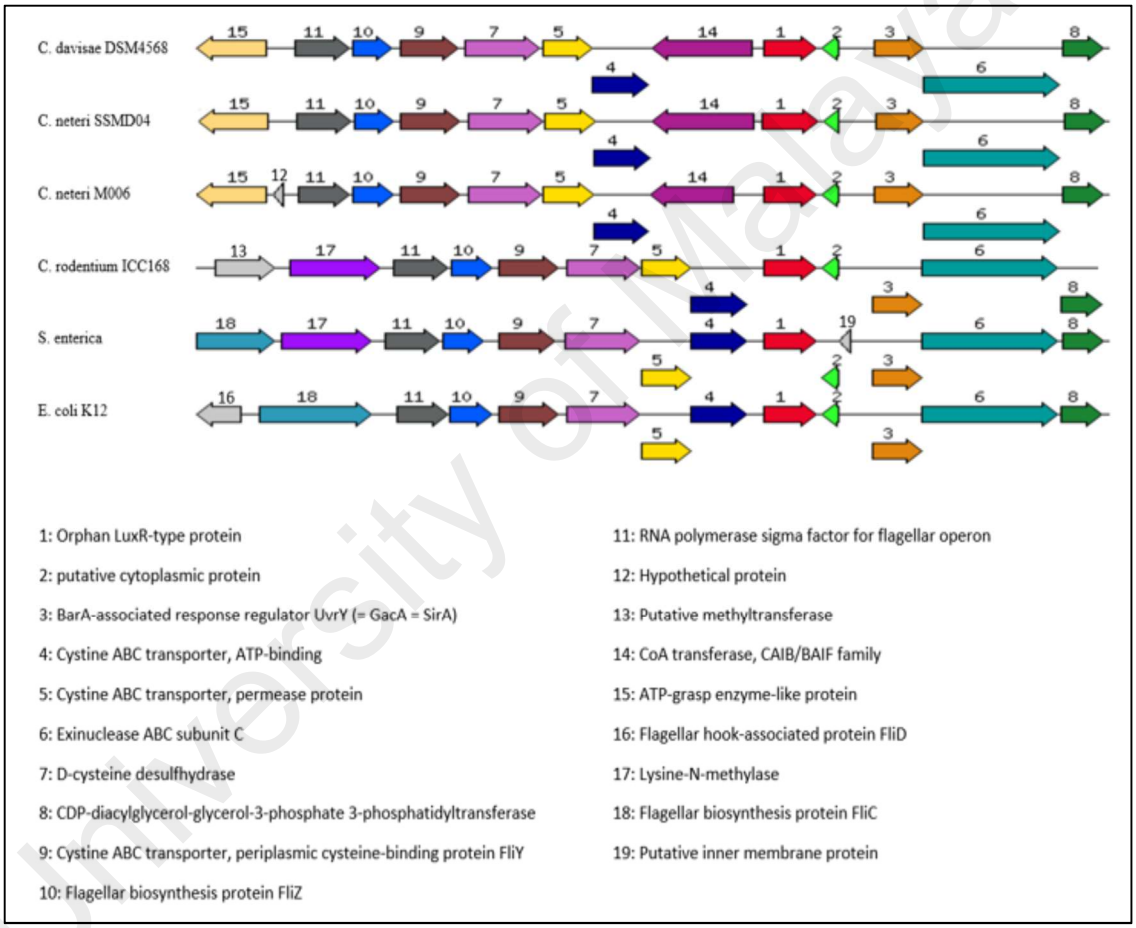

Figure 4.12: Organization of *C. neteri* SSMD04 orphan *luxR* and its flanking genes in comparison with other selected species: *C. davisae* DSM4568, *C. neteri* M006, *C. rodentium* ICC168, *Salmonella enterica* subsp. *enterica* serovar Choleraesuis str. SC-B67 and *E. coli* K12 (GenBank ID: 513473511, 690276415, 282947233, 62178570, 556503834, respectively.

BLAST search has shown that very few known sequences share high degree of similarity with CneI. The homologous gene in *C. rodentium* was originally found to share the highest identify with CneI, but a newly added genome sequence of *K. michiganensis* has

changed this result. *K. michiganensis* possess a pair of *luxI/R* homologue as well. However, due to the fact that *K. michiganensis* was a newly described species (Saha et al., 2013), its AHL activity was not reported. Studies on a large number of LuxI homologues have shown that LuxI homologues are not conserved apart from ten invariant residues (Fuqua et al., 2001). This was shown in the alignment of the amino acid sequences of LuxI homologues retrieved from GenBank that shares the highest similarity with CneI. CneI, a predicted LuxI homologue has the conserved amino acid residues as with other LuxI homologues (Figure 4.13).

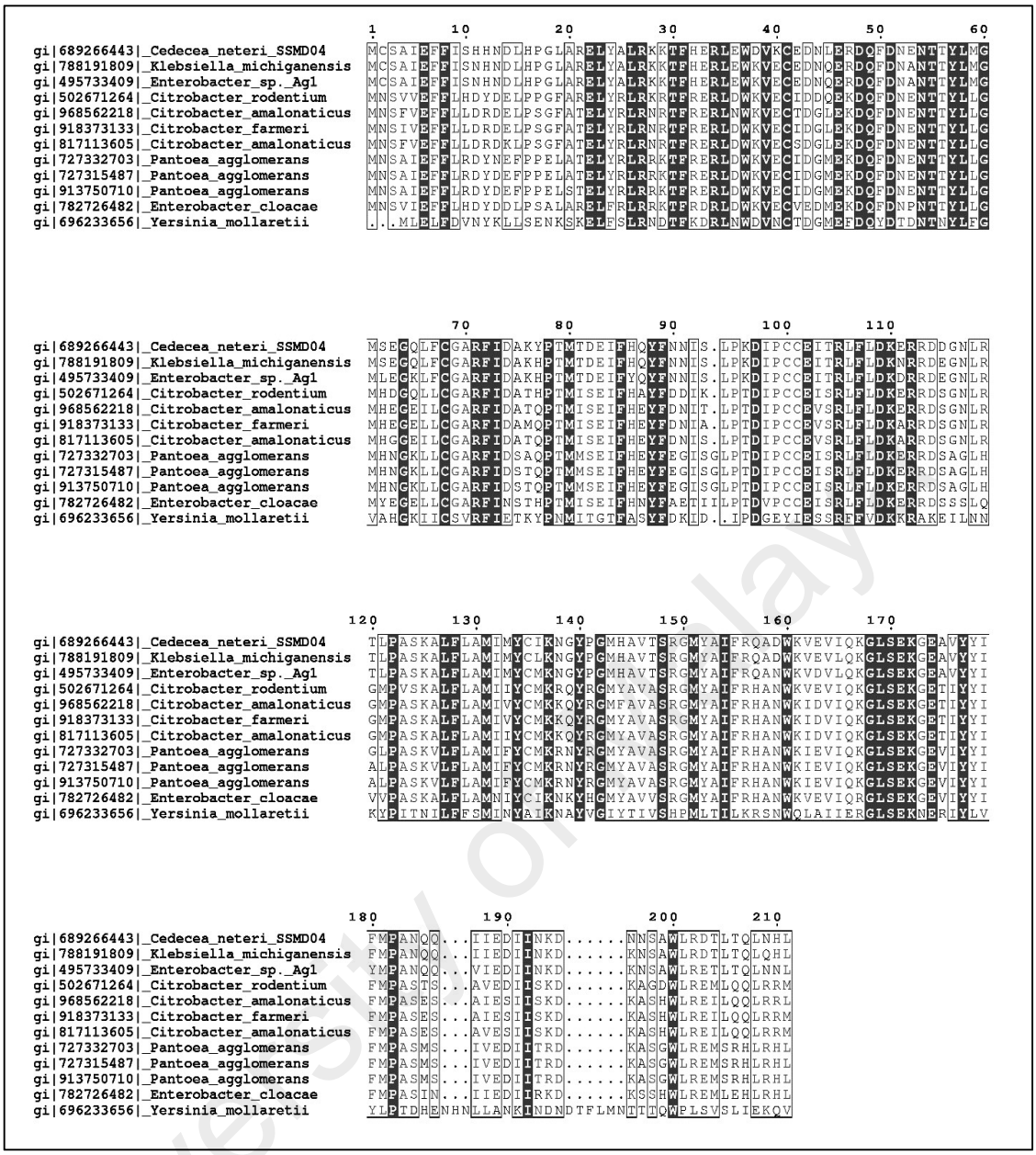

Figure 4.13: Multiple sequence alignment of CneI and other LuxI homologues that share the highest homology with CneI. Identical residues are shown in vertical filled bar and the conserved residues are shown in outline bar. The ten invariant residues in LuxI homologues are at position 27, 31, 37, 46, 48, 51, 71, 85, 102, and 105.

The alignment shown above was then used to construct a phylogenetic tree to show the relationship between these sequences. As shown in Figure 4.14, CneI is most closely related to the LuxI homologue of *K. michiganensis* and *Enterobacter* sp. Ag1 instead of that of *C. rodentium* as previously thought. Moreover, the conservation of LuxI homologue in the *Citrobacter* genus can be seen as all LuxI homologues from this genus formed a single cluster.

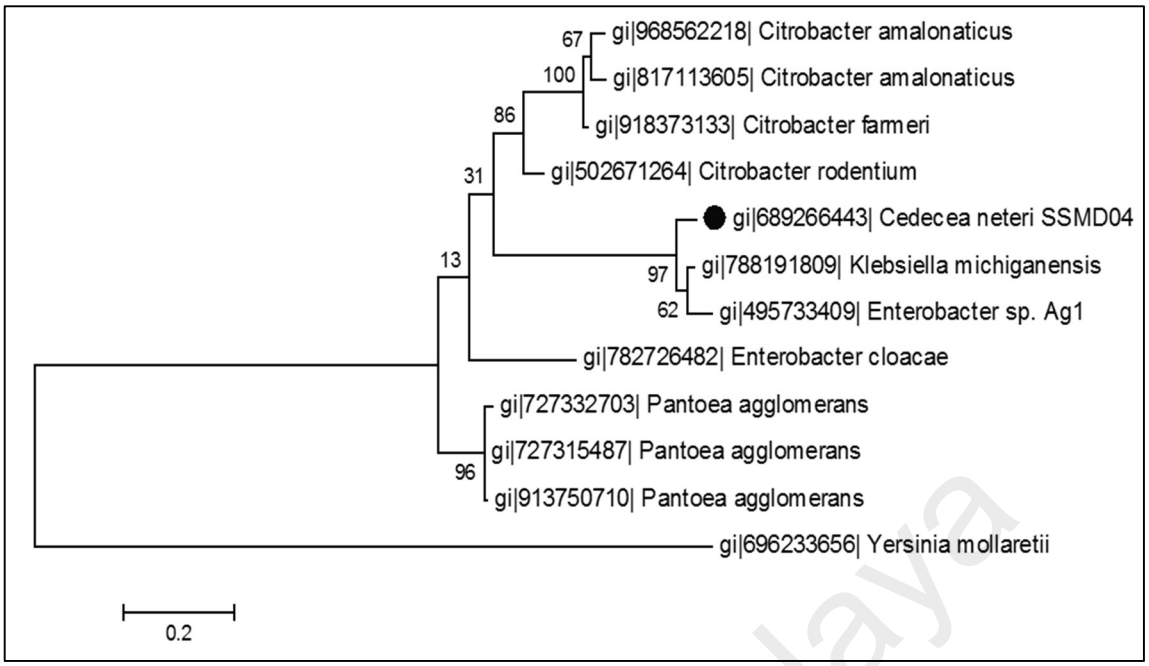

Figure 4.14: Phylogenetic tree built from the alignment of CneI (black circle) and ten other LuxI homologues. The maximum likelihood tree was built using Jones-Taylor-Thornton (JTT) model with gamma distribution. Then, 1000 bootstrap replication was used and the bootstrap values are represented at the nodes. Bar denotes the number of substitutions per amino acid position. The homologous amino acid sequence from *Yersinia mollaretii* was used as an outgroup.

Similarly, CneR and orphan LuxR in *C. neteri* SSMD04 were blasted to find their closely related sequences. Due to the proximity of CneR to CneI, it would be expected that they mutate at the same rate. Therefore, it was not surprising that the LuxR homologues that were matched with CneR after BLAST search belong to bacterial species that harbor LuxI homologues matched with CneI after BLAST search. For this reason, it is expected that the CneR phylogenetic tree would have similar topology with that of CneI's. However, as seen in Figure 4.15, although CneR clusters closely with homologous proteins from *Enterobacter* sp. Ag1 and *K. michiganensis* similar to CneI phylogenetic tree, the cluster with *Citrobacter* spp. is more closely related to the cluster with *Pantoea* spp. as opposed to the relatedness of *Citrobacter* spp. to *C. neteri* SSSMD04 in CneI phylogenetic tree. The orphan LuxR was found to share the highest sequence similarity with the transcriptional regulator SdiA in *K. michiganensis* (94 %), followed by the homologous gene in *Enterobacter* sp. Ag1 (93 %). The relatedness of the orphan LuxR in *C. neteri*

SSMD04 with SdiA from *K. michiganensis* and *Enterobacter* sp Ag1 is shown in Figure 4.16.

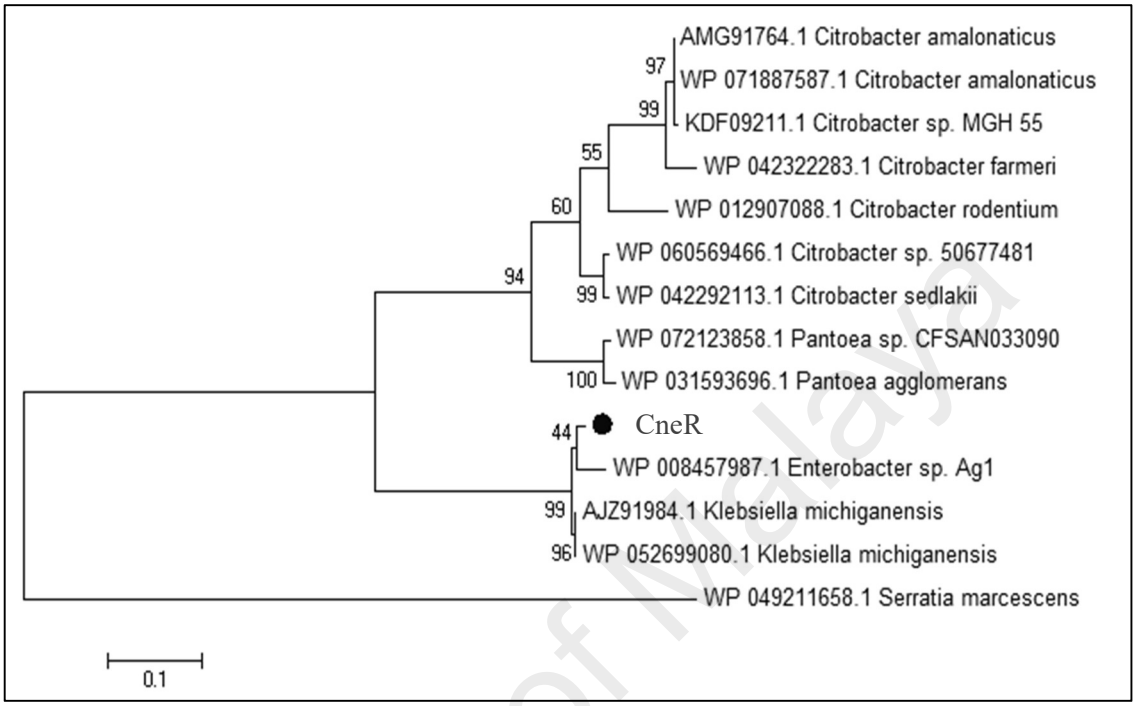

Figure 4.15: Phylogenetic tree built from the alignment of CneR (black circle) and other LuxR homologues. The maximum likelihood tree was built using Le Gascuel 2008 model with gamma distribution. Then, 1000 bootstrap replication was used and the bootstrap values are represented at the nodes. Bar denotes the number of substitutions per amino acid position. The homologous amino acid sequence from *Serratia marcescens* was used as an outgroup.

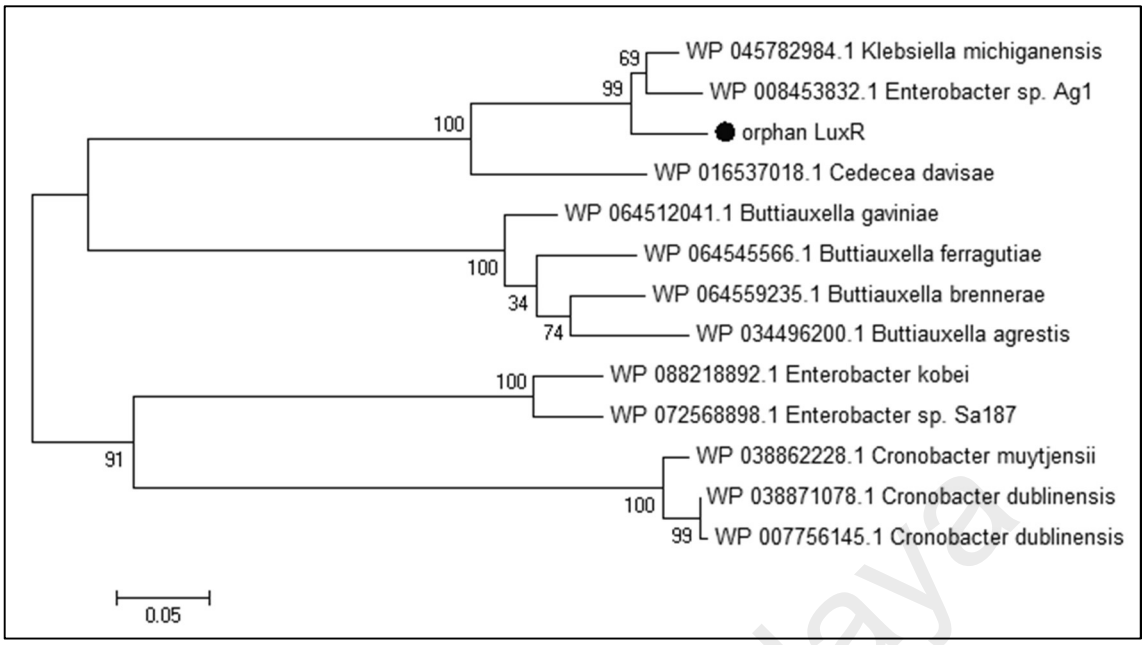

Figure 4.16: Phylogenetic tree built from the alignment of orphan LuxR from *C. neteri* SSMD04 (black circle) and other orphan LuxR. The maximum likelihood tree was built using Le Gascuel 2008 model with gamma distribution. Then, 1000 bootstrap replication was used and the bootstrap values are represented at the nodes. Bar denotes the number of substitutions per amino acid position.

4.7 *cneI* cloning

The complete sequence of *cneI* (636 bp) was amplified from the genomic DNA of *C. neteri* SSMD04 by PCR (Figure 4.17). This amplicon was purified and cloned into an expression vector, pET28a, denoted as pET28a_*cneI*. This amplicon was inserted into the vector downstream of a T7 promoter, but upstream of a 6 × His-tag sequence for subsequent purification purposes. The expression of *cneI* in this expression plasmid was induced by IPTG. Initially, two concentrations of IPTG, 0.5 mM and 1.0 mM were used but 1.0 mM of IPTG was constantly used later as the optimum concentration.

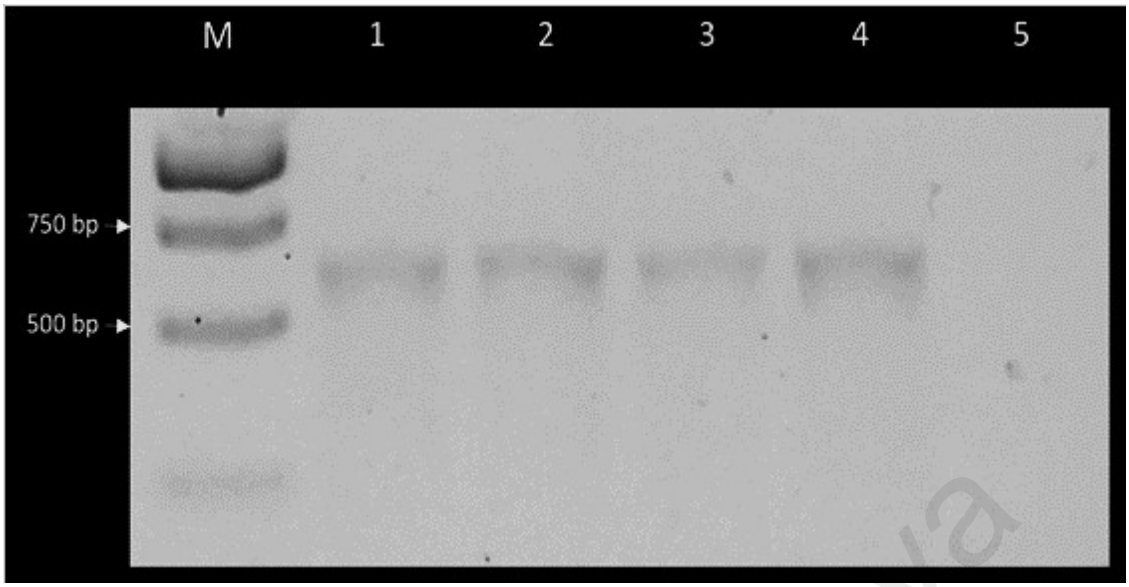

Figure 4.17: PCR amplification of *cneI* from the genomic DNA of *C. neteri* SSMD04. Lane M: 1 kb DNA ladder. Lanes 1-4: the PCR reaction mix (in replicates). Lane 5: negative control. The colour of the figure has been inverted.

E. coli BL21(DE3)pLysS transformed with pET28a_*cneI* was screened for AHL production using the biosensor *C. violaceum* CV026, which would turn purple in the presence of AHL(s). When streaked in proximity, *E. coli* BL21(DE3)pLysS transformed with pET28a_*cneI* activated the purple formation in *C. violaceum* CV026, but *E. coli* BL21(DE3)pLysS transformed with a native pET28a vector did not induce violacein formation in the same AHL biosensor, confirming the function of *cneI* in AHL synthesis (Figure 4.18).

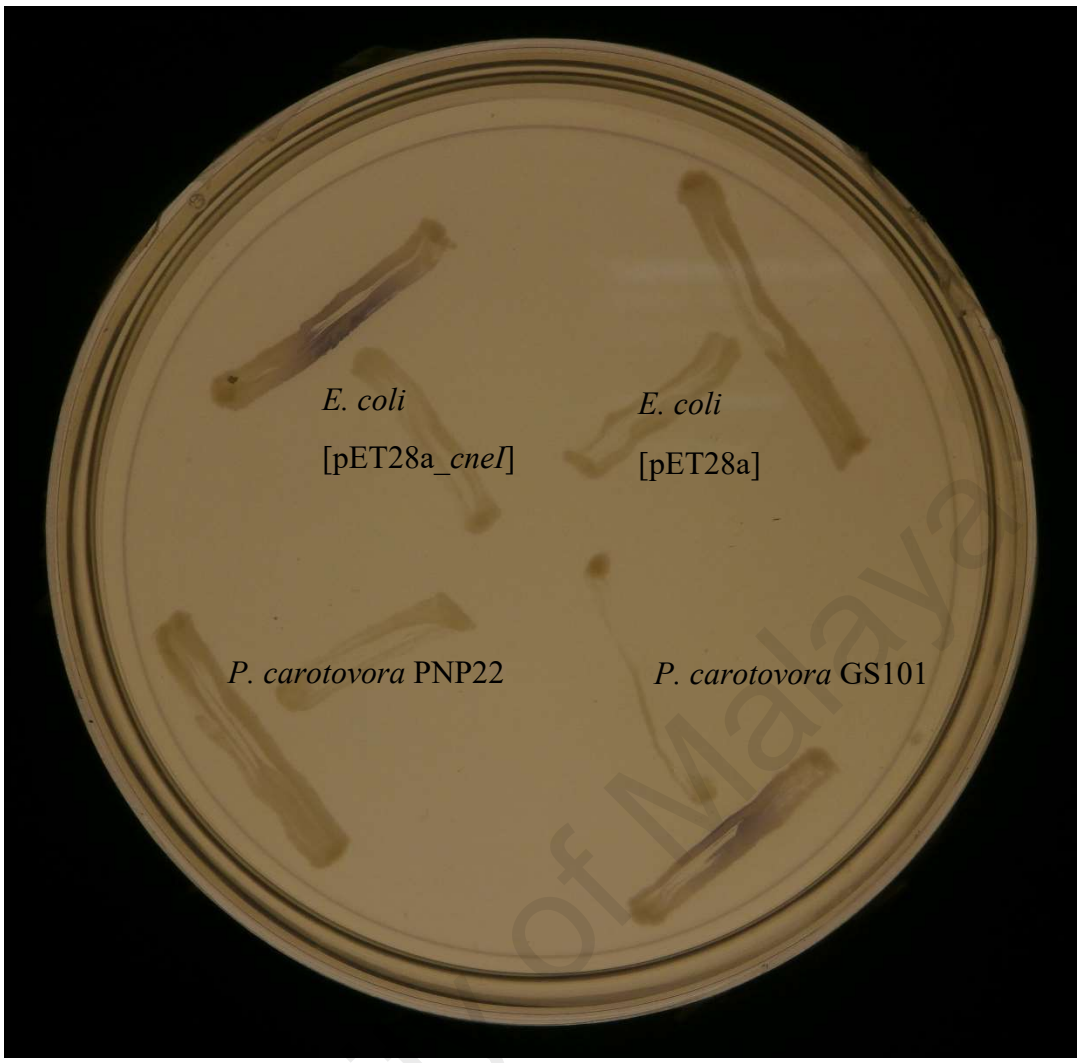

Figure 4.18: AHL synthesis by *E. coli* BL21(DE3)pLysS harbouring pET28a_*cneI*. *P. carotovora* PNP22 and *E. coli* BL21(DE3)pLysS carrying native pET28a served as negative controls, while *P. carotovora* GS101 served as positive control. The AHL biosensor, *C. violaceum* CV026, was streaked perpendicularly against all tested strains at the periphery of the plate.

It is expected that the AHL produced by *E. coli* BL21(DE3)pLysS expressing *cneI* will be identical to that of *C. neteri* SSMD04, since *C. neteri* SSMD04 harbours only one copy of AHL synthase. Triple quadrupole LC/MS was therefore used to investigate the type of AHL produced by *E. coli* BL21(DE3)pLysS after the induction of *cneI* expression. This bacterial strain was induced with the addition of 0.5 mM or 1.0 mM of IPTG to the culture medium and incubated for eight hours after induction. The analysis of the organic extract after induction is shown in Figure 4.19. The EIC of the AHL extract from *E. coli* BL21(DE3)pLysS transformed with pET28a_*cneI* showed a prominent peak that shares

the same retention time with that of synthetic C4-HSL. Similar result was observed regardless of the varying concentrations of IPTG (0.5 mM or 1.0 mM). On the other hand, no AHL can be detected in the spent supernatant of *E. coli* BL21(DE3)pLysS carrying native pET28a. As was mentioned previously, the structure of the AHL produced was determined through the examination of the product ion peaks where the presence of peak *m/z* 102 is indicative of the homoserine lactone ring moiety. The peak *m/z* 172 is indicative of the complete molecule of a C4-HSL (Figure 4.20).

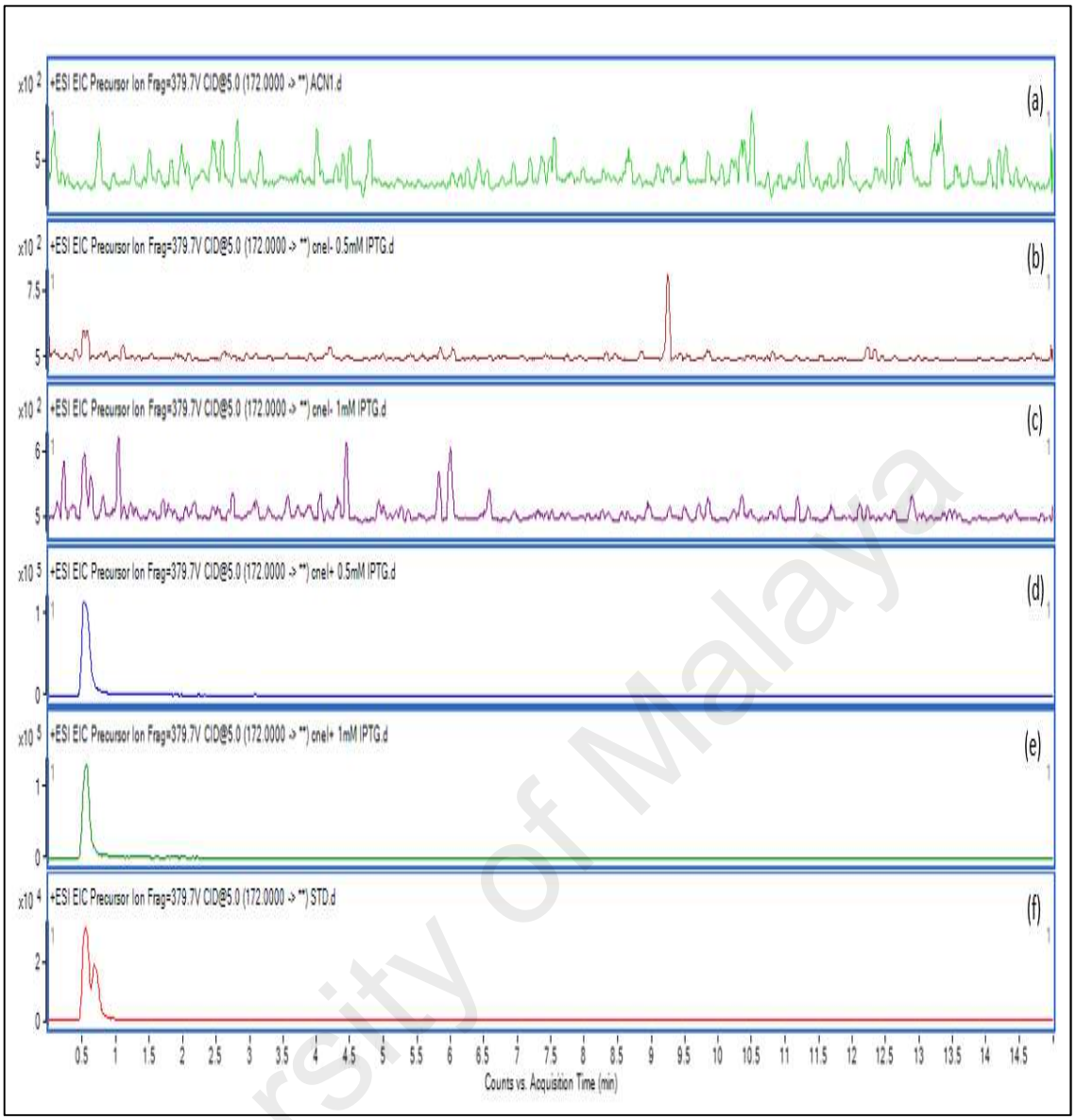

Figure 4.19: EIC of organic extracts of *E. coli* BL21(DE3)pLysS transformed with pET28a_*cneI* or empty pET28a. ACN was used as blank. The mass spectra of *E. coli* BL21(DE3)pLysS harbouring pET28a_*cneI* (d, e) and *E. coli* BL21(DE3)pLysS harbouring empty pET28a (b, c) were compared to that of synthetic C4-HSL (f) at the same retention time.

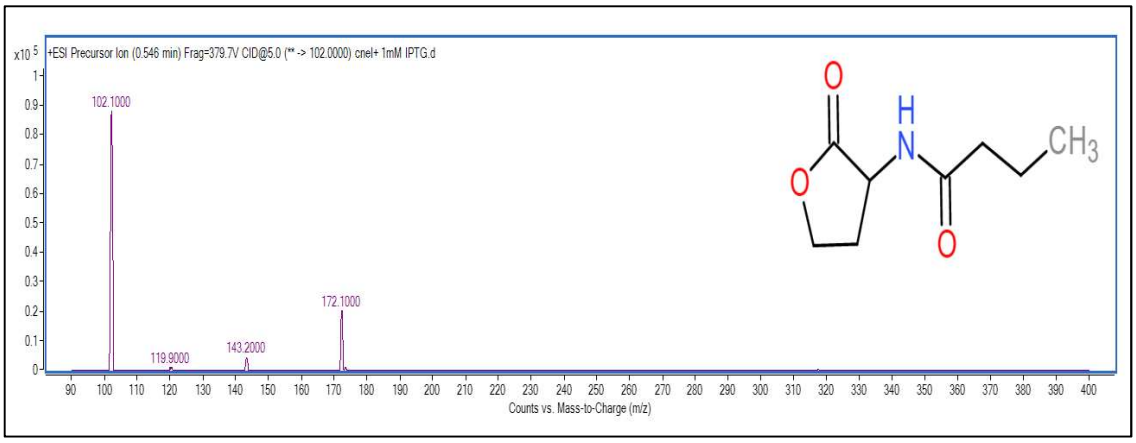

Figure 4.20: Product ion peaks of C4-HSL produced by *E. coli* BL21(DE3)pLysS harbouring pET28a_*cneI*. The presence of peaks *m/z* 102.1000 and *m/z* 172.1000 indicate the presence of C4-HSL. Inset: molecular structure of C4-HSL.

His-tag purification was carried out to isolate the recombinant CneI protein for further SDS-PAGE analysis (Figure 4.21). In comparison to the cell lysate of *E. coli* BL21(DE3)pLysS (Lane 1), cell lysate of *E. coli* BL21(DE3)pLysS harbouring native pET28a (Lane 3), and cell lysate of *E. coli* BL21(DE3)pLysS harbouring pET28a_*cneI* (not induced) (Lane 5), the CneI polypeptide band was not very prominent despite overexpression (Lane 4). Nevertheless, His-tag protein purification using nickel-based affinity column showed a clear band of recombinant CneI that has a molecular weight of slightly lower than 25 kDa.

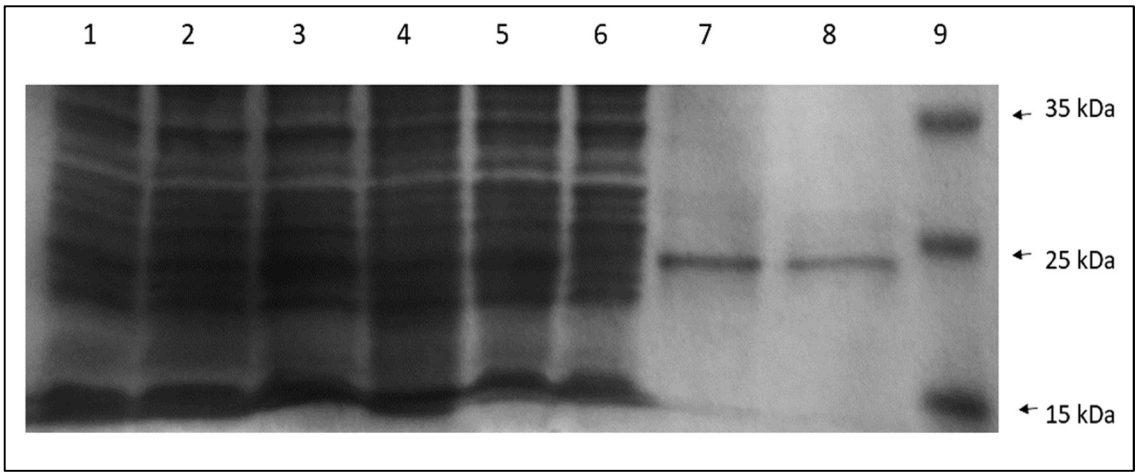

Figure 4.21: SDS-PAGE analysis of CneI overexpression (Lanes 1 to 5) as well as its purification (Lanes 6 to 8) from *E. coli* BL21(DE3)pLysS harbouring pET28a_*cneI*. Lane 1: Cell lysate of *E. coli* BL21(DE3)pLysS. Lanes 2 and 3: Cell lysates of *E. coli* BL21(DE3)pLysS harbouring native pET28a with and without IPTG induction, respectively. Lanes 4 and 5: Cell lysates of *E. coli* BL21(DE3)pLysS harbouring pET28a_*cneI* with and without IPTG induction, respectively. Lane 6: Flowthrough fraction. Lane 7: Wash fraction. Lane 8: Eluted fraction. Lane 9: PageRuler prestained protein ladder (Thermo Scientific, Waltham, MA, USA).

4.8 Mutagenesis

The *cneI* was knockout from *C. neteri* SSMD04 using lambda red recombineering technology, creating the AHL deficient strain, *C. neteri* SSMD04Δ*cneI*. The identity of the mutant strain was confirmed genotypically and phenotypically. Figure 4.22 shows the result of testing *C. neteri* SSMD04Δ*cneI* for its AHL production against the biosensor *C. violaceum* CV026. Triple quadrupole LC/MS was also used to determine if *C. neteri* SSMD04Δ*cneI* still synthesizes C4-HSL. As shown in Figure 4.23, no trace of C4-HSL can be found in the AHL extract of *C. neteri* SSMD04Δ*cneI*.

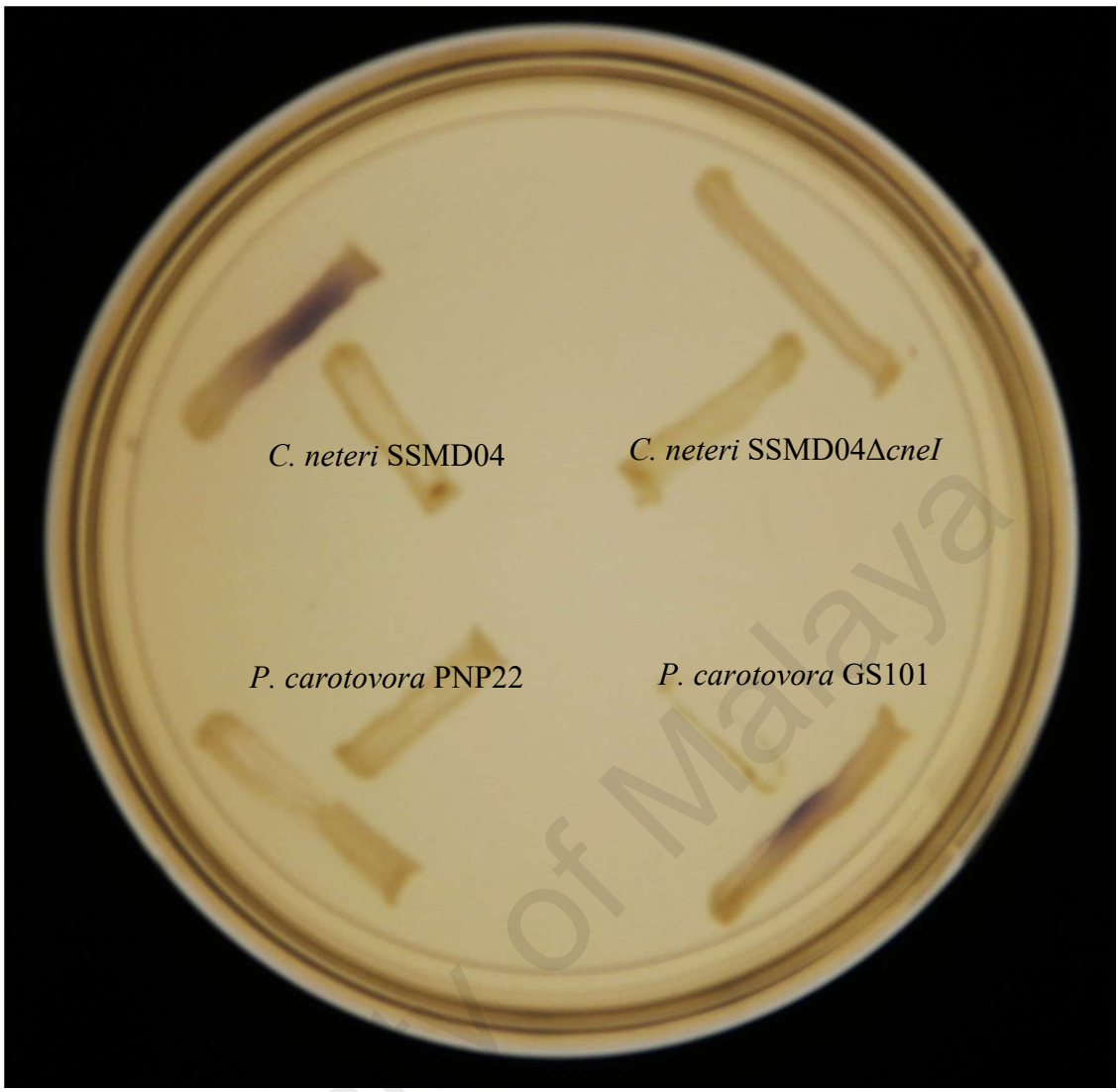

Figure 4.22: Loss of *cneI* led to the loss of AHL producing capability in *C. neteri* SSMD04. *P. carotovora* PNP22 served as negative control, while *P. carotovora* GS101 served as positive control. The AHL biosensor, *C. violaceum* CV026 are positioned perpendicularly to the tested strains.

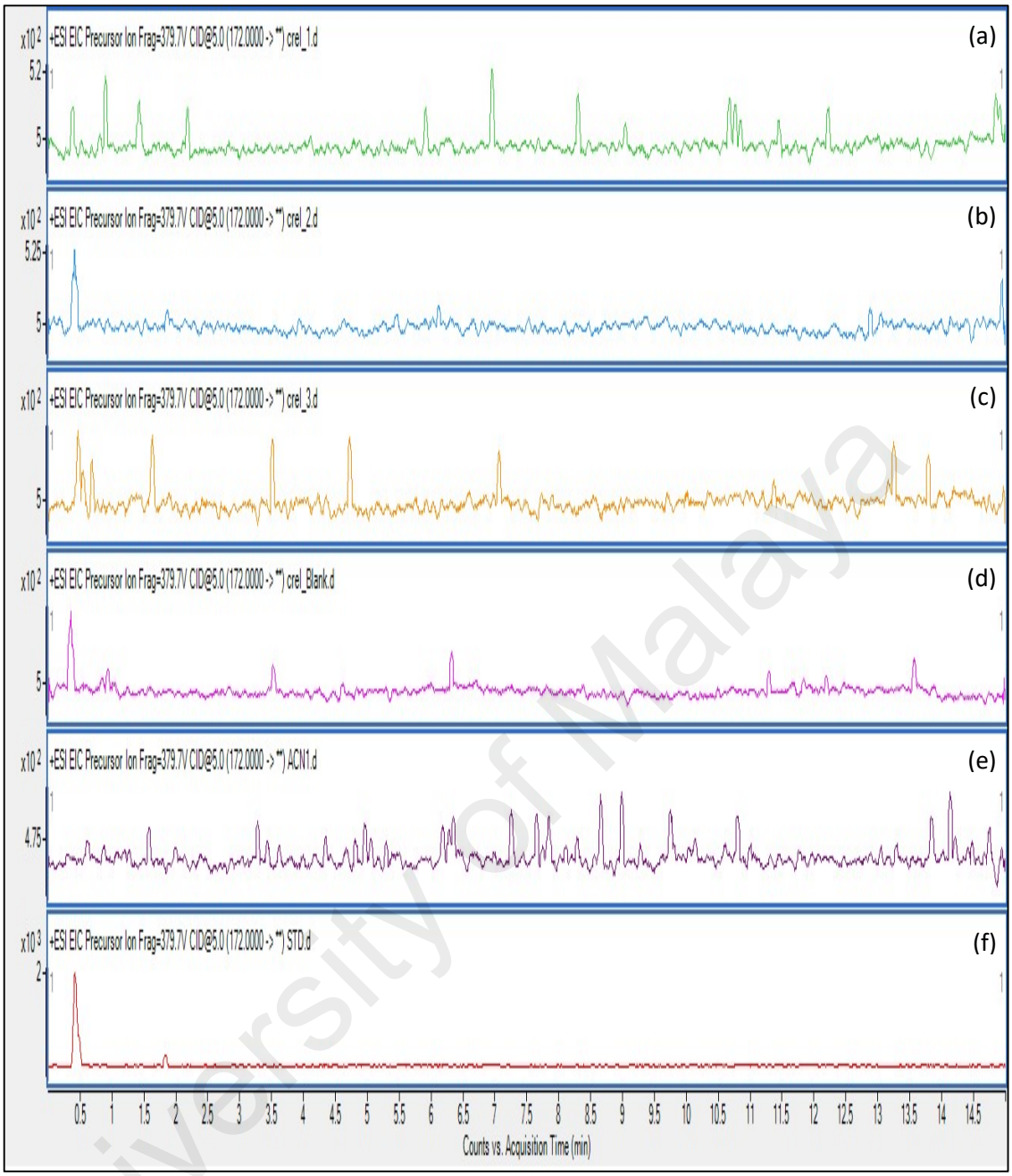

Figure 4.23: EIC of organic extract of *C. neteri* SSMD04Δ*cneI* compared to that of synthetic C4-HSL selected at *m/z* 172.0000. Triplicate of the mutant sample was used (a, b, c), designated as *cneI*. Synthetic C4-HSL was designated as STD (f). *cneI*_Blank is the sterile broth control (d) and ACN1 is the ACN control (e).

We have also investigated if the absence of *cneI* would alter the growth of *C. neteri* SSMD04. However, as seen in Figure 4.24, the growth of the mutant does not differ from that of the wild type.

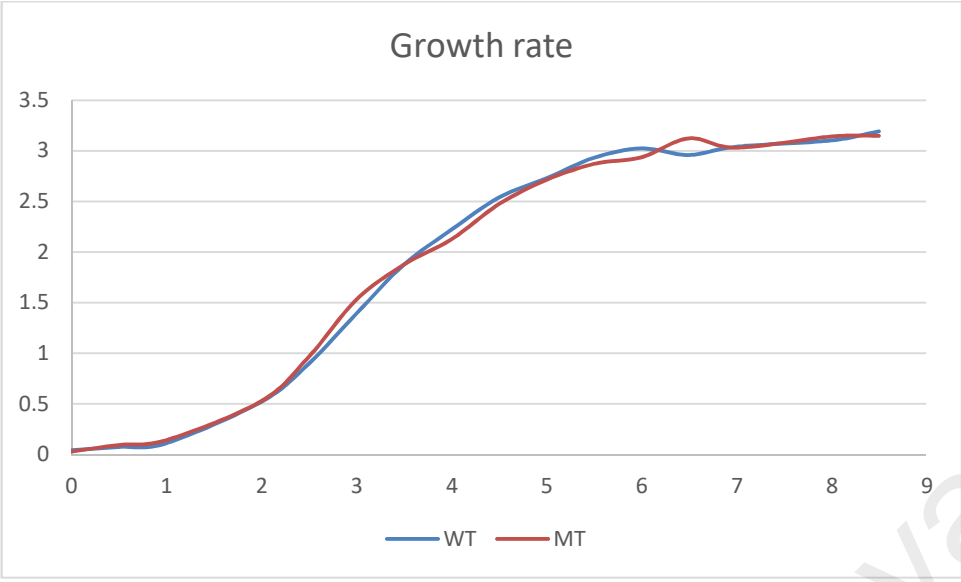

Figure 4.24: Growth rate of *C. neteri* SSMD04 (WT) and *C. neteri* SSMD04Δ*cneI* (MT). The x-axis represents the time (hrs) taken and y-axis represent the OD_{600} reading.

4.9 Differential gene expression analysis

Differential gene expression analysis has resulted in 34 genes significantly differentially expressed, excluding *cneI*, between the wild type and the *cneI* mutant of *C. neteri* SSMD04 (Table 4.6). Seven genes were downregulated and 27 genes were upregulated. Most of the genes were identified from annotation but some were only annotated as hypothetical proteins and thus the functions were not known. BLAST search and domain identification of the hypothetical proteins yielded no information on the functions of the proteins, suggesting the novelty of their functions.

Table 4.6: Gene differentially expressed between *C. neteri* SSMD04 and *C. neteri* SSMD04Δ*cneI*. The genes were filtered for $\log_2$ fold change change > |2| and *q*-value < 0.05.

Protein	$\log_2$ Fold change (Mutant vs. Wild Type)	*p*-value	*q*-value	Locus tag
CneI	-4.320030013	5.53E-96	1.78E-92	JT31_20800
hypothetical protein	-2.602233157	2.72E-45	4.38E-42	JT31_17125
hypothetical protein	-2.275351712	2.68E-24	2.88E-21	JT31_13380
GNAT family acetyltransferase	-1.670344096	1.21E-15	6.52E-13	JT31_17120
hypothetical protein	-1.50685488	7.02E-14	3.23E-11	JT31_02005
hypothetical protein	-1.42720451	2.62E-17	1.69E-14	JT31_01885
type I secretion protein TolC	-0.850759381	0.000276697	0.030741972	JT31_17155
pilus assembly protein PapD	-0.708575911	0.000267587	0.030741972	JT31_21415
cupin	0.674272495	0.000242712	0.030741972	JT31_11015
amino acid dehydrogenase	0.680487337	0.000377235	0.039208105	JT31_22245
transcriptional regulator	0.684355921	7.99E-05	0.015152063	JT31_19465
arginine transporter ATP-binding subunit	0.704848676	0.000515772	0.047480456	JT31_06490
hypothetical protein	0.76934566	0.00020212	0.028314414	JT31_13215
isocitrate lyase	0.789781629	0.000262178	0.030741972	JT31_11170
maltose/maltodextrin transporter ATP-binding protein	0.795034609	0.000426621	0.041653694	JT31_11070
acid phosphatase	0.795888652	5.61E-06	0.001391427	JT31_03800
arginine repressor	0.813685336	0.000100482	0.017528199	JT31_14615
maltoporin	0.825678461	0.000303304	0.032574844	JT31_11065
porin	0.829971393	2.84E-06	0.000831254	JT31_05085
glycine/betaine ABC transporter substrate-binding protein	0.848809956	0.000416547	0.041653694	JT31_22395
5-deoxyglucuronate isomerase	0.84928004	0.000211297	0.028366621	JT31_15695
glucose-1-phosphatase	0.849791275	1.50E-06	0.000483034	JT31_04750
serine/threonine protein kinase	0.866020249	0.000249887	0.030741972	JT31_09800
myo-inositol 2-dehydrogenase	0.877124633	0.000154496	0.022626606	JT31_15680
D-ribose pyranase	0.89563053	3.23E-07	0.000115565	JT31_12445
alanine racemase	0.898986772	6.05E-06	0.001392994	JT31_22250

Table 4.6, continued.

Protein	log_2 Fold change (Mutant vs. Wild Type)	*p*-value	*q*-value	Locus tag
methylmalonate-semialdehyde dehydrogenase	0.904707932	0.000138795	0.021295083	JT31_15650
5-dehydro-2-deoxygluconokinase	0.912835895	0.000103363	0.017528199	JT31_15685
ABC transporter substrate-binding protein	0.914837214	0.000455843	0.043197832	JT31_02890
sugar ABC transporter permease	0.915996912	0.000129105	0.020798841	JT31_15675
sugar ABC transporter substrate-binding protein	1.018456195	1.42E-05	0.003052619	JT31_15665
lipase	1.027740886	6.01E-05	0.012100874	JT31_04985
inositol 2-dehydrogenase	1.047395906	4.12E-06	0.001105497	JT31_15660
D-ribose transporter ATP-binding protein	1.24066212	5.73E-09	2.31E-06	JT31_15670
isocitrate dehydrogenase	2.108539961	9.81E-19	7.90E-16	JT31_11165

4.10 RNA-seq validation

Three genes from the upregulated gene pool (D-ribose transporter ATP binding protein, inositol 2-dehydrogenase, and lipase, represented by ATP, I2D, and LIP respectively) and three from the downregulated gene pool (GNAT family acetyltransferase, type I secretion protein TolC, and pilus assembly protein PapD, represented by GNAT, TolC, and PapD respectively) were selected to validate the result from RNA-seq through qRT-PCR. As shown in Figure 4.25, the differential expression profile of the tested genes were similar to the results seen from RNA-seq data.

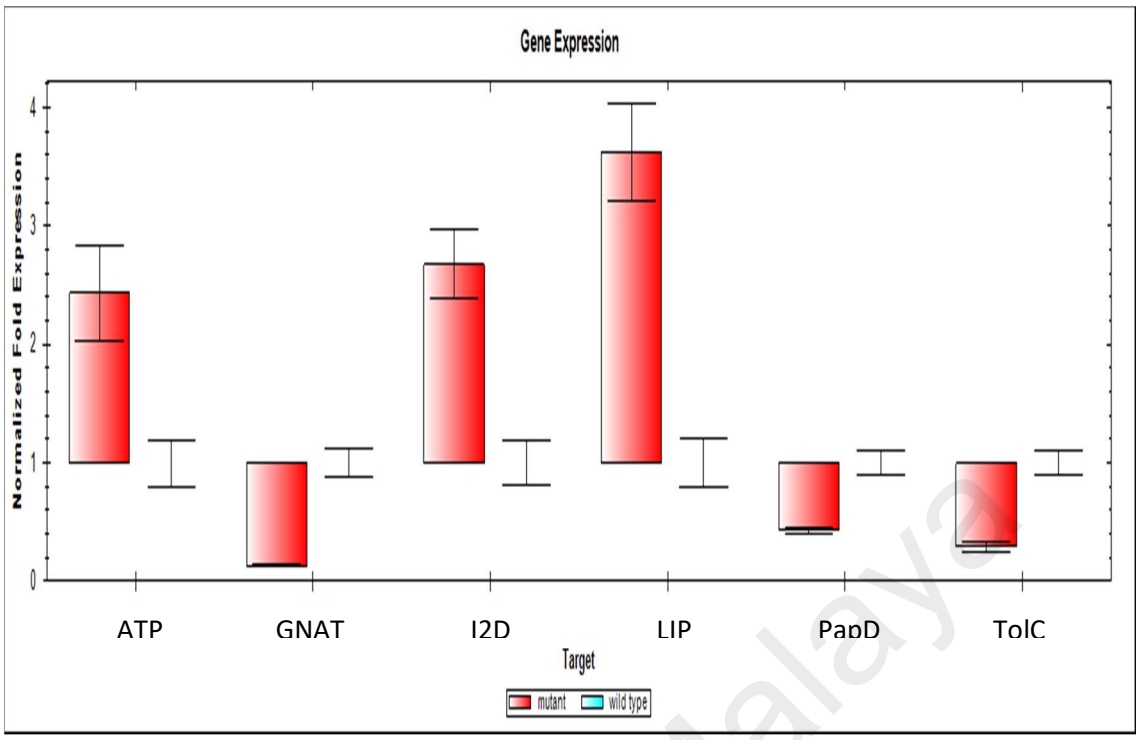

Figure 4.25: qRT-PCR to validate the result of RNA-seq using three selected up-regulated (ATP, I2D, LIP) and three selected down-regulated (GNAT, PapD, TolC) genes. The fold expressions were normalized according to expression of the three housekeeping genes (*recA*, *gyrA*, *ybhF*).

4.11 Carbon source utilization profile comparison

The carbon source utilization profiles of *C. neteri* SSMD04 and its QS deficient mutant, *C. neteri* SSMD04Δ*cneI* were tested on Phenotype Microarray PM1 and PM2 microplates. Out of 190 types of carbon source tested, the wild type and mutant type only showed difference in carbon source utilization in 6 carbon sources, namely glycyl-L-aspartic acid, L-threonine, α-methyl-D-glucoside, L-arginine, glycine, and L-pyroglutamic acid (Figure 4.26). However, the change of utilization after the loss of *cneI* in the mutant strain is not uniform in all six carbon sources. For example, the wild type was not able to grow in the presence of α-methyl-D-glucoside and glycine, but the loss of *cneI* led to the gain of utilization capability of these three carbon sources. On the other hand, the wild type strain was able to grow in the presence of glycyl-L-aspartic acid, L-threonine, and L-arginine, but the growth was slow. The growth rate was, however, increased when *cneI* was knocked out. Nevertheless, the change of utilization of L-

pyroglutamic acid took a different turn in that the mutant strain loss the capability to grow in the presence of L-pyroglutamic acid. This is the only case where the loss of *cneI* led to a loss of function.

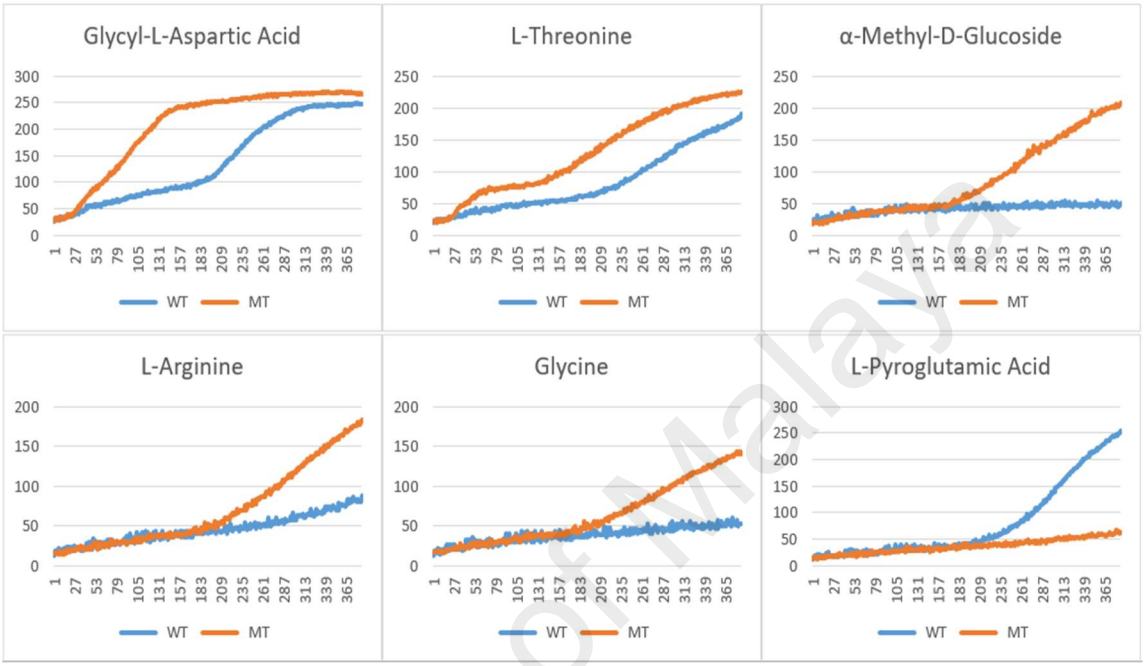

Figure 4.26: Growth kinetics of *C. neteri* SSMD04 (WT) and *C. neteri* SSMD04Δ*cneI* (MT) in the presence of glycyl-L-aspartic acid, L-threonine, α-methyl-D-glucoside, L-arginine, glycine, and L-pyroglutamic acid. The x-axis represents the data point interval (once every 15 mins), and the y-axis represents the absorbance reading at 590 nm.

CHAPTER 5: DISCUSSION

5.1 Identification of *C. neteri* SSMD04

MALDI-TOF MS has been incorporated in microbiology laboratories for the identification of microorganisms due to its ability to identify microorganisms accurately up to species level. It offers a few compelling advantages compared to traditional phenotypic and gene sequencing identification. For example, bacteria identification with MALDI-TOF MS can reach an accurate result within 6 min (Seng et al., 2009) compared to examining growth of bacterial species on different media which would take more than one day. Besides that, the operation of MALDI-TOF MS is easy with little training needed (Croxatto et al., 2012). However, the analysis using MALDI-TOF MS is highly dependent on the manufacturer's database. Studies using the Bruker system have shown that more than 90 % of the bacterial and yeast species tested can be identified accurately (up to 97.7 % correct hit with Enterobacteriaceae). Nevertheless, misidentification and no identification were largely caused by insufficient reference spectra in the database (Seng et al., 2009; van Veen et al., 2010). This can be seen in the identification of *C. neteri* SSMD04 as well, as there is only one reference spectrum available in Bruker's database. Therefore, BIOLOG GEN III microbial identification system as well as 16S rRNA gene sequences phylogenetic analysis were employed to complement the result of *C. neteri* SSMD04 identification from MALDI-TOF MS. Despite the lack of confidence in MALDI-TOF MS, all three identification techniques converged to the same result.

There was no previous publication on *C. neteri* isolation cases from a food source. Despite multiple isolation cases of *Cedecea* spp. from food, such as farmed prawns, aloe vera, eggs, and milk (Musgrove et al., 2008; Gole et al., 2013; Kateete et al., 2013; Akinsanya et al., 2015; Masiello et al., 2016), the *Cedecea* spp. isolated in these studies were not

identified to species level. Therefore, even though *Cedecea* spp. are potentially normal flora found in food, *C. neteri* SSMD04 is the first *C. neteri* strain that was isolated from a food source (Chan et al., 2014) and identified up to species level. Very little is known of the mechanism of pathogenesis in *C. neteri*, and its presence in food has never been studied. Thus, isolation of *C. neteri* SSMD04 from a food source expands the current knowledge on the physiological diversity of *C. neteri*.

5.2 Phenotypic characterization of *C. neteri* SSMD04

Members of the *Cedecea* genus are generally not well studied. Their medical significance can be overlooked despite evidences of their ability to infect human, mainly due to poor understanding of their physiology and etiology. In addition to that, they are potentially clinically challenging pathogens because of their resistance towards a wide range of antimicrobial compounds, such as cephalothin, extended spectrum cephalosporin, colistin, and aminoglycosides (Dalamaga et al., 2008; Mawardi et al., 2010; Abate et al., 2011). The analysis of the complete genome of *C. neteri* SSMD04 using RAST has revealed the presence of multiple antimicrobial resistance genes towards fluoroquinolones, fosfomycin, and beta-lactam antibiotics, a finding in agreement to the resistance nature of *Cedecea* spp. towards antimicrobial compounds.

The result of phenotypic microarray on *C. neteri* SSMD04 provided some insights into the physiology of this bacterium. It has a high adaptability to a wide range of pH and salinity. Environmental pH is a very important aspect of life. Due to the stringency of pH requirement in the aspect of proper protein folding, cellular enzymes quickly lose their activity outside of their optimal pH range. Furthermore, cytoplasmic pH determines the enzyme's activity and its reaction rate, as well as nucleic acids structure, of all which are vital to the survival of a living organism (Slonczewski et al., 2009). The human race has

long been exploiting this knowledge in long term food storage such as seen in the fermentation process of dairy products (Johnson, 1997). Despite this fact, microbes have been found to survive in a wide range of external pH, from as acidic as pH 0 to as alkaline as pH 13 (Nordstrom et al., 2000; Roadcap et al., 2006). Even though the variations of microbial adaptability towards pH is large, the cytoplasmic pH of all bacterial species is, surprisingly, similar, due to homeostasis (Slonczewski et al., 2009). However, each of the bacterial species generally do not grow well in a wide range of pH. There is a widely accepted rule of thumb for the classification of bacteria according to their optimal environmental pH. In general, a given bacterial species that grows within the pH range of 0.5 – 5.0 is considered an acidophile. The ones that grows within pH range of 5 – 9 is classified as neutrophiles. The others that grow within the pH range of 9 – 12 is classified as alkaliphiles (Slonczewski et al., 2009). Most of the non-extremophilic bacteria grow optimally within the external pH range of 5.5 to 9.0 only (Padan et al., 2005). This work, however, has shown that *C. neteri* SSMD04 is able to tolerate a wider range of pH (pH 4 – 10), which led us to speculate its adaptability to a variety of environments of different pH. In fact, its presence in *Shime saba*, a preserved food is a good demonstration of such adaptability. Nevertheless, the upper limit of pH tolerance of *C. neteri* SSMD04 can be higher than 10 as the Phenotype Microarray technology only tests the pH range of 3.5 to 10. Judging from its growth kinetics at pH 10, it is very likely to tolerate a higher pH value.

Shime saba is a traditional Japanese cuisine where the mackerel ('*saba*') is marinated with salt and rice vinegar for the preservation purpose. This is because mackerel, as an oily fish, perishes very quickly and is not ideal to be served as *sashimi* (raw fish). Therefore, it is not surprising that *C. neteri* SSMD04 that was isolated from such environment can tolerate low pH. It came to our surprise that it can tolerate very high pH

as well. Many bacteria are known to grow in an environment with high pH, such as seawater (Harvey, 1955), human pancreatic duct (Evans et al., 1988), and insect guts (Lemke et al., 2003). Most of the *Cedecea* spp. known were not isolated from an alkaline environment, and therefore it was not our expectation for SSMD04's tolerance towards high pH. However, a search into the literature has shown that *Cedecea* spp. have been isolated form the stomach of oriental fruit flies (*Dacus dorsalis*) (Jang & Nishijima, 1990), a testimony to the physiology of members of *Cedecea* spp. of surviving in an alkaline environment.

For centuries, salt has been used to enhance the palate as well as serving the function to retard microbial growth. This is made possible as high salt concentration creates a high osmotic pressure to the microbial cells, thus making growth impossible (Davidson et al., 2013). However, similar to the expectation with its pH adaptability due to its isolation source, strain SSMD04 is expected to grow in the presence of moderately high concentration of salt. The Phenotype microarray platform allowed the investigation of its adaptability in different concentrations of NaCl from 1 to 10 % (w/v). As was shown, *C. neteri* SSMD04 grows in a wide range of NaCl concentrations up to 9 % (w/v). As such, *C. neteri* SSMD04 fits the classification of a halotolerant bacterium (Kushner, 1978).

Previously isolated pathogenic *C. neteri* strains, have demonstrated resistance towards colistin, cephalothin, ampicillin, amoxicillin, amoxicillin/clavulanic, cephalosporins, as well as aminoglycosides (Farmer et al., 1982; Aguilera et al., 1995). Besides that, the genome of *C. neteri* SSMD04 harbours multiple antibiotics resistance genes, as was mentioned (Chan et al., 2014). It is therefore not surprising that *C. neteri* SSMD04 grew well in the range of antibiotics tested, including aminoglycosides, cephalosporins,

glycopeptides, macrolides, penicillins, quinolones, sulfonamides, tetracyclines, polymyxin, and others.

5.3 AHL-type QS activity in *C. neteri* SSMD04

It is known that a wide range of Gram-negative Proteobacteria employ AHL-type QS to regulate gene expression that allows adaptation to the change of population density (Waters & Bassler, 2005). An interesting aspect of QS study is that some bacteria utilizes QS to regulate virulence factors, therefore presenting advantage in terms of invasion to host as the virulence factors are only expressed when the population density is sufficiently large to triumph the hosts' immune system. Coordinated expression of virulence amplifies the effect of invasion (Passador et al., 1993; Brint & Ohman, 1995; McClean et al., 1997; Thomson et al., 2000; Weeks et al., 2010). Regulation of virulence factors by QS has been studied extensively in *P. aeruginosa*. The homologue of LuxR in *P. aeruginosa*, the transcriptional regulator LasR, has been found to upregulate the expression level of virulence genes such as *lasB*, *lasA*, *arA*, and *toxA* in the presence of its activator, the *N*-(-3-oxododecanoyl)-L-homoserine lactone (OC12-HSL) synthesized by LasI, a homologue of LuxI (Gambello & Iglewski, 1991; Toder et al., 1991; Gambello et al., 1993; Pearson et al., 1994). Regulation of virulence by QS has also been well documented and studied in some other bacteria such as *Burkholderia cepacia*, *P. carotovora*, and *Agrobacterium tumefaciens* (de Kievit & Iglewski, 2000).

In this study, *C. neteri* SSMD04 was shown to demonstrate AHL-type QS activity due to the activation of purple pigmentation in *C. violaceum* and bioluminescence in *E. coli* (pSB401). For the identification of the AHL molecules that activated the biosensors, triple quadrupole LC/MS was employed. It was found that C4-HSL was the sole AHL produced by *C. neteri* SSMD04. However, QS has not been reported in *C. neteri*, or any member

of the *Cedecea* genus. Our finding of AHL-type QS activity in *C. neteri* SSMD04 was the first report of QS activity in *C. neteri*.

The whole genome sequence provides valuable information for the study of its genetic basis of QS. This allowed the current study to be carried out in significant ease compared to the traditional method, such as the search of the *luxI/R* homologue sequences in *C. neteri* SSMD04 genome. The discovery of such genes, *cneI/R*, arranged adjacently to each other, is to be expected as it is the common feature of a typical *luxI/R* homologues (Brint & Ohman, 1995; Williamson et al., 2005). As QS has never been reported in any of the *Cedecea* spp., publicly available genomes of other *C. neteri* and *C. davisae* were annotated as well for the search of *cneI* homologues. All *C. neteri* strains (SSMD04, M006, and NBRC105707) harbour *cneI* and *cneR* but not in *C. davisae*. However, since no *C. lapagei* genome was available, it is not known if AHL-type QS is unique in *C. neteri* in *Cedecea* genus.

Since this study is the first documentation of AHL-type QS in *C. neteri*, it is of great interest to find the closest relative of *cneI/R* compared to all other known *luxI/R* pairs. Initially, BLAST search has revealed that they are most similar to the homologous gene pair in *K. michiganensis* RC10, followed by the homologous gene pair, *croI/R*, in *C. rodentium* ICC168. Despite the sequence similarity and homology in the *luxI/R* homologues locus, there is no information available on the QS activity on *K. michiganensis*, as *K. michiganensis* is a newly described species (Saha et al., 2013) and its QS system not studied yet, and thus no functional inference could be made. The AHL-type QS system in *C. rodentium*, however, was described and reported in detail (Coulthurst et al., 2007). The AHL synthase gene in *C. rodentium*, *croI*, produces C4-HSL as the major AHL and C6-HSL as the minor. In comparison, *C. neteri* SSMD04

does not produce detectable levels of C6-HSL. Besides that, the gene arrangements of the chromosomal region of *cneI/R* locus and *croI/R* locus were strikingly different, dismissing the relatedness between the gene pairs. Phylogenetic analysis of *cneI* and *cneR* has shown the same, as both phylogenetic trees showed the *luxI/R* homologues in *Citrobacter* genus belongs to one distinct group. Similarly, both trees has shown that *cneI/R* genes are more closely related to the homologous genes in *K. michiganensis* as well as *Enterobacter* sp. Ag1.

The *cneI/R* locus was aligned to other genomes, including the genomes of *K. michiganensis* RC10 and *C. rodentium* ICC168, in order to investigate if this locus is conserved in other genomes. As *cneI/R* shares the highest sequence similarity to the homologous gene pair in *K. michiganensis*, it was expected that the chromosomal arrangement of this locus is conserved between the two, as well as the genome of another strain of *C. neteri*, M006. Interestingly, when the chromosomal arrangement was made between *C. neteri* SSMD04 and a clinical strain of *C. neteri*, NBRC150707, a large spacer DNA fills the upstream locus of *cneI* in the genome of *C. neteri* NBRC150707, which was vastly different from that of *C. neteri* SSMD04. This suggests possible functional disparity of QS regulation in clinical and non-clinical background by the *cneI/R* system. It would therefore be very interesting to investigate such difference in the future by studying the QS system in *C. neteri* NBRC150707.

However, the locus of the orphan *luxR* of *C. neteri* is highly similar to that of *E. coli* and *Salmonella*, demonstrating the conservation of the orphan *luxR* locus in these bacteria. This led to the speculation that this provides *C. neteri* with the ability to interfere with the AHLs produced by other bacterial species in proximity, thus improving their population

fitness, similar to the function of the orphan *luxR* proposed for *E. coli* and *Salmonella* (Ahmer, 2004).

5.4 Conjecture of AHL-type QS regulation in *C. neteri* SSMD04

AHLs have long been associated with food spoilage via the regulation of proteolytic and lipolytic enzymes (Bruhn et al., 2004; Skandamis & Nychas, 2012). Therefore, it will be interesting to investigate if *C. neteri* SSMD04 plays a role in food spoilage through its lipolytic activity as well as if QS is involved in the regulation of lipolytic pathways. The lipolytic activity of *C. neteri* SSM04 also poses potential biotechnological uses such as the exploitation of lipid-degrading microorganisms as additive in detergent, lipid modification in food industry, removal of pitch from pulp in paper making and so on (Sharma et al., 2001).

Even though the first AHL-type QS study revealed the regulation of bioluminescence in *A. fischeri* (Hastings & Nealson, 1977; Nealson & Hastings, 1979), many other phenotypes have later been found to be controlled by AHL-type QS. Previous QS related studies have proven that virulence and food spoilage traits are phenotypes commonly controlled by QS regulation (Thomson et al., 2000; Weeks et al., 2010; Skandamis & Nychas, 2012), such as can be seen in *P. aeruginosa* (Whiteley et al., 1999), *Serratia proteamaculans* (Christensen et al., 2003), *S. liquefaciens* (Riedel et al., 2001), *E. carotovora* (Pirhonen et al., 1993). The formation of biofilm is a very good example. Many bacterial species encapsulate their colonies within an extracellular matrix called biofilm. This functions to provide resistance towards antimicrobial substances, protecting the colonies (Costerton et al., 1995; Skandamis & Nychas, 2012), which inadvertently enhances their virulence and food spoilage potential. Many bacterial species have been found to employ AHL-type QS to regulate their biofilm formation, such as *Aeromonas*

hydrophila, *Burkholderia cepacia*, *S. liquefaciens*, and *Pantoea stewartii* (Huber et al., 2001; Lynch et al., 2002; Labbate et al., 2004; Koutsoudis et al., 2006).

Apart from biofilm formation, many other genes related to the manifestation of pathogenicity and food spoilage are cell density dependent in their expression, including virulence determinants, proteolytic or lipolytic enzymes, and biosurfactant. One similarity shared by these genes is that they synthesize exo-products, which benefit from cell density dependent regulation as a timed expression of such gene leads to a high production of its products, which would amplify the effects brought about by the secreted products. This is conveniently demonstrated by the diffusion sensing hypothesis (Redfield, 2002).

Similar to *C. neteri* SSMD04, some bacterial species have been found to produce C4-HSL as the major signaling molecule as well. *A. hydrophila*, for instance, harbours a *luxI* homologue called *ahyI* which synthesizes C4-HSL. It was shown that AHL-type QS in *A. hydrophila* is used to regulate its biofilm formation and extracellular production (Swift et al., 1999; Lynch et al., 2002). These two traits, interestingly, are closely relevant to its pathogenicity as well as food spoilage potential. Despite the fact that AHL-type QS has not been explicitly shown to regulate its food spoilage trait, literatures have shown evidence of the presence of C4-HSL in several food sources, such as in the aerobically chilled stored ground beef and commercial bean sprouts, which was found to be originated from some *Aeromonas* sp. responsible for food spoilage. (Skandamis & Nychas, 2012). AHL-type QS is therefore very likely to be responsible for food spoilage trait in *A. hydrophila*. The discovery of similar AHL produced by *C. neteri* SSMD04 suggests such association.

Besides that, *S. liquefaciens*, a close relative of the *Cedecea* genus, is also known to produce C4-HSL as its signaling molecule. Study has shown that QS mutant of *S. liquefaciens* loses the ability to produce biosurfacant, a component vital for the swarming motility in *Serratia* spp. (Lindum et al., 1998). Meanwhile, AHL-type QS also positively regulates the expression level of the *lipBCD* operon, which is involved in the synthesis of Lip exporter, a type I secretion protein machinery that is responsible for the secretion of metalloprotease, lipase, and *S*-layer protein (Riedel et al., 2001). With these examples, we were therefore led to hypothesize that AHL-type QS in strain SSMD04 is likely involved in the regulation of exo-products expression or their secretion, which will inadvertently affect its virulence and food spoilage potential.

The LuxI homologue in *C. neteri* SSMD04, CneI, was initially thought to be most closely related to the homologue in *C. rodentium*, CroI (Coulthurst et al., 2007) through a BLAST search against GenBank database. The genome sequence of a newly characterized bacterium, *K. michiganensis* was added to the GenBank database. A LuxI homologue was found in *K. michiganensis* which showed a much higher sequence similarity to CneI, thus changing our result. *K. michiganensis* is a new member of the *Klebsiella* genus first reported in 2013 (Saha et al., 2013). However, its genome sequence was not published until two years later. In this study, CneI was aligned and compared with ten LuxI homologues that share the highest sequence similarity with CneI. However, only a few of the QS circuits involving those LuxI homologues have known regulatory functions. For example, *C. rodentium* regulates its surface attachment through QS regulation as its QS mutant was found to adhere weaker than its wild type counterpart (Coulthurst et al., 2007). Another QS circuit with known regulatory function is found in gall forming *Pantoea agglomerans*. This bacterium uses its QS system to regulate its gall formation in plants. Loss of QS regulation resulted in the reduction of gall size (Chalupowicz et al., 2008).

Surprisingly, both LuxI homologues in *C. rodentium* and *P. agglomerans* are known to produce C4-HSL as the major AHL. Nevertheless, the regulatory roles of the LuxI homologues from other bacterial species used in this study remains to be discovered.

5.5 Functional characterization of CneI

A large collection of LuxI homologues have been identified and studied since the discovery of QS (Case et al., 2008). Nevertheless, the study of their sequences has not made it possible to predict the nature of AHL(s) produced by a given LuxI homologue based on its sequence alone. Therefore, it is essential to express the luxI homologue in *E. coli* to determine the AHL produced in the *E. coli*, or comparing the wild type parent against a corresponding *luxI* mutant in order to determine the type of AHL(s) produced by a given LuxI (Ortori et al., 2007). *E. coli* does not synthesize AHL even though it possesses an orphan LuxR, the SdiA (Kanamaru et al., 2000), thus making it an ideal host for functional studies of LuxI homologues. The AHL synthase gene, *cneI*, was cloned into an expression vector, pET28a, which was subsequently expressed in *E. coli* BL21(DE3)pLysS. The expression of CneI in *E. coli* BL21(DE3)pLysS led to the synthesis of C4-HSL, establishing that CneI is indeed the AHL synthase of *C. neteri* SSMD04. SDS-PAGE analysis conducted after CneI purification has shown that CneI is a protein with a molecular weight of approximately 25 kDa, which is in agreement with the prediction by bioinformatics tools (24.68 kDa). Unlike other organisms that harbor more than one QS systems such as *B. thailandensis* (Majerczyk et al., 2014), *B. cenocepacia* (O'Grady et al., 2009), and *P. aeruginosa* (Schuster et al., 2003), *C. neteri* SSMD04 possesses only one copy of AHL synthase and was found to produce only C4-HSL, indicating the specificity of this AHL in regulating its physiological processes.

5.6 Lambda red recombineering

Genetic manipulation by homologous recombination is a routine molecular biology work and *E. coli* have frequently been used as the host for such manipulation. However, due to the presence of intracellular RecBCD exonuclease, linear DNA fragments introduced into the host would be degraded very quickly, thus inhibiting homologous recombination (Benzinger et al., 1975). One option would be to use RecBCD nuclease-deficient strains but this is not universally applicable. Furthermore, gene replacement events in such strains occur at a very low frequency. Lambda red system has a recombination rate of 70-fold higher than RecBCD nuclease-deficient background (Murphy, 1998). One of the most commonly used recombination systems involves the use of bacteriophage recombination system called the lambda red recombination system. Lambda red recombination is a useful technique for targeted insertion, deletions, or point mutations that requires very short homology, as few as 35 bp, for homologous recombination (Thomason et al., 2007). Most importantly, although initially used exclusively in *E. coli* (Murphy, 1998), lambda red recombineering (recombination-mediated genetic engineering) is widely applicable and used in many other bacterial species, such as *Salmonella* (Karlinsey, 2007), *Shigella* (Ranallo et al., 2006), *Yersinia* (Derbise et al., 2003), *Aeromonas*, *Edwardsiella* (Hossain et al., 2015), *Pseudomonas* (Lesic & Rahme, 2008), and *Pantoea* (Katashkina et al., 2009). The wide applicability of this recombineering system prompted its use on *C. neteri* SSMD04 for the generation of *cneI* knockout mutant, and it was successful without toxicity issues (Katashkina et al., 2009).

The bacteriophage lambda red recombination system consists of three critical proteins: Bet, Exo, and Gam. Exo is a 5' to 3' double stranded DNA exonuclease that degrades linear DNA molecules from their 5' ends (Little, 1967). Beta binds to the protruding 3' ends and assists in annealing complementary ssDNA strands, which facilitates strand

invasion into homologous regions (Muniyappa & Radding, 1986). Host RecBCD exonuclease activity is inhibited by Gam, allowing linear DNA to persist within the cell for eventual recombination event (Karu et al., 1975). Interestingly, the mechanism of recombination was initially believed to be occurring in a strand invasion manner (Thaler et al., 1987), but recently the recombination was proposed to be largely processed through a full length single stranded DNA intermediate, where one strand of the dsDNA is completely digested by Exo, leaving only a ssDNA for recombination (Mosberg et al., 2010).

The selection of appropriate antibiotic resistance marker is critical in the success of mutant generation. Since *C. neteri* SSMD04 is susceptible to tetracycline and chloramphenicol, tetracycline resistance gene was used as the replacement template for homologous recombination with *cneI*, and pSIM9, the vector that carries the genes required for lambda red machineries as well as a chloramphenicol resistance marker, were used. Successful mutated clones were selected based on their genotypes and phenotypes, that is, sequencing of the replacement junctions to identify the loss of *cneI* and determination of AHL production by AHL detection assays (AHL biosensor C. violaceum CV026 and triple quadrupole LC/MS). As *cneI* is the only copy of AHL synthase gene in *C. neteri* SSMD04, the *cneI* mutant could not synthesize C4-HSL, thus no reaction was observed when tested with *C. violaceum* CV026, and no AHL molecules detected by triple quadrupole LC/MS. The wild type *C. neteri* SSMD04 and the mutant type *C. neteri* SSMD04Δ*cneI* were grown on LB agar for inspection of any phenotypic differences, such as colony morphology, colour, and size, but no observable change was seen.

A typical bacterial cell growth goes into stationary phase after an exponential growth phase, where cell division is slowed and the population density maintains at a certain

level. An experiment on *E. coli* cultures has shown that this transition, although typically thought to be a result of mere nutrient limitation, is also caused by cell to cell signaling. The cells restrict the growth accordingly to the limiting nutrient, thus prolonging the stationary phase (Carbonell et al., 2002). AHL-type QS was also seen to negatively impact the growth of *Vibrio harveyi* (Nackerdien et al., 2008) and *Rhodospirillum rubrum* (Carius et al., 2013), demonstrating the existing mechanism of AHL-type QS in the regulation of population growth. A comparison of growth was therefore made between the *C. neteri* SSMD04 and *C. neteri* SSMD04Δ*cneI* to examine if such mechanism operates in *C. neteri* SSMD04. However, there was no observable difference between the growth of wild type and mutant strain. Therefore, it is unlikely that AHL-type QS regulate cellular growth in *C. neteri* SSMD04.

5.7 Comparative transcriptomics

As QS is a growth phase dependent regulatory mechanism, the QS regulated genes are not activated in the early growth phase (low cellular density). The activation starts when the culture reaches late log phase or early stationary phase where the cellular density is high. Therefore, it was necessary to identify when to extract the cellular transcript that represents the QS profile truthfully for the downstream global comparative transcriptomic study in *C. neteri* SSMD04 and its QS deficient mutant. The OD_{600} reading of *C. neteri* SSMD04 culture reaches 3.0 before plateauing, but OD_{600} = 2.5 was used as the quality of the rRNA depleted transcript using total transcript extracted when the cells reaches plateau phase was not satisfactory, with an abundant of small sized RNA molecules present. RNA extracted when OD_{600} = 2.5 gives intact RNA molecules with minimal small sized RNA molecules in the Bioanalyzer trace.

From the differential gene expression study, several genes involved in cellular metabolism such as isocitrate dehydrogenase kinase, D-ribose transporter ATP-binding protein, inositol 2-dehydrogenase, sugar ABC transporter substrate-binding protein, sugar ABC transporter permease, 5-dehydro-2-deoxygluconokinase, methylmalonate-semialdehyde dehydrogenase, D-ribose pyranase, myo-inositol 2-dehydrogenase, glucose-1-phosphatase, 5-deoxyglucuronate isomerase, glycine/betaine ABC transporter substrate-binding protein, maltoporin, maltose/maltodextrin transporter ATP-binding protein, isocitrate lyase, arginine transporter ATP-binding subunit, amino acid dehydrogenase were found to be upregulated in the mutant, which suggests that AHL-type QS restrict cellular metabolism on several sugars and amino acids in *C. neteri* SSMD04. As QS is dependent on the density of the population, the restriction takes effect only when the population density is high. Therefore, restriction on the use of energy sources imposed by QS helps in the conservation of limiting energy and would likely slow down its growth, prolonging its survival in nutritionally harsh environment, such as when the population is crowded and nutrients become scarce (plateau phase). This led us to speculate that QS system in *C. neteri* is involved in its reduction of metabolic activity when the population is crowded as a mean of survival. This phenomenon is not unique, as it can also be seen in *Burkholderia glumae*, a rice pathogen that causes rice panicle blight. The study by An and his colleagues (An et al., 2014) have shown that *B. glumae* utilizes QS to modulate its nutrient utilization and cellular metabolism. Under quorate conditions, the QS downregulate glucose uptake, key substrate level, oxidative phosphorylation, and *de novo* nucleotide biosynthesis.

It is worth noting that *C. neteri* is likely to depend on two mechanisms to reduce its metabolism: reducing expression of genes related to the breakdown of the substrates as well as the influx of the substrates. This is because even though some genes are directly

related to the catabolism of substrates such as isocitrate lyase, isocitrate dehydrogenase, inositol 2-dehydrogenase, and D-ribose pyranase, many other genes are related to transport function, such as arginine transporter ATP-binding subunit, maltose/maltodextrin transporter ATP-binding protein, maltoporin, porin, and glycine/betaine ABC transporter substrate-binding protein.

Literatures have also revealed that other bacteria such as *P. aeruginosa*. *A. fischeri*, and *B. thailandensis* have their carbon utilization related phenotypes regulated by QS (Schuster et al., 2003; Studer et al., 2008; Chandler et al., 2009), despite different underlying mechanisms of the regulation. However, in the case of *B. thailandensis* (Chandler et al., 2009), QS did not regulate the metabolic diversity, but merely the level of metabolic activity. *C. neteri* SSMD04, on the other hand, has its metabolic diversity and metabolic activity changed when it losses *cneI*. For example, the *cneI* mutant has increased respiration rate when grown with glycyl-L-aspartic acid as the sole carbon source compared to its wild type, suggesting that QS was repressing the metabolic activity on the utilization of glycyl-L-aspartic acid, an example of change of metabolic activity. On the other hand, the mutant type was able to grow in the presence of glycine but the wild type could not, demonstrating a gain of function from the loss of QS regulation, an example of change of metabolic diversity.

Other than these genes, a gene encoding lipase was also upregulated in the mutant, which was speculated as was previously described (Tan et al., 2015) . Therefore, it would be expected that the mutant has higher lipase activity compared to that of the wild type. However, a comparison using plate assay with corn oil as the substrate did not show a noticeable difference. A search from the genome annotation by RAST has revealed that *C. neteri* SSMD04 harbours multiple copies of lipase genes. Therefore, it is likely that

when one copy of the lipase genes is upregulated in the mutant, the phenotype is not changed, or that the change is not noticeable using the plate assay.

The genes downregulated by QS in *C. neteri* SSMD04 gives a less clear picture of its phenotypic modulation. This is because a number of genes were not identified from their sequences and thus their functions not know. Protein domains search as well as BLAST search yielded no information on their functions, suggesting the novelty of these genes. It will therefore be very interesting to investigate the functions of these genes in the future. Nevertheless, type I secretion protein TolC is positively regulated by QS. TolC is a ubiquitous protein in Gram negative bacteria that plays a role in expulsion of a diverse range of molecules, such as toxins and antibacterial drugs (Koronakis et al., 2004). This led to the prediction that QS increases the resistance phenotype of *C. neteri* SSMD04 towards certain toxic compounds or antibiotics. Besides that, pilus assembly protein PapD is also positively regulated by QS. PapD is responsible for the biogenesis of P pili in uropathogenic *E. coli*, which is used for surface attachment to urinary tract epithelium (Lindberg et al., 1989). Although it is not known if this gene plays a role in the pathogenesis in *C. neteri*, it is likely that the loss of QS would lead to loss of surface attachment phenotype. Even though not validated phenotypically, it is hypothesized that the upregulation of these two genes in quorate conditions strengthened the pathogenicity in *C. neteri* SSMD04. However, further work is required to prove this hypothesis.

5.8 Future work

Although comparative transcriptomics has given an insight into the regulatory roles played by AHL-type QS in *C. neteri* SSMD04, it is not a complete picture as genetic regulation is a complicated network of genes interacting with each other to give rise to a phenotype. Identification of genes regulated by QS is not a direct evidence of the

phenotype regulated. It is therefore important to identity the phenotype regulated by QS in *C. neteri* SSMD04 in the future. Besides that, many of the genes regulated by QS in *C. neteri* SSMD04 were annotated as hypothetical proteins with unknown functions. It would therefore be very interesting to investigate the functions of these genes through gene cloning or creation of knockout mutant. This would lead to the discovery of novel proteins or novel phenotypes regulated by AHL-type QS. Furthermore, this work encompassed only the *cneI/R* pair without research into the role played by the orphan *luxR* found in the genome of *C. neteri* SSMD04 as AHL-type QS was the focus of this study. However, the orphan *luxR* gene possibly constitute a completely different or even novel signaling system. It is only sensible if this gene is to be included in the study of this bacterium in the future.

CHAPTER 6: CONCLUSION

The early part of this study aimed at the profiling and characterization of AHL-type QS in *C. neteri* SSMD04, and it was established that *C. neteri* SSMD04 produced C4-HSL. This is the first report of AHL-type QS activity in *C. neteri*. Unlike some other known bacteria that harbours more complicated QS systems, *C. neteri* SSMD04 possess only one pair of *luxI/R* pair, of which was named *cneI/R*. There is also another orphan *luxR* with no cognate *luxI* within its genome. Its autoinducer synthase gene, *cneI*, was subsequently cloned and characterized. The study of *cneI* helps in providing more information for the elucidation of QS properties in *C. neteri* SSMD04, as well as paving the way for future mutagenesis and comparative transcriptomic study. Comparative transcriptomics and differential gene expression analysis showed that QS in *C. neteri* SSMD04 is responsible for the decrease of cellular metabolism, as was evident in the reduction of carbon source metabolism. Besides that, QS also potentially increases resistance to toxic and antibacterial compounds as well as promoting surface attachment in *C. neteri* SSMD04.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

A. List of publications

Tan, K. H., Tan, J. Y., Yin, W. F., & Chan, K. G. (2015). Genome analysis of quorum sensing *Cedecea neteri* SSMD04 leads to identification of its novel signaling synthase (*cneI*), cognate receptor (*cneR*) and an orphan receptor. *PeerJ*, *3*, e1216.

Tan, K. H., How, K. Y., Tan, J. Y., Yin, W. F., & Chan, K. G. (2017). Cloning and characterization of the autoinducer synthase gene from lipid-degrading bacterium *Cedecea neteri*. *Frontiers in Microbiology*, *8*, 72.

Chan, K. G., **Tan, K. H.**, Yin, W. F., & Tan, J. Y. (2014). Complete genome sequence of *Cedecea neteri* strain SSMD04, a bacterium isolated from pickled mackerel sashimi. *Genome Announcements*, *2*(6), e01339-14.

B. List of presentations

Tan, K. H., Tan, J. Y., Yin, W. F., & Chan, K. G. (2015). *Genome analysis of quorum sensing Cedecea neteri SSMD04 leads to identification of its novel signaling synthase (cneI), cognate receptor (cneR) and an orphan receptor*. Oral presentation at the 20th Annual Biological Sciences Graduate Congress – Chulalongkorn University (Bangkok, Thailand).