

**CLONING, EXPRESSION AND CHARACTERIZATION OF AHL-
INACTIVATING *aiiA* GENE HOMOLOGUE IN *Escherichia coli***

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By

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I dedicate this dissertation to my beloved late Mum. I am who I am today because of your love and inspiration. This is for you ma. Finally, I made you proud! For giving me the endless spirit, determination and strength in exploring things as well as surpass all the trials that the study encountered, foremost gratitude to dear God.

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LIST OF SYMBOLS AND ABBREVIATIONS

μg	microgram
μL	microliter
bp	base pair
BSA	Bovine Serum Albumin
DNA	deoxyribonucleic acid
EDTA	ethylene diaminetetraacetic acid
g	gravity
IPTG	isopropyl β -D-1-thiogalactopyranoside
kb	kilo base
kDa	kilo Dalton
LB	Luria Bertani
M	molar
MgCl_2	magnesium chloride
MgSO_4	magnesium sulphate
mL	mililiter
NA	nutrient agar
NaOH	sodium hydroxide
NB	nutrient broth

OD	optical density
PBS	phosphate buffer solution
PCR	polymerase chain reaction
PEG	polyethylene glycol
rDNA	ribosomal deoxyribonucleic acid
Rpm	revolutions per minute
TEMED	tetramethylethylenediamine

PENGLONAN, PENGEKSPRESIAN DAN PENCIRIAN GEN HOMOLOG *aiiA* DALAM *Escherichia coli*

ABSTRAK

Laluan komunikasi antara sel-sel mikrob bersel tunggal biasanya diselenggarakan oleh penderiaan kuorum yang dikoordinasikan oleh molekul isyarat. Sistem ini bergantung kepada kepadatan sel dan memainkan peranan penting dalam penyelarasan and pengekspresan gen kebanyakan bakteria patogenik. Sebaliknya, gangguan laluan isyarat ini melalui sistem pelindapkejutan kuorum akan menjadi satu strategi dalam mengawal jangkitan bakteria. Dalam kajian ini, isolat tanah tempatan *Bacillus cereus* sp SM01 dikenalpasti mempunyai potensi tinggi dalam mengganggu struktur cincin lakton yang merupakan komponen penting dalam molekul isyarat AHL. Fenomena ini menjadikan molekul isyarat tersebut tidak layak untuk mencetuskan penderiaan kuarum. Sifat khas SM01 ini mendorong pengajian yang mendalam untuk menguji keupayaannya dalam pelindapkejutan kuorum. Homolog gen AHL-lactonase (*aiiA*) yang bersaiz 770 bp yang diamplifikasikan daripada genom SM01 kemudiannya diklon ke dalam sistem pengekspresan pCold I DNA untuk mengekspreskan protein rekombinan yang dikehendaki. Pencirian protein rekombinan yang bersaiz 29 kDa mendedahkan bahawa ia berpotensi mengurangkan pembentukan biofilm secara drastik apabila diuji atas *Burkholderia pseudomallei* UKMS-01, sejenis patogen haiwan. Menariknya, protein rekombinan AHL-lactonase telah menunjukkan kestabilan sehingga suhu setinggi 85°C di mana kebanyakan enzim akan menyahaktif. Ia juga menunjukkan kestabilan pH yang luas iaitu daripada pH 5 sehingga 11. Semua ciri-ciri ini menunjukkan bahawa protein

rekombinan AHL-lactonase mempunyai potensi untuk digunakan sebagai enzim komersial untuk mengawal aktiviti jangkitan bakteria patogenik.

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ABSTRACT

Cell-to-cell communication pathway of single-celled microbes is usually coordinated by quorum sensing (QS) system mediated by signal molecules. This cell density-dependent system plays a vital role in functional coordination of most pathogenic bacteria. Antagonistically, interruption of this signal pathway by inactivating the signal molecules through quorum quenching (QQ) could be an alternative strategy to control bacterial virulence. In this study, a local soil isolate identified as *Bacillus cereus* SM01, has been shown to possess the capability of enzymatic inactivation of acyl homoserine lactones (AHLs). This special trait of SM01 prompted the interest in testing its potential quorum quenching ability. Hence, the amplified 770 bp long AHL-lactonase gene homologue (*aiiA*) of SM01 was subsequently cloned into pCold expression system in order to express the desired recombinant AHL-lactonase protein. Further characterization of that the overexpressed 29 kDa recombinant protein revealed that it drastically decreased biofilm formation of *Burkholderia pseudomallei* UKMS-01 strain, an animal pathogen. Interestingly, the recombinant AHL-lactonase protein was observed to be stable up to 85°C in temperature where most of the enzymes will be inactive. Nevertheless, it showed wide range of pH stability, from pH 5 up to 11. All these characteristics suggest that this recombinant AHL-lactonase has the potential to be used as a commercial enzyme for the control of pathogenic bacterial infection.

CHAPTER 1 - INTRODUCTION

Over the years, discovery of vaccines and invention of antimicrobial agents to treat fatal diseases that infect human, animal and also plants has been an uphill struggle. Besides being life-threatening, these infectious diseases brought enormous personal and economic ruins. Emergence and evolution of antibiotics in 1920s sets a start of active control of infectious diseases and saved mankind (Morens et al. 2004).

Although the discovery of antibiotics has been a juncture in the medicinal history, later on, it became a non-sustainable strategy when the pathogenic microbes acquire resistance to ordinary antibiotics used for treatment purposes (Defoirdt et al. 2004). This phenomenon happens because of the nature of antibiotics that impose a selective pressure on microorganisms by killing the susceptible bacteria and allowing only the antibiotic-resistant bacteria to survive and multiply further (Levy 2001). Moreover, conventional antibiotics do enforce a selection pressure on fatal bacteria by interrupting its crucial housekeeping operations that indirectly suppress the growth of bacteria. This unavoidably results in development of antibiotic – resistant pathogenic microbes (Spratt 1994).

As the concern of preventing the development of resistance among dangerous pathogens towards hardly discovered antibiotics in mind, invention of alternative therapeutic agents were needed globally to treat the emerging fatal diseases (Livermore 2004). Hence, a natural antipathogenic mechanism without raising any selection pressure on the survival of infectants is targeted. Since interruption of cell-to-cell communication pathway could influence the vital housekeeping functions of pathogens (Dong and Zhang 2005) without promoting any antibiotic resistance, it's

chosen to be studied in detail to develop new therapies to treat diseases (Kim et al. 2005).

Quorum sensing (QS) in bacteria acts as a sophisticated circuit to detect and respond to the environmental changes as well as to group up the cells together by cell-cell communication within or between the species (Waters and Bassler 2005). Various bacteria use this communication pathway to coordinate their biological activities and regulate target gene expression in a population density dependent manner (Ulrich 2004). Plus, QS is responsible in coordinating numerous biological functions including replication, biofilm formation and virulence factor (González and Keshavan 2006).

Since pathogenic bacteria evolved this kind of special communal genetic behavior, it is not surprising, if their competitors in population possess certain unique pathways to disrupt QS mechanism in order to enhance their competitive advantages. As such signal interference mechanism or so called quorum quenching (QQ) is widespread in both prokaryotes and eukaryotes, it is known to be a the suppressor of QS system of pathogens (Dong et al. 2007). By inactivating the signal molecules of QS system, it disturbs the communication circuit between pathogen population as well as abolishing the expression of virulence related genes (Dong et al. 2002).

As mentioned earlier, although application of antibiotics or even commercial chemical drugs sounds efficient to control diseases caused by pathogenic bacteria, it led to emergence of resistant bacterial strains (Spratt 1994, Levy 2001). QQ promises an alternative infection control without any selection pressure on pathogens. It interrupts the bacterial communication circuit by blocking the signal molecule generation, accumulation or reception pathway (Defoirdt et al. 2004). Since the

concentration of AHL plays an important role in virulence of QS mediated pathogens, this mechanism can be utilized as an alternative approach to manipulate new disease prevention way. By controlling the production of signal molecules or by making them unsuitable for the communication, it is possible to shut down the communication pathway of pathogens.

Among the identified QQ enzymes, lactonase is mainly found in Gram-positive *Bacillus sp.* This microbial origin enzyme efficiently breakdown the homoserine lactone ring moiety of acyl-homoserine lactones (AHLs), the common type of signal molecule produced by bacteria. Structural change in AHL molecules makes them unfit to trigger signal pathway (Liu et al. 2005). This eventually inactivates the expression of QS controlled genes as well as providing competitive advantages for survival of other environmental bacteria.

Compared to other QQ enzymes such as AHL-acylase and paraoxonases, AHL-lactonase has distinct specificity for the substrate and acts efficiently in hydrolyzing the AHL signal molecules. Hence, it prompted the interest of scientists to study this compound at its molecular level as a promising strategy to inhibit communication pathway of pathogenic bacteria. The main objectives of this study are listed below:-

- a) To isolate and identify quorum quenching bacilli from local soil samples
- b) To clone AHL-lactonase gene homologue into the expression vector
- c) To express and purify the recombinant AHL-lactonase enzyme
- d) To determine the potential activity of the recombinant AHL-lactonase enzyme

CHAPTER 2 - LITERATURE REVIEW

2.1 Quorum sensing – An overview

The ability of single-celled bacteria to communicate within or between one another has enabled them to function as multicellular organism (Dong et al. 2000, Dong and Zhang 2005, Kaufmann et al. 2005). The term ‘quorum sensing’ projects the capability of certain bacteria to inspect their own population number in order to attune prioritized gene expressions (Fuqua et al. 2001, Coulthurst et al. 2002).

A range of Gram-negative and Gram-positive bacterial communities employ such communal behavior to proliferate within the confined environment (Diggle et al. 2007). This mass-action of bacteria to attain survival advantages by synchronizing expression of genes related to its essential phenotypes is mediated by low weight chemical signal molecules known as autoinducers (Drobniewski 1993, Waters and Bassler 2005). The production and release of these tiny hormone-like chemical signal molecules depends either on the cell density of bacteria, environmental changes or stages of its growth phase (Diggle et al. 2007, Jayaraman and Wood 2008).

Different bacterial species synthesize different autoinducers. Distinct types of signal molecules that have been identified include acylhomoserine lactones (AHL), hydroxyl-palmitic acid methylester (PAME), cyclic thiolactone (AIP), furanosylborate (AI-2), methyl dodecenoic acid (DSF), bradyoxetin (Dong and Zhang 2005, González and Keshavan 2006, Dong et al. 2007). As illustrated in Figure 2.1, when the cell density of the bacteria expands, the concentration of signal molecules increases as well until reaches a minimal threshold concentration

(Czajkowski and Jafra 2009). At this quorum level, targeted genes or phenotypes are expressed (Waters and Bassler 2005, Williams et al. 2007).

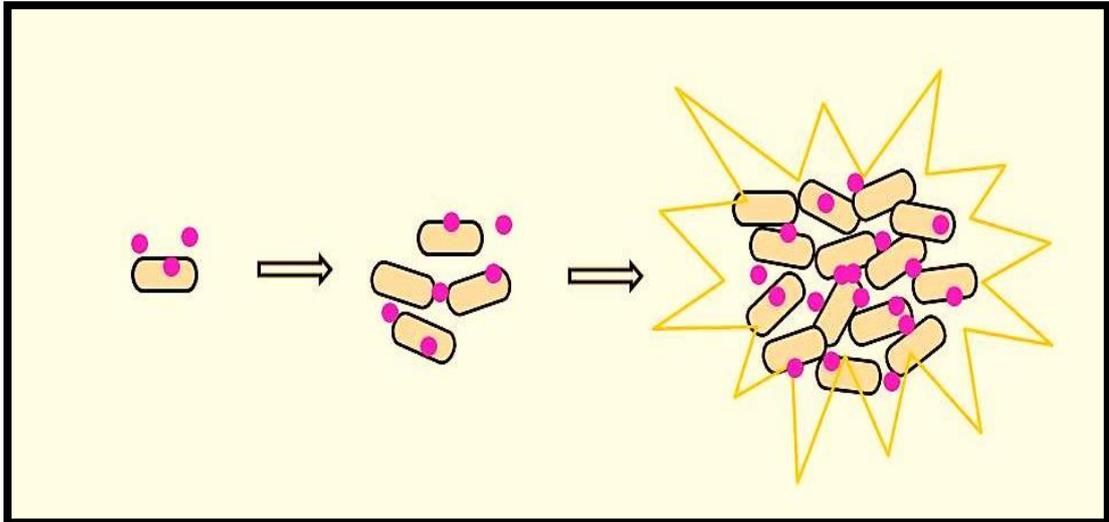


Figure 2.1: Cell density-dependent gene regulation. A single-cell produces low or basal concentration of signal molecules (pink circles), hence the target genes are off. When cell population increases due to clonal growth and aggregates, higher concentration of signal molecules are synthesized. Upon reaching a minimum threshold level, the signal molecules interact with transcription factors in order to regulate expression of QS related genes. Thus, the target genes are on.

A broad range of bacterial behaviors, such as toxin production, biofilm production, virulence factor production, motility, antibiotic production, conjugation, replication, bioluminescence and exoenzyme synthesis are regulated by QS with high specificity and delicate control (González and Keshavan 2006, Czajkowski and Jafra 2009). Expression of such advantageous phenotypes through a signal-based network of cellular transduction mechanism usually promotes proliferation and survival of bacteria under limited growth conditions (Swift et al. 1996).

2.2 Acyl-homoserine lactones: Key players in quorum sensing network

The tiny hormone-like diffusible molecules, termed as ‘quorumones’ are the autoinducers used by bacteria to communicate and exchange information among their population (Kaufmann et al. 2005). These signal molecules can mostly diffuse freely across the cell membrane into the environment by simple diffusion. However, some with relatively long acyl side chains demand efflux pumps that aid their movement outside the cell in order to sense its own population density (González and Keshavan 2006, K Bhardwaj et al. 2013). Among all the identified autoinducers so far, the N-acyl homoserine lactone (AHLs) family plays a vital role in signal pathways of Gram-negative bacteria (Dong and Zhang 2005, Kaufmann et al. 2005).

The typical structure of AHLs contains a homoserine lactone (HSL) ring with an acyl side chain that varies in number of carbons (4 to 18 carbons) as in Figure 2.2 (Czajkowski and Jafra 2009, Fast and Tipton 2012).

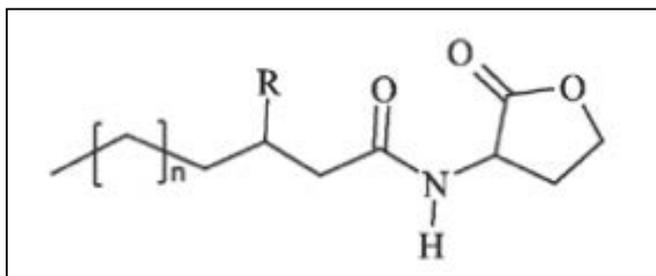


Figure 2.2: The basic structure of typical N-Acyl Homoserine Lactones (AHLs). Almost all AHLs contain a homoserine lactone ring moiety and an acyl side chain that differ in length. Presence of substituents (R) creates variation in the side chains.

Classification of AHLs into short-chain (4 to 8 carbons) or long-chain (10 to 18 carbons) molecules basically depends on the length of the acyl side chain or number of carbons. Besides, the oxidation state of AHLs and the degree of substitution and saturation that differs for every AHL molecule suggest presence of

abundance of AHL derivatives exist in nature (Fuqua et al. 2001, Dong and Zhang 2005).

LuxI is the AHL synthase enzyme that is responsible for synthesis of AHLs in Gram-negative bacteria. It catalyzes the formation of amide bond between S-adenosylmethionine (SAM) and the acyl-acyl carrier proteins to form a complete AHL molecule (Whitehead et al. 2001). Since the LuxI of most QS bacterial species are homologous to the LuxI of *Vibrio fischeri*, the pathway of AHL synthesis are noted to be almost similar (K Bhardwaj et al. 2013). In addition, the nature of AHL signal molecule with hydrophilic lactone ring moiety and hydrophobic acyl side chain conceptualize it as an amphipathic molecule. This aids the movement of tiny AHLs across the phospholipid bilayer of cell membrane (Fuqua et al. 2001).

Although different bacteria produce different signal molecules, some strains may secrete the same type of AHLs that modulate different phenotypes. Furthermore, some bacteria consist of multiple QS system and secrete more than a type of AHLs like *Pseudomonas aeruginosa* (Fuqua et al. 2001). In order to promote signal specificity for QS system employed by these kind of bacteria in a mixed population, variation in acyl side chain by presence or absence of hydroxyl group at C-3 region plays an essential role (McClellan et al. 1997).

2.3 Quorum sensing in Gram-negative bacteria

Basically, bacterial cells were known to be the independent single-cells in the population without any reliance on other cells to regulate its gene expressions. Upon the discovery of bacterial communal behavior of the marine bacterium, *Vibrio fischeri* and *Vibrio harveyi*, the ability of bacterial cells to act as multicellular

organism was established (Nealson et al. 1970, Fuqua et al. 2001). Research conducted on the bioluminescence response of the symbiotic organism, *V. fischeri* over three decades ago has proved that Gram-negative bacteria potentially can trigger QS mechanism (Zhang and Dong 2004).

In 1970, researchers from Harvard University, Kenneth H. Nealson and John W. Hastings revealed that *V. fischeri* that also known as *Photobacterium fischeri* do not exhibit luminesce before attaining a high cell density (10^{10} cells/ml) (Fuqua et al. 2001, González and Keshavan 2006). As detailed in Figure 2.3, in a population density-dependent manner, *V. fischeri* secretes a type of molecular messengers that connects the entire species of cells. These signal molecules are known as autoinducers responsible for regulation of bioluminescence related genes that are mediated by QS pathway (González and Keshavan 2006). Advance research on this marine bacterium has confirmed that those autoinducers produced by *V. fischeri* belongs to the family of N-acyl homoserine lactones (AHLs) (Czajkowski and Jafra 2009).

QS happens to be an extremely specific kind of communication due to the existence of signal molecules with diverse set of fatty acyl-side chains. Since each of the LuxI-type proteins possesses an acyl-binding pocket which only fits with the correct side-chain moiety, it provides specificity in signal production. Moreover, bacteria seldom rely on one LuxIR QS circuit because most of LuxI-type proteins can produce more than a type of AHLs. Yet, LuxR proteins that packaged with specific binding pockets allow each of the AHLs to bind and activate it precisely. All these characteristics make a species of bacteria even in a mixed population

environment able to distinguish and respond to its original signal molecules (Waters and Bassler 2005).

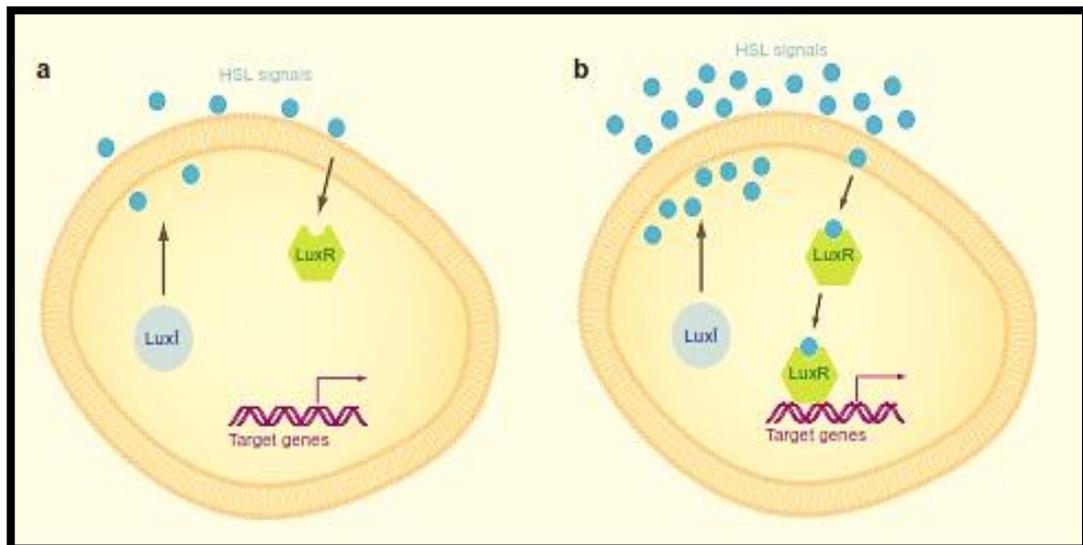


Figure 2.3: Typical quorum sensing mechanism exploited by Gram-negative bacteria. LuxI enzyme homologue is responsible for the production of acyl-homoserine lactone signal molecules (HSL) (blue circles). HSLs will activate LuxR protein regulator homologues by binding to it. **(a)** Low cell density: The concentration of signal molecules produced by LuxI is low at both in and out of the cell environment. Activation of LuxR homologues is minimal at this state. **(b)** High cell density: Concentration of HSLs reaches minimal threshold level (quorum state) where HSLs will activate the LuxR by binding to it specifically. The complex of LuxR-HSLs will then bind to the promoters of target genes in order to initiate their expression.

A very much like QS systems have been identified so far in more than 70 various Gram-negative bacteria (Dong et al. 2007, Williams et al. 2007) including *Rhizobium* spp, *Pseudomonas* spp, *Agrobacterium* spp, *Erwinia* spp, *Burkholderia* spp and *Brucella* spp (McClellan et al. 1997, Bassler 1999). Quite a number of these bacteria rely upon QS to exhibit its pathogenicity against the host. For instance, *P. aeruginosa*, an opportunistic human pathogen utilizes QS mechanism for biofilm formation, regulation of swarming motility and virulence factor production using LasR/I and RhlR/I, the response regulator/ AHL synthase components (Passador et

al. 1993, Brint and Ohman 1995). Mostly these QS related virulent phenotypes in *P. aeruginosa* are distinct when it exists as a community and not as a single bacterium.

Another significant plant pathogen, *Agrobacterium tumefaciens*, infects the host cell nucleus using oncogenic Ti plasmid that is responsible for secretion of opines in the invaded plants that promotes tumor formation (Miller and Bassler 2001). Conjugal transfer of Ti plasmid between bacteria is controlled by regulatory components TraI and TraR of *A. tumefaciens* QS system that located on the transmissible Ti plasmid (Piper et al. 1993). These examples articulate that it is reasonable to look for a disruption mechanism to combat virulence factors of fatal pathogens mediated by QS system in order to create a strong target for effective antimicrobial therapy.

2.4 Quorum quenching

2.4.1 Introduction to the quenching world

While some bacterial species synchronize their phenotypes using cell to cell communication, it is not surprising if the competitive bacteria evolve some special traits to demolish such AHL-dependent interaction. Such signal interference mechanism is recognized as quorum quenching (QQ) and adapted by environmental bacteria to win the constant arm race in a polymicrobial kinship.

Previous researches demonstrated various potential QQ ways that aid signal interference. One of them is the natural substances that can interrupt the signal perception by imitating the AHLs structure. This is inclusive of synthetic derivatives that targets regulatory proteins to attenuate QS regulated communication pathway (Park et al. 2003). Halogenated furones from *Delisea pulchra* and the synthetic AIP

and AHL analogs are the common examples of such synthetic inhibitors that mimic the structure of QS signals (Givskov et al. 1996, Lyon et al. 2000). These small chemical inhibitors substantially compete with corresponding QS signals of respective bacteria in order to bind with the target receptor by displacing the original signal molecule from its reporter protein (Givskov et al. 1996).

Moreover, QQ can also be achieved by blocking the synthesis of QS signals (Parsek et al. 1999). By inhibiting the crucial elements or enzymes involved in signal molecule generation such as acyl-acyl carrier protein (ACP) that catalyze synthesis of acyl side chains, the LuxI homologous proteins and S-adenosylmethionine synthase that plays a role in biosynthesis of AHLs can prohibit synthesis of signal molecules (Parsek et al. 1999, Park et al. 2008).

Besides that, the higher plants interfere with the QS system of microbes by secreting secondary metabolites that imitate bacterial signal molecules. During active development, various compounds that are identical to bacterial signal molecules are secreted through its root in order to interrupt the rhizosphere bacterial gene expressions (Gao et al. 2003). This fact was proven by a recent study on South Florida plant extracts, *Callistemon viminalis*, *Bucida buceras* and *Conocarpus erectus* excluded an inhibitory effect on QS system of *P. aeruginosa* by inhibiting its virulence factor production (Adonizio et al. 2008).

Last but not least, the AHL-inactivating enzymes known as QQ enzymes plays vital role in destructing the QS signal molecules and make them unfit to trigger accumulation (Dong et al. 2000). These enzymes are stable in diverse conditions and highly specific for the QS- regulators. Nevertheless, QQ enzymes only interfere with the signal pathway by cleaving the signal molecules enzymatically without

disturbing any other basal metabolic processes of target bacteria (Romero et al. 2012, K Bhardwaj et al. 2013).

2.4.2 The quorum quenchers: AHL-lactonase and AHL-acylase

As illustrated in Figure 2.4, although the chemical structure of AHLs proposed 4 distinct enzymatic mechanisms for AHL inactivation; lactonase, acylase, decarboxylase and deaminase, only two types of enzymes mentioned above have been notified so far: AHL-lactonase and AHL-acylase (Dong and Zhang 2005).

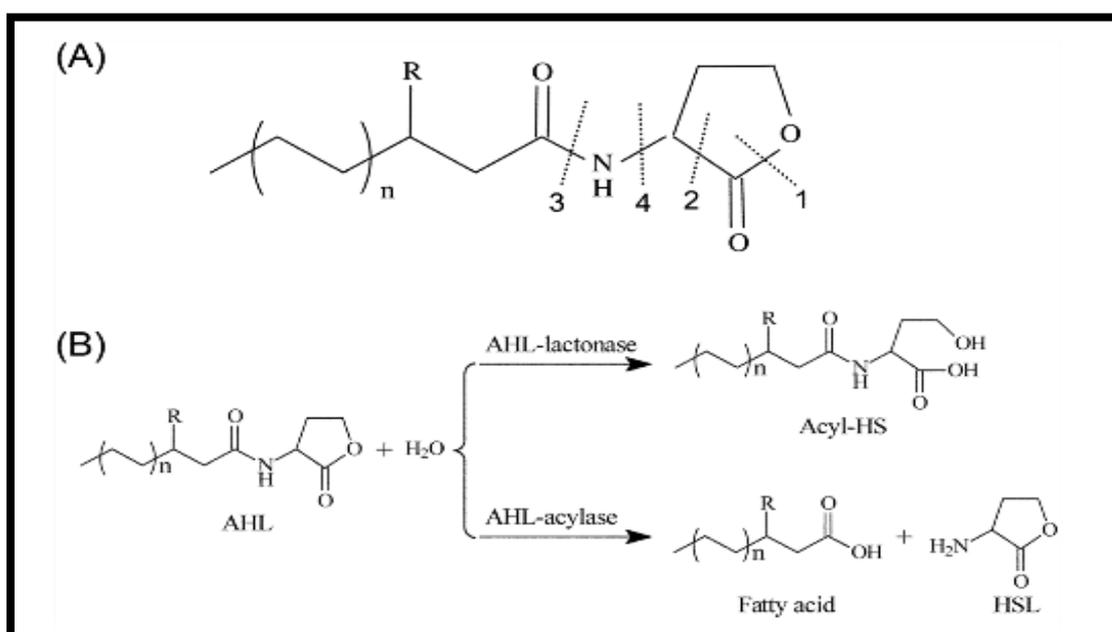


Figure 2.4: Adapted from Dong and Zhang (Dong and Zhang 2005). (A) Four types of AHL inactivating enzymes that cleave the AHL signal molecule enzymatically at specific positions. Lactonase and decarboxylase could potentially hydrolyze at the sites marked as 1 and 2 while acylase and deaminase could break the acyl side chain and homoserine lactone ring moiety at positions marked as 3 and 4 respectively. **(B) The mechanism of AHL inactivation using AHL-lactonase and AHL-acylase.** AHL-lactonase degrades the signal molecule by hydrolyzing the lactone bond while AHL-acylase hydrolyzes the amide linkage between the acyl side chain and lactone ring.

AHL-lactonase way of signal degradation was initially accounted in year 2000 with the discovery of AHL-lactonase from *Bacillus thuringiensis* and AHL acylase from *Variovorax paradoxus* (Dong et al. 2000, Leadbetter and Greenberg 2000). Subsequently, a spectrum of bacterial strains able to produce QQ enzymes were discovered from plants, soil samples and laboratory cultures (Dong and Zhang 2005).

Surprisingly, some of them possess both types of AHL degrading enzymes to aid in its QQ metabolism (González and Keshavan 2006). In some AHL signal molecule producing bacteria, the ability to secrete QQ enzymes were also noted, probably to regulate the QS pathway. For example, QS bacteria such as *P. aeruginosa* PAO1 employs *pvdQ*, homolog of AHL-acylase gene of *Ralstonia* that is responsible for monitoring AHL mediated gene expressions (Huang et al. 2003). These facts prove that genes encoding AHL inactivating enzymes are widely spread in bacterial ecosystem as a defense mechanism against antibiotic-producing bacteria as well as a regulator of QS system.

As shown in Table 2.1, up to date, numerous bacteria that originate from various genera have been identified to possess AHL inactivating activity and hence known as the most potent approach for silencing QS mechanism of pathogenic bacteria.

TABLE 2.1: Adapted from Czajkowski and Jafra 2009. Table shows the list of AHL-inactivating enzymes of various species of bacteria. Two different groups of AHL-inactivating enzyme have been identified so far; AHL lactonase and AHL acylase. Bacterial strains show distinct enzymatic activity which does not seem to be related with its taxonomic classification.

Bacterial strain	Gene/protein	Protein size (kDa)	Number of amino acids	Enzymatic activity	REFERENCES
<i>Bacillus</i> sp.	<i>aiiA/AiiA</i>	28	250	AHL lactonase	Dong <i>et al.</i> , 2000; 2002 Wang <i>et al.</i> , 2004
<i>B. thuringiensis</i>	<i>aiiA/AiiA</i>	28	250	AHL lactonase	Dong <i>et al.</i> , 2002 Lee <i>et al.</i> , 2002
<i>Arthrobacter</i> sp.	<i>ahlD/AhlD</i>	31	273	AHL lactonase	Park <i>et al.</i> , 2003
<i>A. tumefaciens</i>	<i>attM/AttM</i>	29	264	AHL lactonase	Zhang <i>et al.</i> , 2002
<i>A. tumefaciens</i>	<i>aiiB/AiiB</i>	nd.	nd.	AHL lactonase	Zhang <i>et al.</i> , 2002 Carlier <i>et al.</i> , 2003
<i>Rhodococcus</i> sp.	nd.	nd.	nd.	AHL lactonase	Park <i>et al.</i> , 2006
<i>R. erythropolis</i>	<i>qsdA/QsdA</i>	36	323	AHL lactonase	Uroz <i>et al.</i> , 2008
<i>V. paradoxus</i>	nd.	nd.	nd.	AHL acylase	Zhang <i>et al.</i> , 2002 Carlier <i>et al.</i> , 2003
<i>R. eutropha</i>	<i>aiiD/AiiD</i>	84	794	AHL acylase	Lin <i>et al.</i> , 2003
<i>P. aeruginosa</i>	<i>pvdQ/PvdQ</i>	80	762	AHL acylase	Huang <i>et al.</i> , 2003 Sio <i>et al.</i> , 2006
<i>P. aeruginosa</i>	<i>quiP/QuiP</i>	90	847	AHL acylase	Huang <i>et al.</i> , 2006
<i>Anabaena</i> sp.	<i>all3924/AiiC</i>	nd.	847	AHL acylase	Romero <i>et al.</i> , 2008
<i>Streptomyces</i> sp.	<i>ahlM/AhlM</i>	86	804	AHL acylase	Park <i>et al.</i> , 2005
<i>Comamonas</i> sp.	nd.	nd.	nd.	AHL acylase	Uroz <i>et al.</i> , 2006
<i>R. erythropolis</i>	nd.	nd.	nd.	AHL acylase	Uroz <i>et al.</i> , 2005
<i>Acinetobacter</i> sp.	nd.	nd.	nd.	nd.	Kang <i>et al.</i> , 2004
<i>Delftia</i> sp.	nd.	nd.	nd.	nd.	Jafra <i>et al.</i> , 2006b
<i>Ochrobactrum</i> sp.	nd.	nd.	nd.	nd.	Jafra <i>et al.</i> , 2006b

nd., not determined

2.5 Quorum quenching by AHL-lactonase

2.5.1 The gene homologue encoding AHL-lactonase, *aiiA*

The *aiiA* gene homologue encoding AHL-lactonase enzyme was initially discovered in the year 2000 by Dong through his research on soil bacterial isolates. Upon screening more than 500 soil and laboratory isolates to detect their AHL-inactivating activity, 24 isolates exhibited different ranges of activity in terminating AHL signal molecules (Dong et al. 2000).

Out of them, a Gram-positive *Bacillus* species strain 240B1 indicated the strongest enzymatic AHL inactivation activity that could be manipulated as a strategy for biocontrol. Due to its ability in reducing production of cell wall-degrading enzymes controlled by pathogenic bacteria, AHL inactivation activity of this strain gained interest. Moreover, expression of *aiiA* gene in *Erwinia carotovora* has led to decline in production of disease causing enzymes up to 10% and downturn the soft rot disease symptoms in plants (Dong et al. 2000). Further characterization studies revealed that the enzyme coded by *aiiA* homologue is an AHL hydrolase due to its tendency in degrading AHLs by hydrolyzing the lactone bond in AHLs without disturbing the rest of the molecule structure (Dong et al. 2001).

In terms of specificity and substrate preference, AHL-lactonase is found to be the most specific AHL inactivating enzyme to date. Unlike AHL-acylases which possess broader range of substrate preference, AHL-lactonases are responsible in hydrolyzing both short- and long-chain AHLs with the same efficiency. Another research carried out by Molina (Molina et al. 2003) showed that the enzyme encoded by *aiiA* gene of *Bacillus* sp. strain can offer protection to plants as it can decrease the

potato tuber soft rot disease caused by *E. carotovora* almost to 15% and crown gall disease in tomato caused by *A. tumefaciens* to about 10%.

AiiA_{240B1}, the product of *aiiA* gene homolog of *Bacillus* sp. 240B1 is the well-characterized AHL-lactonase to date (Dong et al. 2000, Dong et al. 2001). Further lactonase studies on *Bacillus* genus marked identification of many with *aiiA* homologue and most of them shared high nucleotide sequence similarity that was greater than 90% (Dong et al. 2002, Lee et al. 2002, Kim et al. 2005). This includes *Geobacillus caldoxylosilyticus* YS-8 (Seo et al. 2011) and *Geobacillus kaustophilus* HTA426 (Chow et al. 2010), the partners of Bacilaceae, were observed to produce thermostable lactonase.

Besides *Bacillus* genus, varied number of bacterial strains with respective lactonase such as AttM and AiiB of *A. tumefaciens* (Zhang et al. 2002, Haudecoeur et al. 2009), AhlK of *Klebsiella pneumonia* (Park et al. 2003), AhlS of *Solibacillus silvestris* (Morohoshi et al. 2012), AidH of *Ochrobactrum* (Mei et al. 2010), AhlD of *Anthrobacter* sp. (Park et al. 2003) and QsdA of *Rhodococcus* sp. (Park et al. 2006) were observed to demonstrate potential AHL-lactonase activity.

2.5.2 AHL-lactonase: A sibling of metallo- β -lactamase superfamily

AHL-lactonases can be classified into three different divisions that exhibit homology to protein superfamilies namely paraoxonases, amidohydrolases and metallo- β -lactamases. Out of the three superfamilies, proteins from metallo- β -lactamases gained the attention to be studied in detail due to its active nature of catalytic system (Fast and Tipton 2012).

Sequence alignment and mutagenesis analysis on AHL inactivating lactonases of various *Bacillus* strains later revealed the presence of a small conserved motif with catalytic importance (Dong et al. 2000, Dong et al. 2002). This HXDH~H~D motif was noted to be conserved in all AiiA and also AttM clusters that code for lactonases and highly resembles the Zn²⁺ binding motif of metalohydrolases where most members of metallo-β-lactamases superfamily are anticipated to bind zinc ion (Dong et al. 2000, Dong and Zhang 2005). Metallo-β-lactamases superfamily displays metal requirement and conserve a special site for binding of metal ions, HXHXDH~H metal binding motif (Fast and Tipton 2012). Identification of such conserved sequence motif in 250-residue-long *aiiA* amino acid sequence prompted the interest of researchers to study in detail the metal requirement of AHL-lactonases in AHL-inactivating activity.

The initial study conducted by Wang on AiiA_{240B1} perceived that AHL-lactonases do not depend upon metal ions for catalytic activity and therefore, it might not be a metalloprotein. Yet, later researches discovered that presence of the signature motif, HXHXDH~H in AHL lactonases, represent a brand new catalytic module that play a role in AHL degradation. Analytical study on the crystal structure of AHL-lactonase of *Bacillus thuringiensis* subsp. *Kurstaki* (AiiA_{BTK}) indicated presence of two zinc ions in the active site of the enzyme which is essential for its enzyme activity (Kim et al. 2005, Liu et al. 2005).

In addition, subsequent work on another *Bacillus* strain, *Bacillus thuringiensis* BGSC4A3 showed that AHL-lactonase is a dinuclear metalloprotein where two Zn²⁺ ions bound adjacently on the active site (Thomas et al. 2005). These metal ions were found to be involved in the activity of detaching ester bonds on the

lactone rings harbored by AHL signal molecules as well as easing the enzyme folding (Momb et al. 2008). Moreover, site directed mutagenesis analysis on the *aiiA* homologue implied that the histidine and aspartate residues present in the zinc-binding motif play a crucial role in AHL-lactonase activity (Dong et al. 2001).

2.5.3 Mechanism of AHL-lactonase to hydrolyse AHL signal molecules

The first attempt to characterize AHL-lactonase in order to study its enzyme kinetics and substrate specificity was done by Wang (Wang et al. 2004). In association with that, the recombinant AHL-lactonase enzyme and its other four variants were purified and analyzed. Eventually, the analysis declared that AHL-lactonase displayed prominent enzyme activity towards the tested AHL signal molecules, regardless of their nature of substitution at C3 position and length of the acyl side chain. AHL-lactonase was also verified as inactive towards other non-acyl lactones and noncyclic esters. Such specification for substrates by this AHL inactivating enzyme is based on the presence of amide group and ketone at C1 position of the acyl-chain.

Coupled with that, mutation analysis done by Wang showed that substitution of histidine and aspartate as the essential amino acids present in the conserved metal binding motif of AHL-lactonase enzyme had caused conformational changes to the motif. Substitution of His-106, Asp-108, His-109 or His-169 with serine was recognized to led to notable loss of AHL-lactonase activity (Wang et al. 2004). Moreover, another essential residue for the catalytic activity of AHL-lactonase, Tyr-194, was discovered through research conducted on *Bacillus cereus* strain Y2 (Lu et al. 2006).

Besides the interest on biochemical nature of AHL-lactonases, its ring opening mode and the role played by metal ions in the catalytic mechanism also caught attention of scientists. In conjunction, studies on crystallographic structure of the enzyme reported that AHL-lactonase holds two Zn^{2+} ions where the first one (Zn1) controlled by His-104, His-106 and His-169 and the second one (Zn2) coordinated by His-109, His-235 and Asp-108. Both these ions were noticed to be adjacently bridged by hydroxide ion of a water molecule and the OD2 atom of Asp-191. When the enzyme comes in contact and reacts with AHL signal molecule, the hydroxyl ion of Tyr-194 became the donor in the hydrogen bond as it perform as a general acid protonating the leaving group (Kim et al. 2005, Liu et al. 2005).

According to the catalytic pathway of AHL-lactonase that was proposed by Kim (Kim et al. 2005) upon analyzing the three-dimensional structures of the enzyme, its enzyme-substrate complex as well as studying structures of other enzymes classified under metallo- β -superfamily showed that the reaction involves a nucleophilic attack by water. The hydroxide from water molecule connects the two metal ions on the substrate's carbonyl carbon. This makes the lactone ring and carbonyl oxygens of the AHL signals unite correspondingly with the Zn^{2+} ions, Zn1 and Zn2. Subsequently, this interaction leads to intensified polarization of carbonyl bond as well as making it more susceptible towards nucleophilic attack (Kim et al. 2005).

2.6 Significance of quorum quenching

2.6.1 Conventional therapeutic targets

There has been tremendous upsurge in the records of treatment complexities and withdraws by virtue of antibiotic resistance development. This scenario simply indicates that the recommended dose of drug or antibiotic that is supposed to demolish the evolvement of the bacteria miss to do so and leaving the disease untreated. Such resistances towards antibiotics occur either due to over-usage of that particular drug or misapplication of it.

Therefore, the morbidity and mortality of the pathogenic bacteria eventually reduced when it evolves greater virulence and resistance towards antibiotics. On the other hand, QQ mechanism was found to be a prominent strategy in demolishing QS circuit of microbial pathogens. This directed the interest of scientists to study more on the prospects of QQ to fight bacterial pathogenicity (Chen et al. 2013).

Relevant to that, a study reported that QS plays an essential role in development of biofilm in lungs of patients with cystic fibrosis (Chen et al. 2013). Biofilm is formed basically from assemblage of microbial cells that hardly detach from one another. This closely tied matrix acts as a barrier of protection and contributes to bacterial resistance as it can lower the penetration of antibiotics across the membrane into the cell environment (Donlan 2002). Hence, bacteria in form of biofilm survive much longer when treated with antibiotics compared to the ones found in suspensions (Stewart and William Costerton 2001).

As biofilm formation is regulated by QS system, interference of this signal pathway will make the microbial biofilm more susceptible to clinical antibiotics and

immune response of the host. With that, a new alternative approach of monitoring biofilm formation using biofilm-disrupting QQ compounds coupled with potential antibiotics will simultaneously treat the QS mediated diseases (Estrela and Abraham 2010).

2.6.2 Anti - biofouling strategy

Gradual accumulation of microorganisms, algae, plants and also animals on wet surfaces is known as biofouling. It happens mostly on surfaces that often in contact with water such as pipes, ship hulls, tanks, membranes of bioreactors and mechanical equipment. Biofouling channels the formation of undesired bacterial colonization and contamination on those surfaces as well as corrode the machine parts that in contact with water. This ceases the efficiency of the machine to function up to its optimum level (Choudhary and Schmidt-Dannert 2010).

Besides the high cost of recovery, biofouling increases the consumption of fuels due to high frictional drag caused by biofouling (Schultz et al. 2011). Studies showed that per year, around USD56 million is spend by US Navy to treat hull fouling (Yebra et al. 2004). Since usage of toxic organotin tributyltin-based paint products that previously used as the anti- biofouling agents were banned, the need for environmental friendly anti-biofouling agents immersed (Dobretsov et al. 2006).

In the approach of inhibiting biofouling using anti-biofouling compounds, it was detected that QQ compounds, furanones and betonicine has the potential to lower the attachment of bacteria and decrease formation of biofilm (Dobretsov et al. 2011). A study conducted on *P. aeruginosa* exhibited that the cultures of the respective opportunistic pathogen treated with furanones showed reduction in

expression of its virulence factors and forms a thin layer of biofilm compared to the normal wildtype cultures (Hentzer et al. 2002). This testifies that addition of QS inhibitors on the targeted surface is a possible way of reducing biofouling caused by *P. aeruginosa*.

Moreover, production of AHLs in some of the marine bacteria has been a chance to block their QS pathway that will indirectly reduce microfouling among marine microorganisms. Since usage of biocidal compounds is harmful for the marine ecosystem, QS pathway that contributes in biofilm formation is chosen as an alternative target in treating biofouling (Olsen et al. 2007). Either natural or synthetic inhibitors of QS are added to the anti-fouling coatings with the aim of reducing macrofouling (Choudhary and Schmidt-Dannert 2010). This can be a useful strategy to prevent marine transportation issues caused by biofouling.

2.6.3 Agricultural usage

Inefficiency of current bactericides constantly develops into major losses in agricultural industry. In order to combat the common microbial diseases caused by phytopathogens, QQ bacteria are used as the biocontrol agents. QQ compounds or enzymes attenuate the QS mechanism of this crop infecting microbes by inactivating their signal molecules (Hong et al. 2012). For example, a study conducted on transgenic potato and tobacco plant that expresses AHL-lactonases showed that the plants are viably guarded from harm of *E. carotovora*, a Gram-negative plant phytopathogen (Dong et al. 2000). This demonstrated the ability of QQ enzymes in controlling infectious diseases in plants without depending fully on bactericides.

CHAPTER 3 - MATERIALS AND METHODS

3.1 Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 3.1.

TABLE 3.1: Bacterial strains and plasmids used in this study

Bacterial Strain or Plasmid	Description	Source or Reference
<i>Escherichia coli</i> JM109	<i>supE44 endA1 hsdR17 gyrA96 relA1 thiΔ(lac-proAB)recA1, [F' [traD36proAB⁺ lacI^q lacZΔM15]</i>	Promega
<i>Escherichia coli</i> BL21(DE3)pLysS	F ⁻ , <i>ompT</i> , <i>hsdSB</i> (rB ⁻ , mB ⁻), <i>dcm</i> , <i>gal</i> , λ(DE3), pLysS, Cm ^r	Promega
<i>Escherichia coli</i> BL-Bel (pBel10)	<i>E. coli</i> BL21 strain harboring pBel10 recombinant plasmid	This study
<i>Chromobacterium violaceum</i> CV026	Violacein production-based biosensor, <i>kan^R</i> , produces pigment violacein in the presence of exogenous acyl homoserine lactones (AHLs), AHL-negative double mini Tn5 mutant strain	Gift from Dr Paul Williams, Nottingham University
<i>Burkholderia pseudomallei</i> UKMS-01	Wild-type, local isolate from sheep, displaying symptoms of melioidosis	Prof. Dr. Sheila Nathan, UKM
<i>Bacillus cereus</i> SM01	Soil isolate positive for acyl homoserine lactone inactivation activity	This study

pGEM-T	Linearized vector with a single 3'-terminal thymidine at both ends, enables blue-white selection of recombinants, <i>amp^R</i>	Promega
pCold I DNA	Cold expression vector, <i>cspA</i> gene promoter, translation enhancing element (TEE), his-tag sequence, factor Xa cleavage site, <i>amp^R</i>	Takara Bio. Inc.
pBcl16S	Recombinant pGEM-T plasmid harbouring insertion of 1.4 kb 16s rDNA amplified PCR fragment	This study
pBcl10	Recombinant pCold I DNA plasmid harbouring insertion of 770 bp amplified AHL- lactonase gene homologue	This study.
