Chapter One

Introduction

1.1 *Pseudomonas aeruginosa*

P. aeruginosa is a Gram-negative rod-shaped bacteria species, with size varying from between 0.5-0.8 µm by 1.5-3.0 µm. It belongs to the family Pseudomonadaceae and can be found singularly, in pairs, or in short chains. This bacteria species is non-sporing, non-capsulate and usually motile with polar monotrichous flagella. Typical *P. aeruginosa* cultures are characterised by its greenish-blue appearance due to the production of the soluble phenazine pyocyanin. It also emits an easily recognised sweet grape-like odour and can grow over a wide temperature range- up to 42 ºC. *P. aeruginosa* can grow and survive in almost any environment, although having a preference for moist surroundings (Govan et al., 2003). In industrial settings, its biofilms can contribute to fouling and corrosion of the heat transfer systems. It presents a challenge in clinical settings because it tends to colonise hospital reservoirs, which include respiratory equipment, catheters, and even disinfectants. Hence, *P. aeruginosa* was hailed as a significant cause of hospital acquired infections, especially since hospitalisation can lead to greatly increased colonisation rates (Lyczak et al., 2000). In spite of having a large arsenal of virulence factors, *P. aeruginosa* does not possess the genetic traits to cause disease in healthy individuals with intact immune systems. However, the bacterium is capable of chronically infecting immunocompromised patients. It is a notable cause of infections of the skin, lungs, eyes, blood as well as the genitourinary tract. Often such immunocompromised individuals are treated with antibiotics as a means of defence against the infecting organisms. Unfortunately, such

treatments inadvertently destroys and host's natural microflora, which is the primary biological barrier against subsequent colonisation. In addition to that, *P. aeruginosa* is innately resistant to many of the conventional antibiotics that are used for treatments (Martinez and Baquero, 2002).

1.1.1 Virulence determinants of *P. aeruginosa*

Being a pathogen, *P. aeruginosa* is equipped with the means to not only invade and grow within a host, but also to cause disease. It has the ability to damage host tissues, leading to symptoms and signs of disease, before finally disseminating through the host and/or to another host. In order to carry out this multistage adaptive process *in vivo*, *P. aeruginosa* produces a vast variety of virulence determinants. Flagella, pili and rhamnolipids are motility factors that allow transport of the bacteria to favourable environments. These cell-associated factors also help to mediate adherence of the organism to the mucosal surfaces, thus allowing initial colonisation of host tissues. Toxins and proteases, such as exotoxin A, pyocyanin, elastase and exoenzyme S, are some of the virulence factors that are secreted via transport systems. These can damage the host by disrupting host immune defence and also providing nutrients for the pathogen (Van Delden, 2004). Table 1.1 summarises the functions of some of the main virulence factors produced by *P. aeruginosa*.

Table 1.1 Examples of virulence determinants of *Pseudomonas aeruginosa* and their biological effects (Van Delden, 2004).

Virulence Determinant	Biological Function
Alginate	1. Increases adherence to respiratory epithelia. 2. Inhibit phagocytosis.
Alkaline protease	Inactivation of protease inhibitors and complement proteins.
Elastase	Degrades elastin, resulting in breakdown of host tissues.
Exoenzyme S	1. ADP-ribosylation of GTP binding protein. 2. GTPase activating protein. 3. Inhibits phagocytosis.
Exotoxin A	Inhibition of protein synthesis via ADP-ribosylation of elongation factor 2.
Flagella	1. Adherence to cell surfaces 2. Swimming and swarming motility
Hydrogen cyanide	Inhibits aerobic respiration in host cells.
Lipase	1. Cleaves lipids. 2. Degrades platelets, granulocytes and monocytes.
Pili	1. Adherence to cell surfaces. 2. Twitching and swarming motility.
Pyocyanin	1. Redox-active phenazine pigment. 2. Interferes with ion transport. 3. Stimulates inflammatory response.
Rhamnolipids	1. Biosurfactant for the solubilisation of lung phospholipids and Alkyl Quinolones (AQ). 2. Haemolysin.

1.2 Quorum sensing

Historically, bacterial cells were believed to be asocial organisms that did not rely on other members of the population in the synchronization of gene expression. Bacteria were believed to exist as individual cells that sought mainly to find nutrients and multiply. This perception was however changed when it was discovered that the Gram-negative marine bacterium *Vibrio fischeri* was able to induce and regulate bioluminescence at an early growth stage due to the production of an autoinducing substance known as an *N*-acylhomoserine lactone (AHL) (Nealson et al., 1970, Eberhard et al., 1981). The term "quorum sensing" (QS) was coined to describe the ability of bacteria to interact with each other and their surroundings by coordinating gene expression in accordance with population density (Fuqua et al., 1994). The transcription of QS target genes is density-dependent, and will be activated at a certain population density known as the "quorum size"- the number of bacteria required to activate the QS system (Fuqua et al., 1994, Salmond et al., 1995). Hence, the phenomenon of QS or cell-to-cell communication is based on the principle that when a single bacterium releases autoinducers (AIs) into the open environment, the (AIs) concentration is too low to be detected. But when sufficient bacteria are present, AIs concentration reach a threshold level that allows the bacteria to sense a critical cell mass and react to it by activating or repressing target genes. The most well studied signal molecule is AHLs, which is produced by Gram-negative bacteria. Using the *Vibrio fischeri* system as an example, the signal molecule is synthesized by a synthase protein, called LuxI. At a critical concentration, it binds to the receptor, Lux R. This complex then binds to a regulatory region known as the *lux* box, thus allowing concerted expression of a subset of QS controlled genes. A positive feedback mechanism exists, in which the bound AHL-LuxR complex further activates expression of the LuxI protein, leading to the production of more AHLs. Hence, there is increased transcription of the

of the downstream genes *luxCDABE*, which enables the bacteria to produce the luciferase and fatty acid reductase complex required for light production (Figure 1.1). QS allows individual cells to detect when the situation is conducive to partake in the production various energy-expensive phenotypes (i.e. light in *V. fischeri*), which is only effective at the population level in a confined environment.

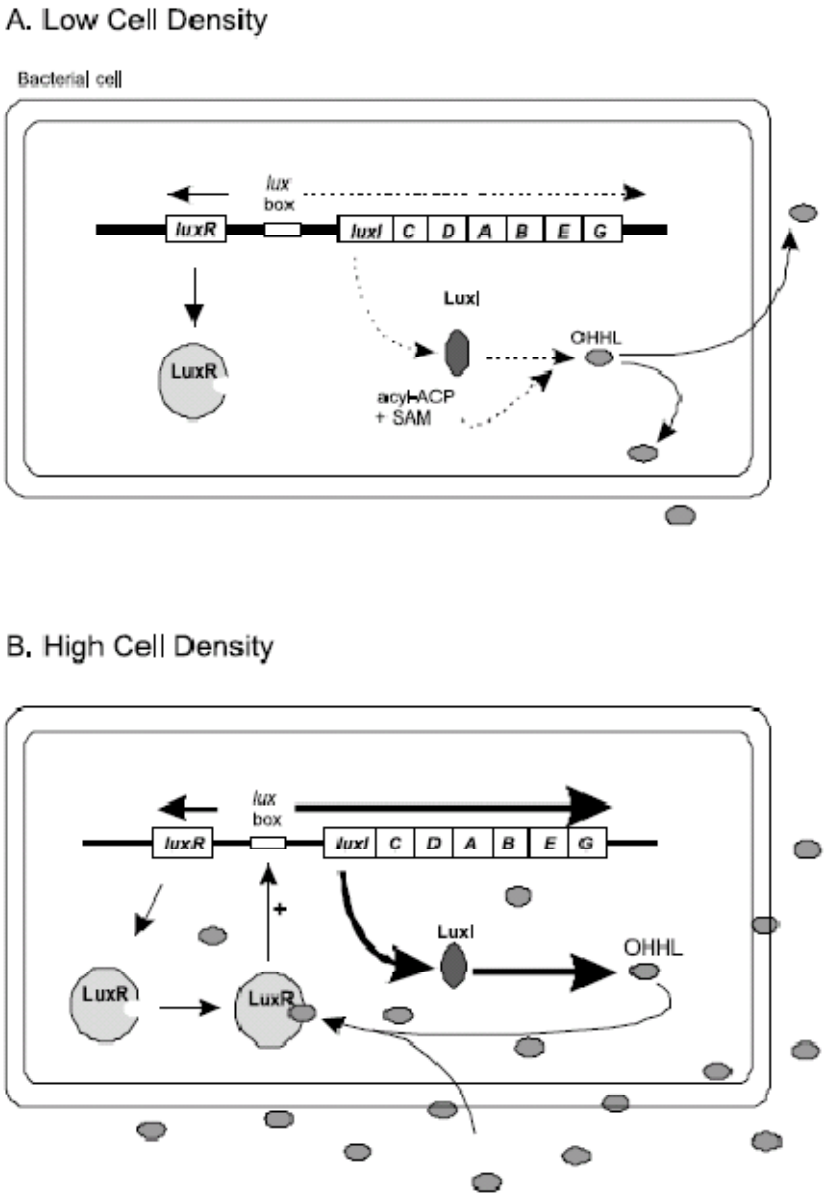

Figure 1.1 The regulation of bioluminescence in *V. fischeri*: the QS paradigm (Whitehead et al., 2001).

1.2.1 QS in *Pseudomonas aeruginosa*

Microarray studies have suggested that around 6 % of the *P. aeruginosa* genome is regulated by the QS hierarchy (Schuster et al., 2003, Wagner et al., 2003) of which many genes code for extracellular enzymes and toxins. The QS system of *P. aeruginosa* consists of two sets of cognate 'R' and 'I' proteins that are involved in virulence regulation, biofilm development and other processes. The first of these sets is termed the *las* system and consists of *lasI*, encoding the AHL synthase LasI (Passador et al., 1993), and lasR, encoding the transcriptional activator LasR (Gambello and Iglewski, 1991). LasI directs synthesis of the autoinducer *N*-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) (Pearson et al., 1994). The second of these systems is termed *rhl* and is composed of *rhlI*, which encodes the AHL synthase RhlI, and *rhlR*, encoding the transcriptional activator RhlR (Brint and Ohman, 1995, Latifi et al., 1995). RhlI directs the synthesis of *N*-butanoyl-L-homoserine lactone (C4-HSL) (Pearson et al., 1995, Winson et al., 1995). It has been suggested that the response regulator proteins LasR and RhlR detect and respond to their signal molecules by binding to palindromic sequences of around 20 base pairs termed '*las-rhl* boxes' in the promoters of target genes (Rust et al., 1996, Pessi and Haas, 2000, Whiteley and Greenberg, 2001). Figure 1.2 shows the structures of the primary AHL signal molecules used by *P. aeruginosa*. These two systems are arranged in a hierarchical manner, with the *las* system exerting positive control over the *rhl* system (Latifi et al., 1996).

At low population densities, 3-oxo-C12-HSL is produced at a basal rate. As the population of bacteria increases, the concentration of 3-oxo-C12-HSL reaches a threshold level, at which point it binds to LasR to form an active complex which in turn activates the transcription of numerous virulence factors such as elastase (Passador et

al., 1993), pyoverdin (Stintzi et al., 1998), and also of *lasI* (creating a positive feedback loop) and *rhlR* (Latifi et al., 1996). The *rhlI* gene is subsequently transcribed and its gene product RhlI synthesises the second autoinducer C4-HSL (Pearson *et al*, 1995; Winson *et al*., 1995). When C4-HSL reaches a sufficient concentration, C4-HSL binds to RhlR and this in turn activates transcription of numerous other virulence factors such as rhamnolipid biosurfactants, cytotoxic lectins, pyocyanin and elastase (Brint and Ohman, 1995, Pearson et al., 1997). Note that some phenotypes such as elastase are regulated by both *las* and *rhl* and in combination; these two systems regulate subsequent activation of genes in the two regulons. Many of these studies were conducted in *E. coli*, however, in *P. aeruginosa*; the hierarchy is complicated by the existence of other regulators of the QS system. The addition of either the AHL signalling molecules of PQS to cultures of *P. aeruginosa* has been shown to enhance, although not necessarily to advance the expression of a large number of genes (Diggle et al., 2003, Wagner et al., 2003), since many QS controlled genes were only expressed towards the stationary phase of growth and many QS controlled genes were induced only in the transition between logarithmic and stationary phase of growth (Schuster *et al*., 2003).

The importance of QS to the pathogenicity of *P. aeruginosa* has been demonstrated in various models. In a burned mouse model, *P. aeruginosa* PAO1 strains deficient in *lasR*, *lasI*, *rhlI* or both lasI and *rhlI* showed reduced virulence *in* vivo (Rumbaugh et al., 1999), and the total number of bacteria recovered from the spleens, livers and skin of infected mice was also reduced in lasI and rhlI mutants. Complementation of *lasI*, *rhlI* or both on a multicopy plasmid restored both virulence and ability to spread (Rumbaugh *et al*., 1999). Pearson *et al*. (2000) obtained similar results in a neonatal pneumonia mouse model. QS is also implicated in *P. aeruginosa* biofilm formation. A PAO1 mutant deficient in 3-oxo-C12-HSL production revealed biofilm growth that was both

thinner and without the associated three-dimensional architecture of the parent strain (Davies et al., 1998). The mutant biofilm also displayed much less resistance to the detergent sodium dodecyl sulphate (SDS), but when grown with exogenously added 3-oxo-C12-HSL, the mutant biofilm resembled the wild type and was resistant to the detergent. Interestingly, some studies have shown that AHLs may themselves play a role in the *in vivo* pathogenicity of *P. aeruginosa*. 3-oxo-C12-HSL has been shown to have immunomodulatory activity (Smith et al., 2002) and both vasorelaxant and bradycardiac (Gardiner et al., 2001) cardiovascular effects.

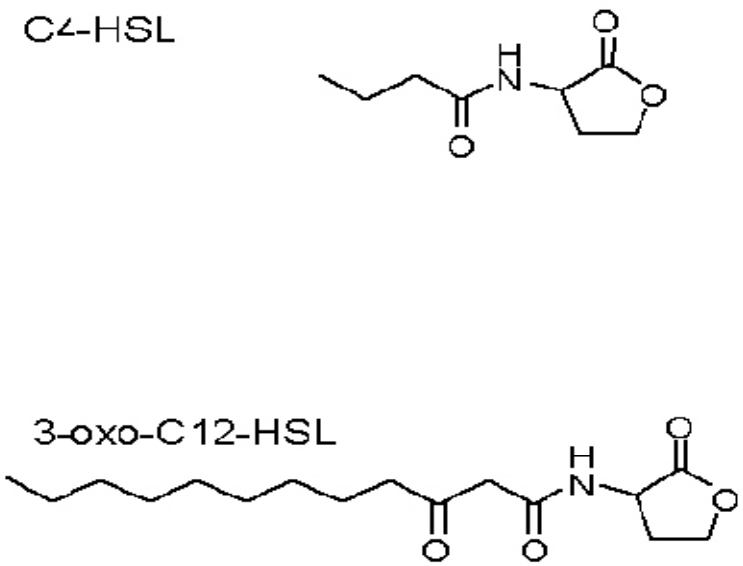

Figure 1.2 Structures of AHLs used by *P. aeruginosa*- C4-HSL and 3-oxo-C12-HSL.

1.2.2 Alkyl quinolone signalling in *P. aeruginosa*

A novel non-AHL QS signalling system in *P. aeruginosa* that utilises a 2-alkyl-4-quinoline (AQ) molecule as the primary signal was subsequently discovered (Pesci et al., 1999). The revelation that *P. aeruginosa* utilised a new, major, non AHL-mediated QS signalling pathway, coupled with the identification of its main signalling molecule-2-heptyl-3-hydroxy-4-quinoline (also known as the *Pseudomonas* quinoline signal-PQS), marked a new way of thinking about QS in pseudomonas. Although this was the first time that AQs have been shown to be involved in QS in a *Pseudomonas*, this was not the first discovery of these molecules in this genus. Other major molecules belonging to this family are 2-heptyl-4-quinolone (HHQ), 2-nonyl-4-quinolone (NHQ) and 2-heptyl-4-quinolone *N*-oxide (HHQNQ). Figure 1.3 shows the structures of a selection of AQ molecules.

PQS was finally described as a QS signal molecule by Pesci *et al*., in 1999 from observations on the expression of the *lasB* (elastase) gene. It was found that addition of spent culture media extract from a *P. aeruginosa* PAO1 caused a major induction of expression of *lasB* in a PAO1 *lasR* mutant. This was interesting as this induction could not have been due to AHLs because the mutant was fully AHL deficient. This therefore strongly suggested that a third non-AHL signal was present, capable of activating *lasB* expression. This signal required LasR and 3-oxo-C12-HSL, due to the lack of *lasB* induction in a *lasR* mutant. Additionally, spent culture extract from PAO1 failed to induce *lasB* expression in a PAO1 *rhlR/ rhlI* double mutant, suggesting that the *rhl* system was required for the bioactivity of the new signal. HPLC separation and mass spectroscopy (MS) of spent PAO1 media extract confirmed that the molecule was 2-heptyl-3-hydroxy-4-quinolone (PQS). PQS represented a new class of AQ signal

molecules, whose mechanism of regulation and effect was interwoven with that of the two previously discovered AHL-based QS systems.

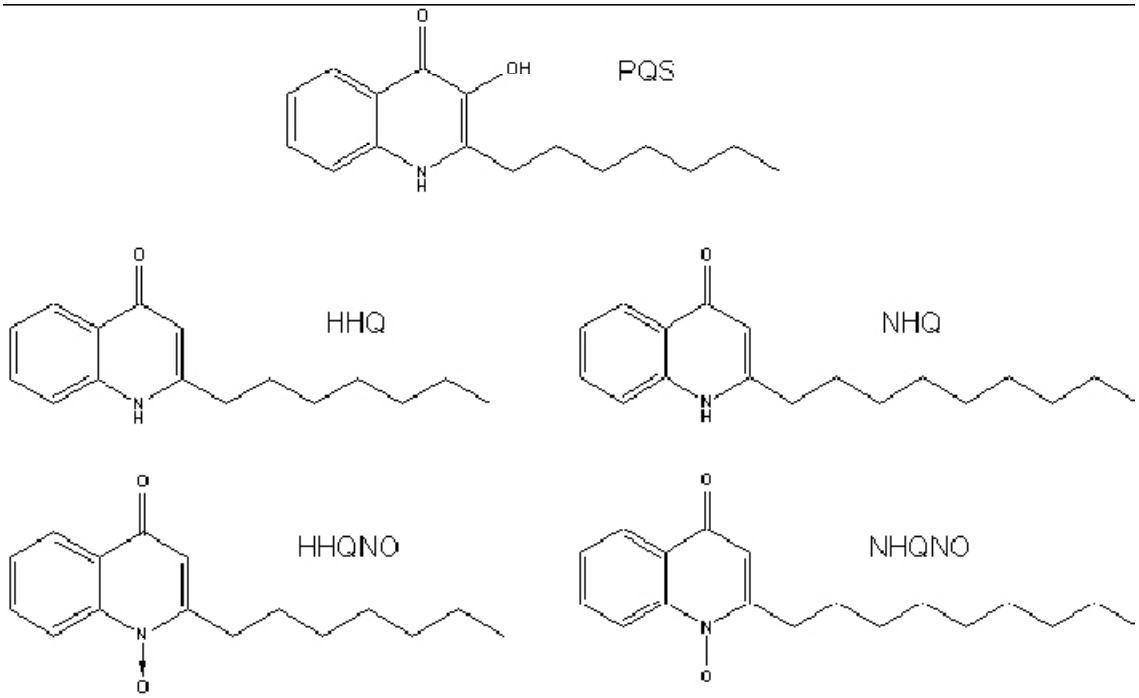

Figure 1.3 Structure of the major AQ molecules synthesized by *P. aeruginosa*- 2-heptyl-3-hydroxy-4-quinolone (PQS), 2-heptyl-4-quinolone (HHQ), 2-nonyl-4-quinolone (NHQ), 2-heptyl-4-quinolone *N*-oxide (HHQNO) and 2-nonyl-4-quinolone *N*-oxide (NHQNO).

1.3 Properties of AQs

AQs have tautomeric properties, with both 2-alkyl-4-quinolone and 4-hydroxy-2-alkyl-quinoline isomers being its possible form depending on the pH. Nonetheless, it has been demonstrated using pK_a values for methyl-PQS , that over the physiological pH ranges (4-6), the neutral 4-quinolone form is the predominant species (Diggle et al., 2007) hence, this is the terminology that will be adopted throughout the report. AQs are lipophilic molecules with a low aqueous solubility. PQS has a solubility of around 1 mg/L in water (Lépine et al., 2003) and due to this hydrophobic nature; a large amount is attached to the cell membrane of the bacteria. *P. aeruginosa* overcomes this obstacle by producing rhamnolipid biosurfactants which increases the solubility of PQS in water. This increased solubility correlates with the ability of PQS to induce *lasB* expression, although too much rhamnolipid is apparently counter-productive, as PQS is possibly sequestered in micelles (Calfee et al., 2005).

AQs were initially thought to be produced primarily during the late log phase. However, using *lasB* expression as an indirect way of monitoring PQS production, negligible amounts were detected in late log phase cultures and maximal production of PQS was detected in late stationary phase after 30-42 h (McKnight et al., 2000). However, two other independent studies on the timing of PQS production, using more sensitive and direct detection methods, have both demonstrated that substantial levels of PQS are produced in late log/ early stationary phase cultures. Using a stable isotope dilution method coupled to mass spectrometry (LCMS), PQS (and HHQNO) production was detected near the end of the log phase and was maximal at the onset of stationary phase, reaching a final concentration of 16 µM (Lepine et al., 2003). A second study using thin layer chromatography (TLC) in conjuction with a synthetic PQS standard estimated that

at the onset of stationary phase, PQS concentrations were approximately 5-10 μM, increasing to 25 μM in late stationary phase (Diggle et al., 2003). It appears that PQS, and presumably other AQs are produced maximally at late log/early stationary phase, but accumulate to higher concentrations later in growth.

1.3.1 Synthesis of AQs

The genes responsible for AQ biosynthesis and the regulation of this biosynthesis were identified in *P. aeruginosa* relatively soon after the discovery of PQS (Cao et al., 2001, Gallagher et al., 2002). This was done somewhat serendipitously by isolation and characterisation of mutants that were defective in the production of the phenazine pigment pyocyanin. It was found that most of these transposon mutants were actually defective in genes involved in the regulation of phenazine biosynthesis, rather than the specific genes responsible for biosynthesis, rather than the specific genes responsible for biosynthesis (*phz*) themselves. These mutants were also PQS-negative, suggesting that they were responsible for PQS biosynthetic functions. Further investigation revealed a five-gene operon under the control of a putative transcriptional regulator. This operon was termed *pqsABCDE* (PQ0996-PA1000) and the transcriptional regulator (PA1003) was re-named *pqsR* (Gallagher et al., 2002).

The *pqs* operon is located on the same chromosomal strand with the exception of *pqsR*, which runs in the opposite orientation. The *pqsABCDE* operon is situated in the proximal 5' position, to which next are located the *phnAB* genes, and next to which lastly, running 3' to 5', resides *pqsR*. The *pqsABCDE* operon forms a polycistronic operon, but with no contiguous run through after *pqsE* (McGrath et al., 2004) Gel shift analysis has shown that *pqsR* binds to the *phnAB* promoter independently of *pqsABCDE*

(Cao et al., 2001). Other *pqs* genes reside on distant parts of the chromosome. Another strain with a mutation in a gene encoding a predicted FAD-dependent mono-oxygenase was found to be negative for PQS production and this gene was termed *pqsH* (PA2587) (Gallagher et al*.,* 2002).

When labelled PQS was added to wild type *P. aeruginosa* culture, no extra peaks were identified, indicating that PQS is either the end product or is not substantially converted to another compound (Déziel et al., 2004), probably via the mono-oxygenase PqsH that is needed for synthesis and a mutant of which does not produce PQS (Gallagher et al., 2002). Surprisingly, PQS can be produced in significant amounts in a *lasR* mutant at higher cell densities (Diggle et al*.,* 2003), suggesting that either production of PqsH can be controlled in a LasR-independent manner or that there are other enzymes which can convert HHQ into PQS. The *pqsA* mutant is unable to make HHQ but is able to utilise any presented to it from an external source to make PQS. Conversely, a *pqsH* mutant is able to make HHQ but is unable to convert it to PQS. Pyocyanin production results when the *pqsA* mutant utilises the HHQ produced by the *pqsH* mutant and converts it into PQS, which then activates expression of the *phz* genes, and showing HHQ is able to act as an extracellular signal. Figure 1.4 shows the proposed mechanism of synthesis of PQS and HHQ.

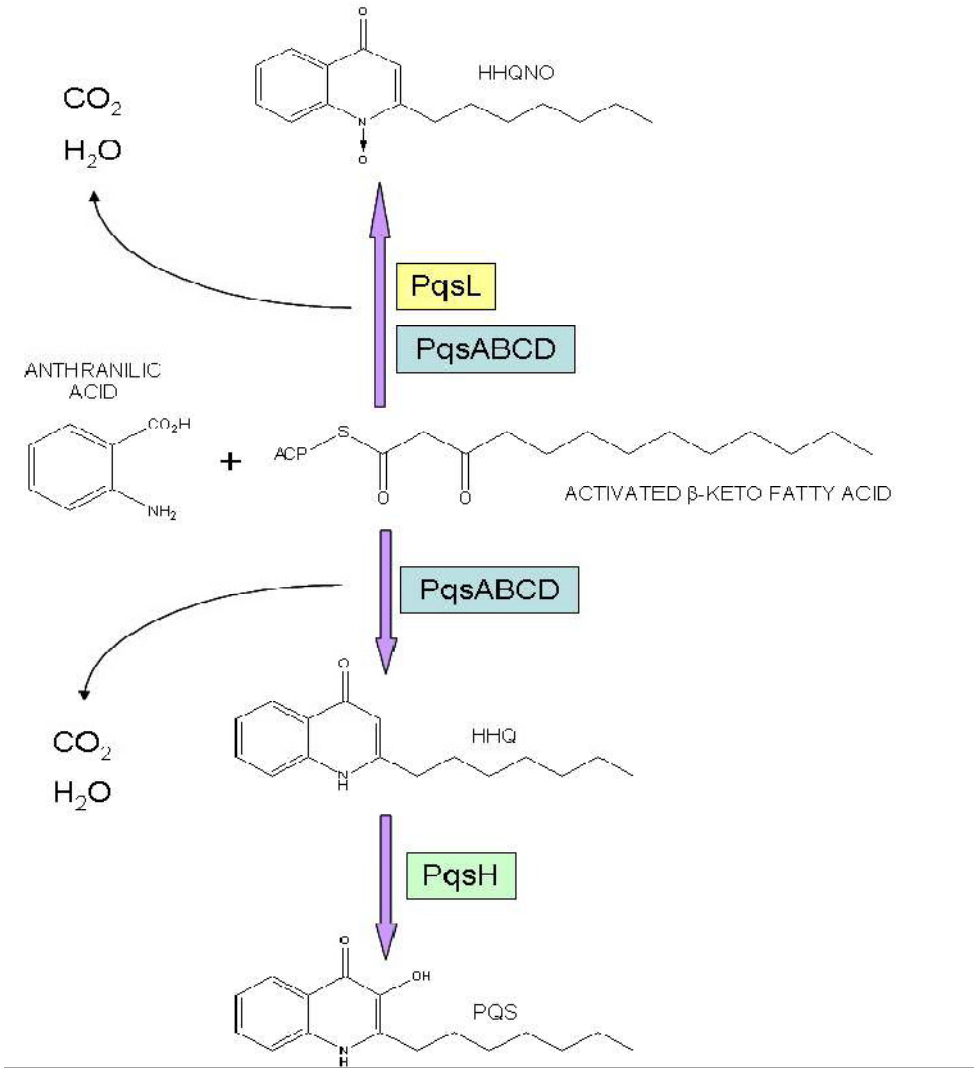

Figure 1.4 Mechanism of synthesis of PQS, HHQ and HHQNO in *P. aeruginosa*. Anthranilate and an activated β-keto fatty acid are condensed via the PqsABCD enzymes to HHQ, releasing CO_2 and water. The monooxygenase PqsH then converts HHQ to PQS. HHQNO is derived from the same starting products as HHQ but utilises the additional oxygenase enzyme PqsL. HHQ is not a precursor for HHQNO. ACP=Acyl Carrier Protein.

1.3.2 Autoregulation of AQs

PqsR is proposed as the transcriptional regulator for the *pqsABCDE* biosynthetic operon (Gallagher et al., 2002). The *PqsR* promoter was mapped by primer extention analysis and this showed that two primary transcriptional start sites exists for *pqsR* 190 and -278 base pairs upstream from the *pqsR* start codon. PqsR is a LysR type regulator, which usually contains a conserved DNA-binding domain without a variable co-inducer binding domain, They typically bind the promoter region without a co-inducer and bind another nearby site with the co-inducer present. A very large decrease of *pqsA* expression in a *pqsR* mutant is evident; therefore *pqsR* is required for *pqsA* transcription (McGrath et al., 2004). *PqsR* binds to the *pqsA* promoter in DNA mobility shift assays in the absence of PQS but when PAO1 extract was added in combination, enhanced binding to the *pqsA* promoter was seen (McGrath et al., 2004). When synthetic PQS was added, binding of PqsR to the promoter was greatly enhanced; therefore PQS is a co-inducer for PqsR. Two bands on the gel were seen, suggesting that PqsR and PqsR-PQS bind to two different locations in the *pqsA* promoter region (Wade et al., 2005).

Interestingly, PQS is seemingly not the only AQ capable of upregulating AQ expression via PqsR. It was revealed that HHQ is also capable of inducing a conformational change in the PqsR protein, and enhances PqsR binding to the *pqsA* promoter *in vitro*, although not as much as that seen by PQS. In a *pqsAH* mutant, PQS was 100 times more potent at activating the expression of *pqsABCDE* than HHQ. This finding was confirmed by another study using a novel AQ bioreporter to show activation of *pqsA* by HHQ in a *pqsAH* mutant (Diggle et al., 2007).

1.3.3 Effects of AQs on gene expression and virulence factor production

It has been previously discussed that a function of PQS is to up-regulate the *rhl* system and co-operatively regulate expression of virulence genes via this pathway. Several studies have demonstrated this effect. In *pqsE* and *pqsR* mutants, pyocyanin (Cao et al., 2001, Gallagher et al., 2002, Diggle et al., 2003), phzA1 (Deziel et al., 2005), lectin (Diggle *et al.,* 2003), elastase and rhamnolipids productions were considerably reduced compared to the wild type (Cao et al., 2001; Diggle et al., 2003). Addition of PQS to PAO1 at concentrations greater than 100 μM caused an extended lag phase and reduced stationary phase optical densities, however, the onset of expression of *lecA* was enabled at lower cell densities and therefore maximal *lecA* expression was always at the onset of stationary phase. These effects were not seen with HHQ or formyl-HHQ (Diggle et al*.,* 2003). PQS was however able to both advance and enhance elastase and pyocyanin production into the logarithmic phase. Addition of PQS could not restore *lecA* in an *rhlR* or *rpoS* mutant. PQS was also able to overcome the repression of *lecA* by *MvaT* and *RsmA* and increased *lecA* expression in mutants overexpressing these repressors (Diggle et al., 2003). Whole genome expression studies have examined the subset of genes regulated by the AQ system (Déziel et al., 2005). A *pqsR* mutant displayed 22 genes whose expression had been repressed by *pqsR*, with another 121 genes whose expression was enhanced by *pqsR* compared to the wild type. Expression of the *pqs* and *phn* operons was abolished and that of *pqsR* reduced. These studies show that AQs are therefore important for both full expression of *P. aeruginosa* virulence and the normal regulation of the genome. Interestingly, PQS has been suggested to balance life and death in *P. aeruginosa* populations. This was carried out by inducing a protective response in some cells while eliminating the damaged cells via pro-and antioxidant activities (Häussler and Becker, 2008).

1.3.4 The role of AQs in infection

P. aeruginosa is a major source of nosocomial infections and lung infections in patients with CF amongst others (Govan and Deretic, 1996). A role for AQs in establishing infection, regulating virulence factors and enhancing the severity of infection has been implicated by several studies. Clinical isolates from CF patients have been shown to produce HHQNO, NHQNO and UHQNO plus their mono-unsaturated derivatives, HHQ (Machan et al., 1992) and also PQS (Collier et al., 2002). Levels of PQS correlated with population density of the sample. PQS was also found in isolates from infants with CF and early stage *Pseudomonas* infection (Guina et al., 2003) and it has been shown that CF sputum is a good growth medium for *P. aeruginosa* and supports high population densities (Palmer *et al.,* 2005). It is likely that AQs are at least partly utilised as antibiotics in early infection to wipe out any competing strains as AQs inhibited the growh of *S. aureus* and *C. albicans* (Machan et al., 1992). Genes responsible for production of pyocyanin and hydrogen cyanide, also capable of acting as antibiotics, were expressed at a higher level in CF medium (Palmer et al., 2005). These findings may explain the absence of many competing species of bacteria such as *S. aureus* in the lungs of CF patients colonised by *P. aeruginosa* after around three to four years of age due to *P. aeruginosa* establishing infection, out competing and wiping out other co-habiting organisms. Coupled with their iron chelating properties and effects on virulence factor expression, it is likely that AQs contribute to providing *P. aeruginosa* with a highly favourable environment in which to grow in the CF lung.

The importance of the AQs for regulation of virulence and contributing to infection severity has also been demonstrated. A *phnAB* mutant was 4-fold less virulent than the wild type in a moth larvae (*G. Mellonella*) model (Jander et al., 2000). *pqsA* and *pqsE*

mutants also have reduced virulence in mice with mortality rates similar to that of the *pqsR* mutant (Déziel et al., 2005). In the lung, *P. aeruginosa* forms a biofilm which protects the bacterial cels from adverse environmental conditions including the host immune system and antimicrobial agents. AQs have been shown to be important in maintaining and establishing these biofilms. Addition of 60 µM exogenous PQS to a growing culture of PAO1 enhanced biofilm development, possibly due to an increased effect on lectin production (Diggle et al., 2003).

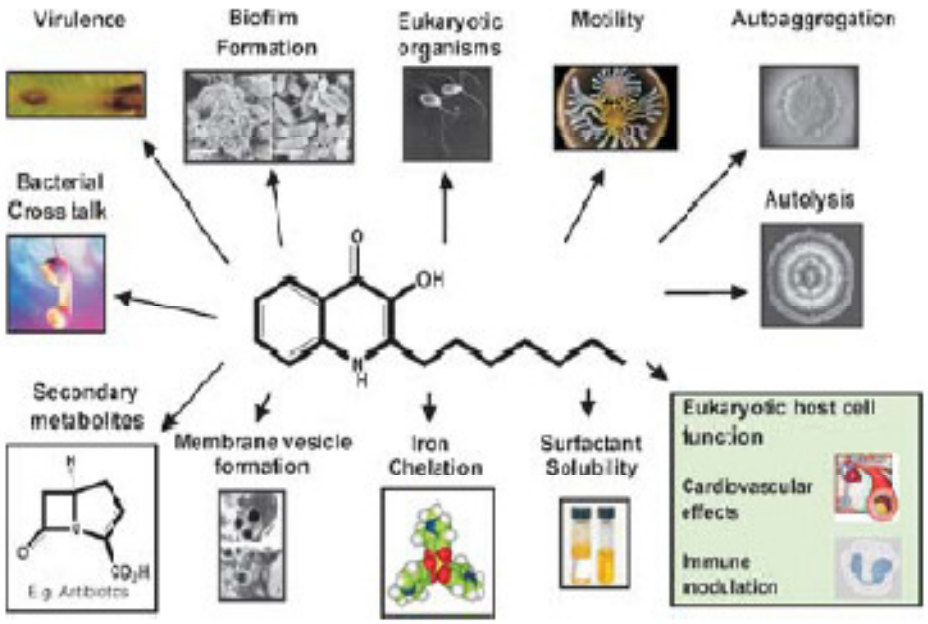

Figure 1.5 Phenotypes regulated by PQS in *P. aeruginosa* (Dubern and Diggle, 2008)

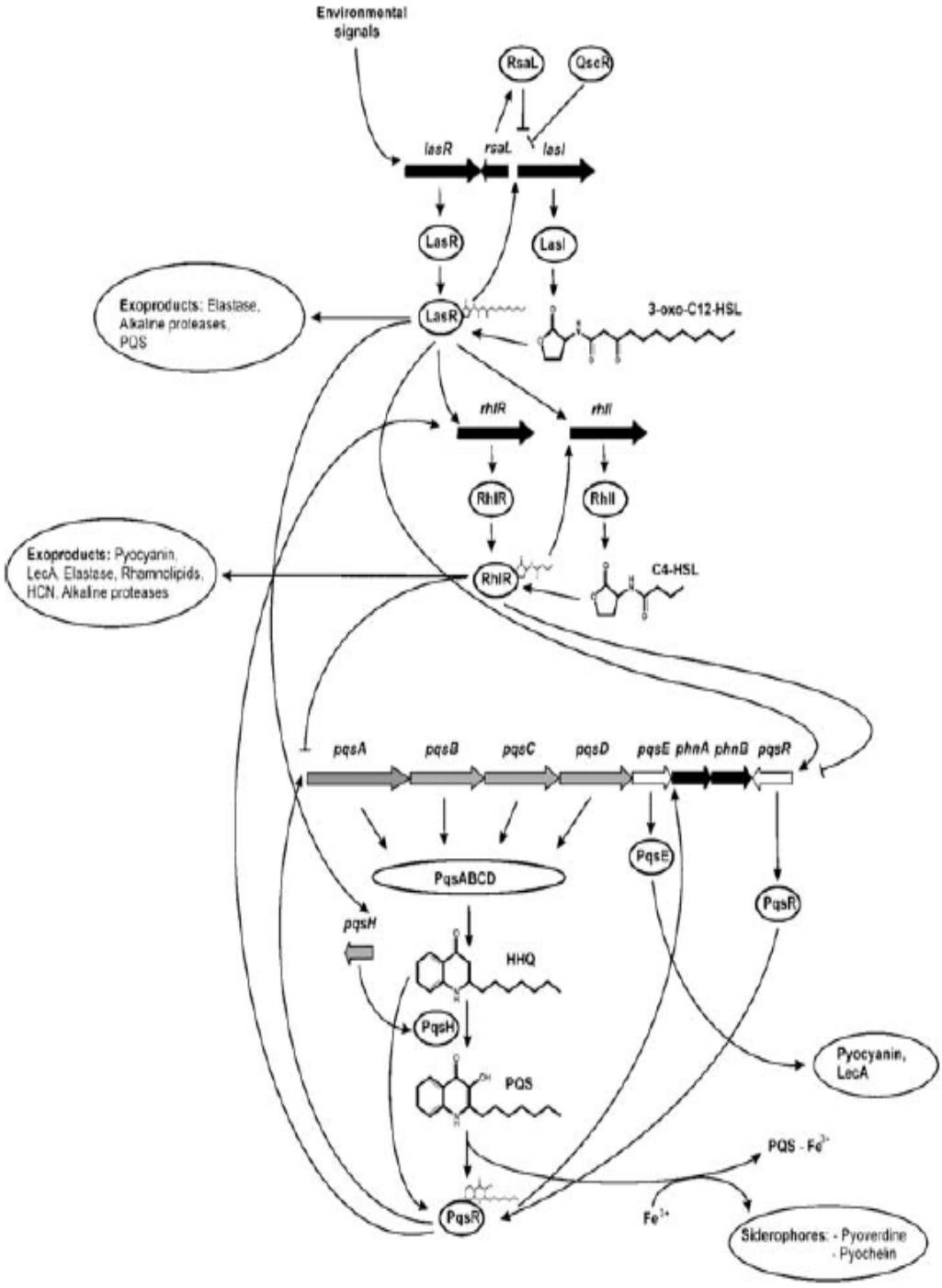

Figure 1.6 AHQ and AHL-dependent quorum sensing in *P. aeruginosa*. LasR/3-oxo-C12-HSL is required for full expression of *pqsH*, while *pqsR* is positively regulated by LasR/3-oxo-C12-HSL. Both pqsA and pqsR are repressed by the RhlR-C4-HSL system. Both PQS and HHQ induce the expression of pqsA in a PqsR-dependent manner. ⊣ Represent positive regulation; → represent negative regulation. (Dubern and Diggle, 2008)

1.4 Quorum Quenching

The use of antibiotics in the active control and prevention of microbial diseases have been used extensively since its discovery in the 1920s. Antibiotics are usually bactericidal or bacteriostatic, thus imposing selective pressure on pathogens. This gives rise to the emergence of multidrug resistant bacteria, also known as 'superbugs'. QS is essential for synchronizing microbial activities of many pathogenic strains, particularly in the regulation of infection-related traits. Hence, quenching microbial quorum sensing (termed quorum quenching) appears to be a promising disease control strategy. Quorum quenching can occur via three main strategies. First of which is the prevention of QS signal production. Secondly, by inactivating the signalling molecule and the third strategy is by means of interfering with the signal receptor.

1.4.1 Prevention of QS signal production

There are several substrate analogues, highlighted by Parsek *et al*., 1999, which includes the holo-ACP, L/D-S-adenosylhomocysteine, sinefungin and butyryl-*S*-adenosylmethionine (butyryl-SAM), that have been discovered to have the ability to block AHL signal production *in vitro*. Moreover, an 3-oxo-C12-HSL analogue, 3-oxo-C12-(2-aminocyclohexanone) was shown to inhibit both the lasI and rhlI AHL synthases in *P. aeruginosa* (Smith et al., 2003) . In the PQS signalling system of *P. aeruginosa*, it has been demonstrated that increasing concentrations of methyl anthranilate caused a concentration-dependent decrease in PQS production as well as elastase activity in a wildtype strain (Calfee et al., 2001). A more recent publication

reported on the use of halogenated anthranilate analogs that inhibited the synthesis of HHQ and PQS, thus restricting *P. aeruginosa* systemic dissemination and mortality in mice without affecting bacterial viability (Lesic et al., 2007). This proved that a compound that is an analog of a precursor for a QS signal can inhibit the synthesis of the signal molecule, leading to the decreased expression of a virulence factor controlled by the signal molecule.

1.4.2 Inactivation of signal molecules

The second strategy for quorum quenching involves the inactivation or degradation of the generated signal molecules. This impairs QS by decreasing the active signal molecule concentration in the environment. QS signal decay can occur via nonenzymatic chemical degradation, enzymic destruction or metabolism of the signalling molecule. Lactonolysis, also known as the ring opening of AHL signal molecules due to high pH is a classic example of chemical intervention of the signal through alkaline hydrolisis (Yates *et al*., 2002; Byers *et al*., 2002). Lactonolysis of AHLs can also be carried out by the AiiA enzyme, which has been found to be produced by members of the genus *Bacillus*, namely *B. cereus*, *B. mycoides* and *B. thuringiensis*. This enzyme is effective in decreasing the amount of bioactive AHL signal molecules by catalyzing the hydrolysis of AHL molecules (Dong et al., 2000, Wang and Leadbetter, 2005, Lee et al., 2002, Dong et al., 2001). The expression of *aiiA* in transgenic tobacco plants made them much less vulnerable to infection by *Erwinia carotovora* compared to their wildtype counterparts, indicating that enzymatic degradation of AHLs can be a useful means of biocontrol. There are several other species of bacteria that have been found to produce AiiA homologues, such as *P.*

aeruginosa, *Arthrobacter sp., Klebsiela pneumoniae*, *Ag. tumefaciens* and *Rhodococcus* sp. (Uroz et al., 2003, Carlier et al., 2003, Park et al., 2003, Huang et al., 2003). In other studies, both *Variovorax paradoxus* and *P. aeruginosa* PAI-A were shown to be able to metabolize AHLs as sole source of energy, and nitrogen. These bacteria produce an amino acylase which cleaves the peptide bond of the signal molecule and utilizing the side chain as a carbon source. The nitrogen from the amide bond is made available as ammonium by the lactonases whilst the ring part is used as the energy donor (Huang et al., 2003, Leadbetter and Greenberg, 2000).

1.4.3 Interference with the signal receptor

The third quorum quenching strategy is via the prevention of the signal from being perceived by the bacteria, either by blockage or destruction of the receptor protein. One particularly widely explored method is to block the receptor with an AHL signal molecule analogue. QSIs of the third strategy have been identified by screening of random compound libraries. Rasmussen *et al*., (2005) identified several QSI compounds with structures unrelated to the signal molecules, including 4-nitro-pyridine-*N*-oxide (4-NPO), indole, *p*-benzoquinone, 2,4,5-tribromoimidazole, indole and 3-nitrobenzene sulphone amide, with the first being the most effective. 4-NPO was found to block the *P. aeruginosa* established las*B-gfp* fusion. The much-investigated group of QSIs from a natural source would be the halogenated furanone compounds produced by the macroalga *D. Pulchra* (Givskov *et al*., 1996). These molecules are secreted to the surface of the fronds and have the ability to prevent bacterial colonization, and thereby macrofouling due to the interference with QS-controlled motility.

Table 1.2 General steps and key components of AHL-type QS systems as well as the prospective quorum-quenching strategy (Dong et al., 2007).

Quorum sensing process		Key component	Prospective quorum-quenching strategy
Low-population density	1. Basal signal generation	Proteins and enzymes involed in biosynthesis of acyl chain and *S*-adenosylmethionine (SAM); LuxI-type (I) protein	Fatty acid biosynthesis inhibitor; SAM biosynthesis inhibitor, I protein inhibitor
	2. Signal accumulation	Proteins involved in long-chain signal active efflux	AHL signal degradation enzyme; active efflux inhibitor
High-population density	3. Signal reception	LuxR-type (R) transcription factor; putative influx system for long-chain AHL signal	R protein inhibitor; influx inhibitor
	4. Autoinduction and activation of quorum sensing regulon	R and I proteins involved in boosted AHL signal production; quorum-sensing-dependent transcription factors	AHL signal degradation enzyme; inhibitors for I and R proteins
	5. Signal decay	AHL degradation enzyme and its regulatory mechanisms	Chemical inducing early expression of AHL degradation enzyme

1.5 Project aims

Given the existence of a complex quorum sensing hierarchy that encompasses AHL and AQ signalling in *P. aeruginosa*, quenching of the AQ signalling system would be a helpful supplement in the development of new treatment strategies.

Specifically, the project aims to:

1. Enrich and isolate PQS degrading bacteria from soil samples collected from Rimba Ilmu (University of Malaya, Malaysia).

2. Develop a biosensor for the screening of PQS quorum sensing inhibitors.