GENETIC REGULATION OF QUORUM SENSING IN *PSEUDOMONAS AERUGINOSA*

SEET QIHUI

(B.Sc. (Hons), Imperial College)

A THESIS SUBMITTED

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

NUS GRADUATE SCHOOL FOR INTEGRATIVE SCIENCES

AND ENGINEERING

NATIONAL UNIVERSITY OF SINGAPORE

2010

Acknowledgements

I would like to thank Prof. Lian-Hui Zhang for giving me the opportunity to carry out my graduate training in his lab and for his guidance in all aspects of research.

Many thanks to Dr. Yi Hu Dong and Dr. Chao Wang for their critical and helpful suggestions and advice.

I greatly appreciate the help given by Dr. Portia Loh and other members from Assoc. Prof. Song Hai Wei's lab for protein purification.

I am also very thankful to all past and present members of Zhang Lian-Hui's lab who have made these last 5 years a memorable experience.

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Summary

In human pathogen *Pseudomonas aeruginosa*, quorum sensing (QS) is crucial in regulating the expression of a large number of genes, especially those encoding virulence factors. Consequently, there is a need to understand how QS is regulated. QS systems in *P. aeruginosa* consist of the Las and Rhl systems and these two systems are also linked to *Pseudomonas aeruginosa* quinolone signal (PQS) signalling systems. It has already been reported that QS is regulated at the transcriptional and post-transcriptional level by many factors, but how QS threshold is modulated remains obscure. In this study, a genetic screen of transposon mutants with changed QS phenotypes was carried out and this led to the discovery of anti-activators QslA and QslH.

In *qslA* null mutant, there was enhanced PQS signalling and greater production of virulence factors compared to wild type. Conversely, overexpression of QslA abolished QS, PQS signalling and virulence factor production. QslA was determined to inhibit LasR post-transcriptionally and it was found using co-immunoprecipitation analysis that QslA inhibited QS by protein-protein interaction with LasR. Electrophoretic mobility shift analysis (EMSA) analysis showed that QslA disrupted LasR activation of gene expression by impeding LasR binding to DNA.

In addition to its control of QS response, QslA also influenced the QS activation threshold. In *qslA* mutant, 9 times less QS signals was sufficient to activate QS-dependent virulence factor production. This finding indicates that QslA is responsible for raising the QS “threshold hurdle” so that QS is activated at a high QS threshold concentration or a high bacterial cell density.

In the same transposon mutant screen, another anti-activator named QslH, was also identified. When *qslH* was overexpressed, QS and PQS signalling systems as well as virulence factor production were inhibited. Using co-

immunoprecipitation analysis, QslH was found to interact with LasR and PqsR. QslH interaction with LasR was verified by studying LasR activity in *Escherichia coli* and by EMSA. Results from bacterial two-hybrid analysis also confirmed that QslH interacts with PqsR.

Null mutant of *qslH* did not differ from the wild type in its QS and PQS signalling phenotype. Thus, QslH inhibited QS and PQS signalling only when overexpressed. Hence, it was hypothesized that QslH was not expressed at adequately high levels to affect QS and PQS signalling in the wild type under the experimental conditions used in this study. Transposon mutagenesis was carried out and it was found that *qslH* expression was increased in the absence of *mvaT*. In *mvaT* mutant, enhanced level of QslH inhibited production of virulence factors. Taken together, the results demonstrate that QslH plays a role in QS regulation at the downstream of MvaT-dependent regulatory networks.

Identification of QslA and QslH demonstrates that QS activation in *P. aeruginosa* is modulated by protein-protein interaction. The results from this study present a further understanding of the sophisticated molecular mechanisms of bacterial QS signalling systems.

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Chapter 1 – Introduction

1.1 *Pseudomonas aeruginosa* is a clinically important human pathogen

Pseudomonas aeruginosa is a Gram-negative bacterium from the γ subclass of proteobacterium. It is a ubiquitous opportunistic pathogen that is adapted to highly variable environmental conditions in soil, marshes, coastal marine habitats, plants, and mammalian tissue. It has a relatively large genome size of 6.3 Mb made up of 5570 open reading frames in which a significant percentage is dedicated to regulatory genes that enable the bacterium to optimise its gene expression for survival (Stover *et al.*, 2000). *P. aeruginosa* isolates are found in opportunistic infections in hospitals (Van Delden & Iglewski, 1998) as well as in chronically infected cystic fibrosis (CF) patients, where *P. aeruginosa* significantly increases morbidity and mortality (Frederiksen *et al.*, 1997, Murray *et al.*, 2007). Therefore, it is imperative that treatment options are available for such infections. Infection by *P. aeruginosa*, however, is not easily treated (Anderson & O'Toole, 2008), and this is mainly due to occurrence of antibiotic resistance strains (Pitt *et al.*, 2003) and antibiotic tolerance of biofilm-embedded bacteria (Costerton *et al.*, 1999). Hence, alternative treatment targeting bacterial virulence has been suggested (Bjarnsholt & Givskov, 2007).

1.2 Quorum sensing controls *P. aeruginosa* virulence

Virulence of *P. aeruginosa* is mediated by multiple factors including secreted enzymes and toxins as well as more complex type III and type VI secretion systems. These factors clear the way for *P. aeruginosa* invasion and also help in hindering host immune defence system. Elastase and protease, for example, can disrupt the complement system (Kharazmi, 1991). Pyocyanin disrupts neutrophil-mediated immune response (Allen *et al.*, 2005) and host cellular respiration (Lau *et*

al., 2004). Type III and type VI secretion systems are implicated in acute and chronic infections, respectively, and they function by forming “needle-like machines” on eukaryotic host membranes (Hauser, 2009, Hood *et al.*, 2010). Bacterial effector proteins that are released directly into host tissue and immune cells by these secretion systems compromise the host functions (Hauser, 2009, Hood *et al.*, 2010).

Many of these virulence mechanisms are controlled chiefly by acyl-homoserine lactone (AHL) dependent quorum sensing (QS). QS is a form of bacterial cell-cell communication that positively regulates type VI secretion as well as the production of virulence factors such as elastase, alkaline protease, exotoxin A, hydrogen cyanide and pyocyanin as well as (Gambello & Iglewski, 1991, Gambello *et al.*, 1993, Latifi *et al.*, 1995, Lesic *et al.*, 2009, Singh *et al.*, 2010). Type III secretion system, on the other hand, is negatively regulated by QS (Hogardt *et al.*, 2004, Bleves *et al.*, 2005). *P. aeruginosa* quinoline signal (PQS) system is linked to QS and this system, together with Rhl QS system, controls production of virulence factors such as pyocyanin, elastase and PA-IL lectin (Pesci *et al.*, 1999, Diggle *et al.*, 2003).

QS also play a possible role in biofilm formation. It was observed that when QS was disrupted in *P. aeruginosa*, biofilm formed was flat, undifferentiated and susceptible to SDS treatment, unlike the structured biofilm of wild type which contains mushroom-like features (Davies *et al.*, 1998). PQS signalling contributes to *P. aeruginosa* biofilm structure as well by controlling release of DNA, which is a biofilm constitutent (Yang *et al.*, 2009). However, the role of QS in activation of biofilm formation is dependent on nutritional and hydrodynamic conditions, and QS is not involved in modulating formation of biofilm when bacteria are grown using glucose and glutamate as sole carbon source, or when bacteria are grown under

turbulent flow where signalling molecules are unable to accumulate (Purevdorj *et al.*, 2002, Shrout *et al.*, 2006).

The importance of QS and PQS systems during pathogenesis was also demonstrated using various infection models. PQS was present in the lungs of *P. aeruginosa* infected CF patients, suggesting that PQS signalling pathway is functional during host infection (Collier *et al.*, 2002). The importance of PQS signalling system in virulence was demonstrated using *Caenorhabiditis elegans, Arabidopsis* and mice models (Cao *et al.*, 2001, Gallagher & Manoil, 2001). The importance of QS in the pathogenesis of *P. aeruginosa* is also verified by studies using infection models, including that of *C. elegans* and mice, which have shown that inhibition of AHL-dependent QS reduced *P. aeruginosa* virulence (Preston *et al.*, 1997, Rumbaugh *et al.*, 1999, Pearson *et al.*, 2000, Wu *et al.*, 2001). Because of the importance of QS in regulation of virulence in *P. aeruginosa*, QS has been studied extensively.

1.3 QS systems in bacteria

1.3.1 Discovery of QS system in *Vibrio fischeri*

The model of QS is formulated based on work done in marine bacterium *Vibrio fischeri*. It was discovered that bioluminescence in *V. fischeri*, which is only activated at critical bacterial population density (quorum), could also be activated at low population density by addition of cell-free supernatant from high cell density cultures (Nealson *et al.*, 1970). The diffusible QS signal molecule (autoinducer) responsible for activation of bioluminescence was subsequently identified as N-3-oxohexanoyl-homoserine lactone (Eberhard *et al.*, 1981) and high concentration of this QS molecule is achieved at high cell density and can activate QS-regulated bioluminescence in *V. fischeri*. QS was later established to be a signal-dependent

mechanism for cell-density dependent gene regulation (Fuqua *et al.*, 1994, Fuqua *et al.*, 1996, Lazdunski *et al.*, 2004).

1.3.2 QS mechanism

The types of autoinducers used for QS vary widely among bacteria. In many Gram-negative bacteria, QS is mediated by acyl-homoserine lactone (AHL) while oligopeptides are more common in Gram-positive bacteria. For AHL-dependent QS systems, QS molecules are typically produced by LuxI-type synthases and released into the extracellular environment. QS molecules are continually synthesized along with bacterial proliferation and would accumulate as bacterial cell density increases. Gradual diffusion of these molecules back into the cells occurs and QS system is activated when a threshold concentration of QS molecule is reached. These molecules then bind to their cognate LuxR-type regulator, which would activate the expression of QS-regulated processes and sometimes also *luxI* to generate a positive feedback response (autoinduction). AHL molecules produced in general are specific to individual bacterial species and they differ in their acyl side-chain length, saturation and substituent groups.

QS allows the bacterial population to synchronise its activation of processes that are advantageous when executed in large numbers. Group behaviours such as pathogenesis in *P. aeruginosa* (Smith & Iglewski, 2003), bioluminescence in *V. fischeri* (Nealson *et al.*, 1970), plasmid conjugation in plant pathogen *Agrobacterium tumefaciens* (Zhang *et al.*, 1993) and symbiosis in *Rhizobium* species (Gonzalez & Marketon, 2003) are known to be regulated by QS.

1.3.3 Role of anti-activators in QS

In the classical QS model, activation of QS depends simply on the regulator and the accumulation of QS signals. The discovery that anti-activators, which are

proteins that interact with LuxR-type regulators, can influence QS activation reveals that additional factors are involved in the control of QS activation. TraM-type QS anti-activators have been identified in *A. tumefaciens* as well as *Rhizobium*, and in *A. tumefaciens*, LuxR-type regulator TraR activation of QS is inhibited by one or few anti-activators, including TraM, TraM2 and TrlR, in the absence of conjugal opines (Piper & Farrand, 2000, Chai *et al.*, 2001, Wang *et al.*, 2006). But in the presence of opines, TraR protein expression is induced and the increased TraR protein level counteracts inhibition by anti-activators (Hwang *et al.*, 1995, Oger *et al.*, 1998, Zhu & Winans, 1998, Wang *et al.*, 2006). As a result, QS threshold is dependent on the availability of conjugative opines through the action of these anti-activators.

Among these known QS anti-activators, TrlR is a truncated version of TraR without the C-terminal DNA-binding domain due to a frameshift mutation and TrlR dimerises with TraR to form an inactive protein complex (Chai *et al.*, 2001). TraM and TraM2, on the other hand, are *Agrobacterium* species-specific 11 kDa proteins with no homology to LuxR-type regulators (Hwang *et al.*, 1995, Wang *et al.*, 2006). Based on mutational analysis of TraR (Hwang *et al.*, 1999, Luo *et al.*, 2000, Swiderska *et al.*, 2001, Qin *et al.*, 2007) and proteolytic fingerprint of TraR-TraM complex (Qin *et al.*, 2007), it was predicted that TraM interacts with TraR at a region adjacent to TraR DNA-binding domain which results in a change of TraR conformation and loss of its DNA-binding ability (Chen *et al.*, 2007, Qin *et al.*, 2007).

1.4 QS in *Pseudomonas aeruginosa*

1.4.1 QS in *P. aeruginosa* consists of the Las and Rhl systems

In *P. aeruginosa*, QS is coordinated independently by the Las and Rhl systems (Fig. 1.1). LasI and RhlI synthases produce N-3-oxododecanoyl-homoserine lactone (3-oxo-C12-HSL) (Pearson *et al.*, 1994) and N-butanoyl-homoserine lactone (C4-HSL), respectively. These synthases generate the homoserine lactone ring from S-adenosyl-methionine (SAM) and link it to fatty acyl chains on acyl-acyl carrier proteins (More *et al.*, 1996, Parsek *et al.*, 1999). 3-oxo-C12-HSL and C4-HSL differ in the number of carbons on the fatty acyl side chains, and 3-oxo-C12-HSL has an oxo group substitution at position C3 on the acyl side chain. The carbon chain length of the autoinducers synthesized by LasI and RhlI is determined by the synthase acyl-chain binding pocket which can restrict the acyl-chain length of the substrate, thus ensuring only certain signals are synthesized (Gould *et al.*, 2004).

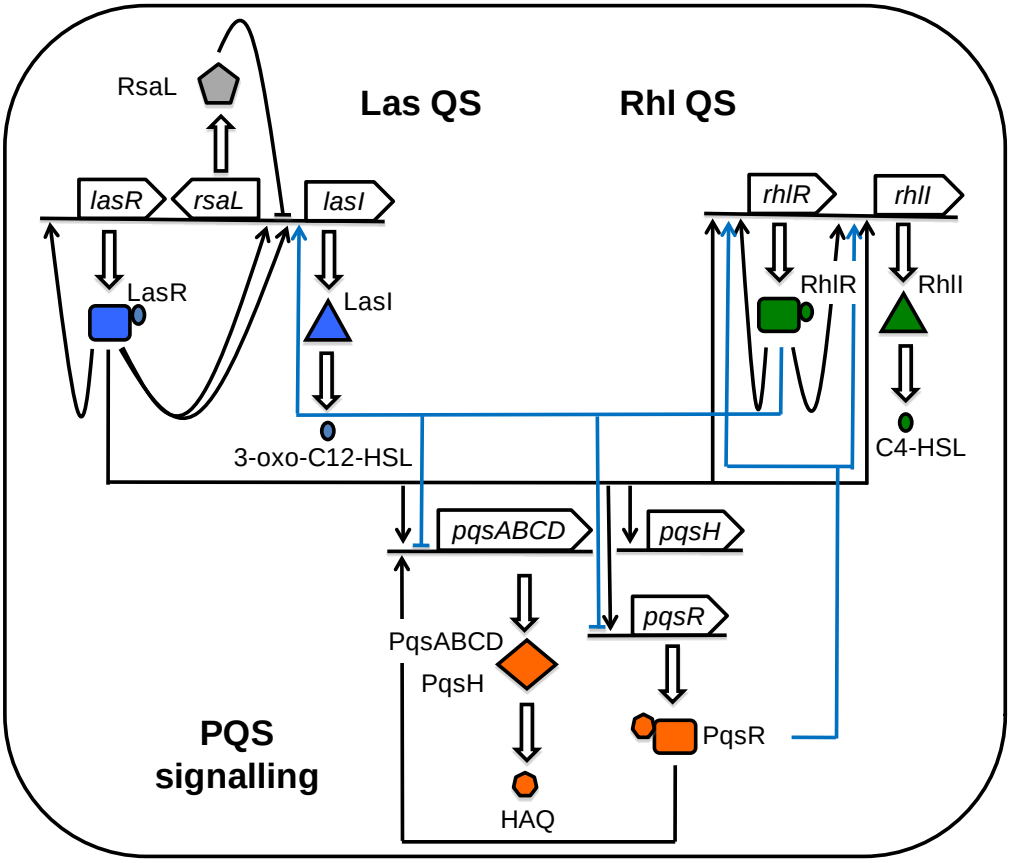


Fig. 1.1 QS and PQS signalling in *P. aeruginosa*.

Diagram showing the Las and Rhl QS and PQS signalling system network in *P. aeruginosa*. Open arrow indicates biosynthesis of proteins or signalling molecules, black and blue solid arrow indicate regulatory controls of the signalling components during growth of bacteria in LB medium either at exponential phase or at stationary phase respectively. The model is revised and updated based on previous publications (Ledgham *et al.*, 2003, Venturi, 2006, Gilbert *et al.*, 2009, Williams & Camara, 2009).

3-oxo-C12-HSL is actively transported out of cells by the MexAB-OprM multi-drug efflux pump but its uptake is not dependent on active transport and it is unclear how 3-oxo-C12-HSL diffuses back into cells (Pearson *et al.*, 1999). On the contrary, C4-HSL is freely permeable (Pearson *et al.*, 1999). 3-oxo-C12-HSL and C4-HSL, respectively, activate their cognate transcriptional regulators LasR (Gambello & Iglewski, 1991) and RhlR (Latifi *et al.*, 1995, Pearson *et al.*, 1995) at threshold concentrations. In this auto-induction loop, QS is activated after diffusion of autoinducers 3-oxo-C12-HSL and C4-HSL back into the same bacterial species (Seed *et al.*, 1995, Latifi *et al.*, 1996). Las and Rhl QS systems are not compatible, so C4-HSL synthesized by RhlI does not activate LasR (Gray *et al.*, 1994, Passador *et al.*, 1996) and similarly, 3-oxo-C12-HSL synthesized by LasI does not activate RhlR (Pearson *et al.*, 1995, Pearson *et al.*, 1997). 3-oxo-C12-HSL binds to LasR with a regulator : autoinducer stoichiometric ratio of 1:1 (Schuster *et al.*, 2004).

In the presence of 3-oxo-C12-HSL, activated LasR dimerises and binds to the promoters of target genes (Kiratisin *et al.*, 2002, Schuster *et al.*, 2004), while RhlR dimerises and binds to DNA both in the presence and absence of BHL (Medina *et al.*, 2003b, Ventre *et al.*, 2003). In the absence of C4-HSL, RhlR binds to the promoter of *lasB* (Anderson *et al.*, 1999) and *rhlR* (Medina *et al.*, 2003a) in a conformation that represses their expression.

1.4.2 Positive feedback and homeostasis control of QS

3-oxo-C12-HSL/LasR and C4-HSL/RhlR complexes, respectively, auto-regulate the expression of 3-oxo-C12-HSL synthesis gene *lasI* and C4-HSL synthesis gene *rhlI* (Passador *et al.*, 1993, Pearson *et al.*, 1994). Las and Rhl QS systems are organised in a hierarchy under growth in rich medium, whereby earlier production of 3-oxo-C12-HSL activates LasR and 3-oxo-C12-HSL/LasR complex then stimulates the expression of *rhlI* and *rhlR* (Pesci *et al.*, 1997, de Kievit *et al.*,

2002). RhlR also induces *rhlI* expression but LasR has a dominant regulatory role (de Kievit *et al.*, 2002). The control of Rhl QS system by LasR is, however, dependent on growth and environmental conditions. During stationary phase growth or growth in phosphate-limited medium, Rhl QS is induced independent of Las QS system (Medina *et al.*, 2003a, Dekimpe & Deziel, 2009). RhlR is also able to activate expression of LasR-dependent genes such as *lasI* (Dekimpe & Deziel, 2009).

When optimal QS response is reached, a homeostatic mechanism mediated by RsaL is triggered to limit 3-oxo-C12-HSL synthesis (Rampioni *et al.*, 2007b), and in the absence of *rsaL*, there is unchecked production of 3-oxo-C12-HSL (Rampioni *et al.*, 2006). RsaL also controls the expression of genes in a QS-independent manner and it modulates the secretion of virulence factors and motility, biofilm formation and antibiotics sensitivity (Rampioni *et al.*, 2007b, Rampioni *et al.*, 2009). RsaL, which belongs to the tetrahelical superclass of HTH proteins, limits 3-oxo-C12-HSL synthesis by binding to *lasI* promoter at a distinct binding site from LasR and inhibiting *lasI* transcription even when LasR is bound to the promoter (de Kievit *et al.*, 1999, Rampioni *et al.*, 2007a, Rampioni *et al.*, 2007b). The *rsaL* gene is located between *lasI* and *lasR* genes in the reverse orientation and it shares the same promoter region as *lasI* (de Kievit *et al.*, 1999). LasR activates the expression of both *lasI* and *rsaL* from this bi-directional promoter and in this way, LasR activates *lasI* expression directly and at the same time, it also indirectly suppresses *lasI* expression through induction of *rsaL* expression. The positive and negative regulation of *lasI* by LasR is balanced to allow fine-tuning of *lasI* expression.

1.4.3 Global regulation of gene expression by QS

A global view of the genes that are regulated by QS in *P. aeruginosa* was carried out using microarray analysis and proteomic analysis. According to results

from microarray studies, around 2.9 – 11.1% of genes in *P. aeruginosa* PAO1 genome are QS-dependent (Hentzer *et al.*, 2003, Schuster *et al.*, 2003, Wagner *et al.*, 2003). The deviations of QS-dependent genes observed in the different studies are likely due to the different growth conditions used. QS-dependent genes are either separately activated by only the Las QS system or Rhl QS system, or are induced by both Las and Rhl QS systems. Since *rhlR* expression is dependent on LasR, the genes that are regulated by both Las and Rhl QS systems could be either co-regulated by LasR and RhlR or just regulated by RhlR. A significant proportion of the genes regulated by QS are those which encode for secreted products and this is in agreement with findings from proteome analysis of extracellular proteins regulated by QS (Nouwens *et al.*, 2003), where 13 proteins including the secreted virulence factors LasB elastase and LasA protease are found to be greatly induced by QS (Nouwens *et al.*, 2003).

1.4.4 Recognition of promoters by LasR and RhlR

The promoters of genes that are directly regulated by LasR were analysed and this led to the identification of a specific LasR-binding DNA consensus sequence called *las* boxes (CT-N_{12}-AG motif) (Whiteley *et al.*, 1999, Schuster *et al.*, 2003, Wagner *et al.*, 2003, Gilbert *et al.*, 2009). Binding of LasR to the promoters can occur in a cooperative or non-cooperative manner, depending on the promoters. Cooperative interaction is likely due to binding of protein to low affinity sites which have no sequence homology with the consensus sequence (Strauch, 1995) since there were no obvious sequence differences in the promoters which exhibit these two types of DNA-binding (Gilbert *et al.*, 2009). A small fraction of the genes (7 – 22%) identified in the microarray have *las* boxes in their promoter (Schuster *et al.*, 2003, Wagner *et al.*, 2003), suggesting that expression of most of the QS-dependent genes were indirectly regulated by LasR. Only genes which are

activated, but not repressed, by LasR are found among the genes which are directly regulated by LasR in the LasR chromatin immunoprecipitation study (Gilbert *et al.*, 2009). This suggests that LasR acts only as an activator.

The consensus sequence for RhlR is not as well characterised as that of LasR but it is predicted that RhlR also recognises las box sites (Schuster *et al.*, 2004). This is due to the fact that RhlR binds to the *las* box in *rhlAB* promoter (Medina *et al.*, 2003b) and that it activates the expression of LasR-regulated genes such as *lasI*, *lasA* and *lasB* (Dekimpe & Deziel, 2009). Nonetheless, there might be distinctive characteristics in the DNA-binding affinities of LasR and RhlR because genes such as *lasB* and *rhlAB* operon that are co-regulated by both regulators showed preferential gene expression activation for either LasR or RhlR (Pearson *et al.*, 1997, Medina *et al.*, 2003b). Hence, other features of the promoters besides the *las* box consensus sequence may determine the differences in the binding of LasR and RhlR.

1.4.5 Timing of QS activation

Gene expression activation by QS is not strictly dependent on signal molecules only and expression of some QS-dependent genes in *P. aeruginosa* is not advanced by addition of exogenous QS signals. The growth phase dependence in their activation by QS could be due to co-regulation of genes by growth-dependent factors such as RpoS and RhlR, but these factors do not completely account for all the growth- and QS- dependent genes (Whiteley *et al.*, 1999, Schuster & Greenberg, 2007). Multiple factors such as MvaT, GacA, RsmA, QscR, and QteE have also been reported to influence the timing of QS activation (Reimmann *et al.*, 1997, Chugani *et al.*, 2001, Pessi *et al.*, 2001, Diggle *et al.*, 2002, Siehnel *et al.*, 2010). It was also reported that complex medium contains inhibitors that prevent early induction of QS-dependent genes (Yarwood *et al.*, 2005).

1.5 PQS signalling in *P. aeruginosa*

1.5.1 Synthesis of signals involved in PQS signalling

PQS signalling system is integrated with QS network by cross-regulation and co-regulation of virulence genes (Fig. 1.1) (Pesci *et al.*, 1999). 4-hydroxy-2-heptylquinoline (HHQ), which is the precursor molecule of PQS (3,4-dihydroxy-2-heptylquinoline), is synthesized from anthranilate by the enzymes encoded from *pqsABCD* operon (Gallagher *et al.*, 2002, Lepine *et al.*, 2004). Anthranilate is involved in several biochemical pathways and about 12% of it is converted to PQS (Calfee *et al.*, 2001). Anthranilate is produced by either PhnA/PhnB (Essar *et al.*, 1990a) or TrpE/TrpG synthases (Essar *et al.*, 1990b). HHQ is converted to PQS by PqsH (Gallagher *et al.*, 2002). Both HHQ and PQS belong to a family of antimicrobial signal family of 4-hydroxy-2-alkylquinolines (HAQs) that are chemically distinct from QS AHL molecules. The production of PQS is initiated at the end of the logarithmic growth phase and is maximal at the onset of the stationary growth phase (Lepine *et al.*, 2003). HHQ and PQS activate the LysR-type transcriptional regulator PqsR (also known as MvfR) (Xiao *et al.*, 2006), which induces the transcription of *pqsABCDE* and *phnAB*, resulting in autoinduction of these signals (Wade *et al.*, 2005).

1.5.2 Undefined role of PqsE

PqsE is related to the metallo-β-lactamase superfamily and although it does not affect PQS synthesis, it is necessary for pyocyanin and cyanide production (Gallagher *et al.*, 2002). It was found that PqsE induces the production of these virulence factors by enhancing RhlR activity in a HHQ and PQS signal independent manner (Farrow *et al.*, 2008). PqsE also represses the expression of PQS signalling genes independent of RhlR, so it was suggested that PqsE is involved in the homeostatic regulation of PQS signalling system (Hazan *et al.*, 2010, Rampioni

et al., 2010). PqsE is responsible for the control of swarming motility, biofilm formation, virulence in *C. elegans*, and infection of lettuce leaf and mouse in a HAQ-independent manner (Rampioni *et al.*, 2010). An attempt to determine the mechanism of PqsE activity was carried out by testing whether compounds such as PQS and AHL were substrates of PqsE based on their binding affinity, but it failed to provide answers as to what the substrate and function of PqsE are (Yu *et al.*, 2009).

1.5.3 Vesicular transport of PQS signals

Unlike QS signal molecules 3-oxo-C12-HSL and C4-HSL which diffuse freely or through active transport by pump into extracellular environment, release of hydrophobic HHQ and PQS signal molecules outside of cells is mediated by vesicles (Mashburn & Whiteley, 2005). This process is controlled directly by PQS but not HHQ, whereby interaction of PQS with the lipid A component of lipopolysaccaride in cell outer membrane will induce formation of membrane vesicles carrying these signal molecules (Mashburn & Whiteley, 2005, Mashburn-Warren *et al.*, 2008).

1.5.4 QS and PQS signalling systems are linked

Both Las and Rhl QS systems influence PQS signalling (Pesci *et al.*, 1999), where LasR positively regulates *pqsA*, *pqsH* and *pqsR* (Gallagher *et al.*, 2002, Lepine *et al.*, 2004, Gilbert *et al.*, 2009) while RhlR exerts negative control (McGrath *et al.*, 2004). PQS production is dependent on LasR only at early growth phases, since significant amount of PQS was detected in *lasR* mutant after 24-hour growth (Diggle *et al.*, 2003). In addition, PQS signalling system upregulates the Rhl system through PqsR (McKnight *et al.*, 2000) and PqsE (Farrow *et al.*, 2008, Hazan *et al.*, 2010). The interaction of these three different signalling systems is dynamic

and is probably dependent on the growth phase and conditions (McGrath *et al.*, 2004). Because of the regulatory links between AHL-dependent QS and PQS signalling, there is a substantial overlap in their regulon and about 55% of PqsR-regulated genes are also regulated by QS (Deziel *et al.*, 2005).

1.5.5 Iron chelating effect of PQS

Besides acting as the ligand for PqsR, PQS (but not HHQ) depletes iron from the growth medium and the iron-limiting conditions caused by exogenous addition of PQS induces production of pyochelin and pyoverdine siderophore as well as the genes involved in oxidative stress response (Bredenbruch *et al.*, 2006). The iron-chelating effect of PQS also causes increased *rhlR* and *pqsA* expression as well as biofilm formation due to reduced iron concentrations and not because PqsR-iron complex has enhanced activity (Bredenbruch *et al.*, 2006, Diggle *et al.*, 2007, Yang *et al.*, 2007, Hazan *et al.*, 2010).

1.6 Regulation of QS

Since QS is an integral regulatory component of *P. aeruginosa*, its expression and signalling amplitude have to be tightly and finely regulated according to environmental conditions. Some of the QS-dependent genes may be individually co-regulated by QS and other regulatory factors at their own specific promoters. On the other hand, regulatory systems could also fine-tune QS signal output level, thereby controlling the expression of all QS-dependent genes (Schuster & Greenberg, 2006). Many studies have thus been conducted to identify the factors that modulate QS.

1.6.1 Transcriptional regulation – Summary

In *P. aeruginosa*, transcriptional regulation of QS by different factors have been described in numerous reports (Schuster & Greenberg, 2006, Venturi, 2006, Williams & Camara, 2009) and some of these factors, such as VqsR, Vfr, MvaT as well as the sigma factors RpoN and RpoS, have been studied in greater details and would be described below. Transcriptional regulators that regulate QS directly include VqsM which affects both QS and PQS signalling by acting upstream of VqsR (Dong *et al.*, 2005), AlgQ which directly regulates *lasR* and *rhlR* expression (Ledgham *et al.*, 2003a), AlgR which binds to *rhlI* and *rhlA* promoters and inhibit their expression in a biofilm-specific manner (Morici *et al.*, 2007), PmpR which inhibits pyocyanin production, biofilm formation and swarming by repressing the expression of *pqsR* directly (Liang *et al.*, 2008), and PhoB which upregulates the expression of *rhlR* and *pqsR* under phosphate-limited condition (Jensen *et al.*, 2006). In addition, there are transcriptional regulators that have been found to regulate QS but whether they do so directly is still being investigated. These regulators include PtxR which positively regulates LasR QS but negatively regulates Rhl QS and PQS signalling (Carty *et al.*, 2006), and PA1196 which induces Rhl QS and PQS signalling (Liang *et al.*, 2009).

1.6.2 Transcriptional regulation – VqsR

VqsR is a LuxR-type regulator that is transcriptionally regulated by LasR (Li *et al.*, 2007). VqsR, instead of inhibiting QS like QscR which is another LuxR-type regulator, is a positive regulator of QS. Mutation of *vqsR* in *P. aeruginosa* TB strain led to loss of autoinducer production, decreased pyocyanin, elastase and protease levels, and reduced virulence in *C. elegans* killing model (Juhas *et al.*, 2004). Modulation of the expression of these QS-regulated genes by VqsR was also observed by microarray analysis (Juhas *et al.*, 2005). Besides QscR, the other

LuxR-type regulators in PAO1 genome (PA1136 (25% protein identity), VqsR (24% protein identity), PA4074 (23% protein identity)) showed less protein identity with LasR (and RhlR) than QscR.

1.6.3 Transcriptional regulation – Vfr

Vfr, which was first discovered as a virulence factor regulator necessary for exotoxin A and protease production, is about 67% identical at amino acid level to *Escherichia coli* catabolite gene activator protein (CAP; also known as CRP) (West *et al.*, 1994). CAP is involved in controlling carbon source utilisation in *E. coli* by responding to intracellular cAMP levels. Similar to CAP, Vfr regulatory activity is dependent on binding of cAMP ligand but Vfr is not involved in catabolite regulation in *P. aeruginosa* (Suh *et al.*, 2002). Moreover, cAMP levels in *P. aeruginosa* are not altered by changes in carbon source (Siegel *et al.*, 1977, Phillips & Mulfinger, 1981).

Instead, Vfr has been reported to modulate QS, twitching motility and type III secretion system in *P. aeruginosa* (Beatson *et al.*, 2002, Wolfgang *et al.*, 2003). Transcription of *lasR* is directly activated by Vfr and consensus binding sequence of Vfr is found on *lasR* promoter (Albus *et al.*, 1997). Promoter analysis of *rhlR* showed that Vfr recognition sequence is also located at downstream of P4 promoter start site and *rhlR* transcription initiated from this promoter is reduced by deletion of *vfr*. However, more evidence is necessary to determine whether Vfr directly regulates *rhlR* transcription (Medina *et al.*, 2003a). Vfr not only regulates QS genes, but also modulates the expression of QS-regulated genes such as *exoA* and *lasB* through PtxR (Ferrell *et al.*, 2008). The expression of *ptxR* is upregulated by Vfr, and PtxR itself is a positive regulator of *lasI* expression and an inhibitor of PQS and Rhl QS signalling systems (Carty *et al.*, 2006).

1.6.4 Transcriptional regulation – MvaT

MvaT represent a novel class of H-NS-like proteins that are functionally similar to H-NS in *E. coli* despite the lack of sequence similarity (Tendeng *et al.*, 2003). MvaT in PAO1 is homologous to P16 subunit of *P. mevalonii* heteromeric transcriptional regulator, which regulates mevalonate catabolism (Rosenthal & Rodwell, 1998). MvaT inhibits pyocyanin production, 3-oxo-C12-HSL and C4-HSL levels but enhances elastase and protease production, and it also delays QS activation in the presence of exogenous 3-oxo-C12-HSL and C4-HSL (Diggle *et al.*, 2002). MvaT, together with MvaU, binds to AT-rich motifs and inhibits transcription initiation from these regions (Castang *et al.*, 2008). However, MvaT was not found to bind to the promoters of QS genes, hence it is likely that MvaT indirectly regulates QS gene expression.

MvaT also transcriptionally regulates multiple QS-modulatory genes such as *ptxS* and *mexEF-oprN* operon (Westfall *et al.*, 2004, Westfall *et al.*, 2006). MvaT induces the expression of *ptxS* (Westfall *et al.*, 2004) which interferes with activation of QS by PtxR (Colmer & Hamood, 1998). QS is also inhibited by MvaT through small RNA *rsmZ.* RsmZ positively modulates QS by inhibiting negative regulation of QS by RsmA (Pessi *et al.*, 2001, Heurlier *et al.*, 2004). Expression of *rsmZ* is repressed by MvaT (Brencic *et al.*, 2009). MvaT also positively modulates QS by repressing the expression of *mexEF-oprN* operon (Westfall *et al.*, 2006), which inhibits the synthesis of C4-HSL signals (Kohler *et al.*, 2001). Thus, MvaT affects QS through transcriptional regulation of various proteins.

1.6.5 Transcriptional regulation – RpoN RpoS

Alternative sigma factors, specifically RpoN and RpoS, recognise different subsets of gene promoters and QS is influenced by these sigma factors. However, the consensus binding sites of RpoN and RpoS are not found or are non-functional

in the promoters of QS genes. RpoN recognition sequence is found in *rhlI* promoter, but it does not display a functional role (Heurlier *et al.*, 2003), so QS is probably indirectly regulated by RpoN and RpoS. In the absence of *rpoN*, there is increased transcription of *lasI*, *lasR*, *rhlI* and *rhlR* as well as increased 3-oxo-C12-HSL and C4-HSL production (Heurlier *et al.*, 2003). In minimal medium, however, *rhlI* expression is positively regulated by RpoN (Thompson *et al.*, 2003). The transcription of *rhlI* and as well as RhlR-regulated genes such as *hcnA* and *phzA* in addition to production of BHL and pyocyanin are enhanced in the absence of *rpoS* (Whiteley *et al.*, 2000). This contradicts an earlier study where RhlR was found to activate rpoS transcription (Latifi *et al.*, 1996).

1.6.6 Post-transcriptional regulation – small RNA

Post-transcriptional regulation of QS could be controlled at the mRNA level by small RNAs mediated by RsmA and GacA. GacA upregulates the expression of small RNAs (sRNAs) *rsmZ* and *rsmY* (Kay *et al.*, 2006), which interacts with RNA-binding protein RsmA and inhibit its activity (Heurlier *et al.*, 2004). RsmA activity is also inhibited by regulatory RNA *rsmB* (Burrowes *et al.*, 2005). RsmA is a negative regulator of Las and Rhl QS system, which inhibit QS signal production and reduced QS-dependent production of protease and elastase (Pessi *et al.*, 2001). RsmA is a homologue of CsrA in *E. coli* and CsrA is hypothesized to inhibit translation by blocking the ribosome-binding site (RBS) and by altering mRNA stability (Romeo, 1998). It is not known whether RsmA directly binds to the mRNA of QS genes, but it was found that RsmA binds to the RBS of *hcnA* which is also transcriptionally regulated by QS (Pessi & Haas, 2000, Pessi & Haas, 2001), thus RsmA affects QS-dependent gene expression directly and indirectly.

GacA is a two-component system regulator and is activated when phosphorylated. Activation of GacA, which was determined by measuring *rsmZ*

expression, was found to be positively regulated by GacS and LadS and negatively regulated by RetS (Goodman *et al.*, 2004, Ventre *et al.*, 2006). GacS, LadS and RetS belong to the family of two-component sensor kinases. GacS activates GacA by directly phosphorylation and this is disrupted by RetS, which interacts with GacS and inhibits GacS autophosphorylation (Goodman *et al.*, 2009). The kinase activities of the two-component system sensor kinases are possibly modulated by binding of extracellular stimulatory ligands, but the natural ligands for these sensors have not been identified.

1.6.7 Post-transcriptional regulation – Lon protease

QS is also regulated at the post-translational level by Lon protease. Lon protease is an ATP-dependent protease and it negatively regulates QS by specifically degrading LasI and RhlI synthases. Hence, the expression of *rhlI* and *rhlR*, as well as C4-HSL production, were increased in *lon* mutant compared to wild type (Takaya *et al.*, 2008). QS regulation by proteases was also reported in *A. tumefaciens* where TraR was degraded by ClpXP in the absence of autoinducer (Zhu & Winans, 2001). The stimulus for degradation by Lon protease is not known, and its role may be to ensure protein turnover so that autoinducers are synthesized only when necessary.

1.6.8 Post-transcriptional regulation – lactonase and acylase

QS is inhibited by quorum quenching whereby QS signal molecules are degraded by acylases and lactonases (Dong *et al.*, 2002, Lin *et al.*, 2003, Wang *et al.*, 2004). Acylases cleaves the homoserine lactone ring from the acyl side chain while lactonases hydrolyses the homoserine lactone ring. Genes encoding acylases are present in *P. aeruginosa* genome and these include *PvdQ*, *QuiP*, *PA0305* and *PA1893*. Overexpression or exogenous addition of PvdQ was found to reduce 3-

oxo-C12-HSL molecules and reduce elastase and pyocyanin production (Sio *et al.*, 2006). Similarly, overexpression of QuiP also prevents the accumulation of 3-oxo-C12-HSL (Huang *et al.*, 2003). Although PvdQ and QuiP have not been shown to regulate QS, their abilities to affect accumulation of QS signals suggests that they might potentially affect QS signalling. QuiP, but not PvdQ, PA0305 and PA1893, is also necessary for bacteria grown with decanoyl-HSL as sole carbon source (Huang *et al.*, 2006).

1.6.9 Post-transcriptional regulation – GidA

GidA, which encodes for a flavin adenine dinucleotide -binding protein that modifies tRNA, post-transcriptionally regulates Rhl QS system (Gupta *et al.*, 2009). In the *gidA* mutant, production of rhamnolipid, pyocyanin and protease are abolished due to reduced RhlR protein levels. Las QS system and *rhlR* transcription are not influenced by GidA. Based on the results, GidA was postulated to affect RhlR translation and this is possibly due to its function in uridine tRNA modification that is necessary for specific pairing at the wobble position for certain amino acid codons. In the absence of *gidA*, the translation of proteins that have a higher percentage of such amino acids would be affected.

1.6.10 Unconfirmed mode of QS regulation – RelA, Ppk, PtsP

RelA is responsible for the synthesis of guanosine 3′,5′-bisdiphosphate (ppGpp) in *E. coli*, and this stringent response is triggered under nutritionally stressed conditions. When *relA* is overexpressed in *P. aeruginosa*, transcription of *lasR* and *rhlR* as well as production of 3-oxo-C12-HSL, C4-HSL and elastase are induced earlier (van Delden *et al.*, 2001). This occurs independent of the effect of RelA on expression of *rpoS* (van Delden *et al.*, 2001). Conversely, QS-regulated elastase production and virulence in *Drosophila melanogaster* feeding assay was

reduced in null mutant of *relA* (Erickson *et al.*, 2004). DksA increases the binding affinity of RNA polymerase to rRNA promoters in the presence of ppGpp and forms an inactive RNA polymerase complex that inhibits rRNA transcription (Perron *et al.*, 2005). The regulatory effect ppGpp has on QS might be mediated by DksA. In addition, DksA was previously demonstrated to activate the translation of QS-dependent genes *lasB* and *rhlAB* and the production of elastase and rhamnolipids (Jude *et al.*, 2003).

Polyphosphate kinase 1 (Ppk1) is responsible for the polymerisation of polyphosphate from ATP (Ahn & Kornberg, 1990), while Ppk2 synthesizes polyphosphate using GTP (Zhang *et al.*, 2002a). *ppk1* mutant exhibits reduced production of 3-oxo-C12-HSL, C4-HSL, elastase and rhamnolipids (Rashid *et al.*, 2000b), which may be related to its decrease in motility (Rashid & Kornberg, 2000, Rashid *et al.*, 2000a, Fraley *et al.*, 2007) and biofilm formation (Davies *et al.*, 1998). The effect of Ppk2 on QS has not been reported.

PtsP, which encodes for phosphoenolpyruvate-protein phosphotransferase EINtr, was found to positively regulate expression of QS negative regulator *qscR* which leads to the inhibition of *lasI* and *rhlI* expression (Xu *et al.*, 2005). In *E. coli*, EINtr, together with NPr and IIANtr, was suggested to modulate the transcriptional regulation of carbon and nitrogen metabolism through its phosphate relay (Reizer *et al.*, 1996). Hence, it is possible that PtsP might also indirectly regulate QS in *P. aeruginosa* in this manner.

1.6.11 Probable anti-activators in *Pseudomonas aeruginosa*

In *P. aeruginosa*, fluorescence anisotropy and cross-linking studies suggests that an orphan LuxR-type regulator QscR inhibits QS by anti-activation of LasR and RhlR (Ledgham *et al.*, 2003b). Null mutation of *qscR* results in early induction of QS signal production, enhanced virulence in a *Drosophila*

melanogaster feeding assay and increased elastase production (Chugani *et al.*, 2001). QscR, however, also has a functional DNA-binding domain and regulates the expression of a distinct set of genes when bound to 3-oxo-C12-HSL (Lee *et al.*, 2006, Lequette *et al.*, 2006). Thus, QscR effects on QS may also be a result of 3-oxo-C12-HSL sequestering or through its own gene regulation. The precise mechanisms of QscR activity remain to be investigated.

QteE in *P. aeruginosa* also controlled QS activation where expression of QS-dependent genes is induced at earlier growth phase in *qteE* mutant compared to wild type as a result of increased LasR protein levels (Siehnel *et al.*, 2010). Induced expression of *qteE* also abolished QS-dependent production of rhamnolipids, elastase, protease and pyocyanin due to reduced LasR and RhlR protein stabilities. It is likely QteE is a QS anti-activator that mediate LasR and RhlR protein stabilities through protein-protein interaction (Siehnel *et al.*, 2010).

1.6.12 Environmental regulation of QS

Although many factors have been determined to affect QS signalling, the modulation of these regulatory factors by either environmental or intrinsic stimuli is largely not known. QS was demonstrated to be influenced by iron, oxygen, NaCl (Wagner *et al.*, 2003, Kim *et al.*, 2005, Duan & Surette, 2007), but the regulatory components responsible for sensing and translating these changes to changes in QS gene expression are not completely clear.

QS activation is dependent on the accumulation of QS signals that increases as bacterial cell density increases. However, the accumulation of QS signals is not only dependent on cell numbers but also on the environment, as the rate at which QS signals accumulate is governed by factors such as pH changes, degradation enzymes and mass transfer processes (Boyer & Wisniewski-Dye, 2009). Hence, QS has also been proposed to function as diffusion sensing or

efficiency sensing, whereby the environment is monitored for accumulation of QS signals to gauge whether it would be beneficial for the bacteria to produce virulence factor or initiate DNA uptake or other processes which are location-dependent (Redfield, 2002, Hense *et al.*, 2007).

1.7 Aims and Scopes

In *P. aeruginosa*, the timing of QS activation has been found to be regulated by several factors (Section 1.4.5) and many QS regulators has also been identified (Section 1.6). However, the modulation of QS threshold is largely unknown. In this study, a random transposon mutagenesis was carried out to identify potential regulators of QS activation in *P. aeruginosa*, specifically anti-activators. This led to the discovery of anti-activators QslA and QslH, and the effect of these anti-activators on QS and PQS signalling in *P. aeruginosa* was studied using transcriptional assays, signal detection analysis and measurement of QS-dependent virulence factor production. Biochemical studies were also carried out to show the protein-protein interaction of these anti-activators with QS regulators. Finally, the roles of these anti-activators on QS activation and response were examined.

Chapter 2 – Transposon mutants identified in genetic screen

2.1 Introduction

As discussed in Chapter 1, anti-activators play a significant role in the control of QS activation in *A. tumefaciens* and it was speculated that a similar mechanism may be conserved in *P. aeruginosa*. Considering that anti-activators commonly share little amino acid sequence homology, a random transposon mutagenesis approach was taken to identify QS regulators, particularly anti-activators, that modulate QS activation in *P. aeruginosa*. As shown by the flowchart below (Fig. 2.1), these mutants were screened on selection agar plates based on expression level of reporter gene *lacZ*, which was under the control of the promoter of QS gene *rhlR*. From the genetic screen, 20 mutants with altered *rhlR* expression were selected and potential anti-activator genes were identified for further characterisation

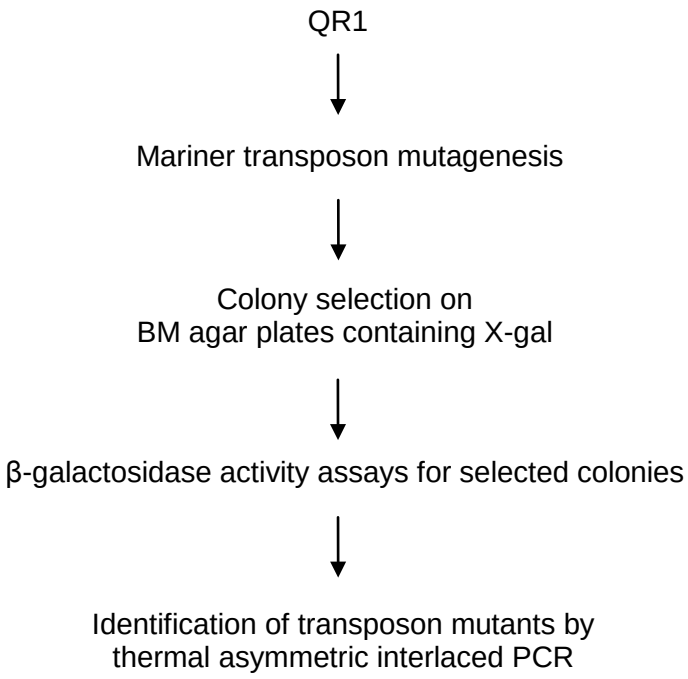


Fig. 2.1 Flow chart showing steps taken to identify QS anti-activators.

2.2 Materials and Methods

2.2.1 Bacterial strains, plasmids, media and growth conditions

Bacterial strains and plasmids used in this chapter are listed in Table 2.1. Basic minimal medium (BM medium) supplemented with 0.2% mannitol (Zhang *et al.*, 2002b) was used in transposon mutagenesis and Luria-Bertani (LB) medium was used in β-galactosidase activity assays. Antibiotics at the following concentrations were added when necessary: gentamicin, 30 μg ml^{-1}; tetracycline, 100 μg ml^{-1} for *P. aeruginosa*; and gentamicin, 5 μg ml^{-1}; tetracycline, 10 μg ml^{-1} for *E. coli*.

Table 2.1 Bacterial strains and plasmids used in Chapter 2.

Strain or plasmid	Relevant genotype or phenotype	Source
P. aeruginosa		
PAO1	Prototrophic laboratory strain	Holloway *et al.*, 1979
QR1	PAO1 containing pUCP-lasRHTH and prhlR-lacZ	This study
E. coli		
DH5α	F^- Φ80*lacZ*ΔM15 *end*A1*hsd*R17 (r_k^- m_k^-) *sup*E44 *thi*$^{-1}$ *gyr*A96 Δ(*lacZYA-argF*)	Laboratory collection
S17-1	*recA pro* (RP4-2Tet::Mu Kan::Tn7)	Simon *et al.*, 1983
Plasmid		
pBT20	*mariner transposon mutagenesis vector,* Gm^R *&* Ap^R	Kulasekara et al., 2005
pME2-lacZ	pME6010 carrying a full-length lacZ	Laboratory
prhlR-lacZ	pME2-lacZ carrying *rhlR* (*PA3477*) promoter fused to *lacZ*	This study
pUCP19	*E. coli – P. aeruginosa* shuttle vector with *lac* promoter (P*lac*), Amp^R/Cb^R	ATCC 87110
pUCP-lasRHTH	pUCP19 containing 505-717 bp of *lasR* (*PA1430*) ORF under the control of P*lac*	This study

2.2.2 Construction of reporter strain

QR1 is a derivative of strain PAO1 containing plasmid pUCP-lasRHTH and prhlR-lacZ. pUCP-lasRHTH was constructed by ligating the last 213 bp (505-717 bp) of *lasR* open reading frame (ORF) into pUCP19 under the control of P*lac*. The prhlR-lacZ transcriptional fusion reporter was constructed by amplifying the promoter region of P*rhlR* (-401 to +66 relative to translational start site) and ligating it to pME2-lacZ vector.

2.2.3 Transposon mutagenesis and identification of transposon insertion site

A mariner-based transposon carried by vector pBT20 was used for mutagenesis of *P. aeruginosa* strain QR1. *E. coli* S17-1 containing pBT20 was used for conjugal mating with recipient *P. aeruginosa* at 37°C for 6 h. BM agar plates containing gentamicin and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Biosynth) were used to select for transconjugants. Gentamicin-resistant colonies that were more or less blue than the parental strain QR1 were selected and their β-galactosidase activities were measured. Thermal asymmetric interlaced PCR (TAIL-PCR) was carried out on the transposon mutants that showed either 20% more or 20% less β-galactosidase activities compared to QR1. Transposon specific primers and arbitrary degenerate primers were used to identify the transposon insertion sites as described previously (Kulasekara et al., 2005). The PCR product was then sequenced and analyzed using NCBI BLAST server.

2.2.4 β-galactosidase activity assays

The cells of *P. aeruginosa* containing *lacZ* transcriptional fusion reporter genes were collected at exponential growth phase (OD_{600} = 1.5) after growth in LB medium and assayed for β-galactosidase activity (Sambrook *et al.*, 1989), each with triplicates.

2.3 Results

2.3.1 Screening of transposon mutants

Reporter strain, QR1, was designed to screen for regulators that control QS activation in *P. aeruginosa*. QR1 contained a pUCP-lasR-HTH plasmid overexpressing the DNA-binding domain of LasR, and a reporter plasmid PrhlR-lacZ where *rhlR* promoter was ligated with reporter gene *lacZ*. The expression of *rhlR* is induced by both Las and Rhl QS system (Pesci *et al.*, 1997, Medina *et al.*, 2003a), so mutation of anti-activators that interact with either LasR and RhlR or mutation of regulators of either Las or Rhl QS system would affect *rhlR* expression. Expression of LasR-HTH enables activation of P*rhlR-lacZ* activity in the absence of QS signal 3-oxo-C12-HSL (Anderson *et al.*, 1999, Kiratisin *et al.*, 2002), and the pUCP-lasR-HTH plasmid was introduced to ensure that regulators that modulate expression of *lasR* and *lasI* as well as *lasR* and *lasI* mutants were not selected in the genetic screen. This is particularly because spontaneous *lasI* and *lasR* mutants develop frequently *in vivo* and *in vitro* (Heurlier *et al*., 2005, D'Argenio *et al.*, 2007) as well as during transposon mutagenesis (unpublished data). Overexpressing whole LasR will not prevent the identification of *lasI* mutants, but this can be overcome by overexpressing LasR-HTH since it can activate gene expression independent of 3-oxo-C12-HSL. Furthermore, QS anti-activators such as TraM and TrlR interact with QS regulators at the DNA-binding domain so it was postulated that QS anti-activators could still be identified in *P. aeruginosa* using reporter strain overexpressing LasR-HTH.

About 15, 000 transposon mutants were screened and 20 of these mutants were selected because their *rhlR* expression levels differed substantially from that in parental strain QR1 (Fig. 2.1). These mutants were grouped according to the

genes in which the transposons were found and the effects the mutations have on *rhlR* expression were described in the following sections.

2.3.2 Mutants with transposons inserted in the intergenic regions or in the gene encoding hypothetical protein

Among the 20 mutants selected, DNA sequencing analysis showed that 11 of these mutants had transposons inserted in the intergenic regions and one mutant had its transposon inserted in a gene encoding a hypothetical protein (Table 2.2). P*rhlR-lacZ* activities of these 12 mutants were measured and 7 of the mutants had reduced P*rhlR-lacZ* activities compared to QR1 (Fig. 2.2). One of these 7 mutants was QRM14, where P*rhlR-lacZ* activity was about 3 folds less than that in QR1. In QRM14, the transposon was inserted between *PA3865* and *PA3866*, which encode putative amino acid-binding protein and putatitive pyocin protein, respectively. The functions of these two proteins, however, have not yet been verified, so it was not clear how *rhlR* expression was affected in QRM14.

In QRM71, P*rhlR-lacZ* activity was also reduced, but only by about 20% compared to QR1. The transposon in QRM71 was inserted between *mexK* and *mexL*. These two genes are part of *mexJKL* operon and MexJK-OprM is involved in antibiotic efflux (Chuanchuen *et al.*, 2005). It is possible that *rhlR* expression was altered in QRM14 because changes in expression of *mexJKL* operon affects OprM availability for 3-oxo-C12-HSL efflux by MexAB-OprM pump (Pearson *et al.*, 1999).

Surprisingly, the transposon in QRM82 was inserted between *PA0263* and *PA0263.1*, which is part of a genetic region encoding proteins involved in type VI secretion system (Blondel *et al.*, 2009). This caused P*rhlR-lacZ* activity in QRM82 to be about half of that in QR1. Although it has been reported that type VI secretion system is regulated by QS (Lesic *et al.*, 2009), converse regulation has not been

reported. Hence, the reason why *rhlR* expression was changed in QRM82 could not be explained without further studies.

Table 2.2 Mutants with transposons inserted in the intergenic regions or in the gene encoding a hypothetical protein.

Strain	Gene	Description
QRM14	251 bp upstream of *PA3865*, 1051 bp downstream of *PA3866*.	PA3865: probable amino acid binding protein, PA3866: probable pyocin protein
QRM22	*PA2228*	PA2228: hypothetical protein
QRM71	8 bp upstream of PA3678 (*mexL*), 88 bp upstream of PA3677 (*mexK*).	PA3678: regulator from tetR family, PA3677: probable Resistance-Nodulation-Cell Division efflux membrane fusion protein precursor
QRM75	102 bp upstream of *rhlA*, 322 bp downstream of *PA3480*.	RhlA: rhamnosyltransferase chain A PA3480: probable dCTP deaminase
QRM82	70 bp downstream of *PA0263.1*, 1186 bp upstream of *hcpC* (*PA0263*).	PA0263.1: tRNA-Arg HcpC: secreted protein
QRM92	767 bp upstream of *PA1244*, 248 bp upstream of *aprX* (*PA1245)*.	PA1244: hypothetical protein, AprX: substrate of the type I secretion system
QRM130	42 bp upstream of *PA1243*, 194 bp downstream of *PA1244*.	PA1243: probable sensor/response regulator hybrid, PA1244: hypothetical protein
QRM168	570bp upstream of *PA2228*, 372 bp upstream of *PA2229*	PA2228: hypothetical protein, PA2229: hypothetical protein
QRM205	4 bp upstream of *dut* (*PA5321*), 4 bp downstream of *coaC* (*PA5320*).	Dut: deoxyuridine 5'-triphosphate nucleotidohydrolase, CoaC: phosphopantothenoylcysteine synthetase/decarboxylase
QRM206	727bp upstream of *PA2228*, 215 bp upstream of *PA2229.*	PA2228: hypothetical protein, PA2229: hypothetical protein
QRM241	55 bp upstream of *rpoC* (*PA4269*), 11 bp downstream of *rpoB* (*PA4270*).	RpoC: DNA-directed RNA polymerase RpoB: DNA-directed RNA polymerase
QRM262	6 bp upstream of *ftsJ* (*PA4752*), 89 bp upstream of *PA4753.*	FtsJ: cell division protein PA4753: hypothetical protein

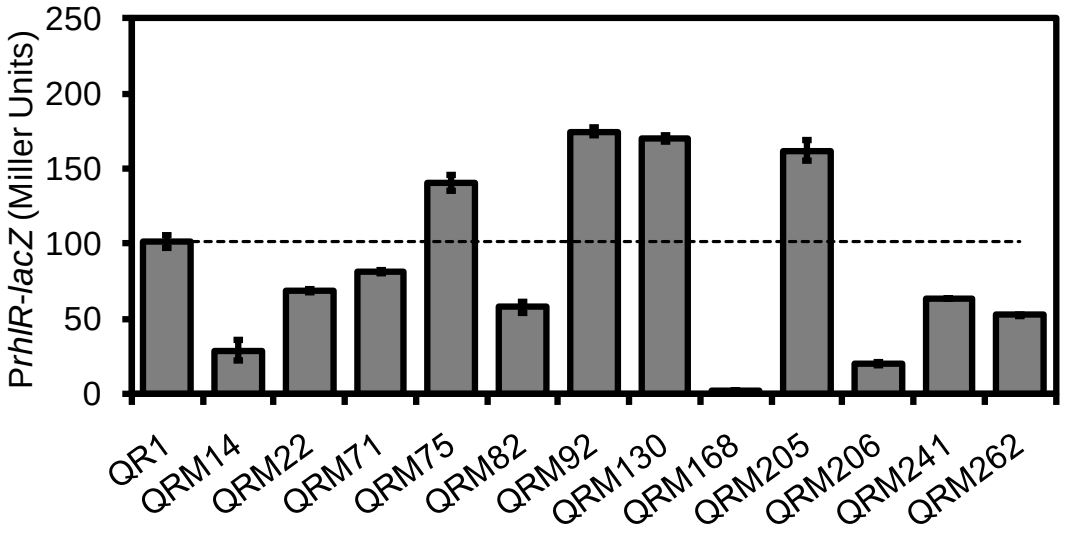


Fig. 2.2 P*rhlR-lacZ* activities of mutants with transposons inserted in the intergenic regions or in the gene encoding a hypothetical protein.
P*rhlR-lacZ* activities were measured at OD_{600} = 1.5. Dotted line indictates level of P*rhlR-lacZ* activity in parental strain QR1. The data shown were means of 3 replicates and error bar, which indicates standard deviation, is not seen when standard deviation is small.

Transposons in QRM168 and QRM206 were found inserted between PA*2228* and *PA2229*, while transposon in QRM22 was inserted in *PA2228,* which encodes a hypothetical protein. P*rhlR-lacZ* activities of these three mutants were less than the parental strain QR1, so it was likely that the changes in *rhlR* expression in these mutants were due to disruption of the same gene. Because both *PA2228* and *PA2229* encode hypothetical proteins, the effect of these genes on *rhlR* expression could not be predicted without further studies.

P*rhlR-lacZ* activities in mutants QRM241 and QRM262 were reduced by about 50% compared to QR1. DNA sequencing analysis showed that the transposon in QRM241 was inserted between *rpoB* and *rpoC*, and these genes encode DNA-directed RNA polymerase. In QRM262, the transposon was inserted between *ftsJ* and *PA4753* and these genes, respectively, encode a cell division protein and a hypothetical protein. It is thus possible that in mutants QRM241 and QRM262, *rhlR* expression were reduced because basal cellular processes such as transcription and cell division were interfered.

P*rhlR-lacZ* activities were increased in QRM75, QRM92, QRM130 and QRM205 compared to QR1. In QRM75, P*rhlR-lacZ* activity was about 40% higher compared to QR1 and DNA sequencing analysis showed that the transposon was inserted upstream of *rhlAB* operon (*PA3478-PA3479*) and *rhlR* (*PA3477*). This might have caused an increase in expression of the adjacent genes and increased expression of *rhlR* would increase P*rhlR-lacZ* activity.

In QRM92 and QRM130, transposons were, respectively, inserted at upstream and downstream of *PA1244* and these caused P*rhlR-lacZ* activities to be increased by about 70%. PA1244 is a hypothetical protein while its adjacent genes *PA1243* and *aprX*, respectively, encodes for a hypothetical protein, which is a probable sensor/response regulator hybrid, and a substrate for type I secretion

system (Duong *et al.*, 2001). The change in *rhlR* expression is likely due to the either PA1243 or PA1244, which are both hypothetical proteins.

In QRM205, DNA sequencing analysis showed that the transposon was inserted between *dut* and *coaC*, which encodes deoxyuridine 5'-triphosphate nucleotidohydrolase (Dut) and phosphopantothenoylcysteine synthetase/decarboxylase (CoaC), respectively. Dut catalyses the hydrolysis of dUTP (deoxyuridine 5'-triphosphate) (Cedergren-Zeppezauer *et al.*, 1992), while CoaC is involved in coenzyme A synthesis (Kupke *et al.*, 2000). It has been reported that increased uridine monophosphate (UMP) synthesis and increased availability of UMP from catalysis of uracil positively influences QS (Ueda *et al.*, 2009). Thus, it was postulated that the increase in P*rhlR-lacZ* activity in QRM205 compared to QR1 was probably due to increased *dut* expression, which would increase UMP levels and upregulate QS signalling.

2.3.3 Mutants with transposons inserted in the genes encoding enzymes

Four of the mutants selected had transposons inserted in the genes encoding enzymes (Table 2.3) and their P*rhlR-lacZ* activities were determined (Fig. 2.3). In QRM67, P*rhlR-lacZ* activity was 1.5 times higher than that in QR1. Mutation of *PA3958*, which encodes for a mutator protein, in QRM67 has been reported to cause an increase of mutation frequency by 3–4 folds (Wiegand *et al.*, 2008), so it is likely that PA3958 did not directly affect QS but accelerate the spontaneous mutations that affect QS signalling. Similarly, mutation of *mfd* was also found to increase mutation frequency (Wiegand *et al.*, 2008) and this mutation was present in QRM234. P*rhlR-lacZ* activity in QRM234, however, was reduced to about 50% of that in QR1.

The gene *mqoB* was disrupted in QRM300 and this caused about 60% increase in P*rhlR-lacZ* activity. MqoB is essential for growth on ethanol or acetate in *P. aeruginosa* (Mellgren *et al.*, 2009), and because metabolism of carbon sources is modulated by Crc which is a QS regulator (Linares *et al.*, 2010), it is possible that mutation of *mqoB* may affect QS through its effect on carbon source utilisation.

P*rhlR-lacZ* activity was reduced in QRM95 compared to QR1 and this was due to the disruption of *moaA2*, which encodes for a protein responsible for synthesis of molybdopterin, an important co-factor for enzymatic reactions. The role of molybdopterin in QS has not been reported, and it is not known whether molybdopterin is required among the enzymes or proteins associated with QS.

P*rhlR-lacZ* activity was also reduced in QRM213 compared to QR1. The gene *pyrE* encoding orotate phosphoribosyltransferase is disrupted in QRM213, and PyrE catalyses orotate to orotidine-5'-phosphate. Orotidine-5'-phosphate is the precursor of UMP, and as mentioned in Section 2.3.2, UMP levels positively influence QS (Ueda *et al.*, 2009). Hence, the reduced *rhlR* expression in QRM213 was probably due to disruption of UMP synthesis.

Table 2.3 Mutants with transposons inserted in the genes encoding enzymes.

Strain	Gene	Description
QRM67	*PA3958*	PA3958: probable endonuclease/exonuclease/phosphatase
QRM95	*moaA2*	MoaA2: molybdenum cofactor biosynthetic protein A2
QRM213	*pyrE* (*PA5331*)	PyrE: orotate phosphoribosyltransferase
QRM234	*mfd* (*PA3002*)	Mfd: transcription-repair coupling protein
QRM300	*mqoB* (*PA4640*)	MqoB: malate:quinone oxidoreductase

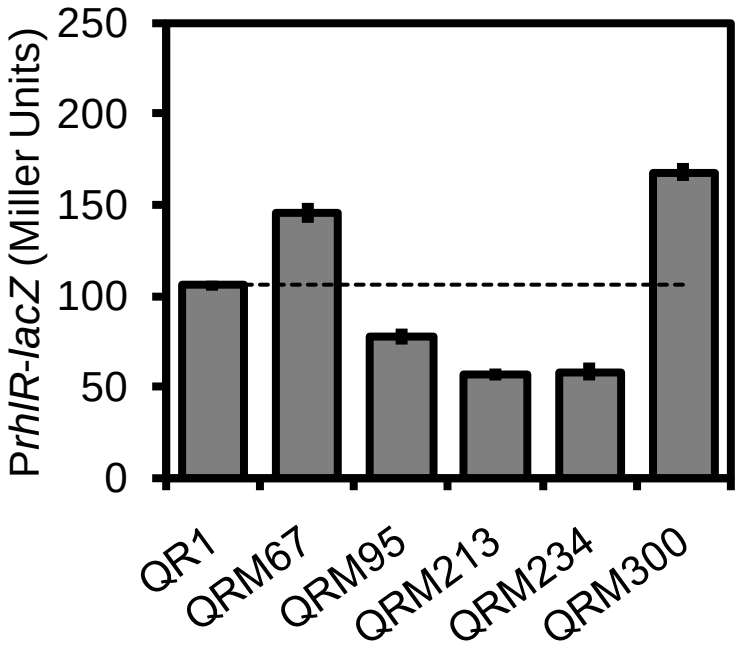


Fig. 2.3 P*rhlR-lacZ* activities of mutants with transposons inserted in the genes encoding enzymes.
P*rhlR-lacZ* activities were measured at OD_{600} = 1.5. Dotted line indictates level of P*rhlR-lacZ* activity in parental strain QR1. The data shown were means of 3 replicates and error bar indicates standard deviation.

2.3.4 Mutants with transposons inserted in the genes encoding regulators

3 of the selected mutants had transposons inserted in genes that encode regulators (Table 2.4) and their P*rhlR-lacZ* activities compared with parental strain QR1 were determined (Fig. 2.4). The gene encoding *rsaL*, which was disrupted in QRM208, is a repressor of QS (de Kievit *et al.*, 1999). Mutation of *rsaL* was expected to increase P*rhlR-lacZ* activity but instead it was reduced by about half. This may be because of the polar effect of inserted transposon that inhibited the expression of neighbouring genes *lasI* and *lasR*, which encode for proteins that positively regulate *rhlR* expression.

P*rhlR-lacZ* activities were about 3 times lesser in QRM110 and QRM137 compared to QR1 due to insertion of transposons at two different sites (843 bp and 649 bp) in *PA5506*. *PA5506* is part of an operon that includes genes encoding enzymes isochorismatase (*PA5507*), glutamine synthetase (*PA5508*) and amidohydrolase (*PA5509*). The change in *rhlR* expression might be due to the enzyme expression changes, which remains to be further investigated.

Table 2.4 Mutants with transposons inserted in the genes encoding regulators.

Strain	Gene	Description
QRM110	*PA5506* (843 bp)	PA5506: RpiR family transcriptional regulator
QRM137	*PA5506* (649 bp)	PA5506: RpiR family transcriptional regulator
QRM208	*rsaL* (*PA1431*)	RsaL: repressor of *lasI* transcription

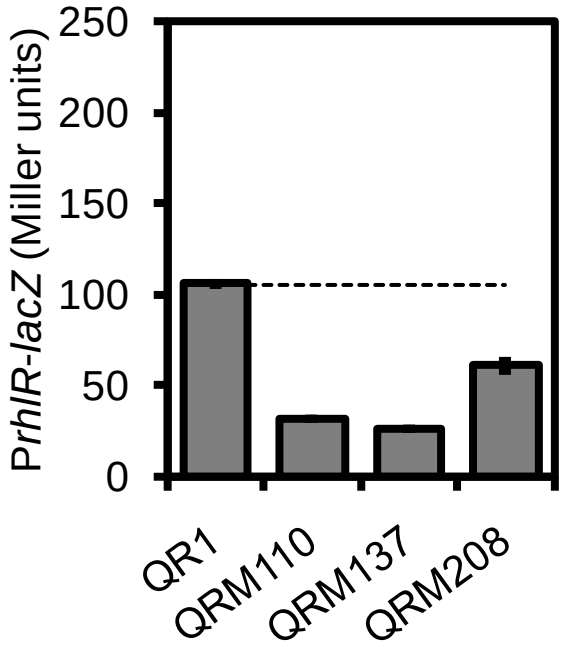


Fig. 2.4 P*rhlR-lacZ* activities of mutants with transposons inserted in the genes encoding regulators.
P*rhlR-lacZ* activities were measured at OD_{600} = 1.5. Dotted line indictates level of P*rhlR-lacZ* activity in parental strain QR1. The data shown were means of 3 replicates and error bar, which indicates standard deviation, is not seen when standard deviation is small.

2.3.5 Identification of genes encoding probable QS anti-activators

Based on knowledge of TraM and TraM2 (Hwang *et al.*, 1995, Wang *et al.*, 2006), QS anti-activator genes are expected to encode for small proteins around 11 kDa and these proteins would not have any known domains or motifs as well as protein export signals. 8 of the 20 mutants had transposons inserted in genes, but these genes either encode enzymes or regulators. It was possible that genes, which are adjacent to the intergenic regions described in Section 2.3.2, might encode for QS anti-activators, so further *in silico* analysis of these genes were carried out. It was found that there were two potential QS anti-activator genes, *PA1244* and *PA2226* (Table 2.5), and these were, respectively, further characterised in Chapter 3 and Chapter 4.

Table 2.5 Probable QS anti-activator genes identified among mutants

Strain	Gene	Probable QS anti-activator gene
QRM92	767 bp upstream of *PA1244*, 248 bp upstream of *aprX* (*PA1245)*.	PA1244 (342 bp) encodes 12.7 kDa protein
QRM130	42 bp upstream of *PA1243*, 194 bp downstream of *PA1244*.	
QRM168	570bp upstream of *PA2228*, 372 bp upstream of *PA2229*	PA2226 (501 bp) encodes 18.3 kDa protein
QRM206	727bp upstream of *PA2228*, 215 bp upstream of *PA2229.*	

2.4 Discussion

In this chapter, transposon mutants were screened for changes in *rhlR* expression so as to identify potential QS regulators of QS activation. A total of 20 mutants were selected from about 15, 000 mutants screened, among them 6 of these mutants had higher P*rhlR-lacZ* activities and 14 mutants had lower P*rhlR-lacZ* activities compared to wild type. It was found that, with the exception of *rsaL*, many of the known QS regulators were not identified in this genetic screen. It is interesting to note that regulators that modulate QS post-transcriptionally were not found in this study. One likely reason is that insufficient mutants were screened. On the other hand, the fact that most of the QS regulators that influence QS transcriptionally were not identified may highlight the feasibility of using this screening method to selectively identify potential genes such as QS anti-activator genes that are responsible for post-transcriptional regulation of QS, which was the objective of reporter design as described in Section 2.3.1.

It is likely that some of the genes identified in this transposon mutagenesis encode proteins that are involved in the regulation of QS activation. PA5506 regulator and enzymes MoaA2 and MqoB have not been reported to be associated with QS signalling and because they are unlikely to be related to QS anti-activation, their roles in QS regulation will not be studied.

Interestingly, more than half of the selected mutants had transposons inserted in the intergenic regions. This might be because mariner transposon used, inserts at the dinucleotide TA (Robertson & Lampe, 1995) and as PAO1 genome is GC-rich (66.6%) (Stover *et al.*, 2000), there is a higher probability that the transposons would insert in AT-rich promoter regions (which are intergenic). Nevertheless, analysis of the mutants with transposons inserted in the intergenic region led to the identification of two probable genes *PA1244* and *PA2226* that may

encode for anti-activators and the effect of these genes on QS will be subsequently examined in Chapter 3 (PA1244) and Chapter 4 (PA2226).

Chapter 3 – Defining the Quorum Sensing Threshold and Response by QslA

3.1 Introduction

In *A. tumefaciens*, anti-activators TrlR, TraM, TraM2 play an important role in regulating QS activation (Piper & Farrand, 2000, Chai *et al.*, 2001, Wang *et al.*, 2006). However, whether similar mechanisms are conserved in other bacterial pathogens remains to be investigated. By using *P. aeruginosa* as a model, a large scale random transposon mutagenesis was conducted and 20 mutants with altered expression of QS gene *rhlR* were selected (Chapter 2). Among these mutants were QRM92 and QRM130, which had higher *rhlR* expression than parental strain QR1. Transposons in both QRM92 and QRM130 were inserted between *PA1243* and *PA1244.* Because of their interesting phenotype and that *PA1244* encodes a small hypothetical protein which is a common feature of known anti-activators, these two mutants were chosen for further studies.

In this chapter, PA1244 (QslA) was found to be responsible for the altered *rhlR* expression in QRM92 and QRM130 and the role of QslA in QS and PQS signalling pathways as well as QS-dependent virulence factor production in PAO1 wild type strain was examined. The effect of QslA on QS and PQS signalling pathways was shown to be due to protein-protein interaction with LasR and this resulted in the loss of LasR DNA-binding activity. QslA was also found to define the QS threshold and plays an important role in the regulation of QS activation. The findings from this chapter depict a complex mechanism by which the anti-activator QslA governs the QS threshold and response in *P. aeruginosa*.

3.2 Materials and Methods

3.2.1 Bacterial strains, plasmids, media and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 3.1. Bacteria were routinely maintained at 37°C in Luria-Bertani (LB) medium. For analysis, overnight starter cultures were diluted to OD_{600} = 0.03 in LB and cultured under the same temperature with shaking at 250 rpm until OD_{600} = 0.3 and 0.6 (lag phase), 1.5 and 2.5 (log phase), and 4.5 (stationary phase). Antibiotics at the following concentrations were added when necessary: gentamicin, 30 μg ml^{-1}; kanamycin, 500 μg ml^{-1}; tetracycline, 100 μg ml^{-1} for *P. aeruginosa*; and kanamycin, 100 μg ml^{-1}; tetracycline, 10 μg ml^{-1}; carbenicillin 100 μg ml^{-1} for *E. coli.*

3.2.2 β-galactosidase activity assays

The *lacZ* transcriptional fusion reporter strains were constructed by amplifying the promoter regions of P*lasI*, P*lasR*, P*pqsA*, P*rhlI*, P*rsaL* and P*qslA* at -174 to +63 (P*lasI*), -345 to +72 (P*lasR*), -454 to +128 (P*pqsA*), -238 to +60 (P*rhlI*), -500 to +157 (P*rsaL*) and -500 to +114 (P*qslA*) relative to their translational start sites and ligating these promoter fragments to pME2-lacZ or pUC18-mini-Tn7T-Gm-lacZ vector, respectively. The construction of P*rhlR-lacZ* transcriptional fusion reporter strain was mentioned in Chapter 2. Unless otherwise stated, the cells of *P. aeruginosa lacZ* transcriptional fusion reporter strains were collected at OD_{600} = 1.5 and assayed for β-galactosidase activities, each with triplicates.

Table 3.1 Bacterial strains and plasmids used in Chapter 3.

Strain or plasmid	Relevant genotype or phenotype	Source
P. aeruginosa		
PAO1	Prototrophic laboratory strain	Holloway *et al.*, 1979
QR1	PAO1 containing pUCP-lasRHTH and prhlR-lacZ	Chapter 2
QRM92	QR1 with transposon inserted 766 bp upstream of *qslA*	Chapter 2
QRM130	QR1 with transposon inserted 191 bp downstream of *qslA*	Chapter 2
QR1(PA1243)	QR1 containing pDSK-PA1243	This study
QR1(PA1244)	QR1 containing pDSK-qslA	This study
QR1(aprX)	QR1 containing pDSK-aprX	This study
PP1	PAO1 *pqsA*::Tn containing ppqsA-lacZ	This study
QF1	PAO1 chromosomal-integrated *qslA* fused with FLAG	This study
ΔqslA	*qslA* in-frame deletion mutant of PAO1	This study
ΔqslA(qslA)	ΔqslA containing pDSK-qslA	This study
ΔlasR	*lasR* in-frame deletion mutant of PAO1	This study
wt(qslA)	PAO1 containing pDSK-qslA	This study
wt(qslA-FLAG)	PAO1 containing pDSK-qslA-FLAG	This study
ΔlasR(qslA-FLAG)	*lasR* in-frame deletion mutant of PAO1 containing pDSK-qslA-FLAG	This study
ΔlasI	*lasI* in-frame deletion mutant of PAO1	This study
ΔlasIΔqslA	*lasI* and *qslA* in-frame deletion mutant of PAO1	This study
E. coli		
DH5α	F^- Φ80*lacZ*ΔM15 *end*A1*hsd*R17 (r_k^- m_k^-) *sup*E44 *thi*$^{-1}$ *gyr*A96 ∆(*lacZYA-argF*)	Laboratory collection
DL1	DH5α containing pUCP-lasR plasI-lacZ	This study
DR1	DH5α containing pUCP-rhlR prhlI-lacZ	This study
S17-1	*recA pro* (RP4-2Tet::Mu Kan::Tn7)	Simon *et al.*, 1983
BL21 Star™	F^- *ompT hsdS*$_B$ (*r*$_B^-$ *m*$_B^-$) *gal dcm rne131 (DE3)*	Invitrogen

Plasmid		
pK18mobsacB	Broad-host-range gene replacement vector, sacB, Gm^R	Laboratory collection
pK18-qslA	pK18mobsacB containing *qslA* flanking region	This study
pK18-lasI	pK18mobsacB containing *lasI* flanking region	This study
pK18-lasR	pK18mobsacB containing *lasR* flanking region	This study
pME2-lacZ	pME6010 carrying a full-length lacZ	Laboratory
plasI-lacZ	pME2-lacZ carrying *lasI* promoter fused to *lacZ*	This study
plasR-lacZ	pME2-lacZ carrying *lasR* promoter fused to *lacZ*	This study
ppqsA-lacZ	pME2-lacZ carrying *pqsA* promoter fused to *lacZ*	This study
prhlI-lacZ	pME2-lacZ carrying *rhlI* promoter fused to *lacZ*	This study
prhlR-lacZ	pME2-lacZ carrying *rhlR* promoter fused to *lacZ*	Chapter 2
prsaL-lacZ	pME2-lacZ carrying *rsaL* promoter fused to *lacZ*	This study
pUC18-mini-Tn7T-Gm-lacZ	Gm^r on mini-Tn*7*T; *lacZ* transcriptional fusion vector	Choi *et al.*, 2006
pqslA-lacZ	pUC18-mini-Tn7T-Gm-lacZ carrying *qslA* promoter fused to *lacZ*	This study
pUCP19	*E. coli – P. aeruginosa* shuttle vector with *lac* promoter (P*lac*), Amp^R/Cb^R	ATCC 87110
pUCP-lasR	pUCP19 containing *lasR* under the control of P*lac*	This study
pUCP-lasRHTH	pUCP19 containing 505-717 bp of *lasR* ORF under the control of P*lac*	Chapter 2
pUCP-rhlR	pUCP19 containing *rhlR* under the control of P*lac*	This study
pDSK519	Broad-host-range cloning vector with *lac* promoter (P*lac*), Km^R	Laboratory collection
pDSK-PA1243	pDSK519 containing *PA1243* under the control of P*lac*	This study
pDSK-aprX	pDSK519 containing *PA1245* (*aprX*) under the control	This study
pDSK-qslA	pDSK519 containing *qslA* under the control of P*lac*	This study
pDSK-qslA-FLAG	pDSK519 containing *qslA* fused with FLAG peptide under the control of P*lac*	This study
pET14b	Protein expression vector, Amp^R	Laboratory collection
pET14b-lasR	pET14b containing *lasR*	This study
pGEX6P1	GST fusion protein expression vector, Amp^R	Laboratory collection
pGEX6P1-qslA	pGEX6P1 containing *qslA*	This study

3.2.3 RNA extraction and reverse transcription-PCR (RT-PCR) analysis

Bacterial cells were collected after 5 h growth or at specific time points as indicated. Total RNA samples were purified using RNeasy miniprep kit (QIAGEN). Genomic DNA was removed by on-column treatment with DNase (QIAGEN) and recombinant DNaseI (Roche). RT-PCR was carried out using OneStep RT-PCR kit (QIAGEN) and band intensities were determined by ImageJ (http://rsbweb.nih.gov/ij/).

3.2.4 DNA manipulation and deletion mutagenesis

The *qslA* in-frame deletion mutant in PAO1 was generated using pK18mobSac ligated to approximately 450 bp upstream and downstream flanking regions of *qslA*. The construct was introduced into PAO1 by conjugal mating using *E. coli* S17-1, and recombination of the plasmid with the genomic DNA resulted in an internal deletion from 1 – 288 bp of *qslA*. The mutant was confirmed by PCR analysis. For complementation, the coding region of *qslA* was amplified from 27 bp upstream of the translational start site to 108 bp downstream of the stop codon. The PCR product was cloned into expression vector pDSK519 and confirmed by DNA sequencing.

3.2.5 FLAG recombinant protein purification and sequencing

The DNA fragment encoding FLAG peptide and glycine spacer was fused in-frame to *qslA* open reading frame at the C-terminal by PCR, and the PCR product was ligated to pDSK519. The *qslA-FLAG* fusion plasmid was transformed into wild-type strain PAO1. Chromosomal-encoded *qslA-FLAG* fusion gene was constructed by ligating the *qslA-FLAG* PCR product to pK18mobSac and carrying out recombination as described (Kulasekara et al., 2005). Expression of the FLAG-fusion protein was confirmed by western blot analysis. Plasmid- and chromosomal-

encoded FLAG-fusion protein were purified from overnight cultures using ANTI-FLAG® M2 Affinity Gel (Sigma) according to manufacturer's protocol. The purified FLAG-fusion protein was resolved by 15% SDS-PAGE, transferred onto PVDF membrane for protein sequencing by Edman degradation analysis.

3.2.6 Assessment of supernatant AHL and HAQ levels

Reporter strains DL1, DR1 and PP1 were used to evaluate 3-oxo-C12-HSL, C4-HSL and HAQ levels, respectively. Cell-free supernatants from PAO1, ΔqslA and ΔqslA(qslA) were added separately to 1:20 diluted overnight cultures of corresponding reporter strains and grown at 37°C with shaking for 2.5 h. β-galactosidase activity was then measured. Each experiment was carried out in triplicate and the representative data from three independent experiments were presented.

3.2.7 Analysis of virulence factor production

Elastase activity was determined by elastin-Congo red assay as previously described with minor modifications (Bjorn et al., 1979). Briefly, 500 µl of culture supernatant was added to tubes containing 1 ml of 5 mg ml^{-1} of elastin-Congo red (Sigma) in ECR buffer (0.1 M Tris-Cl pH 7.2, 1 mM $CaCl_2$). Tubes were shaken at 37°C for 2 h. The unreacted elastin Congo-red was pelleted down and the supernatant was measured at OD_{495}. Elastase activity units were determined using the equation: 1 unit of elastase activity = (OD_{495}/OD_{600}) × 100. Proteolytic activity assay was carried out according to the method previously described (Denkin & Nelson, 2004). Briefly, 100 µl of culture supernatants was incubated with 100 µl of 5 mg ml^{-1} azocasein dissolved in protease buffer (50 mM Tris-Cl pH 8.0, 0.04% NaN_3) for 40 min at 37°C. Reaction was stopped by addition of 10% trichloroacetic acid to a final concentration of 6.7% and unreacted azocasein was spun down.

Supernatants were added to 700 µl of 525 mM NaOH and OD_{442} were measured. Protease activity units were determined using the equation: 1 unit of protease activity = (OD_{442}/OD_{600}) × 100. Pyocyanin levels were measured in supernatants of *P. aeruginosa* strains grown in LB medium as previously described (Dong et al., 2008). Pyocyanin levels were determined according to a previously published method (Essar et al., 1990a).

3.2.8 Co-immunoprecipitation and western blot analysis

Bacteria were grown to OD_{600} = 3.0 and cells were resuspended in lysis buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1% Tween 20, 1 µM 3-oxo-C12-HSL). Cell lysates were obtained by sonication and incubated with ANTI-FLAG® M2 Affinity Gel (Sigma) for 2 h at 4°C. Eluted proteins were resolved by 15% SDS-PAGE and transferred onto a PVDF membrane. Immunoblotting was performed using mouse monoclonal ANTI-FLAG® M2 antibody (Sigma) or LasR rabbit polyclonal antibody (Martin Schuster, Oregon State University).

3.2.9 Expression and purification of LasR and QslA

The *lasR* coding region was amplified and ligated into pET14b (Novagen) using NcoI and XhoI restriction sites to produce pET14b-lasR. Strain BL21 star™ (Invitrogen) transformed with pET14b-lasR was grown in 1 litres of LB medium containing 2 µM 3-oxo-C12-HSL to OD_{600}= 0.5 before being induced with 500 µM of isopropyl-β-d-thiogalactopyranoside overnight at 18°C. Cell pellet was resuspended in 25 ml of LasR purification buffer (LRPB) (25 mM Tris-HCl pH 7.8, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, 0.05% Tween 20, 200 nM 3-oxo-C12-HSL) (Schuster *et al.*, 2004) and lysed by sonication. The cell debris was removed by spinning at 26,000 X g for 1 h and the cell lysates were applied to a 5-ml Hi-Trap Heparin HP affinity column (GE). Bound LasR proteins were eluted using a 100-ml

linear gradient of 150 - 1000 mM of NaCl. Fractions containing LasR were pooled and applied to a 30-ml Mono Q column (GE) and the unbound flow-through fractions were collected and further purified using the Hi-Trap Heparin HP affinity column to obtain the fraction containing >90% pure LasR protein.

For purification of QslA, the PCR product of *qslA* coding region was digested using BamHI and EcoRI and ligated into pGEX6P1 to generate the construct pGEX6P1-qslA. Strain BL21 Star™ carrying pGEX6P1-qslA was grown in 2.5 litres of LB medium to OD_{600} = 0.5 before being induced with 200 µM of isopropyl-β-d-thiogalactopyranoside overnight at 18°C. Cell pellet was resuspended in 25 mM Tris-HCl buffer (pH 7.8) containing 150 mM NaCl and lysed by sonication. GST-QslA fusion protein was purified using Glutathione Sepharose column chromatography. GST tag was cleaved from GST-QslA fusion protein using PreScission™ Protease (GE). The protein purity was judged by SDS/PAGE to be >99% pure.

3.2.10 Electrophoretic mobility shift assay (EMSA)

Biotinylated DNA probe was amplified using 5′-biotin labelled primer (Table S1) to generate a 350 bp DNA fragment. LasR and QslA proteins, at indicated concentrations, were pre-incubated in DNA-binding buffer (10 mM Tris-Cl pH 7.5, 50 mM KCl, 1 mM DTT, 2.5% glycerol, 5 mM MgCl2, 10 ng μl^{-1} poly (dI·dC), 0.05% NP-40, 5 µM 3-oxo-C12-HSL) for 20 min at room temperature and further incubated for 20 min after 1.8 fmol of DNA probe was added. Loading buffer was then added to the reaction mixture and electrophoresis was conducted in native 6% Tris-borate-EDTA polyacrylamide gel at 4°C. DNA probes were detected using LightShift® Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's protocol.

3.2.11 Bioinfomatics analysis

Domain and motif scans were carried out using InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/). Protein homologues were identified using NCBI BlastP. Hydrophobicity plot analysis of QslA was carried out using CLC Main WorkBench (CLC Bio, Denmark). Alignments and amino acid sequence percent identity of QslA, QteE, QscR and TraM were analysed by ClustalW (http://www.ch.embnet.org/software/ClustalW.html) and shaded using Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Protein structures were predicted by Hidden Markov Model (HMM)-based method (http://compbio.soe.ucsc.edu/SAM_T08/T08-query.html) and viewed using PyMol.

3.3 Results

3.3.1 Identification of QslA as a suppressor of *rhlR* expression

The mutants QRM92 and QRM130, which showed substantially higher P*rhlR-lacZ* activities than QR1 (Fig. 2.1), were chosen for further characterization in this study. DNA sequencing analysis showed that the transposons were, respectively, inserted in the region between *PA1243* and *PA1244* and between *PA1244* and *aprX* in QRM130 and QRM92 (Fig. 3.1A). *PA1243* and *PA1244* encode hypothetical proteins of 858 amino acid and 113 amino acid, respectively, whereas *aprX* encodes a 414-amino acid protein substrate of the type I secretion system (Duong *et al.*, 2001). RT-PCR analysis showed that only *PA1244* and *aprX* transcript levels were consistently reduced or increased in both mutants (Fig. 3.1B), suggesting that they might be related to the change in expression pattern of *rhlR* in mutants QRM92 and QRM130.

Further analysis showed that *in trans* expression of *PA1244* in QR1 reduced P*rhlR-lacZ* activity to a basal level, but expression of *PA1243* or *aprX* did not affect the reporter activity (Fig. 3.2). These data suggests that PA1244 could be involved in modulation of QS. For the convenience of discussion, *PA1244* was referred to as *qslA* hereafter according to its role in encoding a quorum sensing LasR-specific anti-activator as described below. To verify that QslA also affects *rhlR* expression in wild type strain, the *qslA* in-frame deletion mutant ΔqslA and its complemented strain ΔqslA(qslA) were generated using PAO1 as the parental strain. As expected, P*rhlR-lacZ* activity was increased in ΔqslA but greatly reduced in the complemented strain ΔqslA(qslA) (Fig. 3.2).

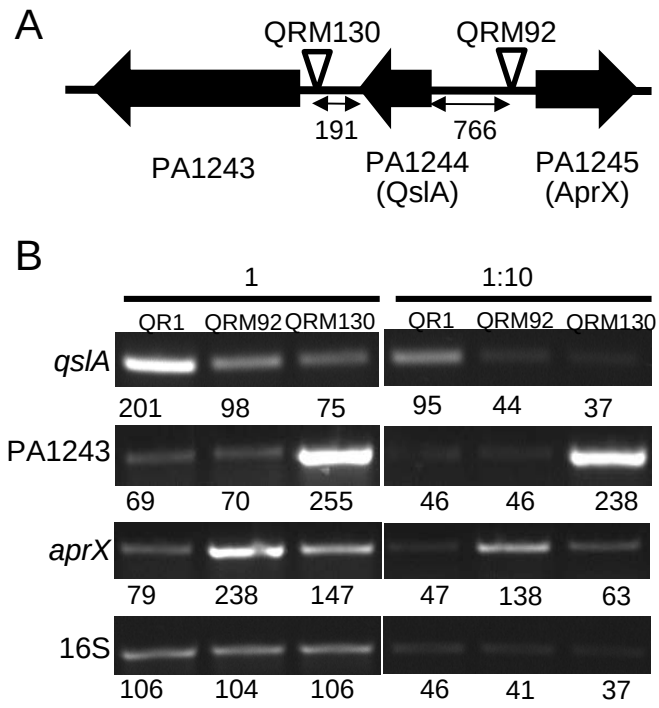


Fig. 3.1 Changed level of QslA affects *rhlR* expression in mutant QRM92 and QRM130.
(A) Genetic organisation of *qslA* and adjacent genes *PA1243* and *aprX*. Transposon insertion sites of mutants QRM92 and QRM130 were indicated by triangles. (B) RT-PCR analysis of *qslA*, *PA1243*, *aprX* and control 16S rRNA in QR1, QRM92 and QRM130. Band intensities listed under the bands were measured by ImageJ.

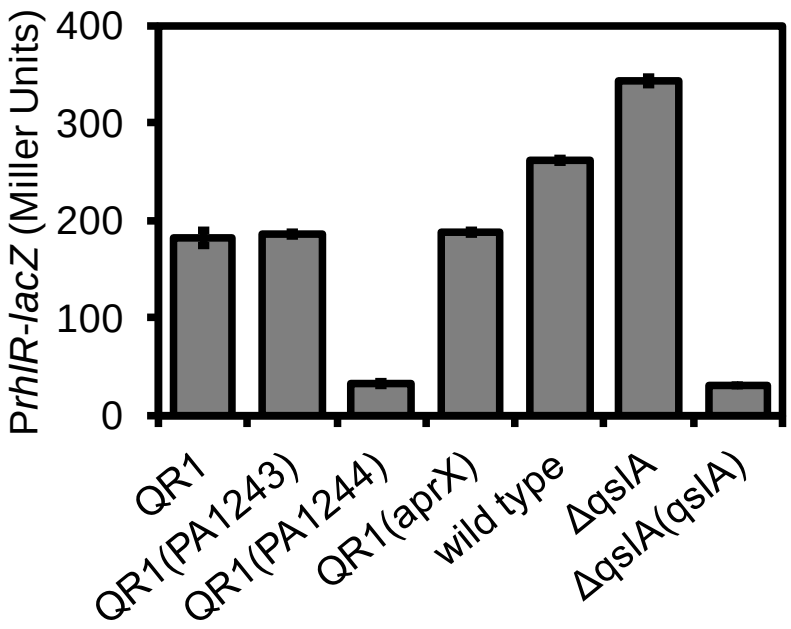
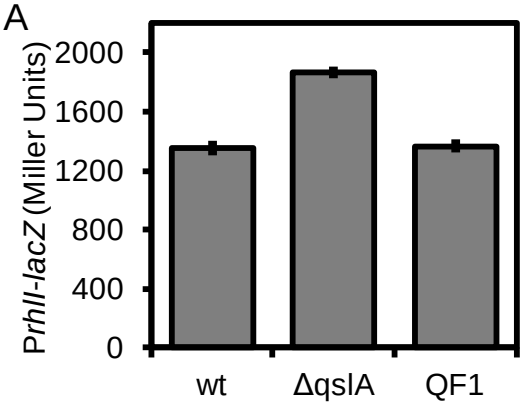


Fig. 3.2 QslA is implicated in regulation of the QS gene *rhlR* expression. P*rhlR-lacZ* activity assays were measured at OD_{600} = 1.5. The data shown were means of 3 replicates and error bar, which indicates standard deviation, is not seen when standard deviation is small.

3.3.2 Translational start site of QslA

QslA was annotated as a putative protein with 113 amino acids in the Pseudomonas Genome Database (www.pseudomonas.com), but *in silico* analysis predicted a translational start site at 27 bp downstream of the original annotated start site and the open reading frame (ORF) would generate a 104-amino acid protein (FGENESB, Softberry). To address this discrepancy, a flag peptide fragment was fused to chromosomal *qslA* at the C-terminal, which was determined not to affect QslA activity (Fig. 3.3A). The purified QslA-FLAG fusion protein was analysed by Edman degradation and the results showed that the first 8 amino acids of the QslA-flag fusion protein was matched to the 10^{th}-17^{th} amino acid of the originally annotated QslA protein (Fig. 3.3B). Due to background noise in the Edman degradation analysis, more than one amino acid peaks were detected at each position and these background peaks are usually from the previous or next amino acid. QslA protein therefore consists of 104 amino acids with a predicted molecular weight of 11.8 kDa.

A



B

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Annotated amino acid	M	T	L	R	N	G	V	P	S	M	T	K	D	E	K	E	T
Sequenced amino acid										M/T	T/K	D/K	E/D	E/D	K/E	K/E	T/K

C

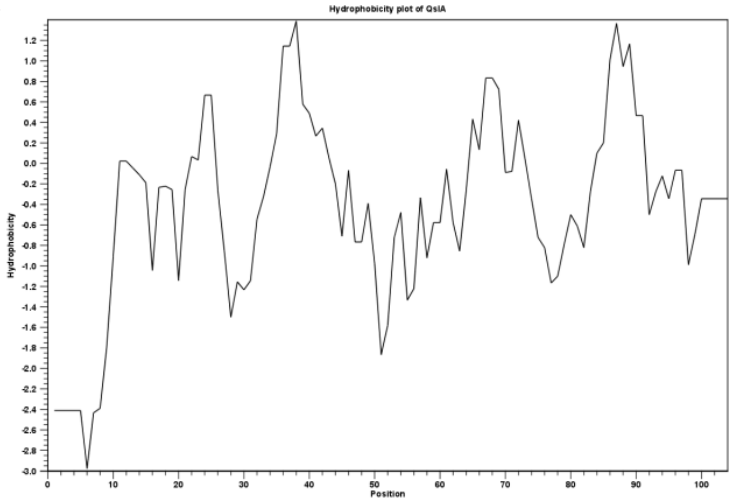


Fig. 3.3 QslA was discovered to alter *rhlR* gene expression in mutant QRM92 and QRM130.
(A) P*rhlI-lacZ* activities of wild type (wt), ΔqslA and wild type strain with chromosomal-integrated qslA fused with FLAG (QF1). The data shown were means of 3 replicates and error bar indicates standard deviation. (B) Alignment of the annotated QslA amino acid sequence with the sequenced amino acids of QslA-FLAG fusion protein. (C) Hydrophobicity plot analysis of QslA by Kyte-Doolittle scale, window size=9 (CLC Main Workbench).

3.3.3 *In silico* analysis of QslA

QslA homologues are found in certain *Pseudomonas* species, including *P. aeruginosa*, *P. mendocina* and *P. fluorescens* (Table 3.2). These homologues share over 30% identity at amino acid level but none of them have been characterised previously. Domain analysis and motif search did not reveal any useful clues. Analysis of QslA by PredictProtein (Rost *et al.*, 2004) showed that it has a highly helical secondary structure (61%) and the helical regions are mainly found in two regions (amino acid 51–70 and 80–96) as predicted by DNASTAR® Lasergene Protean software. These structural features were also identified in QslA predicted protein structure determined using a HMM-based protein structure modelling program (http://compbio.soe.ucsc.edu/SAM_T08/T08-query.html) (Fig. 3.4A). It was also noted that QslA contains a relatively high proportion of hydrophobic amino acid residues (40/104), but the hydrophobicity plot did not identify an obvious hydrophobic region (Figure 3.3C).

The function of QslA was not evident from analysing its protein sequence, and this was expected for TraM-like anti-activator proteins which do not have identifiable motifs or domains (Hwang *et al.*, 1995). As QslA negatively regulates *rhlR* expression, it was very likely that QslA was a TraM-like anti-activator. Thus, the protein sequence of QslA was compared with known and probable QS anti-activators QscR, QteE and TraM, but sequence alignment revealed little amino acid similarity among these proteins (Table 3.3, Fig. 3.5A). However, TraM and QslA were found to be similar in protein size containing 102 amino acids and 104 amino acids, respectively. Moreover, interestingly, protein structure prediction by HMM-based program (http://compbio.soe.ucsc.edu/SAM_T08/T08-query.html) showed that QslA shared conserved alpha helix structural features with TraM and TraM2 (Fig. 3.4A, B), as the three proteins contain two long alpha helices (Chen *et al.*, 2004, Vannini *et al.*, 2004, Chen *et al.*, 2006). In contrast, QslA did not resemble

the predicted structures of QscR and QteE (Fig. 3.4C, D). Although QslA and TraM share some protein structural similarities, superimposition of QslA predicted structure on TraM protein structure (Vannini *et al.*, 2004) using PyMOL (http://www.pymol.org/) resulted in partial overlap only (Fig. 3.5B), and not all of the crucial residues essential for TraM interaction with TraR are present in the overlapping region (Hwang *et al.*, 1999, Swiderska *et al.*, 2001). This is somewhat expected as the LuxR homologues from different bacterial species, such as TraR and LasR, normally share low amino acid sequence homology. The above analyses suggest that QslA may be a QS anti-activator and its effect on QS was further investigated.

Table 3.2 QslA protein homologues.
Proteins which showed amino acid sequence homology to QslA were identified by BLASTP program provided by the National Center for Biotechnology Information (NCBI).

Species	Gene ID	E value	Identities (%)	Positives (%)
P. aeruginosa PAO1	PA1244	5e-54	100	100
P. aeruginosa UCBPP-PA14	PA14_48150	5e-54	100	100
P. aeruginosa 2192	PA2G_00237	5e-54	100	100
P. aeruginosa PACS2	PaerPA_01001720	7e-54	100	100
P. aeruginosa PAb1	PaerPAb_19586	7e-54	100	100
P. aeruginosa LESB58	PLES_40681	3e-53	99	99
P. aeruginosa C3719	PACG_00214	3e-53	99	99
Pseudomonas mendocina ymp	Pmen_3423	2e-11	36	57
P. mendocina ymp	Pmen_1507	5e-08	34	47
Pseudomonas fluorescens Pf0-1	Pfl01_3024	1e-05	41	52
P. fluorescens SBW25	PFLU2003	0.001	37	46
P. fluorescens SBW25	PFLU1925	0.045	42	55

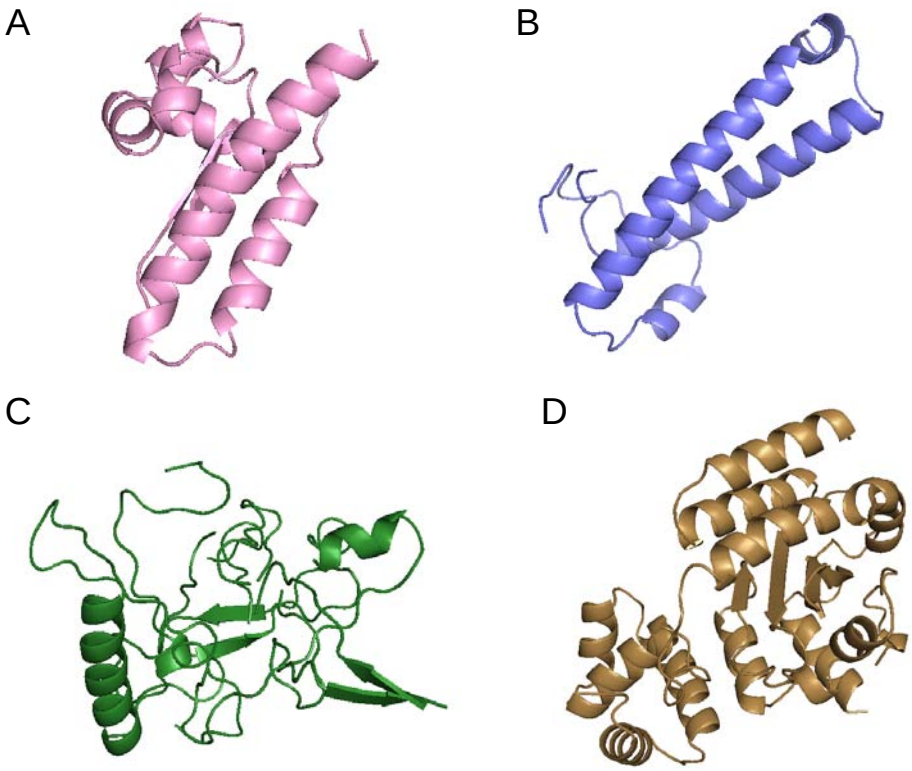


Fig. 3.4 Predicted Protein structure of QslA, QteE and QscR and crystal structure of TraM.
Ribbon structure predicted for QslA (A), QteE (C) and QscR (D) by HMM-based methods (http://compbio.soe.ucsc.edu/SAM_T08/T08-query.html). TraM ribbon structure chain B (B) was obtained from PDB no. 1US6 (Vannini *et al*. 2004).

Table 3.3 Protein sequence identity of QslA, QteE, QscR and TraM.
Proteins were aligned using ClustalW and the percent identities of alignment were shown.

Protein	QslA	QteE	QscR	TraM
QslA		11%	12%	11%
QteE	11%		14%	10%
QscR	12%	14%		9%
TraM	11%	10%	9%	

A

```
QslA     1 MTKDEKEKTHVDAIIERYKDLMVEIPPADRQPGLSLLWPVPF-AQPAIDKGVRQAENWLA
TraM     1 -MELEDANVTKKVELRPLIGLTRGLPPTDLETITIDAIRTHRRLVEKADELFQALPETYK

QslA    59 DQIEGQLWTAFAFGRDSLPTPMQKTAFEVAFLTRLQQRLVAARRSG
TraM    60 TGQACGGPQHIRYIEASIEMHAQMSAVNTLYSILGFIPKVVVN---
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B

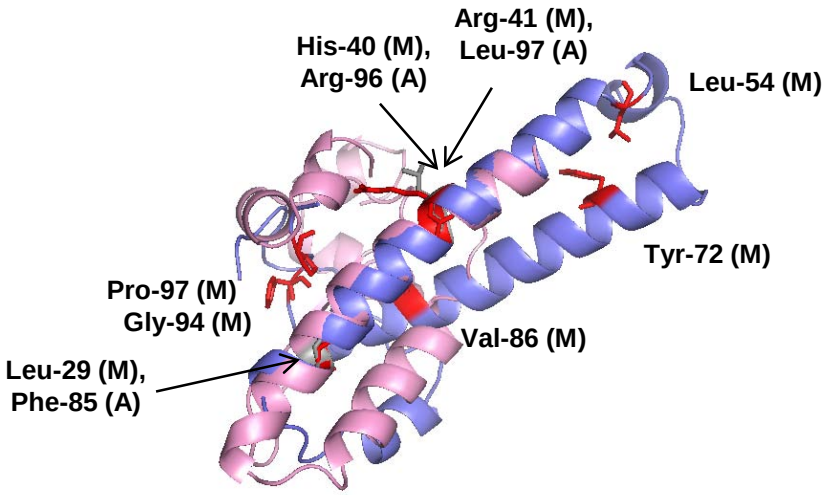


Fig. 3.5 Comparison of QslA and TraM structures.
(A) Alignment of QslA and TraM protein sequence using ClustalW. The identical amino acids are highlighted black and the similar amino acids are highlighted grey. Dashes (-) indicate gaps. The boxed regions indicate residues which were reported to be important for TraM interaction with TraR (Hwang et al., 1999 (B) Superimposition of QslA predicted ribbon structure (pink) on TraM ribbon structure chain B from PDB no. 1US6 (Vannini et al. 2004) (purple). QslA residues (indicated as (A)) Phe-85, Arg-96 and Leu-97 are found overlapping TraM residues (indicated as (M)) Leu-29, His-40, Arg-41, respectively. Side chains of TraM residues Leu-29, His-40, Arg-41, Leu-54, Tyr-72, Val-86, Gly-94 and Pro-97 are shown in red while side chains of QslA residues Phe-85, Arg-96 and Leu-97 are shown in grey.

3.3.4 Effect of QslA on QS gene expression and signal production

The Rhl QS system is linked to the Las QS and PQS signalling systems (Latifi *et al.*, 1996, Pesci *et al.*, 1999, McKnight *et al.*, 2000). As QslA affects *rhlR* expression, the impact of QslA on these three signalling systems was investigated. To this end, the coding sequence of *lacZ* was cloned under the control of the promoters of these signal synthase genes, i.e., *lasI*, *pqsA* and *rhlI*, to generate the reporter fusion genes P*lasI*-*lacZ*, P*pqsA*-*lacZ* and P*rhlI*-*lacZ*. The expression of these signal synthases in wild type PAO1, ΔqslA and ΔqslA(qslA) were determined by measuring P*lasI*-*lacZ*, P*pqsA*-*lacZ* and P*rhlI*-*lacZ* activities at different cell densities (OD_{600}). As a comparison, the corresponding signal production in these strains was also measured. Contrary to the effect on *rhlR* expression, deletion of *qslA* resulted in decreased P*lasI*-*lacZ* activity, and overexpression of *qslA* in ΔqslA abolished P*lasI*-*lacZ* activity (Fig. 3.6A). The transcriptional fusion gene reporter data agreed well with the results of 3-oxo-C12-HSL assay, which showed lesser 3-oxo-C12-HSL molecules in ΔqslA than in wild-type with undetectable level of 3-oxo-C12-HSL being found in the complemented strain ΔqslA(qslA) (Fig. 3.6D).

It is expected that inhibition of 3-oxo-C12-HSL-dependent Las QS would result in loss of HAQ and C4-HSL signals since LasR activates the expression of their synthesis genes (de Kievit *et al.*, 2002, Gallagher *et al.*, 2002). In agreement with this, *in trans* expression of *qslA* in ΔqslA blocked the expression of *rhlI* and *pqsA* (Fig. 3.6B, C), and abolished the production of HAQ and C4-HSL (Fig. 3.6E, F). Consistent with the *qslA* expression results, *pqsA* expression and HAQ production were higher in ΔqslA than in wild type (Fig. 3.6B, E). In contrast, only a modest change in *rhlI* transcription and C4-HSL level were detected in the absence of *qslA* at OD_{600} = 0.6 (Fig. 3.6C, F).

The above results establish the negative regulatory role of overexpression of *qslA* on three signalling systems, i.e., PQS, Las and Rhl, when expressed in

trans. On the contrary, QslA influence of QS and PQS signalling in wild type PAO1 is gene-dependent. The expression of *rhlI* was slightly upregulated in the deletion mutant ΔqslA compared to wild type whereas expression of *pqsA* was induced by 2-3 fold in ΔqslA compared to wild type. It is possible that the expression level of the LasR-dependent genes is related to the LasR affinity to corresponding promoters (Schuster *et al.*, 2004). The promoters such as *pqsA* promoter with relatively low affinity for LasR may not be "saturated" under normal conditions, and thus could direct a substantially higher increase in gene expression than those "saturated" promoters such as *rhlI* promoter when more LasR molecules become available.

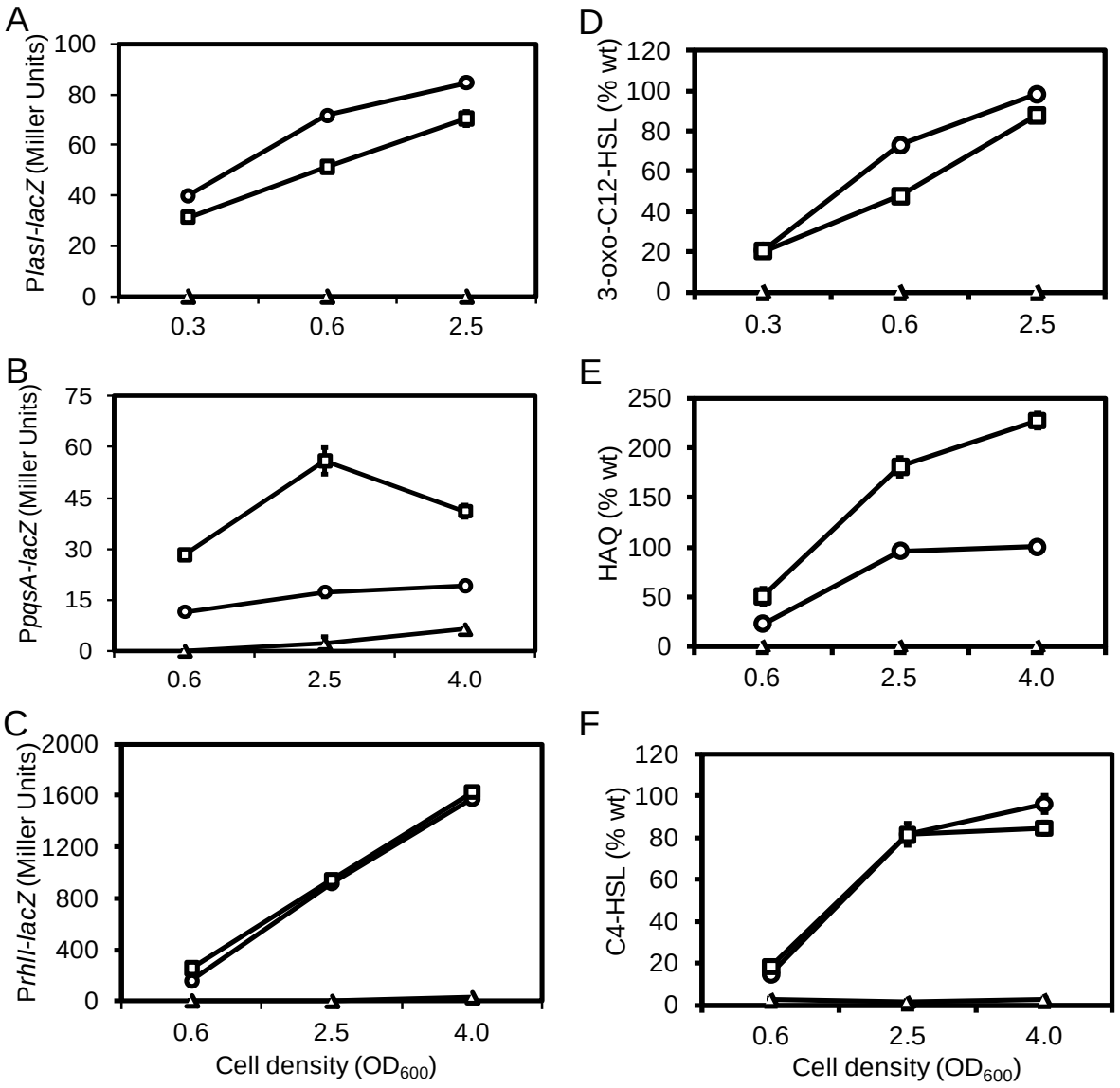


Fig. 3.6 QslA influences the Las and Rhl QS as well as PQS signalling systems. The effects of QslA on expression of lasI (A), pqsA (B) and rhlI (C) were determined by measuring β-galactosidase activities in wild type (○), ΔqslA (□) and ΔqslA(qslA) (Δ). PlasI-lacZ, PpqsA-lacZ and PrhlI-lacZ gene fusion construct were introduced into the strains tested. 3-oxo-C12-HSL (D), HAQ (E) and C4-HSL (F) levels in the supernatant of wild-type (○), ΔqslA (□) and ΔqslA(qslA) (Δ) were determined by β-galactosidase activity assays using reporter strains DL1, PP1 and DR1, respectively. The data shown were means of 3 replicates and error bar, which indicates standard deviation, is not seen when standard deviation is small.

3.3.5 QslA affects virulence factor production

The AHL-dependent QS and PQS signalling systems regulate the expression of genes encoding a range of virulence factors such as elastase, protease and pyocyanin (Latifi *et al.*, 1995, Pesci *et al.*, 1999). Because QslA altered QS and PQS signalling, we reasoned that production of these virulence factors would be similarly affected. The results showed that deletion of *qslA* resulted in about 20 – 200% increase in elastase production compared with the wild type strain PAO1 at three growth stages (Fig. 3.7A). Protease produced in ΔqslA was about 3-fold and 50% more than that in wild-type at OD_{600} = 0.5 and OD_{600} = 3.0, respectively, but at OD_{600} = 5.0, no substantial difference was detected in wild type and the mutant ΔqslA (Fig. 3.7B). Compared to elastase and protease, deletion of *qslA* caused most substantial changes in pyocyanin production. Its level in ΔqslA was increased by about 150 – 280% over the wild type PAO1 at three growth stages from OD_{600} = 3.0 to 5.5 (Fig. 3.7C). In the *qslA* complemented strain, production of three virulence factors were drastically reduced to almost undetectable levels (Fig. 3.7A, B, C).

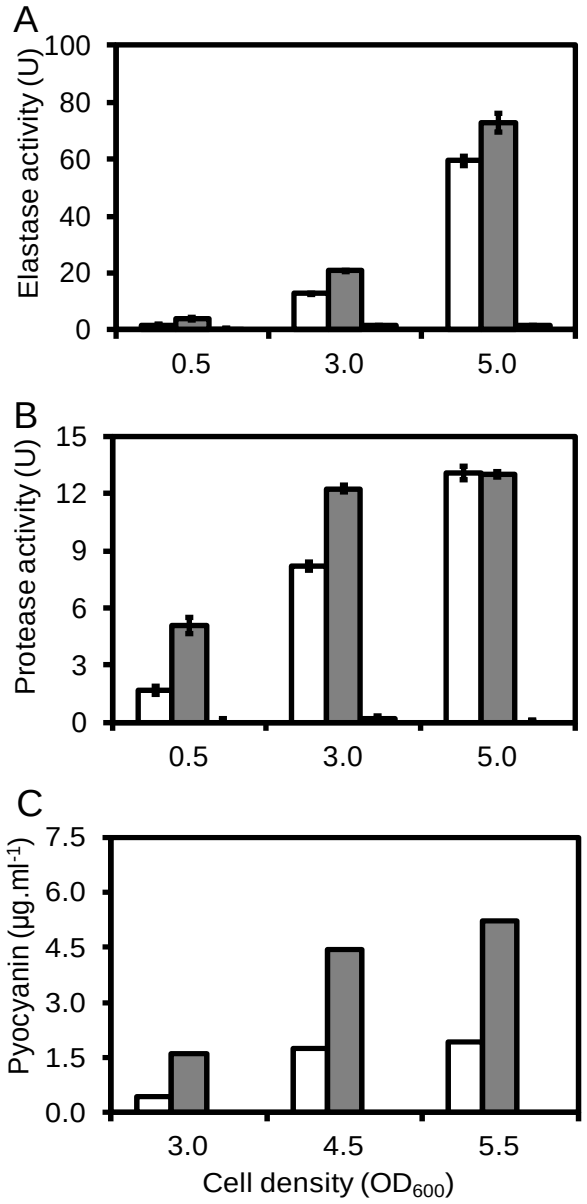


Fig. 3.7 QslA affects virulence factor production.
Production of elastase (A), protease (B), and pyocyanin (C) were determined in wild type (white bar), ΔqslA (shaded bar) and ΔqslA(qslA) (dashed bar). The data shown were means of 3 replicates and error bar indicates standard deviation.

3.3.6 QslA modulates LasR activity post-transcriptionally

To understand whether QslA affects the activity of QS signal receptor LasR, *lasR* expression was first analysed by determining P*lasR*-*lacZ* activities in wild type, ΔqslA and ΔqslA(qslA). The results showed that *lasR* expression was consistently higher in ΔqslA compared to the wild type and this was reduced when *qslA* was expressed *in trans* in ΔqslA (Fig. 3.8A).

The above results indicate that the transcript level of *lasR* is modulated by QslA. Given that QslA does not contain a DNA-binding domain and that *lasR* expression is auto-regulated (Pesci *et al.*, 1997), we reasoned that QslA might control *lasR* transcription through its post-transcriptional effect on LasR. To explore this possibility, *lasR* was fused with the constitutive promoter P*lac* and expressed in strain ΔlasR that contains either the *qslA* expression construct (pDSK-qslA) or the empty vector pDSK. LasR activity was gauged by its activation of P*lasI*-*lacZ* activity. The results showed that QslA inhibited LasR activity when *lasR* was expressed constitutively (Fig. 3.8B), demonstrating that QslA inhibits LasR post-transcriptionally. QslA was also able to inhibit the LasR-HTH activity (Fig. 3.8B), as previously shown for QR1 (Fig. 3.2).

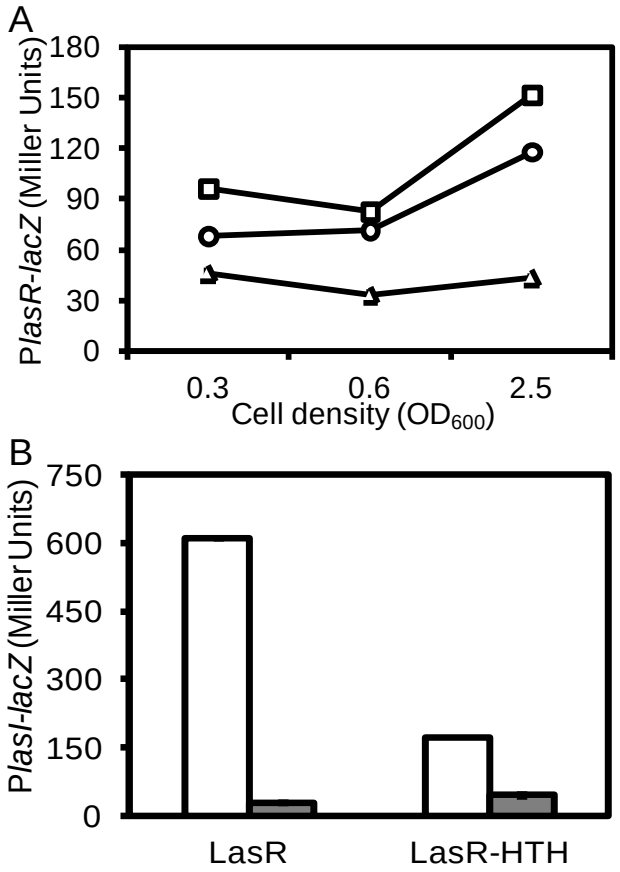


Fig. 3.8 QslA inhibits LasR activity post-transcriptionally.
(A) The effects of QslA on expression of lasR were determined by measuring β-galactosidase activities in wild type(○), ΔqslA (□) and ΔqslA(qslA) (Δ). (B) The influences of QslA on PlasI-lacZ activities in ΔlasR(lasR) and ΔlasR(lasRHTH). The lacZ activities were determined in the absence (white bar) or presence (shaded bar) of in trans expression of *qslA*. The data shown were means of 3 replicates and error bar, which indicates standard deviation, is not seen when standard deviation is small.

3.3.7 QslA inhibits LasR by protein-protein interaction

To determine whether QslA post-transcriptionally modulates LasR activity through protein-protein interaction, QslA-FLAG fusion protein was expressed in strain PAO1 and the deletion mutant Δ*lasR*. As a control, untagged QslA protein was also expressed in PAO1 using the same vector as the QslA-FLAG fusion gene. Immunoprecipitates eluted from anti-FLAG affinity gel were immunoblotted using anti-LasR and anti-FLAG antibodies separately and the results showed that QslA-FLAG co-immunoprecipitated with LasR (Fig. 3.9A, top and middle panels). On the other hand, LasR did not co-immunoprecipitate with untagged QslA although LasR was present in the cell lysate (Fig. 3.9A, top and bottom panels lane 1). The specificity of anti-LasR antibody against LasR was confirmed by the absence of band in the immunoblot of the cell lysates from Δ*lasR*(*qslA*-FLAG) (Fig. 3.9A, bottom panel lane 3). The results demonstrated that QslA formed a heterologous protein complex with LasR *in vivo*.

To test whether DNA-binding ability of LasR is disrupted by QslA and to further verify the interaction between LasR and QslA, electrophoretic mobility shift assay (EMSA) was performed using purified LasR and QslA proteins. Previous study showed that LasR binds to the intergenic promoter region between *lasI* and *rsaL* (Schuster *et al.*, 2004, Gilbert *et al.*, 2009), so the biotinylated DNA probe of this region was generated. EMSA analysis confirmed that LasR formed DNA-protein complexes with the DNA probe (Fig. 3.9B lane 3). But when QslA was added to the reaction mixture, the DNA-binding activity of LasR was disrupted in a dose-dependent manner, with complete disruption of DNA-binding when 800-fold molar excess of QslA over LasR was incubated (Fig. 3.9B lane 4-8). As a control, QslA alone could not form a complex with the probe DNA (Fig. 3.9B lane 1). Interestingly, heat-treatment of QslA reduced but did not abolish the DNA-binding ability of LasR (Fig. 3.9B lane 9), suggesting that either QslA is relatively heat-

stable or denatured QslA remains partially active in interaction with LasR. We further showed that QslA interaction with LasR was not affected by addition of up to 125 μM of 3-oxo-C12-HSL (Fig. 3.9C)

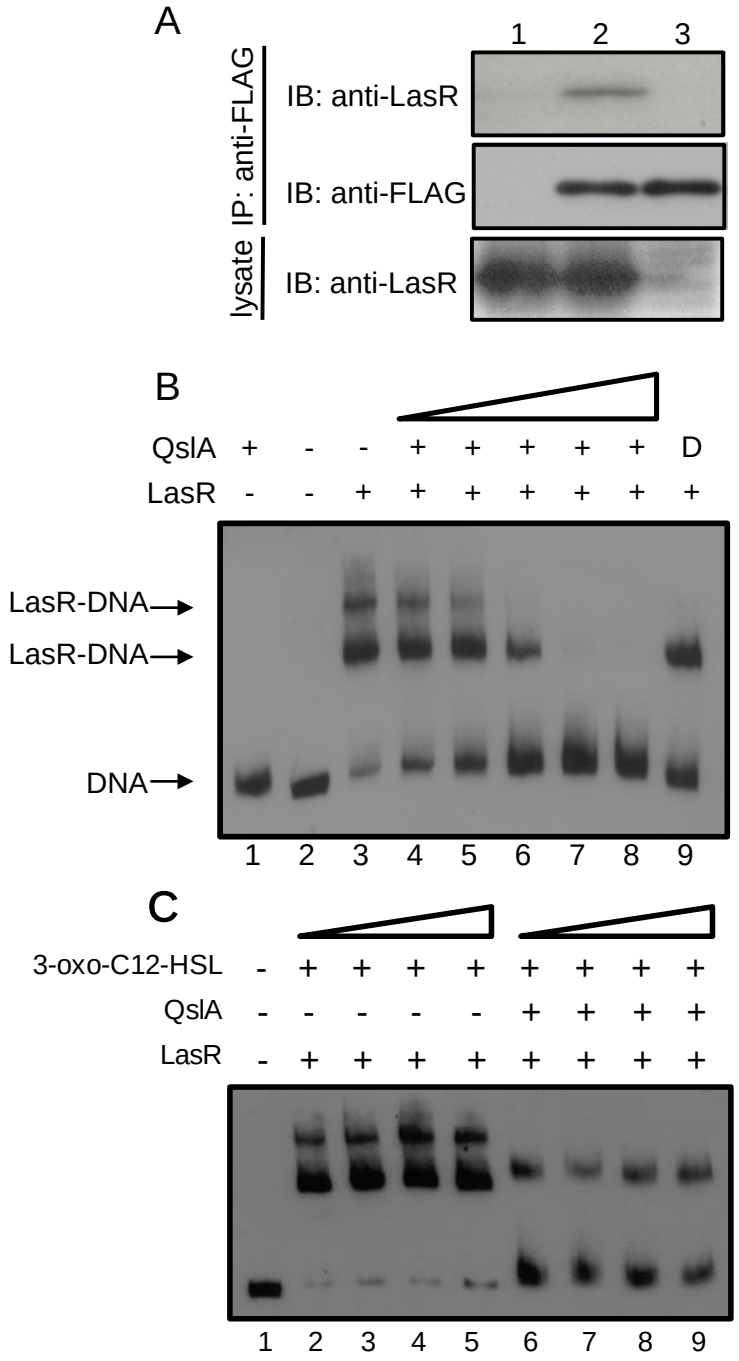


Fig. 3.9 QslA directly interacts with LasR.
(A) The total protein lysates from wt(qslA), lane 1; wt(qslA-FLAG), lane 2; and ΔlasR(qslA-FLAG), lane 3 were immunoprecipitated (IP) separately with anti-FLAG M2 affinity gel. Immunobloting (IB) was carried out using anti-LasR and anti-FLAG antibodies as indicated. (B) EMSA was carried out using biotinylated DNA probe containing *lasI-rsaL* promoter region and 1.8 fmol of DNA probe was used in each reaction. DNA probe was incubated for 20 min with QslA (144 μM), lane 1; LasR (90 nM), lane 3; and LasR (90 nM) pre-incubated with increasing concentrations of QslA (9, 18, 36, 72, 144 μM) for 20 min, lanes 4 – 8. QslA (144 μM) in lane 9 was heat-denatured at 99°C for 15 min before incubation with LasR and DNA probe. Lane 2 contains DNA probe without proteins. (C) Lane 1 contains DNA without proteins. DNA was incubated with LasR (90 nM) (lane 2 – 5) or LasR (90 nM) and QslA (36 μM) (lane 6 – 9) with different concentrations of 3-oxo-C12-HSL: 1 μM (lane 2, 6), 5 μM (lane 3, 7), 25 μM (lane 4, 8) and 125 μM (lane 5, 9).

3.3.8 LasR overexpression promotes negative feedback regulation of *lasI* expression

It is known that LasR activates the expression of *lasI* and 3-oxo-C12-HSL production (Seed *et al.*, 1995), but surprisingly, null mutation of the LasR anti-activator QslA did not cause enhanced *lasI* expression and 3-oxo-C12-HSL production (Fig. 3.6A, D). We speculated that this is due to increased LasR activation of the negative feedback mechanism involving RsaL, which is a *lasI* promoter specific repressor (Rampioni *et al.*, 2007b), in ΔqslA. When *lasR* was overexpressed in wild type strain to mimic ΔqslA by changing the stoichiometry of QslA and LasR and providing more unbound LasR proteins than the wild type containing the expression vector, it was found that *lasI* expression and 3-oxo-C12-HSL production were indeed reduced at late growth stage (Fig. 3.10A, B). To determine whether reduction in Las QS signalling in ΔqslA and in *lasR* overexpression strain was due to increased expression of *rsaL*, *rsaL* transcript level analysis were carried out by fusing the *rsaL* promoter with the *lacZ* reporter gene. The results showed that *rsaL* expression was increased to a comparable level by either deletion of *qslA* or overexpression of *lasR* (Fig. 3.10C), thus it is plausible that the increased level of RsaL in ΔqslA might be the cause of the decreased *lasI* expression and 3-oxo-C12-HSL production in ΔqslA.

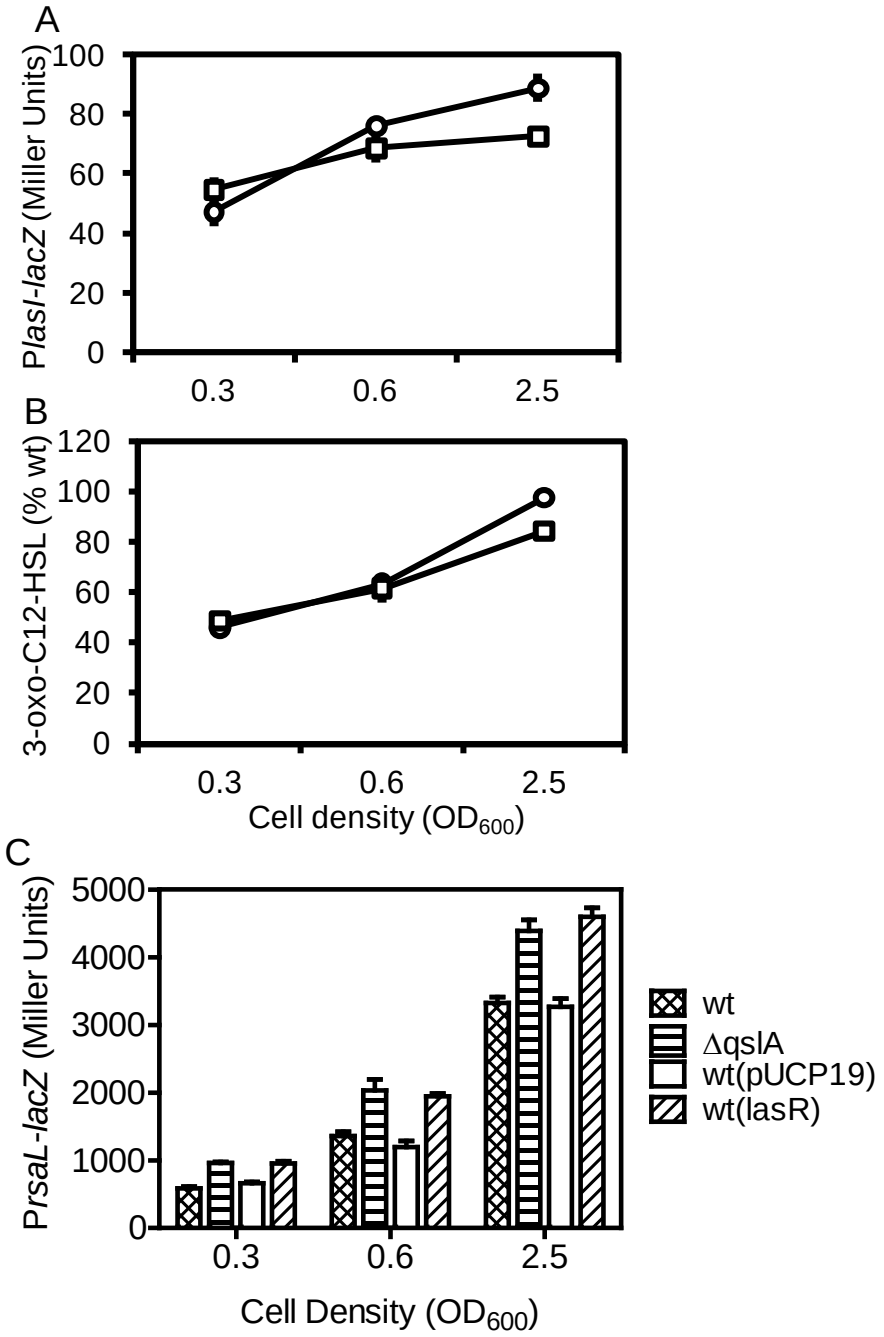


Fig. 3.10 Overexpression of LasR reduces P*lasI-lacZ* expression and 3-oxo-C12-HSL production.
P*lasI-lacZ* activities (A) and 3-oxo-C12-HSL levels (B) of wild type containing the vector pUCP19 (○) and wild type containing pUCP-lasR (□). (C) P*rsaI-lacZ* activities of wild type (wt), ΔqslA, wild type with pUCP19 vector control (wt(pUCP19)) and wild type containing pUCP-lasR (wt(lasR)). The data shown were means of 3 replicates and error bar, which indicates standard deviation, is not seen when standard deviation is small.

3.3.9 RsaL-dependent negative feedback regulation inhibits lasI expression in ΔqslA

To test whether RsaL was responsible for the inhibition of *lasI* expression in ΔqslA, the single deletion mutant ΔrsaL and double deletion mutant ΔrsaLΔqslA were generated. As expected, deletion of *rsaL* from PAO1 substantially increased the *lasI* expression level (Fig. 3.11A). In the absence of *rsaL*, null mutation of *qslA* caused an increase of about 25 – 40% in the transcriptional expression level of *lasI* than the single deletion mutant ΔqslA (Fig. 3.11A). Similarly, the transcriptional expression level of *rhlI* was significantly higher in ΔrsaLΔqslA compared to ΔrsaL (Fig. 3.11B). Taken together, the above findings suggest that the increased level of RsaL in ΔqslA plays a role in negative feedback inhibition of *lasI* expression. However, the likelihood that *lasI* expression is also inhibited by other mechanism(s) in the absence of QslA cannot be ruled out at this stage, as the expression of *lasI* is not necessarily dependent on LasR (Duan & Surette, 2007).

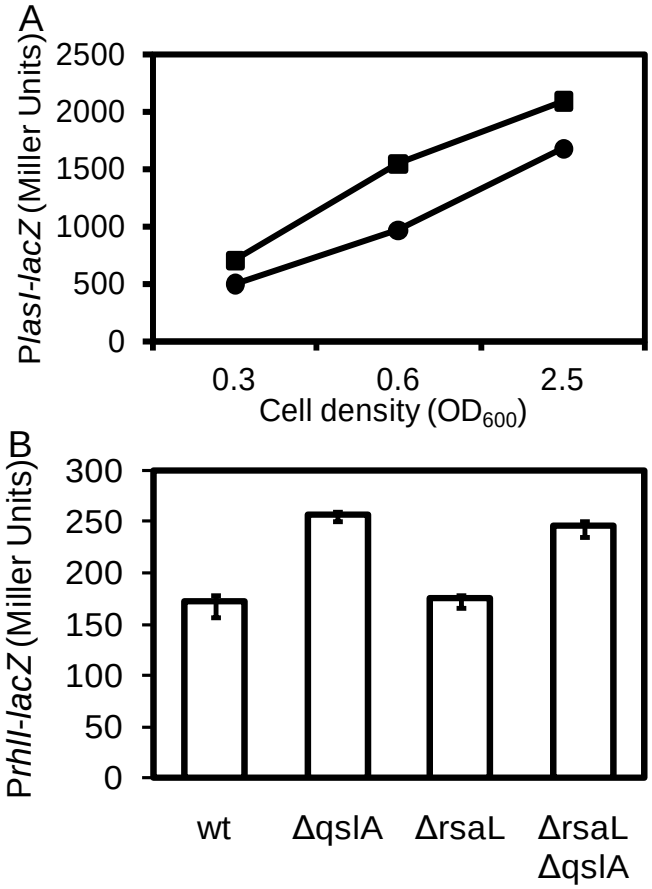


Fig. 3.11 RsaL is implicated in QslA modulation of *lasI* expression.
(A) Null mutation of QslA resulted in increased transcriptional expression of *rsaL.* Symbol: open bar, wild type; filled bar, ΔqslA. (B) Deletion of *rsaL* led to decreased transcriptional expression of *lasI* in the QslA null mutant. Symbol: slash bar, ΔrsaL; grill bar, ΔrsaLΔqslA.The data shown were means of 3 replicates and error bar indicates standard deviation. Statistically significant level is shown by "**", which indicates p value < 0.01, or "#", which indicates p value < 0.05.

3.3.10 3-oxo-C12-HSL sensitivity is modulated by QslA

It was shown in Fig. 3.6 that deletion of *qslA* resulted in increased *pqsA* and *rhlI* expression, which are known to be LasR-dependent (de Kievit *et al.*, 2002, Gilbert *et al.*, 2009), despite reduced 3-oxo-C12-HSL level. This led us to question whether the bacterial population could become more sensitive to 3-oxo-C12-HSL in the absence of QslA. To test this intriguing possibility, single deletion mutant ΔlasI and double mutant ΔlasIΔqslA were generated and the amount of exogenous 3-oxo-C12-HSL necessary for induction of QS-dependent elastase and protease production in these mutants were determined. Results showed that in the absence of *qslA*, less than 200 nM 3-oxo-C12-HSL was sufficient to trigger production of elastase and protease to wild-type levels (Fig. 3.12A, B). This was about 9 times less than the 3-oxo-C12-HSL concentration needed in ΔlasI (Fig. 3.12A, B).

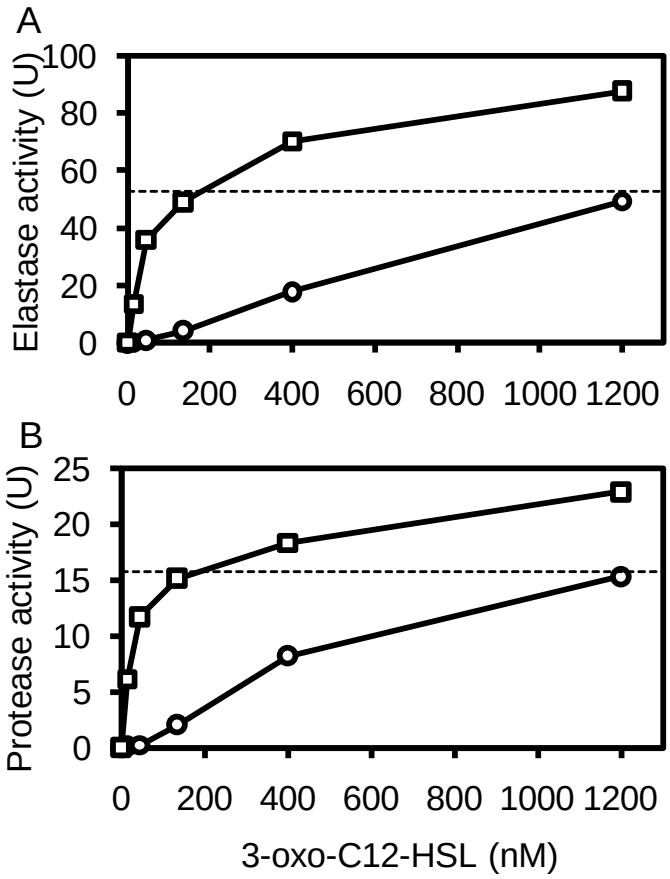


Fig. 3.12 QslA controls the 3-oxo-C12-HSL threshold concentration needed for QS activation.
Elastase (A) and protease (B) production by mutants ΔlasI (○) and ΔlasIΔqslA (□) were determined in LB supplemented with various concentrations of 3-oxo-C12-HSL as stated. The dotted line indicates the level of elastase or protease in wild type strain PAO1 grown without exogenous 3-oxo-C12-HSL. The data shown were means of 3 replicates and error bar, which indicates standard deviation, is not seen when standard deviation is small.

3.3.11 Expression of *qslA* is not QS-regulated and is constitutive

To determine whether QslA effect on QS could be regulated by controlling *qslA* expression levels, transcriptional levels of *qslA* was analysed. The promoter region of *qslA* is adjacent to that of *aprXDEF* operon which was identified to be QS-regulated in microarrays (Schuster *et al.*, 2003, Wagner *et al.*, 2003) as well as in ChIP-chip study (Gilbert *et al.*, 2009). But, our transcriptional studies by P*qslA*-*lacZ* activity assays and RT-PCR analysis showed that *qslA* expression is not regulated by LasR or RhlR (Fig. 3.12A, B), which dismissed the possibility that QslA may be involved in negative feedback of LasR activity. RT-PCR analysis of *qslA* also showed that its expression is constitutive throughout growth (Fig. 3.12C), suggesting that it is not involved in growth-dependent regulation of QS.

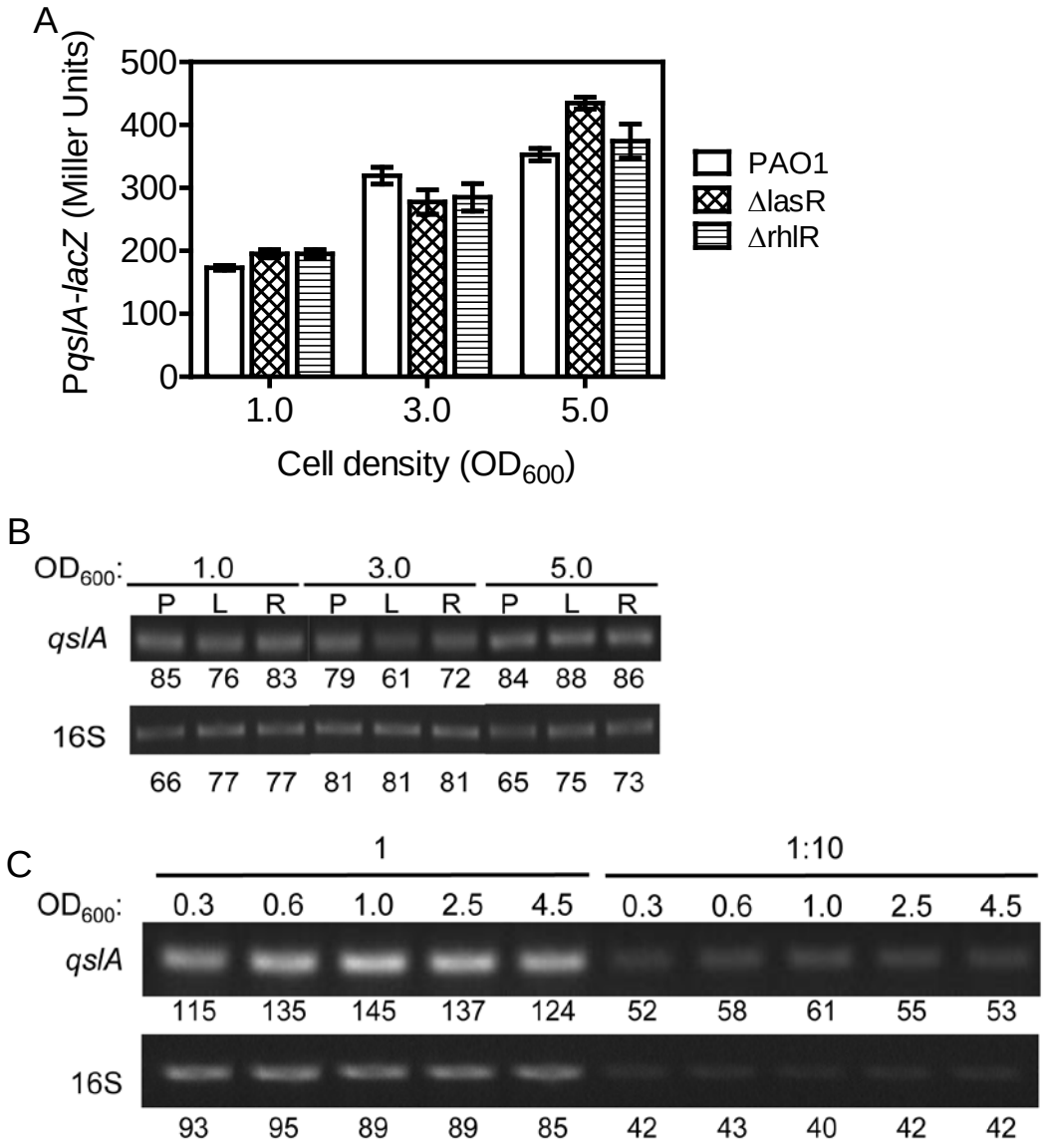


Fig. 3.13 Expression of *qslA* is not QS-dependent and is constitutive.
(A) The influence of LasR and RhlR on P*qslA-lacZ* activities were determined at different cell densities (OD_{600}). The data shown were means of 3 replicates and error bar indicates standard deviation. (B) RT-PCR analysis of *qslA* and control 16S rRNA in wild type (P), ΔlasR (L) and ΔrhlR (R). (C) RT-PCR analysis of *qslA* and control 16S rRNA at different cell densities (OD_{600}). Band intensities listed under each band were measured by ImageJ.

3.4 Discussion

In this chapter, evidence that a hypothetical protein PA1244, which was designated as QslA, is a key negative regulatory factor in the QS and PQS signalling pathway of *P. aeruginosa* was presented. In the absence of *qslA*, there was enhanced secretion of QS-regulated signal molecules and virulence factors including HAQ, pyocyanin, elastase and protease (Fig. 3.2, 3.6, 3.7). Significantly, *in trans* expression of *qslA* in the deletion mutantΔqslA substantially reduced or even abrogated the biosynthesis of AHL and HAQ signals as well as production of QS-regulated virulence factors (Fig. 3.6D-F; Fig. 3.7). In addition, we showed that QslA inhibited QS and virulence factor production through counteracting the QS signal receptor LasR. Co-immunoprecipitation analysis indicated that QslA formed a protein-protein complex with LasR under *in vivo* conditions (Fig. 3.9A). The finding was further verified by EMSA study, which showed that QslA prevented LasR from binding to its target promoter (Fig. 3.9B). Thus, it was established that QslA is an anti-activator, which is structurally and functionally similar to TraM, and acts by modulating QS through molecular interaction with the QS signal receptor LasR in bacterial pathogen *P. aeruginosa*.

Identification of QslA adds a new member to the list of negative regulatory factors in the QS signalling pathway of *P. aeruginosa*, which includes QscR and QteE (Ledgham *et al.*, 2003b, Siehnel *et al.*, 2010). Expression of these regulatory factors in trans led to inhibition of QS-dependent phenotypes such as protease, elastase and pyocyanin as well as OdDHL and BHL (Chugani *et al.*, 2001, Siehnel *et al.*, 2010). The available data, albeit limited, seem to suggest different mechanisms of regulation among these three regulatory factors. Contrary to QslA, which negatively regulates *lasR* transcription (Fig. 3.8A), expression of *lasR* is not affected by QteE. In addition, both QscR and QteE were shown to inhibit RhlR activity (Ledgham *et al.*, 2003b, Siehnel *et al.*, 2010), which is a homologue of LasR

in *P. aeruginosa*. But when we expressed both QslA and RhlR in *E. coli*, QslA was not found to inhibit RhlR transcriptional activation from *rhlI* promoter (data not shown). Furthermore, 3-D structure modelling revealed different structural features for these three regulatory factors (Fig. 3.4). Moreover, loss of QscR or QteE resulted in advanced activation of QS gene expression (Chugani *et al.*, 2001, Siehnel *et al.*, 2010), which was not observed for QslA since expression of QS genes and production of QS dependent virulence factors in the QslA deficient mutant were initiated at the same time as the wild type (Fig. 3.6, 3.7). This was not due to growth-dependent expression of *qslA,* which was expressed constitutively throughout bacterial growth as revealed by RT-PCR analysis (Fig. 3.12C). In contrast, instead of controlling QS threshold by influencing the timing of QS activation, our data showed that QslA defines the QS threshold by governing the sensitivity of bacterial cells to QS signals. Null mutant of QslA in *P. aeruginosa* substantially reduced the amount of 3-oxo-C12-HSL signal molecules necessary for QS activation (Fig. 3.11).

Why would QslA-deficient mutant of *P. aeruginosa* become more sensitive to QS signals? This was probably due to the role of QslA in raising the “threshold hurdle” for QS activation to a higher 3-oxo-C12-HSL concentration (Fig. 3.13). In the wild type, QslA inhibits and sequesters LasR/3-oxo-C12-HSL complex at 3-oxo-C12-HSL concentration below threshold level. In the absence of QslA, the “hurdle” is removed and QS threshold is lowered because QS can be activated by uninhibited LasR/3-oxo-C12-HSL complex formed at low 3-oxo-C12-HSL concentration. At higher 3-oxo-C12-HSL concentration, the increased QS response in ΔqslA compared to wild type is also because of increased amount of LasR/3-oxo-C12-HSL complex. Hence, QslA-deficient mutant was more sensitive to QS signals because in the absence of QslA inhibition, LasR/3-oxo-C12-HSL complex formed at lower 3-oxo-C12-HSL concentration could activate QS.

The increased 3-oxo-C12-HSL sensitivity, however, did not lead to advanced QS activation. This may not be surprising since expression of most QS-regulated genes in *P. aeruginosa* is not advanced by addition of exogenous QS signals (Whiteley *et al.*, 1999, Schuster *et al.*, 2003). The non-quorum nature of these QS-regulated genes was suggested to be due to complex promoter architecture such that expression of QS-regulated genes requires other growth-dependent factors as well as removal of inhibitors in medium (Yarwood *et al.*, 2005, Schuster & Greenberg, 2006, Schuster & Greenberg, 2007). Furthermore, studies have found that the timing of QS activation is dependent on multiple factors such as MvaT, GacA, RsmA, QscR, and QteE (Reimmann *et al.*, 1997, Chugani *et al.*, 2001, Pessi *et al.*, 2001, Diggle *et al.*, 2002, Siehnel *et al.*, 2010).

Identification of QslA provides further insight into the sophisticated QS regulatory mechanisms in *P. aeruginosa*. The pathogen may recruit QslA to prevent premature QS activation at low bacterial population density by raising the QS threshold, and this anti-activation mechanism could serve an important role in ensuring that virulence factors are only produced at optimal bacterial quorum when they can overwhelm host defence responses. Moreover, the presence of QslA in *P. aeruginosa* and its functional analogue TraM in *A. tumefaciens* may suggest that modulation of QS by anti-activator(s) could be a fairly conserved mechanism in Gram-negative bacteria.

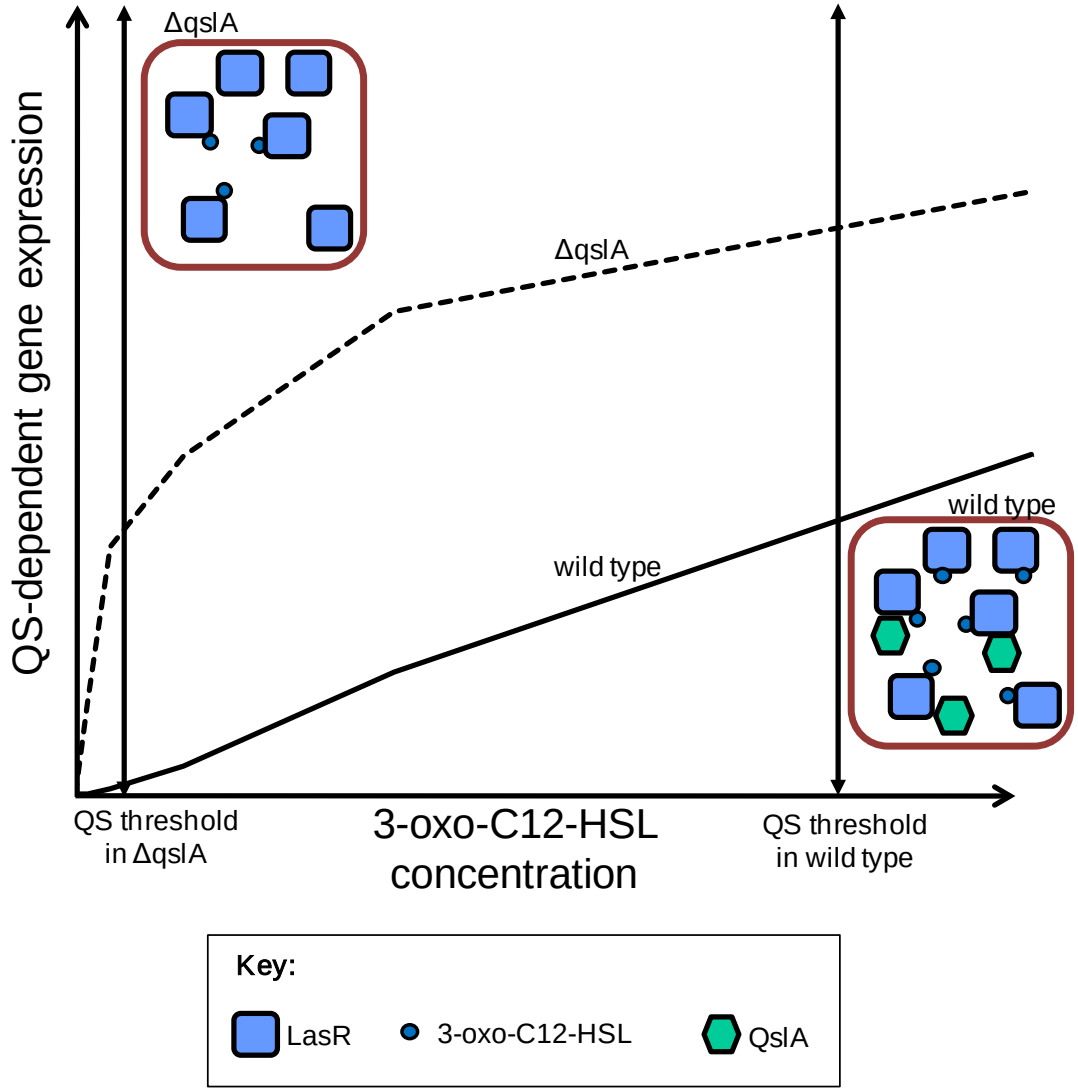


Fig. 3.14 Schematic model which shows QslA role in raising the QS threshold. QS-dependent gene expression is plotted against 3-oxo-C12-HSL concentration for wild type strain (solid line) and ΔqslA (dotted line). The 3-oxo-C12-HSL concentration at which QS threshold occurs in wild type and ΔqslA are indicated by lines with double arrows. In ΔqslA, QS threshold is at a lower 3-oxo-C12-HSL concentration because activity of 3-oxo-C12-HSL/LasR complex at such 3-oxo-C12-HSL concentration is not inhibited in the absence of QslA.

Chapter 4 – Identification of a MvaT-regulated anti-activator QslH

4.1 Introduction

Anti-activators regulate QS by interacting with LuxR-type regulators. In *A. tumefaciens*, two types of QS anti-activators, TrlR and TraM, have been discovered (Piper & Farrand, 2000, Chai *et al.*, 2001), and certain strains such as A6 and Ach5 also possess a *traM* homologous gene encoding TraM2 (Wang *et al.*, 2006). In the previous chapter, QslA of *P. aeruginosa* was discovered to fine-tune QS response and activation by counteracting the LasR-dependent activation of QS. But, QslA might not be the sole anti-activator of QS. Homology search of QslA in *P. aeruginosa* PAO1 genome did not reveal other QslA-like proteins and this suggests that the genes encoding QS anti-activators, if they are present, are not easily identified by sequence homology analysis.

In Chapter 2, a large scale random mutagenesis was conducted to identify potential genes involved in QS regulation by anti-activation, which led to the identification of 20 mutants with changed QS gene expression patterns. In this chapter, characterisation of the mutant QRM168 and QRM206, which showed decreased expression of QS gene *rhlR*, led to the identification of a novel anti-activator QslH. QslH acts by interacting with both LasR and PqsR. Overexpression of *qslH* resulted in inhibition of QS-dependent elastase, protease and pyocyanin production in *qslH* overexpression strain. Evidence is also provided that *qslH* expression was negatively regulated by MvaT, which is a global regulator implicated in regulation of QS, pyocyanin synthesis, biofilm formation, arginine metabolism and prophage activation (Diggle *et al.*, 2002, Vallet *et al.*, 2004, Li *et al.*, 2009). The results provided further evidence to support the hypothesis that anti-activators are an integral part of QS systems.

4.2 Materials and Methods

4.2.1 Bacterial strains, plasmids, media and growth conditions

Bacterial strains and plasmids used in this chapter are listed in Table 4.1. Bacteria were routinely maintained at 37°C in LB medium as described in Section 3.2.1. PA medium (also known as King's A medium (King *et al.*, 1954)) is a phosphate-limiting medium used in PpqsA-lacZ activity assay when indicated (Dong *et al.*, 2008).

4.2.2 β-galactosidase activity assays

β-galactosidase activities in *P. aeruginosa* were assayed following the method described previously in Section 3.2.2. P*lasI-lacZ* and P*rhlI-lacZ* activities in *E. coli* were measured when, respectively, grown with exogenous 3-oxo-C12-HSL (250 nM) and C4-HSL (5 µM).

4.2.3 RNA extraction and RT-PCR analysis

RNA extraction and RT-PCR analysis were carried out as described in Section 3.2.3.

4.2.4 DNA manipulation and deletion mutagenesis

The *qslH* in-frame deletion mutant in PAO1 was generated using pK18mobSac ligated to approximately 500 bp upstream and downstream flanking regions of *qslH*. The construct was introduced into PAO1 by conjugal mating using *E. coli* S17-1, and recombination of the plasmid with the genomic DNA resulted in the 55 – 447 bp deletion of *qslH*. The mutant was confirmed by PCR analysis. For complementation, the coding region of *qslH* was amplified from the start site to 93 bp downstream of the stop codon. The PCR product was cloned into expression vector pDSK519 and confirmed by DNA sequencing.

Table 4.1 Bacterial strains and plasmids used in Chapter 4.

Strain or plasmid	Relevant genotype or phenotype	Source
P. aeruginosa		
PAO1	Prototrophic laboratory strain	Holloway et al. (1979)
QR1	PAO1 containing pUCP-*lasRHTH* and P*rhlR-lacZ*	Chapter 2
QRM168	QR1 with transposon inserted 572 bp upstream of PA2228 coding region	Chapter 2
QRM206	QR1 with transposon inserted 729 bp downstream of PA2228 coding region	Chapter 2
QR1(PA2225)	QR1 containing pDSK-PA2225	This study
QR1(qslH)	QR1 containing pDSK-qslH	This study
QR1(vqsM)	QR1 containing pDSK-vqsM	This study
QR1(PA2228)	QR1 containing pDSK-PA2228	This study
QR1(PA2229)	QR1 containing pDSK-PA2229	This study
QR1(PA2230)	QR1 containing pDSK-PA2230	This study
QEGP	PAO1 containing P*PA2228-lacZ*	This study
QEG15	QEGP transposon inserted at 139 bp of *mvaT* ORF	This study
QEG20	QEGP transposon inserted at 18 bp of *mvaT* ORF	This study
PP1	PAO1 *pqsA*::Tn containing ppqsA-lacZ	Chapter 3
ΔqslH	*qslH* in-frame deletion mutant of PAO1	This study
ΔqslH(qslH)	ΔqslH containing pDSK-qslH	This study
ΔlasR	*lasR* in-frame deletion mutant of PAO1	Chapter 3
ΔrpoS	PAO1 *rpoS*::Tn	This study
ΔmvaT	*mvaT* in-frame deletion mutant of PAO1	This study
ΔqslHΔmvaT	*qslH* and *mvaT* in-frame deletion mutant of PAO1	This study
wt(vqsM)	PAO1 containing pDSK-vqsM	This study
ΔqslH(qslH)	ΔqslH containing pDSK-qslH	This study
ΔqslH(qslH-FLAG)	ΔqslH containing pDSK-qslH-FLAG	This study
wt(qslH)	PAO1 containing pDSK-qslH	This study
wt(qslH-FLAG)	PAO1 containing pDSK-qslH-FLAG	This study
ΔlasR(qslH-FLAG)	*lasR* in-frame deletion mutant of PAO1 containing pDSK-qslH-FLAG	This study

QHF1	PAO1 chromosomal-integrated *qslA* fused with FLAG and chromosomal-integrated *qslH* fused with FLAG	This study
HF1	PAO1 chromosomal-integrated *qslH* fused with FLAG	This study
MHF1	*mvaT* in-frame deletion mutant of HF1	This study
E. coli		
DH5α	F^- Φ80*lacZ*ΔM15 *end*A1*hsd*R17 (r_k^- m_k^-) *sup*E44 *thi*$^{-1}$ *gyr*A96 ∆(*lacZYA-argF*)	Laboratory collection
DL1	DH5α containing pUCP-lasR plasI-lacZ	Chapter 3
DR1	DH5α containing pUCP-rhlR prhlI-lacZ	Chapter 3
BL21 Star™ (DE3)	F^- *ompT hsdS$_B$ (r_B^- m_B^-) gal dcm rne131 (DE3)*	Invitrogen
S17-1	*recA pro* (RP4-2Tet::Mu Kan::Tn7)	Simon *et al*., 1983
Plasmid		
pK18mobsacB	Broad-host-range gene replacement vector, sacB, Gm^R	Laboratory collection
pK18-qslH	pK18mobsacB containing *qslH* flanking region	This study
pK18-mvaT	pK18mobsacB containing *mvaT* flanking region	This study
pME2-lacZ	pME6010 carrying a full-length lacZ	Laboratory collection
plasI-lacZ	pME2-lacZ carrying *lasI* promoter fused to *lacZ*	Chapter 3
ppqsA-lacZ	pME2-lacZ carrying *pqsA* promoter fused to *lacZ*	Chapter 3
prhlI-lacZ	pME2-lacZ carrying *rhlI* promoter fused to *lacZ*	Chapter 3
prhlR-lacZ	pME2-lacZ carrying *rhlR* promoter fused to *lacZ*	Chapter 2
pPA2228-lacZ	pME2-lacZ carrying *PA2228* promoter fused to *lacZ*	This study
pUCP19	*E. coli – P. aeruginosa* shuttle vector with *lac* promoter (P*lac*), Amp^R/Cb^R	ATCC 87110
pUCP-lasR	pUCP19 containing *lasR* under the control of P*lac*	Chapter 3
pUCP-lasRHTH	pUCP19 containing 505-717 bp *lasR* ORF under the control of P*lac*	Chapter 2
pUCP-rhlR	pUCP19 containing *rhlR* under the control of P*lac*	Chapter 3
pDSK519	Broad-host-range cloning vector with *lac* promoter (P*lac*), Km^R	Laboratory collection
pDSK-PA2225	pDSK519 containing PA2225 under the control of P*lac*	This study
pDSK-qslH	pDSK519 containing *qslH* under the control of P*lac*	This study
pDSK-vqsM	pDSK519 containing *vqsM* under the control of P*lac*	This study

pDSK-PA2228	pDSK519 containing PA2228 under the control of P*lac*	This study
pDSK-PA2229	pDSK519 containing PA2229 under the control of P*lac*	This study
pDSK-PA2230	pDSK519 containing PA2230 under the control of P*lac*	This study
pDSK-qslH-FLAG	pDSK519 containing *qslH* fused with FLAG peptide under the control of P*lac*	This study
pET14b-lasR	pET14b containing *lasR*	Chapter 3
pGEX6P1	GST fusion protein expression vector, Amp^R	Laboratory collection
pGEX6P1-qslH	pGEX6P1 containing *qslH*	This study
pTRG	Vector containing *RNAP*α under the control of the IPTG-inducibe, tandem promoter lpp/lac-UV5	Stratagene
pTRG-Gal11^P	pTRG containing gal11^P	Stratagene
pTRG-qslH	pTRG containing *qslH*	This study
pBT	Vector containing λ-*cI* under the control of the IPTG-inducible *lac-UV5*	Stratagene
pBT-LGF2	pBT containing *lgf2*	Stratagene
pBT-pqsR	pBT containing *pqsR*	This study
pBT-pqsR-LBD	pBT containing 304-999 bp *pqsR* ORF	This study
pBT-pqsR-HTH	pBT containing 1-276 bp *pqsR* ORF	This study

4.2.5 Assessment of supernatant AHL and HAQ levels

3-oxo-C12-HSL, C4-HSL and HAQ were measured using reporter strains DL1, DR1 and PP1 as previously mentioned in Section 3.2.6.

4.2.6 Analysis of virulence factor production

Virulence factors levels in overnight cultures were determined according to the previously mentioned methods (Section 3.2.7).

4.2.7 Co-immunoprecipitation and western blot analysis

The DNA fragment encoding FLAG peptide and glycine spacer was fused in-frame to *qslH* open reading frame at the C-terminal by PCR, and the PCR product was ligated to pDSK519. The *qslH*-FLAG fusion plasmid was transformed into ΔqslH strain. Bacteria were grown to OD_{600} = 3.0, and co-immunoprecipitation and western blot analysis were carried out as stated in Section 3.2.8. Protein bands were characterised by liquid chromatography – mass spectrometry (LC-MS) analysis.

Western blot analysis of QslA-FLAG and QslH-FLAG protein levels was carried out using overnight cultures. The same amount of cell pellets from each strain were resuspended in loading buffer and lysed by incubating at 99°C for 15 min. Cell lysates were obtained by spinning down the cell debris and analysed by immunoblotting with anti-FLAG antibody after electrophoresis by 15% SDS-PAGE and transfer to PVDF membrane.

4.2.8 Expression and purification of LasR and QslH

LasR protein was purified according to previously stated method (Section 3.2.9). The PCR product of *qslH* coding region was digested using *BamHI* and *EcoRI* and ligated into pGEX6P1 to form pGEX6P1-qslH. Strain BL21 star carrying pGEX6P1-

qslH was grown in 2.5 litres of LB medium to OD_{600} = 0.5 before being induced with 200 µM of isopropyl-β-d-thiogalactopyranoside overnight at 18°C. Cell pellet was resuspended in 25 mM Tris-HCl buffer (pH 7.8) containing 150 mM NaCl and lysed by sonication. GST-QslH fusion protein was purified using Glutathione Sepharose column chromatography. GST tag was cleaved from GST-QslH fusion protein using PreScission™ Protease (GE). Protein purity was judged by SDS/PAGE to be >99% pure.

4.2.9 EMSA

EMSA was carried out as previously described (Section 3.2.10) using LasR and QslH protein.

4.2.10 Bacterial two-hybrid assay

Bacterial two-hybrid assay was carried out using plasmids and host reporter strain XL1-Blue MRF´ Kan from BacterioMatch II two-hybrid system vector kit (Stratagene). The coding region of *qslH* was cloned into pTRG vector and partial or whole coding region of *pqsR* was cloned into pBT vector. Protein-protein interaction would induce expression of *HIS3* and *aadA* reporter genes that would, respectively, allow bacterial growth on selective medium plates containing 3-amino-1,2,4-triazole (3-AT) and streptomycin. Non-selective and selective medium plates were prepared according to the manufacturer's protocol.

4.2.11 *In silico* analysis of QslH

Domain and motif scans, protein structure prediction, protein homology search and amino acid sequence identity of QslH with QslA and TraM, as well as amino acid sequence homology of DNA binding domain of LasR and PqsR were carried out as previously mentioned (Section 3.2.11).

4.2.12 Construction of reporter strain QEGP and transposon mutagenesis

QEGP is PAO1 strain containing construct pPA2228-lacZ. The pPA2228-lacZ transcriptional fusion reporter was constructed by amplifying the promoter region of P*PA2228* (-801 to +159 relative to translational start site) and ligating it to pME2-lacZ vector. Transposon mutagenesis was carried out as previous mentioned (Section 2.2.3).

4.3 Results

4.3.1 QslH inhibits *rhlR* expression

Two insertion mutants QRM168 and QRM206 described in Chapter 2 were found to have considerably less P*rhlR-lacZ* activity compared to their parental strain QR1 (Fig. 2.1). The transposon insertion sites in these two mutants were determined to be in the intergenic region between *PA2228* and *PA2229* (Fig. 4.1A). *PA2228* and *PA2229* encode hypothetical proteins and they are predicted to be part of the *PA2228-vqsM-PA2226* operon and the *PA2229-PA2230* operon, respectively. Because the transposons were inserted in the non-coding regions, it was hypothesized that changes in the expression level of the neighbouring genes were responsible for the changes in P*rhlR-lacZ* activities. RT-PCR results showed that the transcript levels of *PA2225* to *PA2230* were increased in QRM168 and QRM206 compared to QR1, but there was no change in transcript level of loading control 16S rDNA in the 3 strains (Fig. 4.1B). *PA2225* to *PA2230* were then individually overexpressed in QR1 and it was found that only overexpression of *PA2226* reduced P*rhlR-lacZ* activities (Fig. 4.2). Thus, PA2226 is a putative regulator that influences AHL-dependent QS and PQS signalling. Given its role as a quorum sensing lasR-binding anti-activator as discussed below, PA2226 was named QslH.

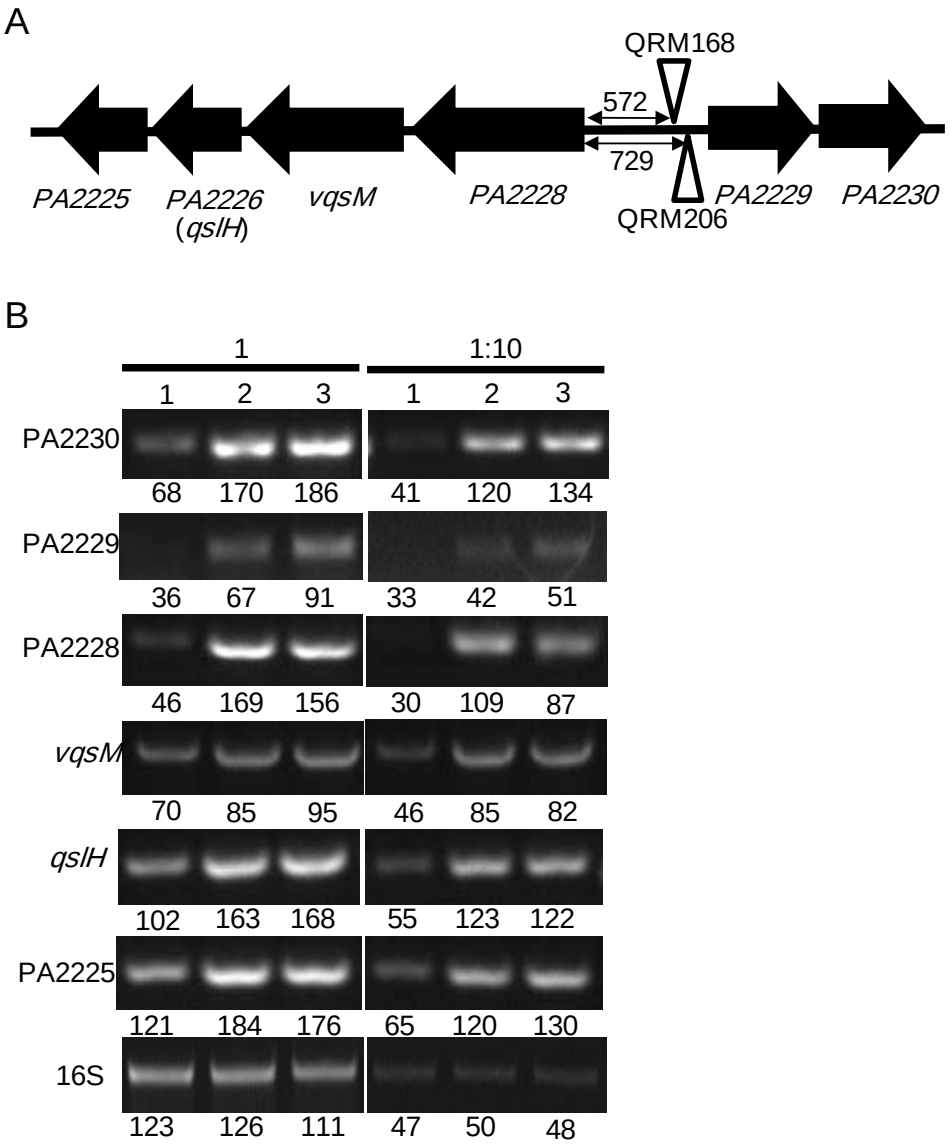


Fig. 4.1 QslH alters *rhlR* gene expression in mutant QRM168 and QRM206.
(A) Genetic organisation of *qslH* and adjacent genes *PA2225* to *PA2230*. Transposon insertion sites of mutants QRM168 and QRM206 were indicated by triangles and their distance from the translation start site of PA2228 was noted. (B) RT-PCR analysis of *PA2225* – *PA2230* and control 16S in QR1 (1), QRM168 (2) and QRM206 (3). Band intensities listed under each band were measured by ImageJ.

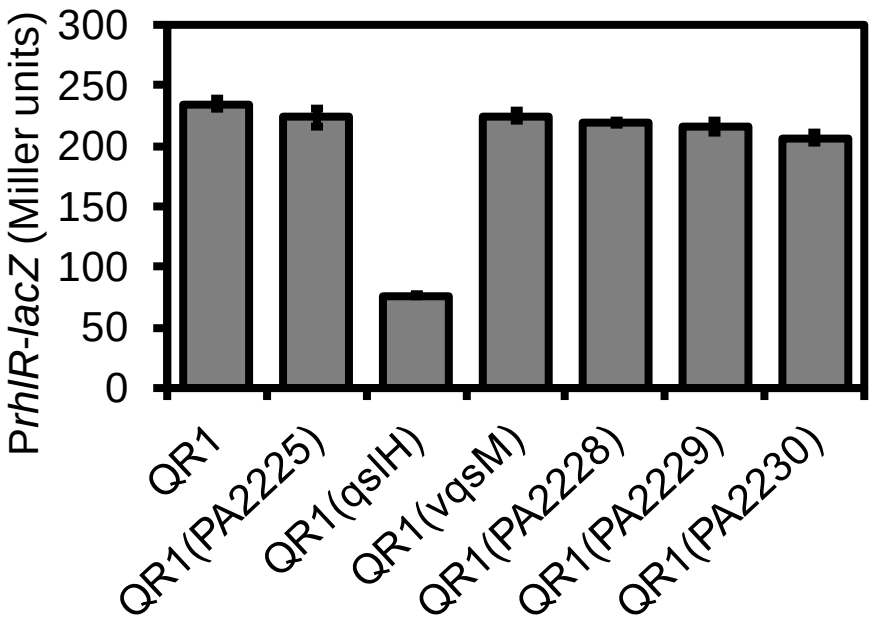


Fig. 4.2 P*rhlR-lacZ* activities of QR1 overexpressing *PA2225* – *PA2230* separately. PA2226 was designated as *qslH*. P*rhlR-lacZ* activities were measured after growth of bacteria in LB until OD_{600} = 1.5. The data shown were means of 3 replicates and error bar indicates standard deviation.

4.3.2 QslH blocks AHL-dependent QS and PQS signalling systems in *P. aeruginosa*

The effect of QslH on AHL-dependent QS and PQS signalling systems was then studied by determining *lasI*, *pqsA* and *rhlI* expression using *lacZ* transcriptional fusion. The production of corresponding signalling molecules, 3-oxo-C12-HSL, C4-HSL and HAQ, were also measured.

It was found that overexpression of *qslH* in wild type blocked the expression of *pqsA* and *rhlI* throughout growth (Fig. 4.3B, C). Consistent with the gene expression data, negligible HAQ signal was detected and C4-HSL production was decreased by 35 – 80% when *qslH* was overexpressed (Fig. 4.3E, F). Inhibition of *lasI* expression and decreased 3-oxo-C12-HSL production in the *qslH* overexpression strain were also observed, but the effect was only evident at early growth phases (OD_{600}=0.4, 1.0) (Fig. 4.3A, D). Thus, QslH substantially inhibits both AHL-dependent QS and PQS signalling systems.

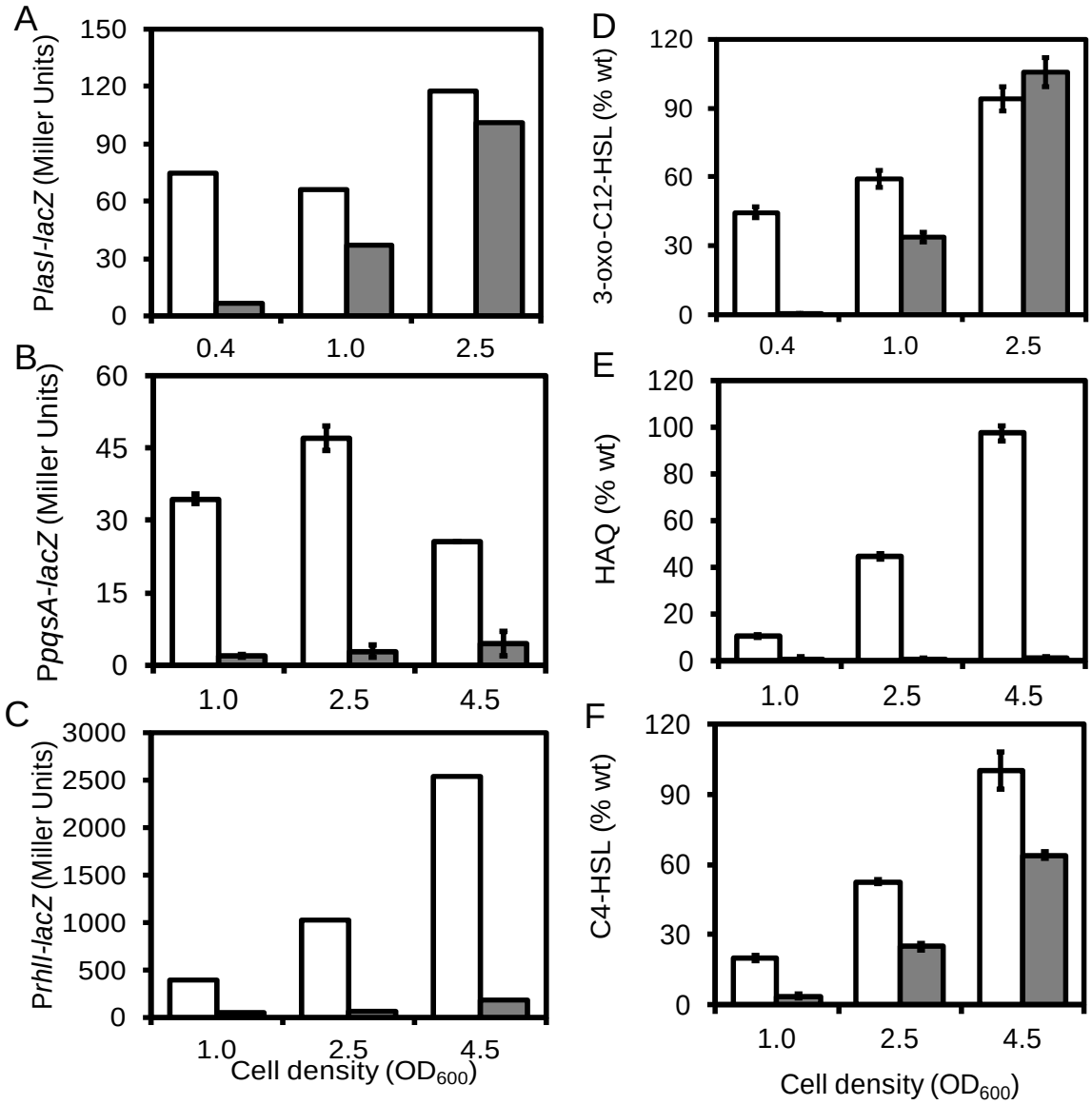


Fig. 4.3 QslH overexpression inhibits AHL-dependent QS and PQS signalling. The effects of QslH on expression of *lasI* (A), *pqsA* (B) and *rhlI* (C) were determined by measuring β-galactosida se activities in wild type strain PAO1 containing pDSK vector control (white bar) and wild type strain overexpressing *qslH* (grey bar). PlasI-lacZ, PpqsA-lacZ and PrhlI-lacZ gene fusion construc ts were separately introduced into the strains tested. 3-oxo-C12-HSL (D), HAQ (E) and C4-HSL (F) levels in the supernatant of wt(pDSK) (white bar) and wt(qslH) (grey bar) were determined by β-galactosidase activity assays using reporter strains DL1, PP1 and DR1, respectively. The data shown were means of 3 replicates and error bar indicates standard deviation.

4.3.3 QslH inhibits virulence factor production in *P. aeruginosa*

The production of elastase, protease and pyocyanin virulence factors are induced by QS (Latifi *et al.*, 1995, Pesci *et al.*, 1999). Hence, it was predicted that inhibition of AHL-dependent QS and PQS signalling systems by overexpression of *qslH* would cause a decline in virulence factor production. In agreement with this, elastase, protease and pyocyanin measured in *qslH* overexpression strain were greatly reduced compared to the vector control (Fig. 4.4). These results confirmed the QS inhibitory effects of QslH.

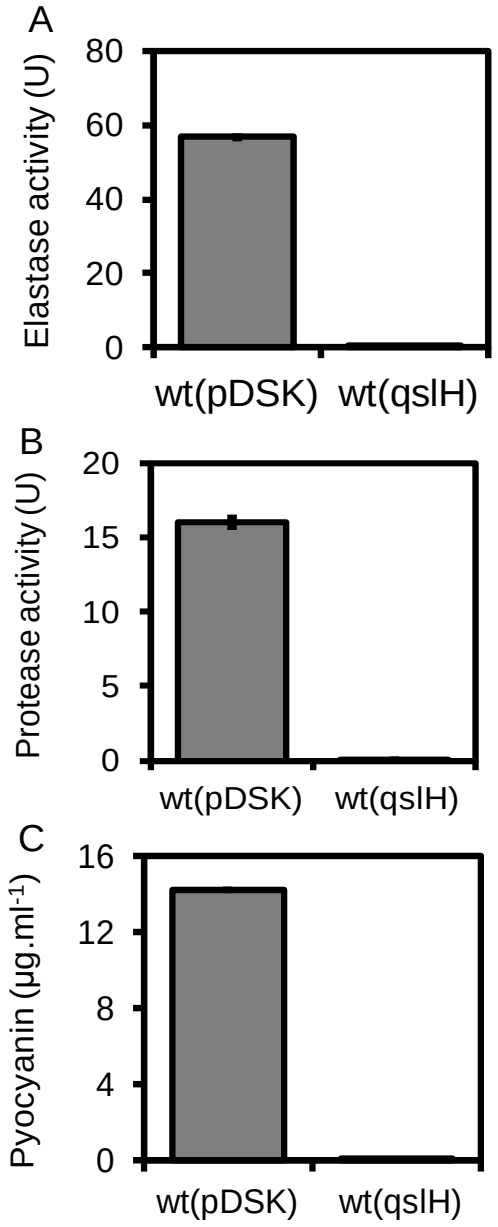


Fig. 4.4 QslH overexpression reduces virulence factor production in *P. aeruginosa*. Production of elastase (A), protease (B) and pyocyanin (C) were determined in wt(pDSK) and wt(qslH). The data shown were means of 3 replicates and error bar, which indicates standard deviation, is not seen when standard deviation is small.

4.3.4 *In silico* analysis of QslH

QslH is a unique 166-amino acid protein that is found only in *P. aeruginosa* strains (Table 4.2). In order to determine QslH functionality, domain analysis and motif scans were carried out but they did not reveal useful clues. QslH protein is mainly made up of hydrophobic residues (47%), but hydrophobicity plot showed that they were not localised at specific regions of the protein (Fig. 4.5A). Prediction of secondary structure of QslH suggests that it consists of 3 α-helices (Fig. 4.5B) approximately at residues 47–61, 69–93 and 96–121. Although the number of helices predicted in QslH was different from that in TraM and QslA and the protein identities between these proteins were not high (Fig. 4.6D), QslH resembles TraM and QslA protein structures as it is mainly made up of α-helices (Fig. 4.6A-C). QslH is predicted to be 18 kDa and is slightly larger than TraM and QslA which are around 11 kDa. To determine whether QslH inhibits QS by anti-activation, its putative protein-protein interaction targets were subsequently examined.

Table 4.2 QslH homologues were found only in strains of *P. aeruginosa*.
Proteins which showed amino acid sequence homology to QslH were identified by BLASTP program provided by the National Center for Biotechnology Information (NCBI).

Species	Gene ID	E value	Identities (%)	Positives (%)
P. aeruginosa PAO1	PA2226	2e-91	100	100
P. aeruginosa C3719	PACG_04962	9e-30	48	66
P. aeruginosa 2192	PA2G_05512	9e-30	48	66
P. aeruginosa PACS2	PaerPA_01000893	3e-29	47	66
P. aeruginosa UCBPP-PA14	PA14_58930	2e-24	46	65
P. aeruginosa PA7	PSPA7_4439	6e-24	46	65
P. aeruginosa PAb1	PaerPAb_24036	9e-21	45	65

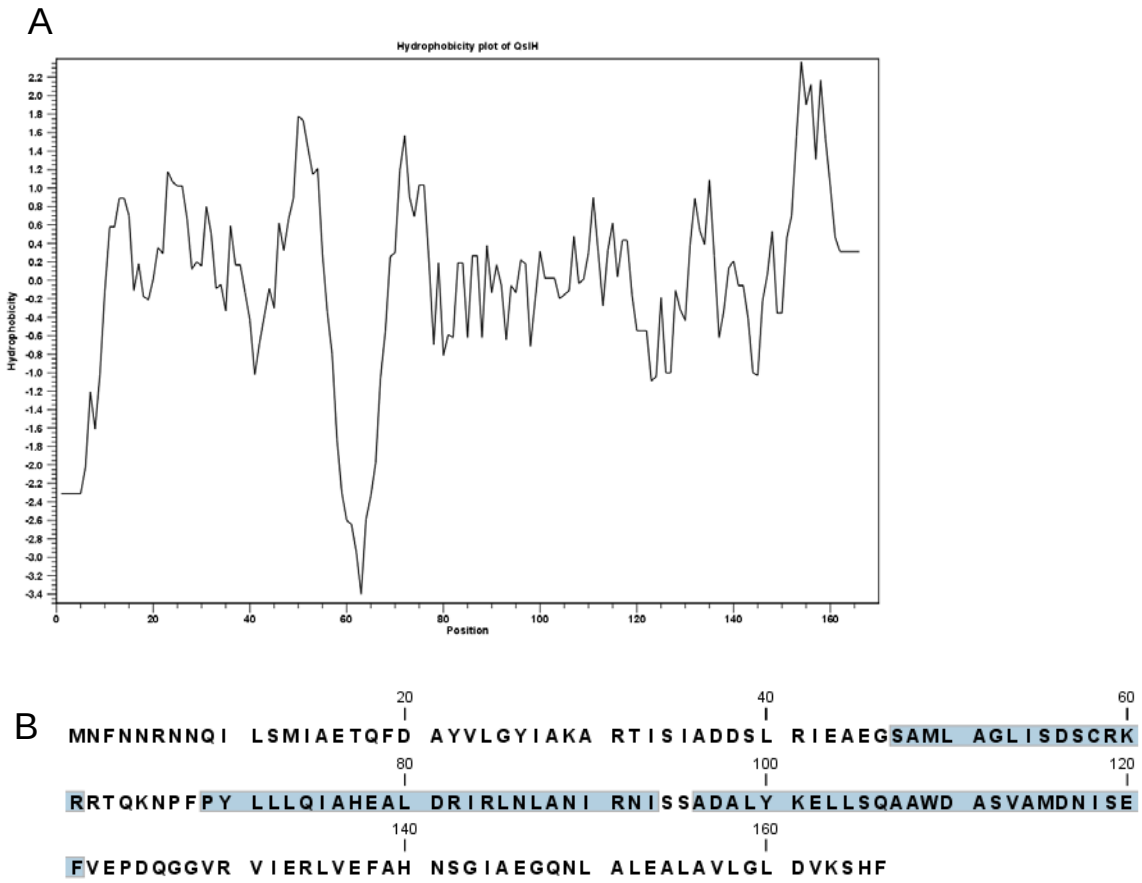
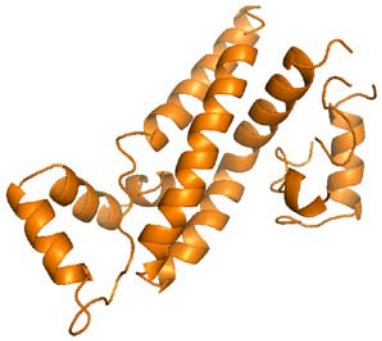
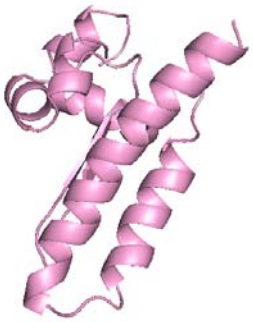


Fig. 4.5 *In silico* analysis of QslH.
(A) Hydrophobicity plot analysis of QslH by Kyte-Doolittle scale, window size=9 (CLC Workbench). (B) Secondary structure of QslH was analysed and highlighted areas denotes alpha helix regions.

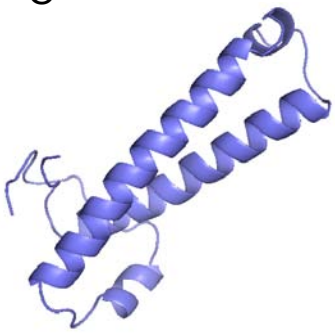
A



B



C



D

Protein	QslH	QslA	TraM
QslH		10%	11%
QslA	10%		11%
TraM	11%	11%	

Fig. 4.6 Comparison of QslH protein structures and amino acid sequence with TraM and QslA.
Ribbon structures predicted for QslH (A), QslA (B) by HMM-based methods (http://compbio.soe.ucsc.edu/SAM_T08/T08-query.html) as well as the crystal structure of TraM (C) (Vannini *et al.*, 2004). (D) The amino acid sequence of QslH, QslA and TraM were aligned by ClustalW and the protein identities of pairwise comparison of these proteins were stated.

4.3.5 QslH interacts with LasR and PqsR *in vivo*

To identify the potential protein-protein interaction targets of QslH, QslH was overexpressed as a FLAG tag recombinant protein, which did not affect QslH activity when expressed in mutant ΔqslH (Fig. 4.7A). QslH-FLAG fusion protein was purified under non-denaturing conditions and putative interacting proteins were co-immunoprecipitated by using anti-FLAG affinity gel. Eluted protein complexes were separated by SDS-PAGE and the protein bands were cut and identified by liquid chromatography-mass spectrometry analysis (Table 4.3). In the negative control where untagged *qslH* was overexpressed in wild type, only GltA, which is a citrate synthase that catalyses oxaloacetic acid and acetyl-CoA to citrate in the TCA cycle, glyoxylate cycle and in anaerobic respiration (Donald *et al.*, 1989), was eluted. Interestingly, LasR and PqsR eluted together with QslH-FLAG fusion protein (Fig. 4.7B, Table 4.3), and because these two proteins are directly involved in QS and PQS signalling, QslH protein-protein interaction with LasR and PqsR were then further characterised. Most of the other proteins that co-immunoprecipitated with QslH-FLAG fusion protein are housekeeping proteins which are expressed in relatively larger amounts, hence it is likely they were unspecifically bound to the anti-FLAG affinity gel or to QslH-FLAG fusion protein. These housekeeping proteins include ribosomal proteins 30S S3 and 30S S2, and chaperone proteins DnaJ, GroEL and DnaK. Proteins that are unspecifically bound are also present in smaller quantities which are insufficient to provide peptide coverage of more than 50% (Table 4.3)

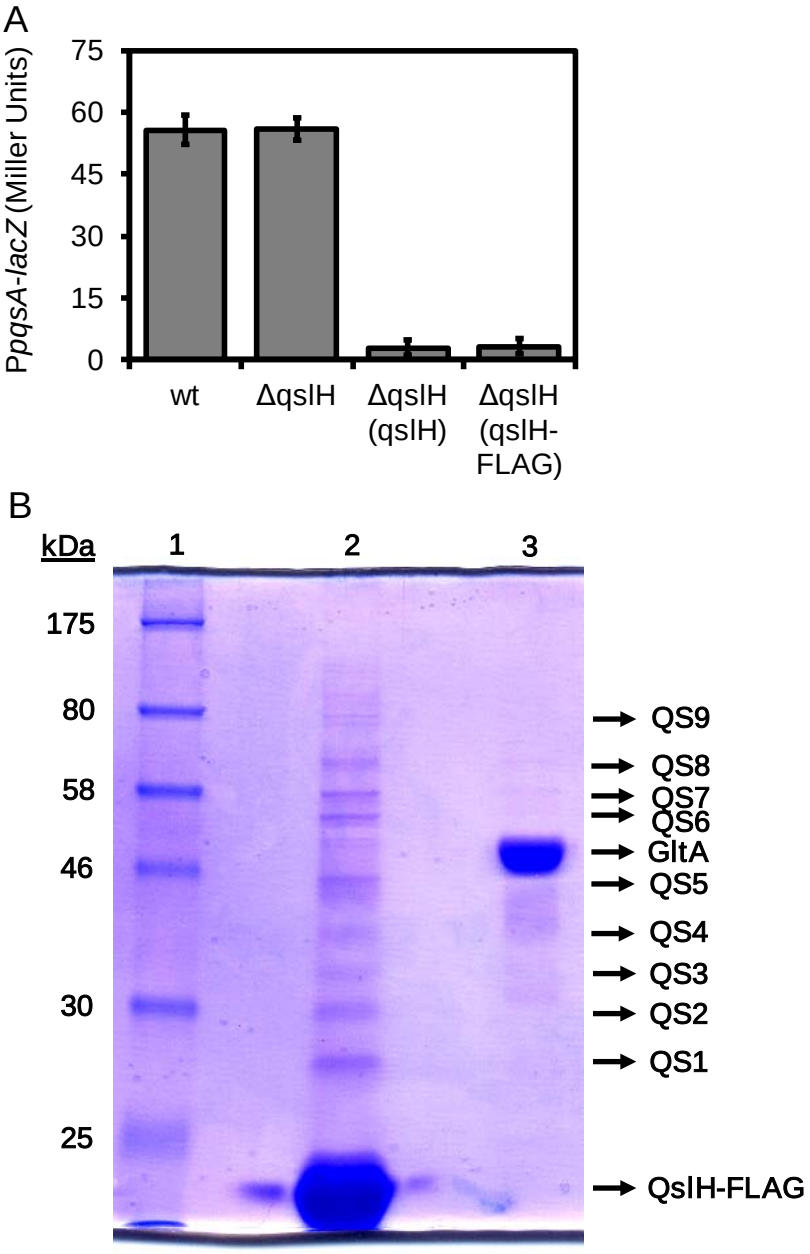


Fig. 4.7 Co-immunoprecipitation analysis of QslH-FLAG fusion protein.
(A) The P*pqsA-lacZ* activities, measured at OD_{600} = 1.0, when wild-type (wt), ΔqslH, ΔqslH(qslH*)* and ΔqslH(qslH -FLAG) were grown in LB. (B) Protein purification from the cell lysates of ΔqslH(qslH-FLAG*)* (lane 2) and ΔqslH(qslH*)* (lane 3) using anti-FLAG M2 affinity gel and analysed by 12% SDS-PAGE. Lane 1 are molecular weight markers (NEB). The distinct protein bands in lane 2 includes QslH-FLAG and unknown protein bands which are arbitrarily named QS1 – QS9; the protein band in lane 3 is GltA.

Table 4.3 Proteins that co-immunoprecipitated with QslH-FLAG fusion protein. The protein bands were characterised by LC-MS analysis.

Band	Peptide coverage (%)	Protein	kDa	Band	Peptide coverage (%)	Protein	kDa
QS1	57	LasR	27	QS5	72	DnaJ	40
QS1	43	30S S3	26	QS5	58	NirJ	44
QS1	38	OprG	25	QS5	49	FtsA	45
QS1	37	50S L3	21	QS5	46	DadA	47
QS1	30	GrpE	21	QS5	37	SpeC	44
QS2	50	30S S2	27	QS5	33	PA2127	48
QS2	42	PA3262	22	QS6	69	AtpD	50
QS2	38	MutM	30	QS6	39	FliC	49
QS2	30	PA5179	33	QS8	59	GroEL	55
QS3	63	PqsR	37	QS9	53	DnaK	68
QS3	44	PA1127	36	QS9	38	PA4595	61
QS3	43	MreB	37	QS9	33	SdhA	64
QS3	30	LtaA	38				
QS4	39	PA1572	41				

4.3.6 QslH inhibits LasR by protein-protein interaction

To find out whether QslH inhibits LasR directly, LasR activity in the presence of QslH was studied in heterologous host *Escherichia coli* DH5α. *lasR,* as well as *lasR-HTH* which encodes the DNA-binding domain of LasR, was fused separately with the constitutive promoter P*lac* and expressed in *E. coli* that contains either the *qslH* expression construct (pDSK-qslH) or the empty vector pDSK. Activities of LasR and LasR-HTH were gauged by activation of P*lasI-lacZ* reporter activity. It was found that QslH inhibited LasR activity in *E. coli* (Fig. 4.8A), suggesting that QslH may interact with LasR directly. QslH also inhibited LasR-HTH activity in *E. coli* (Fig. 4.8A), and this result showed that the DNA-binding domain of LasR (LasR-HTH) was sufficient for protein-protein interaction and that 3-oxo-C12-HSL was not essential for inhibition of LasR activity by QslH.

To confirm the interaction between QslH and LasR, the presence of LasR in the co-immunoprecipitates of QslH-FLAG protein in wild type was tested using anti-LasR antibody. As expected, LasR co-immunoprecipitated with QslH-FLAG but not with untagged QslH although LasR was present in the cell lysates (Fig. 4.8B). These results demonstrated that QslH interacts with LasR *in vivo*.

To evaluate the possibility that QslH and LasR interaction inhibits LasR activity by disrupting its DNA-binding ability, an electrophoretic mobility shift assay (EMSA) using purified LasR and QslH protein was carried out. The results showed that the biotinylated DNA probe containing *lasI-rsaL* promoter was bound by LasR (Fig. 4.8C lane 3) and in the presence of QslH, DNA-binding of LasR was disrupted in a dose-dependent manner, with complete disruption of DNA-binding when 200-fold molar excess of QslH over LasR was incubated (Fig. 4.8C lane 4-9). The 4-fold lesser QslH protein compared to QslA protein needed to completely disrupt LasR binding suggests that the binding affinity between QslH and LasR is stronger that that between QslA and LasR, however this cannot be verified without further

experiments such as isothermal calorimetry. QslH itself did not bind to the DNA probe (Fig. 4.8C lane 1). As a control, heat-denatured QslH protein showed reduced disruption of LasR DNA-binding (Fig. 4.8C lane 10).

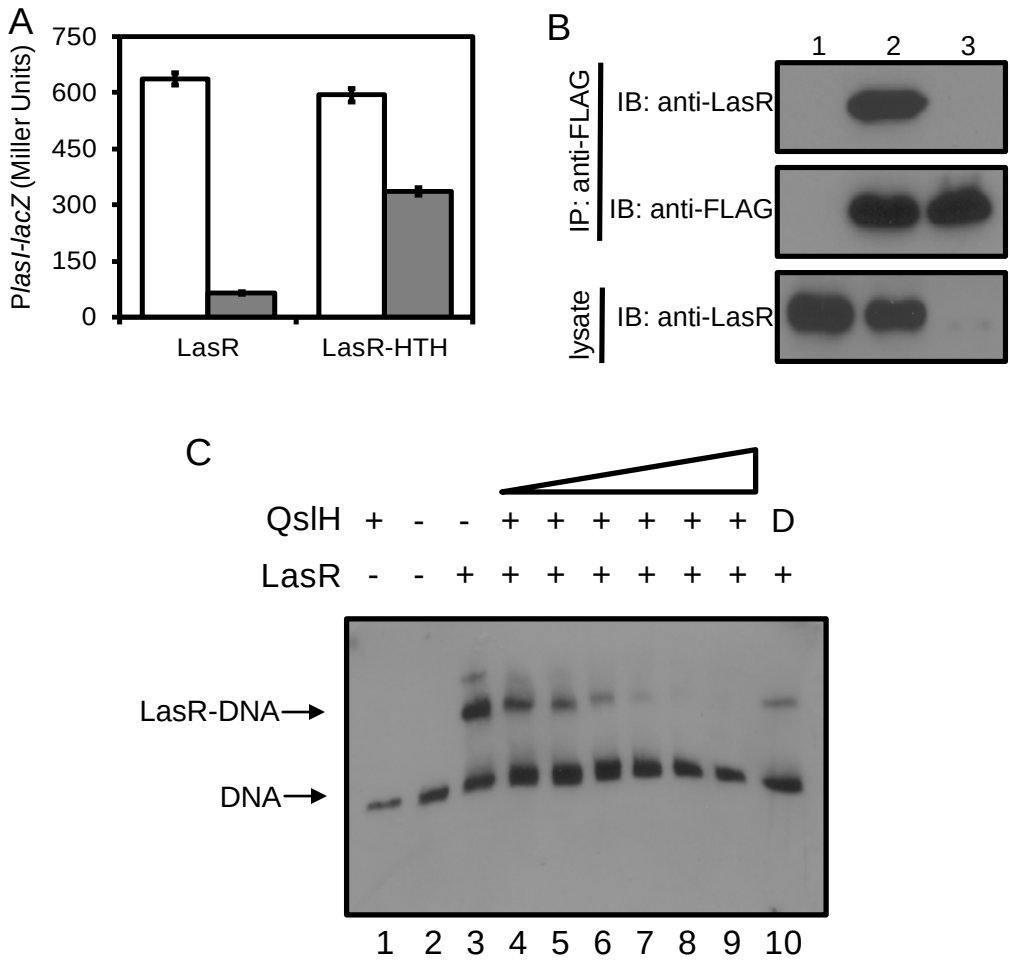


Fig. 4.8 QslH interacts with LasR and inhibits LasR activity by disrupting its DNA-binding ability.
(A) Transcriptional activity of LasR and LasR-HTH in *E. coli* DH5α were determined by P*lasI-lacZ* activities. The *lacZ* activities were determined in the absence (white bar) or presence (grey bar) of overexpressed *qslH*. The data shown were means of 3 replicates and error bar indicates standard deviation. (B) Proteins were immunoprecipitated (IP) using anti-FLAG M2 affinity gel in 1: wt(qslH), 2: wt(qslH-FLAG) and 3: ΔlasR(qslH-FLAG). Immunoblots (IB) of immunoprecipitates were carried out using anti-LasR and anti-FLAG antibodies. (C) EMSA was carried out using biotinylated DNA probe containing *lasI-rsaL* promoter region and 1.8 fmol of DNA probe was used in each reaction. DNA probe was incubated for 20 min with only QslH (72 μM) in lane 1, with only LasR (90 nM) in lane 3, and with LasR (90 nM) pre-incubated for 20 min with increasing concentrations of QslH (2.25, 4.5, 9, 18, 36, 72 μM) in lanes 4 – 9. QslH (72 μM) in lane 10 was heat-denatured at 99°C for 15 min before pre-incubation with LasR. Lane 2 contains DNA probe only without proteins.

4.3.7 QslH inhibits PqsR independently of protein-protein interaction with LasR

Besides LasR, PqsR was also identified as a putative interaction target of QslH (Fig. 4.7B). To verify QslH interaction with PqsR, the PqsR-dependent *pqsA* expression was studied when bacteria were grown in PA medium (Dong *et al.*, 2008). Under the low phosphate conditions in PA medium, *pqsA* expression is activated by PqsR but not LasR (Jensen *et al.*, 2006). It was found that *pqsA* expression was reduced by about 5 folds when *qslH* was overexpressed in Δ*lasR* (Fig. 4.9A), which strongly suggests that QslH is able to inhibit PqsR activity. This also showed that the inhibitory effect of QslH on PqsR was independent of its inhibition of LasR.

Due to the technical difficulties in purifying PqsR protein and the lack of anti-PqsR antibody, protein-protein interaction between QslH and PqsR could not be verified by co-immunoprecipitation or EMSA. Instead, bacterial two-hybrid assay was carried out to confirm the interaction between QslH and PqsR *in vitro*. The results from bacterial two-hybrid assay showed that QslH interacts with the DNA-binding domain of PqsR (PqsR-HTH) only (Fig. 4.9B, sector 7) but not with PqsR ligand-binding domain or full length protein (Fig. 4.9B, sector 6, 8), which was also observed when PQS was added to the agar plates (Fig. 4.10). At this stage, it could not be explained why the full length PqsR did not interact with QslH, one likely explanation is that fusion of bacteriophage λ repressor to PqsR in the pBT vector used for bacterial two-hybrid assay might block proper protein folding to a conformation required for interaction. As a negative control, QslH or PqsR, expressed together with empty vector pBT or PTRG, did not result in bacterial growth (Fig. 4.9B, sector 2 – 5). All the bacterial strains grew on the non-selective medium plate (Fig. 4.9A), excluding the possibility that bacterial growth was

affected. Thus, the results indicate that QslH interacts with PqsR through the DNA-binding domain of the latter.

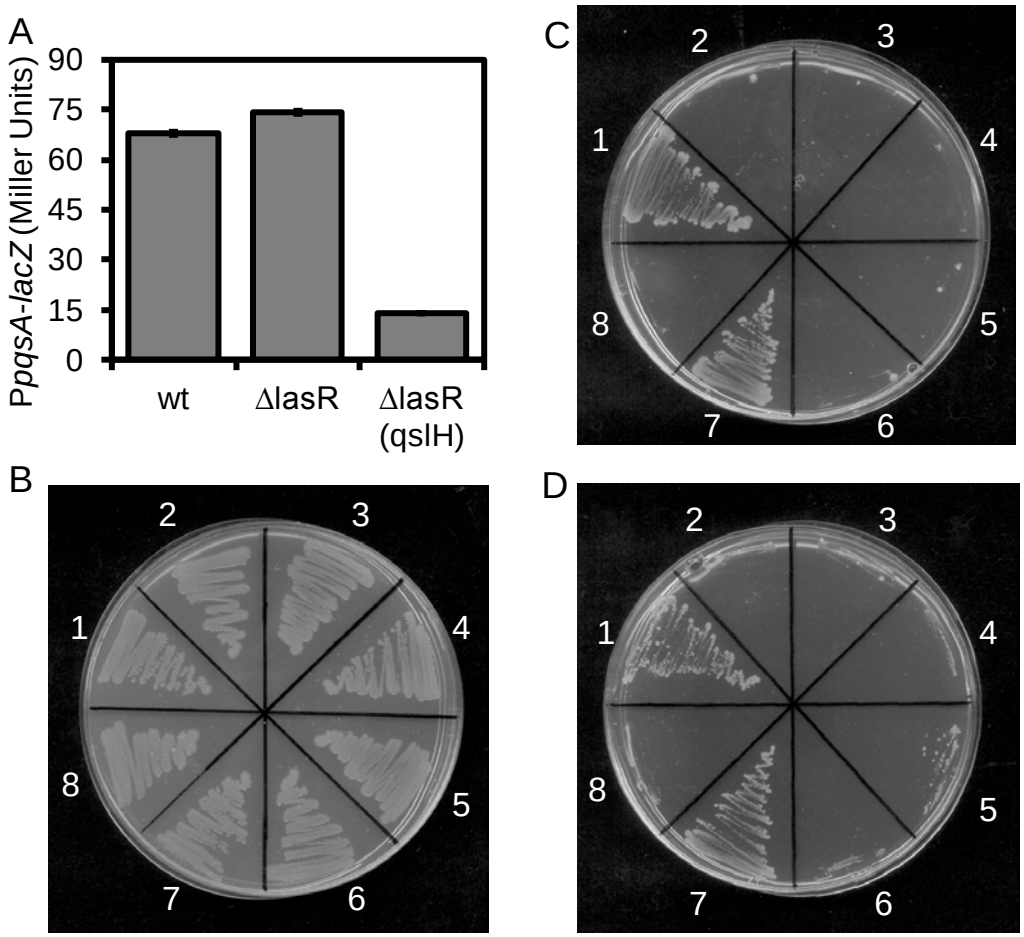


Fig. 4.9 QslH inhibit s PqsR activity by protein-protein interaction.
(A) Bacteria were grown in PA medium until OD_{600}=1.0 and *pqsA* expression was determined by β-galactosidase activity assay. The data shown were means of 3 replicates and error bar, which indicates standard deviation, is not seen when standard deviation is small. (B-D) Bacterial two-hybrid was carried out by plating co-transformants of pBT-LGF2 and pTRG-Gal11^{P} as positive control (1); co-transformants of pBT empty vector and pTRG-qslH (2), pTRG empty vector and pBT-pqsR-LBD (3), pTRG empty vector and pBT-pqsR-HTH (4), pTRG empty vector and pBT-pqsR (5) as negative controls; and co-transformants of pTRG-qslH and pBT-pqsR-LBD (6), pTRG-qslH and pBT-pqsR-HTH (7), pTRG-qslH and pBT-pqsR (8) to determine QslH protein-protein interaction with partial and whole PqsR. The agar plates used were non-selective medium plates (B) or selective medium plates containing 3-AT (C) or containing 3-AT together with streptomycin (D).

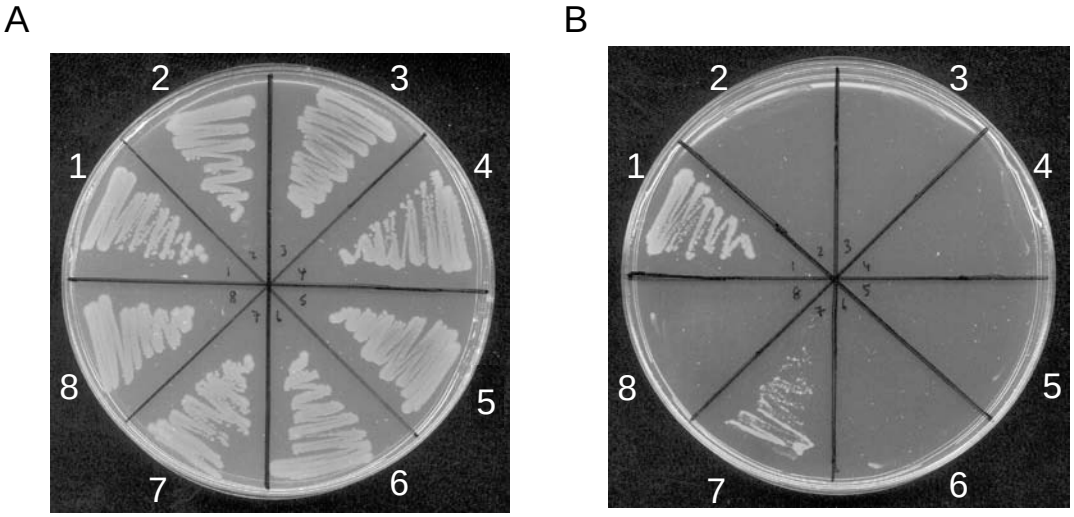


Fig. 4.10 Interaction of QslH and PqsR in the presence of PQS.
Bacterial two-hybrid assay was carried out according to Fig. 4.9B-D. The agar plates used were non-selective medium plates (A) or selective medium plates containing 3-AT (B) and both plates were supplemented with PQS at a final concentration of 1 µM.

4.3.8 Homology of LasR and PqsR

It is interesting to note that QslH interacts with DNA-binding domain of LasR and PqsR although the DNA-binding domain of these two regulators do not share substantial amino acid sequence homology (9% protein identity, 37% protein similarity) (Fig. 4.11A). LasR and PqsR are respectively part of the LuxR and LysR families of transcriptional regulators, which are functionally activated when bound by their cognate ligands (3-oxo-C12-HSL and HHQ/PQS). While the amino acids critical for QslH interaction with LasR and PqsR remain to be investigated, based on the distinct features of the two transcriptional regulators, it is likely that the epitopes for QslH interaction with LasR and PqsR maybe different. RhlR is highly homologous to LasR (47% protein identity, 65% protein similarity) (Fig. 4.11B), however its activity was not substantially inhibited by QslH when these two proteins were overexpressed in *E. coli* (Fig. 4.12).

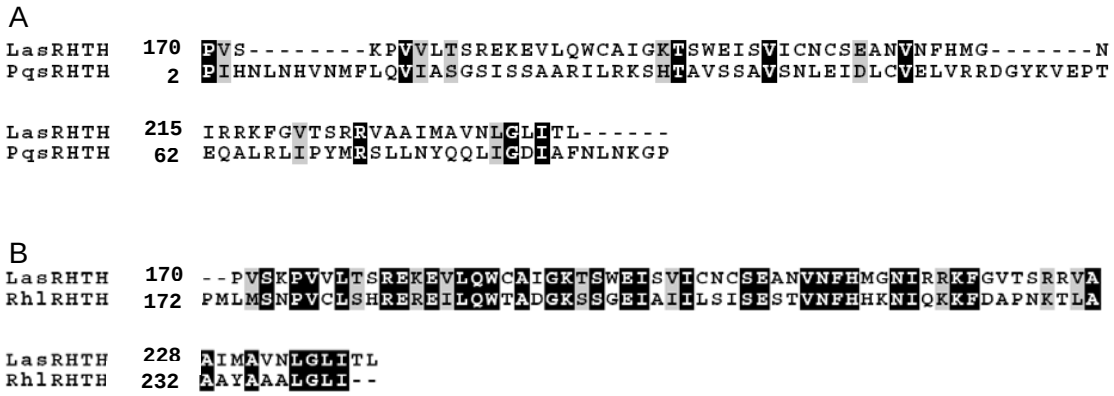


Fig. 4.11 Comparison of amino sequence of LasR, PqsR and RhlR.
Amino acid sequence alignment of DNA-binding domain (HTH) of LasR with that of PqsR (A) and RhlR (B). The identical amino acids are highlighted black and the similar amino acids are highlighted grey. Dashes (-) indicate gaps.

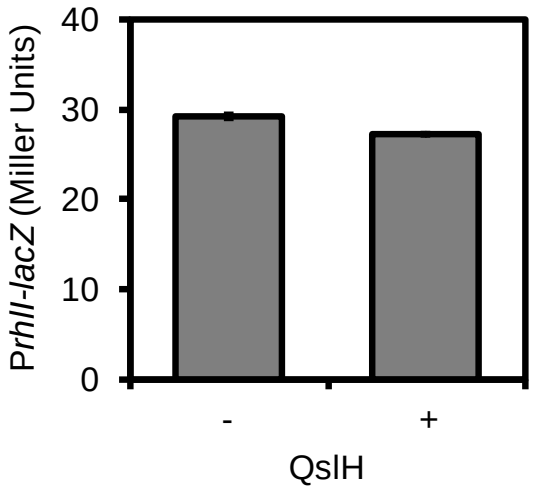


Fig. 4.12 QslH did not inhibit RhlR activity in *E. coli*.
Transcriptional activity of RhlR in *E. coli* was determined by P*rhlI-lacZ* activities. The *lacZ* activities were determined in the absence (-) or presence (+) of overexpressed *qslH.* These values were normalised by deduction of background readings of P*rhlI-lacZ* activity without RhlR. The data shown were means of 3 replicates and error bar, which indicates standard deviation, is not seen when standard deviation is small.

4.3.9 Deletion of *qslH* in wild type strain does not affect AHL-dependent QS and PQS signalling systems

Overexpression of *qslH* inhibited AHL-dependent QS and PQS signalling systems (Fig. 4.3) as well as QS-dependent virulence factor production (Fig. 4.4). Subsequently, the role of QslH in wild type strain was studied by generating an in-frame deletion of *qslH* in PAO1 strain. PlasI-lacZ, PpqsA-lacZ and PrhlI-lacZ constructs were introduced into *qslH* deletion mutant (ΔqslH) and wild type to study the expression of *lasI*, *pqsA* and *rhlI* and it was found that the expression of these genes were not significantly higher in ΔqslH compared to wild type (Fig. 4.13A-C). Furthermore, similar levels of virulence factors were produced in ΔqslH and wild type (Fig. 4.13D-F). Hence, QslH did not affect AHL-dependent QS and PQS signalling in wild type.

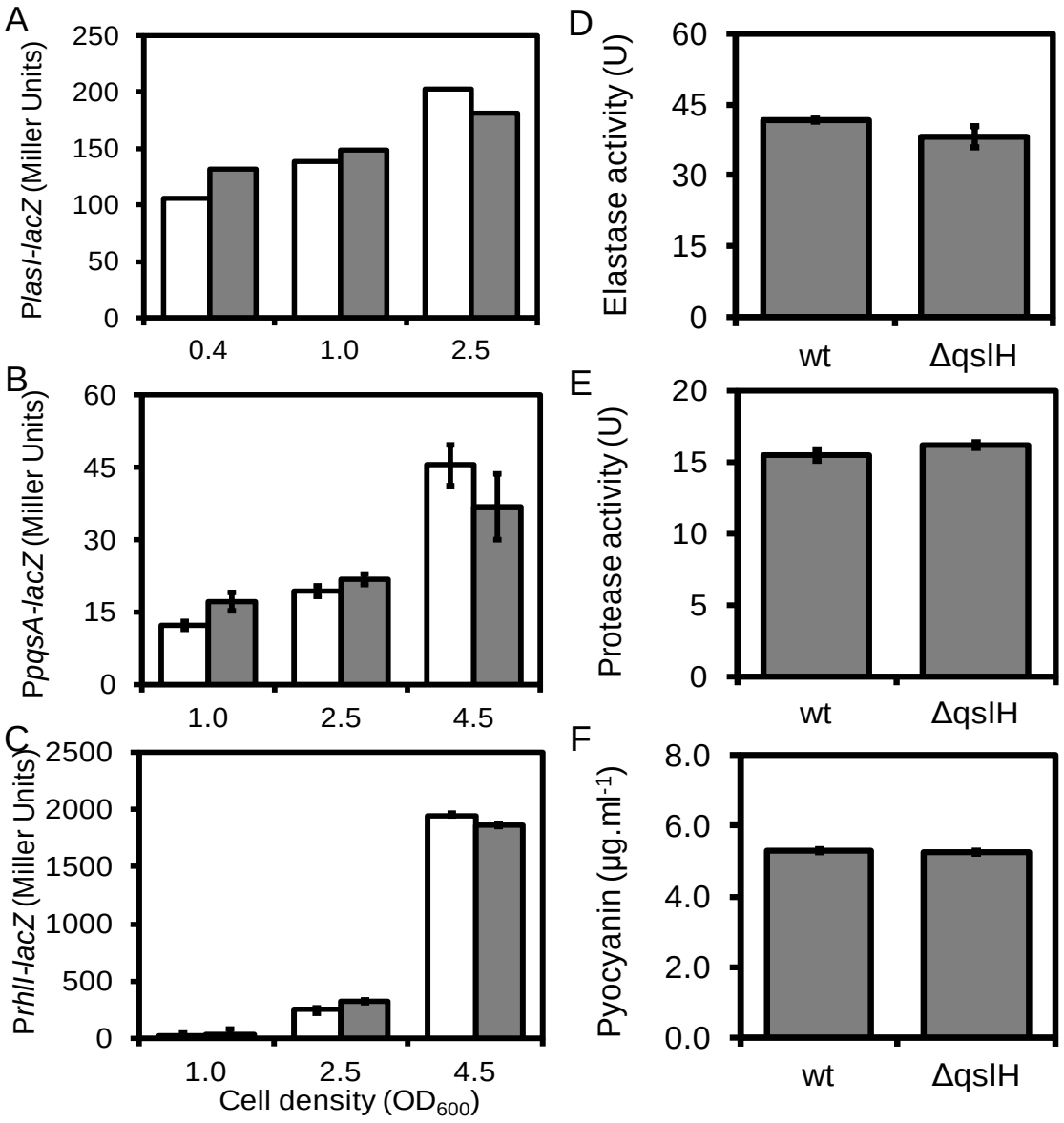


Fig. 4.13 Deletion of *qslH* in wild type strain PAO1 has lit tle effects on AHL-dependent QS and PQS signalling as well as virulence factor production.
The effects of *qslH* null mutation on expression of *lasI* (A) *pqsA* (B) and *rhlI* (C) were determined by measuring β-galactosidase activities in wild type (white bar) and ΔqslH (grey bar). PlasI-lacZ, PpqsA-lacZ and PrhlI-lacZ gene fusion constructs were, respectively, introduced into the strains tested. Production of elastase (D), protease (E) and pyocyanin (F) were determined in PAO1 and mutant ΔqslH. The data shown were means of 3 replicates and error bar, which indicates standard deviation, is not seen when standard deviation is small.

4.3.10 Identification of transposon mutants with increased *qslH* expression

QslH only inhibited AHL-dependent QS and PQS signalling when overexpressed but not at wild-type levels. It was speculated that this was due to the low expression level of *qslH* in the wild type (this was subsequently confirmed by western blot analysis in Fig. 4.16C). Therefore, a transposon mutagenesis screening was carried out to identify putative regulators which control *qslH* expression. *PA2228*, *vqsM* and *qslH* were predicted to belong to an operon (*Pseudomonas* genome database) (Fig. 4.14A) and detection of intergenic transcripts by RT-PCR analysis confirmed this (Fig. 4.14B). *PA2228* encodes for a hypothetical protein while *vqsM* is a known QS regulator. The promoter of *PA2228* was fused with *lacZ* and the construct was transformed into wild type to generate the parental strain QEGP used for transposon mutagenesis. Around 20,000 insertion mutants were screened and 11 mutants with increased P*PA2228-lacZ* activities were selected (Table 4.4). Two of these mutants selected (QEG15 and QEG20) had about 1.5 to 2 times higher P*PA2228-lacZ* activities compared to the parental strain QEGP (Fig. 4.15) and it was found that the transposons were inserted, respectively, at 139bp and 18bp in *mvaT*, suggesting that disruption of *mvaT* might lead to increased *qslH* expression.

MvaT belongs to the H-NS family of transcriptional repressors and it binds to AT-rich DNA regions, inhibiting transcriptional initiation from these sites (Castang *et al.*, 2008). The role of MvaT in QS has been illustrated by its inhibition of QS signals and pyocyanin production and upregulation of elastase and protease production (Diggle *et al.*, 2002). Hence, MvaT effect on QS might possibly be linked to QslH and the association between MvaT and QslH was further characterised.

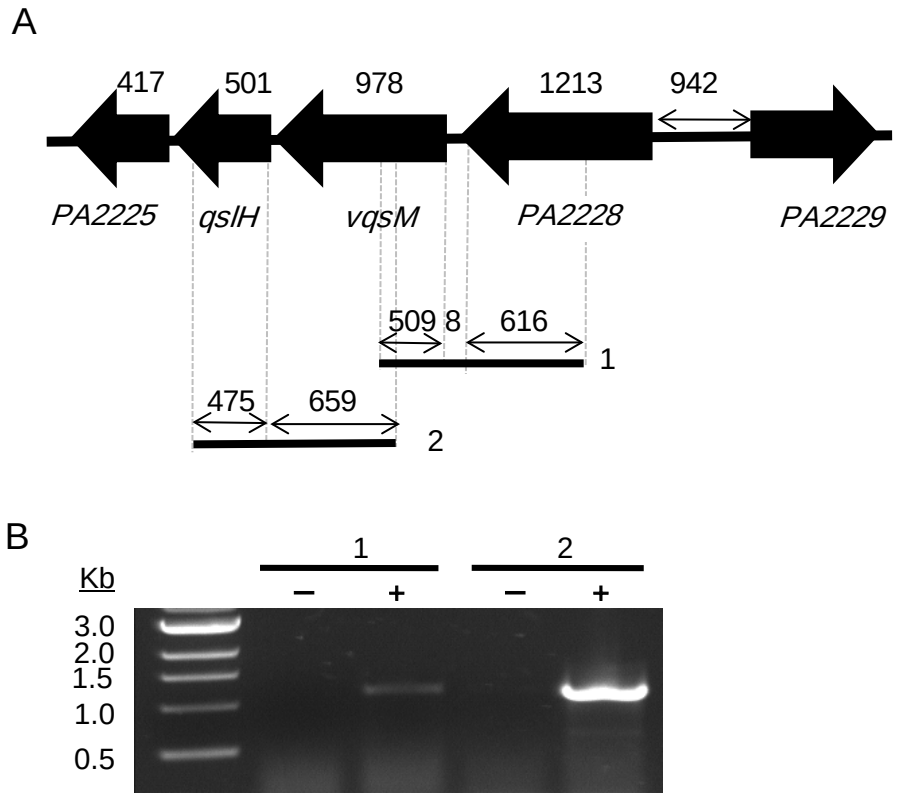


Fig. 4.14 *PA2228*, *vqsM* and *qslH* are part of an operon.
(A) Genome organisation of the *PA2228-vqsM-qslH* operon. Primers were designed to amplify RNA of genetic region 1 and 2. Numbers above single-headed arrows indicate number of base pairs in the open reading frame while numbers aboe double-headed arrows indicate number of base pairs between genes or of DNA fragment. (B) RT-PCR analysis of genetic region 1 and 2 with (+) and without (-) reverse transcription.

Table 4.4 Transposon mutants of QEGP with higher P*PA2228-lacZ* activities than parental strain.

Strain	Gene	Description
QEG13	*PA3054*	PA3054: predicted carboxypeptidase
QEG15	*mvaT* (at 139 bp)	MvaT: transcriptional regulator
QEG17	*PA2228* (at 593 bp)	PA2228: hypothetical protein
QEG18	166 bp upstream of *PA5370*, 120 bp upstream of *PA5371*	PA5370: probable MFS transporter PA5371: probable acyl-CoA hydrolase
QEG20	*mvaT* (at 18 bp)	MvaT: transcriptional regulator
QEG30	*PA2228* (at 1082 bp)	PA2228: hypothetical protein
QEG34	*PA1511* or *PA0262*	PA1511/ PA0262: probable Vgr protein in type VI secretion system
QEG35	807 bp upstream of *PA2228*	Not on pPA2228-lacZ vector
QEG38	*PA1940*	PA1940: probable catalase
QEG47	*PA2228* (at 696 bp)	PA2228: hypothetical protein
QEG52	*PA2228* (at 171 bp)	PA2228: hypothetical protein

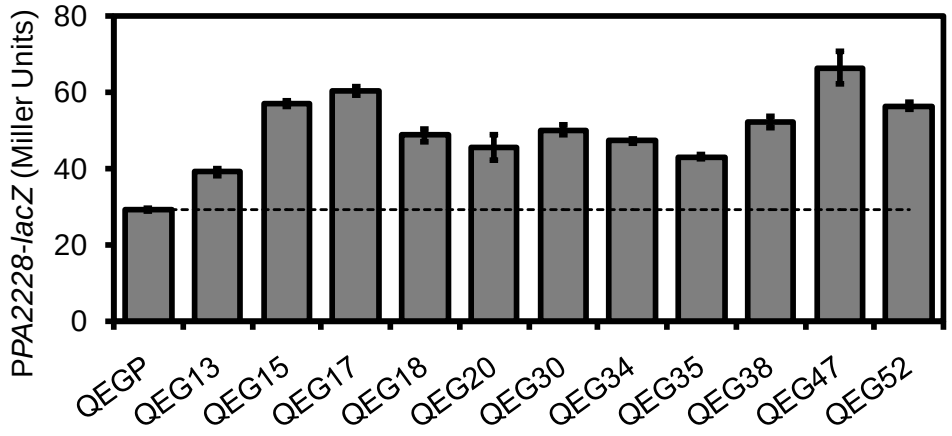


Fig. 4.15 Transposon mutants that were found to have higher P*PA2228-lacZ* activities than the parental strain QEGP.
P*PA2228-lacZ* activities were measured after growth of bacteria in LB to OD_{600} = 1.5. Dotted line indictates level of P*PA2228-lacZ* activity in parental strain QEGP. The data shown were means of 3 replicates and error bar indicates standard deviation.

4.3.11 MvaT suppresses *qslH* expression

To verify that MvaT is involved in modulation of *qslH* expression, *mvaT* was deleted in wild type strain PAO1 to generate ΔmvaT. RT-PCR analysis of *qslH* (Fig. 4.16A) and P*PA2228-lacZ* activities (Fig. 4.16B) showed that *qslH* expression was increased by 2–3 folds in ΔmvaT compared to wild type.

The effect of null mutation of anti-activator QslA in wild type strain PAO1 on AHL-dependent QS and PQS signalling was described in Chapter 3, but mutation of QslH did not show similar phenotype under the same experimental conditions (Fig. 4.13). To determine that this was due to lower QslH protein level compared to QslA in wild type and also to confirm that there were more QslH protein present in ΔmvaT compared to wild type, strains QHF1, HF1 and MHF1 were generated by genetic recombination (Section 3.2.5). QHF1 contains chromosomal-integrated *qslH* fused with FLAG and *qslA* fused with FLAG while HF1 and MHF1 are, respectively, wild type and ΔmvaT strains containing chromosomal-integrated *qslH* fused with FLAG. Fusion of FLAG peptide to QslH and QslA protein did not affect QslH and QslA activities (Fig. 3.3A, 4.7A). Western blot analysis was carried our using anti-FLAG antibody to detect QslH-FLAG and QslA-FLAG fusion proteins. The results showed that QslH was only detected in ΔmvaT but not in the wild type (Fig. 4.16C). In contrast, QslA, the anti-activators characterized in Chapter 3, was present at a substantially higher level in wild type compared to QslH (Fig. 4.16C). This explains why deletion of *qslH*, unlike *qslA*, did not affect QS and PQS signalling systems in the wild type under the experimental conditions used in this study. Consistent with the results from Fig. 4.16, microarray analysis reported previously also showed that *qslH* was upregulated by 3.15 folds in the *mvaT* mutant compared to wild type (Vallet *et al.*, 2004).

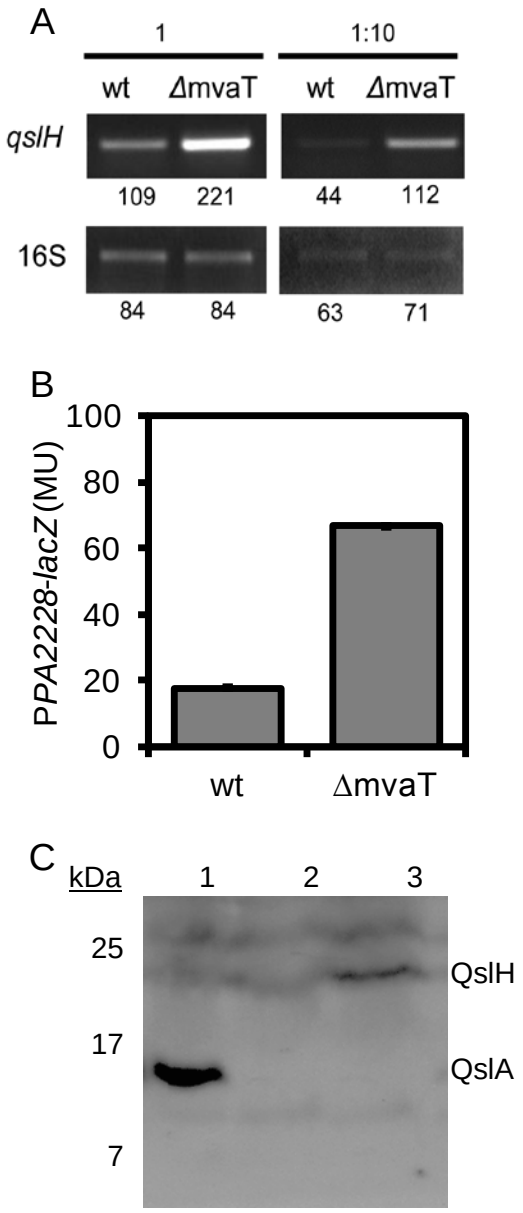


Fig. 4.16 MvaT represses *qslH* expression.
(A) RT-PCR analysis of *qslH* and 16S transcriptional expression and (B) P*PA2228-lacZ* activities were determined at OD_{600} = 1.5. The data shown were means of 3 replicates and error bar indicates standard deviation. (C) Western blot analysis of QslH and QslA in strains wild type strain with chromosomal-integrated *qslA* and *qslH* fused with FLAG, QHF1 (1); wild type strain with chromosomal-integrated *qslH* fused with FLAG, HF1 (2); and ΔmvaT with chromosomal-integrated *qslH* fused with FLAG, MHF1 (3).

4.3.12 QslH inhibits QS-dependent production of virulence factors in ΔmvaT

To determine whether increase in *qslH* expression in ΔmvaT reduced AHL-dependent QS and PQS signalling, the production of virulence factor between ΔmvaT and ΔmvaTΔqslH was compared. The results showed that elastase and protease production in ΔmvaT compared to wild type were reduced, respectively, by about 30% and 10%, consistent with previous reports (Diggle *et al.*, 2002). Deletion of *qslH* in ΔmvaT restored elastase and protease production to wild type levels (Fig. 4.17A, B). These results suggest that QslH plays a role in ΔmvaT by suppressing QS-dependent elastase and protease production. In contrast to elastase and protease production in ΔmvaT, pyocyanin in ΔmvaT was increased by about 50 – 100% compared to wild type (Fig. 4.17C) as previously reported (Diggle *et al.*, 2002, Li *et al.*, 2009). This was possibly attributed to MvaT regulation of expression of other genes, besides QS, that are involved in pyocyanin production (Li *et al.*, 2009) since MvaT positively regulates QS. Pyocyanin produced in ΔmvaTΔqslH was increased by about 30% compared to ΔmvaT when they were grown in LB (Fig. 4.17C), which showed that QslH inhibited QS-dependent regulation of pyocyanin in ΔmvaT. Therefore QslH, when expressed at enhanced levels in ΔmvaT, is responsible for reduced AHL-dependent QS and PQS signalling.

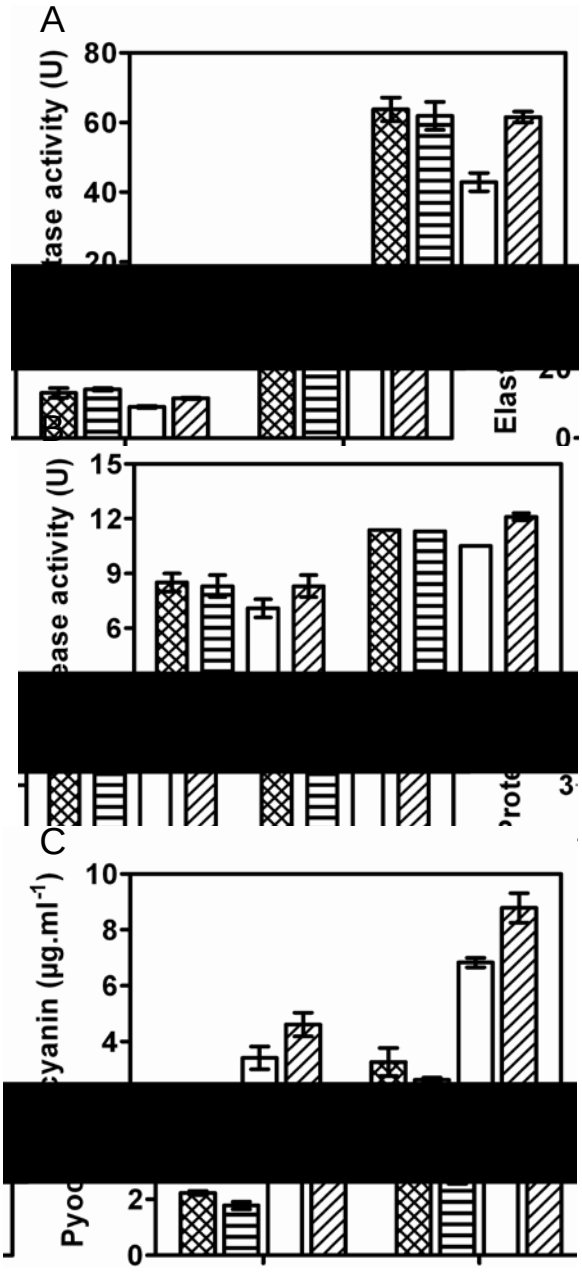


Fig. 4.17 QslH inhibits virulence factor production in ΔmvaT.
Production of elastase (A), protease (B) and pyocyanin (C) in wild type ( ), ΔqslH( ), ΔmvaT ( ) and ΔqslHΔmvaT ( ) were determined at the indicated cell density (OD_{600}). The data shown were means of 3 replicates and error bar, which indicates standard deviation, is not seen when standard deviation is small.

4.4 Discussion

QslH, a QS anti-activator belonging to the MvaT regulatory network, was identified in this study. Overexpression of *qslH* inhibited AHL-dependent QS and PQS signalling due to its interaction with LasR (Fig. 4.8) and PqsR (Fig. 4.9). QslH overexpression disrupted Las QS signalling at early growth phase (Fig. 4.3A, D) and inhibited PQS and Rhl QS signalling throughout bacterial growth (Fig. 4.3B, C, E, F). The effect of QslH on AHL-dependent QS and PQS signalling systems led to substantially attenuated production of virulence factors (Fig. 4.4). QslH interaction with LasR was supported by co-immunoprecipitation and EMSA analysis (Fig. 4.8), while interaction with PqsR was shown by co-immunoprecipitation and bacterial two-hybrid studies (Fig. 4.9).

QslA was previously reported to inhibit AHL-dependent QS and PQS signalling by anti-activation (Chapter 3). However, QslA and QslH do not show sequence homology (Fig. 4.6) although both proteins interact with LasR. TraM in *A. tumefaciens* is also not homologous to the anti-activators in *P. aeruginosa* (Fig. 4.6). This suggests that functionally similar anti-activators do not share sequence homology, instead the secondary structures they share may be crucial for their interaction with LuxR-type regulators. In addition, QslH also interacts with PqsR at its DNA-binding domain and because the DNA-binding domain of PqsR and LasR do not show homology, it was hypothesized that QslH interacts with these two proteins through different epitopes. TraM and predicted structure of QslA have 2 α-helices while QslH has 3, and the presence of an additional α-helix might have introduced a different interaction interface for QslH. However, QslH interaction with LasR and PqsR likely did not take place at the same time, since inhibition of LasR by *qslH* overexpression was alleviated at later growth phase when PqsR was accumulating.

Overexpression of *qslH* substantially inhibited AHL-dependent and PQS signalling, but deletion of *qslH* in wild type did not lead to obvious phenotype changes (Fig. 4.13). This was subsequently found to be partially because of the repression of *qslH* expression by MvaT (Fig. 4.16). MvaT in PAO1 is homologous to P16 subunit of *P. mevalonii* heteromeric transcriptional regulator MvaT, which regulates mevalonate catabolism (Rosenthal & Rodwell, 1998). MvaT is functionally similar to H-NS in *E. coli* (Tendeng *et al.*, 2003) in its ability to bind to AT-rich regions to block transcription initiation from these sites. Instead of recognising a specific consensus sequence on its DNA-binding sites, MvaT interacts specifically with AT-rich regions as shown by chromatin immunoprecipitation (ChIP)-on-chip studies (Castang *et al.*, 2008). *qslH* is located in a region (*PA2221–PA2228*) of significantly lower GC content (49.2%) compared to the rest of *P. aeruginosa* PAO1 genome (66.6%) (Stover *et al.*, 2000), and *qslH* neighbouring gene *PA2220* was also found to be enriched in MvaT ChIP-on-chip and ChIP qPCR results (Castang *et al.*, 2008). As *qslH* is part of an AT-rich region, it is likely that *qslH* expression is directly repressed by MvaT.

QslH is not the only MvaT-regulated protein that affects QS. MvaT is a global repressor and it influences QS both positively and negatively by controlling the expression of other QS-modulatory genes such as *ptxS*, *rsmZ* and *mexEF-oprN* operon (Westfall *et al.*, 2004, Westfall *et al.*, 2006). MvaT induces the expression of *ptxS* (Westfall *et al.*, 2004), which interferes with activation of QS by PtxR (Colmer & Hamood, 1998), hence inhibiting QS in the process. QS is also inhibited by MvaT through small RNA *rsmZ* as *rsmZ* expression is repressed by MvaT (Brencic *et al.*, 2009). RsmZ inhibits negative regulation of QS by RsmA (Pessi *et al.*, 2001, Heurlier *et al.*, 2004). However, MvaT also positively modulates QS by repressing the expression of *mexEF-oprN* operon (Westfall *et al.*, 2006) which inhibits the synthesis of C4-HSL signals (Kohler *et al.*, 2001). MvaT is therefore a pleiotrophic

regulator that exhibits a multi-layered complex regulation of QS-dependent virulence factor production, and QslH is one of the effectors in the MvaT-dependent regulatory networks that plays a role at the downstream of MvaT in control of QS-dependent pathogenesis.

Chapter 5 – General Discussion and Future Directions

Two novel QS anti-activators, QslA and QslH, were identified in this study. Both QslA and QslH interact with LasR and in addition, QslH also interacts with PqsR. Identification of these two novel anti-activators has provided further insight on the sophisticated QS regulatory mechanisms in *P. aeruginosa*. It is surprising that QslH interacts with both LasR and PqsR since they do not share substantial protein sequence similarity. Further biochemical analysis of the amino acid residues of LasR and PqsR that are crucial for interaction with QslH would improve understanding of these interactions and provide clues as to whether QslH interact with these two proteins at similar domain or amino acid motif. These results can also be compared with the LasR residues that are important for QslA interaction. It would be interesting to find out whether the two different anti-activators QslA and QslH interact with LasR at the same residues and whether these residues determine the binding affinity of the protein-protein interaction.

Currently, the crystal structures of LasR and PqsR have not been determined and only the structure of the ligand-binding domain of LasR has been solved (Bottomley *et al.*, 2007, Zou & Nair, 2009). When the protein structures of regulators LasR and PqsR as well as anti-activators QslA and QslH are available, the interaction interfaces of these proteins can be elucidated. These “hot spots” for interaction on LasR and PqsR might be potential targets for inhibitors, provided that the interaction interface is reasonably small and specific. QS inhibitors that have been reported are mostly targeted at the ligand-binding domain of LasR (Muh *et al.*, 2006a, Muh *et al.*, 2006b, Geske *et al.*, 2007, Zou & Nair, 2009), while inhibitors of PqsR have not been reported. The information of QS regulator structures, together with the identification of crucial residues on QS regulators that are found at the interaction interfaces with anti-activators, may mark potential target sites for

inhibition of QS regulators. These sites can be targeted for docking-based screening using small molecule or peptide QS inhibitors (Soulere *et al.*, 2010). The compounds or peptides with strong binding affinity that mimic structure or sequence of QS anti-activators at the interaction interfaces might be developed to act as QS inhibitors.

Besides developing QS inhibitors based on the biochemical information of anti-activation, the *in vivo* effect of anti-activation by QslA and QslH should also be investigated. It is not known whether the heterologous protein complex formed upon anti-activation gets localised to a non-functional region such as in the membrane or whether the regulators are degraded upon interaction with QslA or QslH. If the interaction between these anti-activators and their regulators were reversible, it would also be interesting to find out whether the protein-protein interaction leads to release of the activating ligands from their regulators, which would imply that the turnover of ligand binding is influenced by anti-activation.

Unlike QslA, QslH did not affect QS in wild type strain under the tested experimental conditions. This was found to be due to repression of *qslH* expression by MvaT in wild type so QslH was not present in sufficient levels in the wild type to inhibit QS and PQS signalling (Chapter 4.3.10). Hence, not all the anti-activators that are encoded in the genome are utilised to control QS under standard laboratory conditions and some of these anti-activators may be expressed only under certain environmental conditions to fine-tune QS signalling for adaptation. Inhibition of QS by QslH may therefore only be initiated under MvaT-regulated conditions.

On the other hand, RT-PCR analysis indicates that transcriptional expression of *qslA* appears to be constitutive (Section 3.3.10) and a genetic screen for transposon mutants with changes in expression of *qslA* also did not identify any potential regulators of *qslA* (results not shown). Nonethess, there is a possibility that QslA inhibition of LasR is modulated to allow control of the QS threshold.

It is possible that anti-activation of QslA may be regulated non-transcriptionally by other means. Type III secretion system regulator ExsA in *P. aeruginosa* is controlled by anti-activator and anti-anti-activators proteins (McCaw *et al.*, 2002, Dasgupta *et al.*, 2004), similarly, proteins that can interact strongly with QslA and disrupt its interaction with LasR would affect QS anti-activation by QslA. In addition, small molecules may also disrupt protein-protein interaction between QslA and LasR, as in the case of glycine that inhibits GcvR anti-activation of GcvA regulator in *E. coli* (Heil *et al.*, 2002).

This study has identified two QS anti-activators, QslA and QslH, which share similar protein characteristics as TraM and TraM2 in being small in size and having secondary structures that are mostly made up of α-helices. This differs from the putative *P. aeruginosa* anti-activator QscR that is homologous to LasR. The anti-activators in *A. tumefaciens* inhibit the basal amount of TraR/3-oxo-C8-HSL complex formed in the absence of opine, and this is only overcome when expression of *traR* is induced by OccR or NocR (Fuqua & Winans, 1994, Hwang *et al.*, 1995, Piper & Farrand, 2000, Wang *et al.*, 2006). The activity of *A. tumefaciens* anti-activators thus resembles that of QslA, where basal amount of ligand-bound regulators are inhibited. The control of QS threshold in *A. tumefaciens*, however, has not been tested. Hence, even though expression of *lasR* and *traR* are induced by different mechanisms in *P. aeruginosa* and *A. tumefaciens*, respectively, QS activation in both bacteria are modulated similarly by anti-activation.

TrlR, TraM and TraM2 in *A. tumefaciens* inhibit only LasR homologue, TraR (Hwang *et al.*, 1995, Piper & Farrand, 2000, Wang *et al.*, 2006). Anti-activation, however, is not only targeted at LuxR-type regulators. QslH was found to inhibit PqsR, which is a LysR-type regulator. GcvA, which is a regulator involved in glycine metabolism, is also a LysR-type regulator (Heil *et al.*, 2002), while ExsA is a AraC-type regulator (McCaw *et al.*, 2002), so inhibition of regulator activity by anti-

activation occurs for different types of regulators. RhlR, which is homologous to LasR in *P. aeruginosa*, was not inhibited by QslA or QslH. It may be possible that the anti-activators which interact with RhlR were not identified in this study.

Identification of QS anti-activators in *A. tumefaciens* and *P. aeruginosa* suggests that these anti-activators could be an integral part of QS systems in bacteria with AHL-dependent QS systems. QS anti-activators are likely species-specific because homologues of known anti-activators are only found in strains within the same species. As a result, homology search may not allow identification of QS anti-activators in other bacteria engaged in QS.

In summary, this study has revealed the roles of two novel QS anti-activators in *P. aeruginosa* with specific functions. QslA is instrumental in defining the QS threshold, while QslH allows regulation of QS according to MvaT-regulated conditions. Based on these findings, further investigation of the protein-protein interaction interface of QslA and QslH with QS regulators may facilitate development of QS inhibitors to attenuate *P. aeruginosa* virulence.

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