AN ABSTRACT OF THE DISSERTATION OF

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Martin Schuster

Pseudomonas aeruginosa is an opportunistic human pathogen that infects immunocompromised individuals such as those suffering from burns or the genetic disorder cystic fibrosis. This organism utilizes a cell-cell communication mechanism known as quorum sensing (QS) to coordinate virulence gene expression and biofilm formation. It has three interconnected QS systems, namely *las*, *rhl* and *pqs.* Each system is comprised of autoinducer synthesis genes, *lasI*, *rhlI*, and *pqsABCDH*, and the cognate regulatory genes, *lasR*, *rhlR*, and *pqsR*, respectively. Here, we primarily focused on understanding the regulatory mechanisms of QS, which we investigated at two levels. First, we sought to identify additional activators that regulate QS at the level of the *las* and *rhl* systems, and second, we investigated the regulation of downstream genes, particularly biofilm exopolysaccharide genes, by QS. For the first approach, we employed a mutagenesis screen to identify global QS activators. We screened a non-redundant transposon library for mutants deficient in QS-dependent

phenotypes. We identified a novel regulator, GidA, a glucose-inhibited cell division protein, that selectively controls QS gene expression posttranscriptionally via RhlR-dependent and –independent pathways. For the second part, we established a regulatory link between QS and Pel exopolysaccharide. We showed that the *las* system represses Pel and modulates colony biofilm structure through the *pqs* pathway. LasR mediated colony rugosity via 4-hydroxy-2-alkylquinolines in a PqsR-independent manner, ascribing a novel function to this class of signaling molecules in *P. aeruginosa*. Taken together, our study highlights the complexity of QS, which involves integration of various regulatory pathways to control downstream processes in response to different environmental conditions.

Quorum Sensing Gene Regulation in *Pseudomonas aeruginosa*

by
Rashmi Gupta

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Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

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Rashmi Gupta, Author

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CONTRIBUTION OF AUTHORS

Dr. Martin Schuster contributed in experimental design, scientific discussions, data interpretation, and manuscript preparation for Chapters 2, 3, and Appendix A and B. Timothy R. Gobble performed some experiments for Chapter 2.

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My parents
J. P. Gupta and Sunita Gupta

Quorum Sensing Gene Regulation in *Pseudomonas aeruginosa*

Chapter 1

Introduction and literature review

General introduction

Pseudomonas aeruginosa is a Gram-negative bacterium commonly found in soil and water. *P. aeruginosa* is highly versatile, can live either freely (planktonically) or in surface-associated communities known as biofilms. It possesses a large genome of approximately 6.3 Mbp (Stover *et al.*, 2000). It is an opportunistic pathogen to both plants and animals, including humans. In humans, it particularly affects immunocompromised individuals such as those suffering from burns or cystic fibrosis (CF). *P. aeruginosa* is a major cause of mortality and morbidity in CF patients (Govan & Nelson, 1992). It causes chronic lung infections characterized by biofilm formation in the lung airways, lung damage, respiratory failure, and death (Iglewski & Wagner, 2008). *P. aeruginosa* secretes various virulence factors and is resistant to several antibiotics mainly due to its biofilms lifestyle. This makes the treatment of *P. aeruginosa* infections even more challenging. The success of *P. aeruginosa* as a pathogen is in large part due to a cell-cell communication system known as quorum sensing (QS). Thus, understanding the molecular basis and evolution of QS signaling is central to its exploitation as a target for novel antibacterial agents.

QS systems in bacteria: the *Vibrio fischeri* paradigm and beyond

QS is a cell-to-cell communication mechanism by which bacteria coordinate gene expression as a function of cell density (Fuqua *et al.*, 1994). QS was first discovered in the marine bioluminescent bacterium *Vibrio fischeri* in the early 1970s (Nealson *et al.*, 1970, Eberhard, 1972). *V. fischeri* is a marine bacterium that either lives freely or as a mutualistic symbiont in the light organ of the squid *Euprymna scolopes* (Ruby, 1996). QS in *V. fischeri* controls bioluminescence via the LuxI/LuxR QS system, which is the paradigm for cell-cell communication in Gram-negative bacteria (Fig. 1.1).

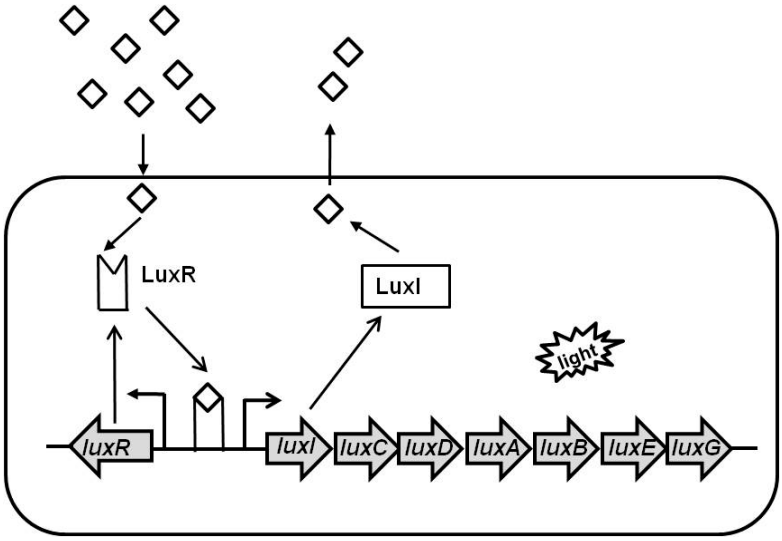

Fig. 1.1: LuxI/LuxR QS in *V. fischeri*. At low cell density, the signal (represented by the diamond symbols) is produced at low levels by LuxI and there is weak transcription of the genes (*luxICDABEG*). At high cell density, a critical concentration of the signal, 3-oxo-C6-HSL, is reached. The signal binds to LuxR and stimulates transcription of *luxICDABEG*, leading to emission of light.

LuxI is an autoinducer synthase which synthesizes the signal 3-oxohexanoyl-homoserine lactone (3-oxo-C6-HSL) (Kaplan & Greenberg, 1985, Nealson & Hastings, 1979), and LuxR is a transcriptional activator that detects and responds to the signal (Engebrecht *et al.*, 1983, Kaplan & Greenberg, 1985). The LuxR protein contains two functional domains. The N-terminal domain interacts with the autoinducer and the C-terminal domain contains a highly conserved helix-turn-helix (HTH) motif involved in DNA binding and transcriptional activation (Hanzelka & Greenberg, 1995, Henikoff *et al.*, 1990). At low cell-density, the signal 3-oxo-C6-HSL diffuses out of the cells but when cell-density increases, the signal accumulates and reaches a critical threshold (Kaplan & Greenberg, 1985, Nealson & Hastings, 1979). At this point, it binds to the LuxR protein and this signal-bound LuxR (LuxR-HSL) then binds to a conserved promoter element termed *lux* box to activate transcription of the *lux* operon containing *luxI* and the genes *luxCDABEG* required for light production (Kaplan & Greenberg, 1985, Nealson & Hastings, 1979, Engebrecht & Silverman, 1984) (Fig. 1.1). This positive autoregulation of *luxI* causes further amplification of 3-oxo-C6-HSL levels and is responsible for the sudden onset of luminescence during *V. fischeri* culture growth (Kaplan & Greenberg, 1985, Nealson & Hastings, 1979). LuxRI-type of QS is found in many other Gram-negative proteobacteria (Boyer & Wisniewski-Dye, 2009) (Table 1.1).

Table 1.1: Examples of bacterial QS systems

Organism	QS system	Target function
Gram-negative bacteria		
Vibrio fischeri	LuxI/LuxR	Bioluminescence (Eberhard *et al.*, 1981, Engebrecht *et al.*, 1983)
Pseudomonas aeruginosa	LasI/LasR RhlI/RhlR	Virulence and biofilm development (Davey *et al.*, 2003, Davies *et al.*, 1998, de Kievit & Iglewski, 2000, Pearson *et al.*, 1995)
Erwinia carotovora	ExpI/ExpR	Exoenzyme synthesis (Jones *et al.*, 1993, Pirhonen *et al.*, 1993)
	CarI/CarR	Carbapenem antibiotics (Bainton *et al.*, 1992)
Agrobacterium tumefaciens	TraI/TraR	Conjugal transfer (Piper *et al.*, 1993, Zhang *et al.*, 1993)
Burkholderia cepacia	CepI/CepR	Protease, Siderophore (Sokol *et al.*, 1999)
Gram-positive bacteria		
Bacillus subtilis	ComP/ComA	Competence (Magnusson *et al.*, 2005, Solomon *et al.*, 1996)
Staphylococcus aureus	AgrC/AgrA	Virulence (Novick & Muir, 1999)
Streptococcus pneumoniae	ComD/ComE	Competence (Dawson & Sia, 1931)

QS in Gram-positive bacteria mainly involves modified oligopeptides as signals instead of acyl-homoserine lactones (acyl-HSL) (Miller & Bassler, 2001). Gram-positive bacteria employ a two–component regulatory system consisting of a membrane-bound sensor kinase and a cognate response regulator (Dunny & Leonard, 1997, Lazazzera & Grossman, 1998) as opposed to LuxR type regulators. The sensor kinase detects the signal, autophosphorylates, and then transmits the phosphoryl group to the response regulator. The phosphorylated, activated response regulator then binds to DNA and regulates target gene expression. Besides acyl-HSL and peptides,

autoinducer-2 is also used as a signal by many Gram-positive and Gram-negative bacteria. Examples of bacterial QS systems are listed in Table 1.1.

QS allows bacteria to coordinate group behavior and to function as quasi-multicellular organisms to perform tasks which otherwise would be difficult for individual cells. This helps them cope with and survive in changing environmental conditions. In bacterial pathogens, QS often regulates factors associated with virulence and infection and is therefore, considered an ideal target for anti-virulence strategies.

The *P. aeruginosa* QS circuitry

P. aeruginosa QS is among the best-studied systems and is mediated by two chemically distinct classes of signal molecules, N-acylhomoserine lactones (acyl-HSL) (Haussler & Becker, 2008) and alkyl quinolones (Pesci *et al*., 1999) (Fig. 1.2). Alkyl quinolones in the lactam form are tautomeric with alkyl quinolines in the hydroxyl form. The predominance of one form over the other depends on pH (Heeb *et al*., 2011).

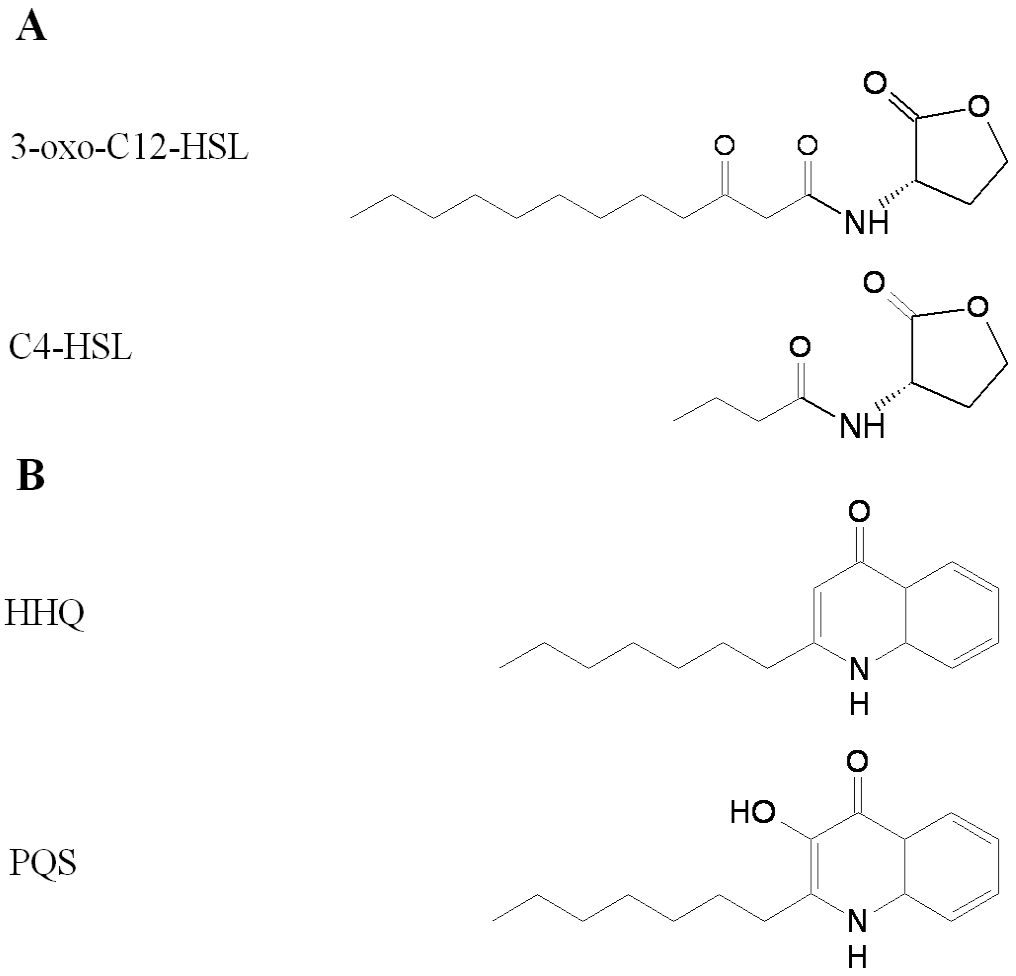

Fig. 1.2: Chemical structures of *P. aeruginosa* QS signal molecules. **A.** Acylhomoserine lactones. 3-oxo-C12-HSL: Oxododecanoyl-homoserine lactone, C4-HSL: Butanoyl-homoserine lactone. **B.** Alkyl quinolones. HHQ: 4-hydroxy-2-heptylquinolone, PQS: 2-heptyl-3,4-dihydroxyquinolone.

Acyl-HSL QS

This group involves the Lux-type QS systems, *las* and *rhl* (Brint & Ohman, 1995, Passador *et al.*, 1993) (Fig. 1.3). The *las* system is comprised of the transcriptional regulator, LasR, and the signal synthase, LasI that produces the signal oxododecanoyl-homoserine lactone (3-oxo-C12-HSL) (Pearson *et al.*, 1994). The signal, 3-oxo-C12-

HSL diffuses slowly and is also actively transported out of the cells by an efflux pump (Pearson *et al.*, 1999). LasR binds to its cognate signal molecule 3-oxo-C12-HSL and regulates expression of specific genes (de Kievit & Iglewski, 2000, Gambello & Iglewski, 1991, Kiratisin *et al.*, 2002). When overexpressed for purification purposes, LasR requires its signal for the expression of soluble, active protein (Schuster *et al.*, 2004). However, when expressed at physiological levels, LasR appears to bind its signal reversibly (Sappington *et al.*, 2011). Transcription of a LasR-dependent gene, *lasI*, was quenched after removal of the signal from the culture medium.

The *rhl* system consists of the transcriptional regulator, RhlR, and the signal synthase, RhlI, that produces butanoyl-homoserine lactone (C4-HSL) (Fig. 1.3) (Pearson *et al.*, 1995). The *las* system regulates the *rhl* system, interconnecting the two in a hierarchical manner (Latifi *et al*., 1996, Pesci & Iglewski, 1997a). The expression of *rhlI* and *rhlR* genes is positively regulated by LasR:3OC12-HSL complex (Latifi *et al*., 1996, Medina *et al.*, 2003a, Pesci *et al.*, 1997b). In contrast to 3-oxo-C12-HSL, C4-HSL diffuses rapidly and is not actively transported (Pearson *et al*., 1999). RhlR binds to C4-HSL to induce expression of specific genes (Fig. 1.3). It also functions as a repressor by binding to the DNA in absence of the signal (Medina *et al*., 2003b).

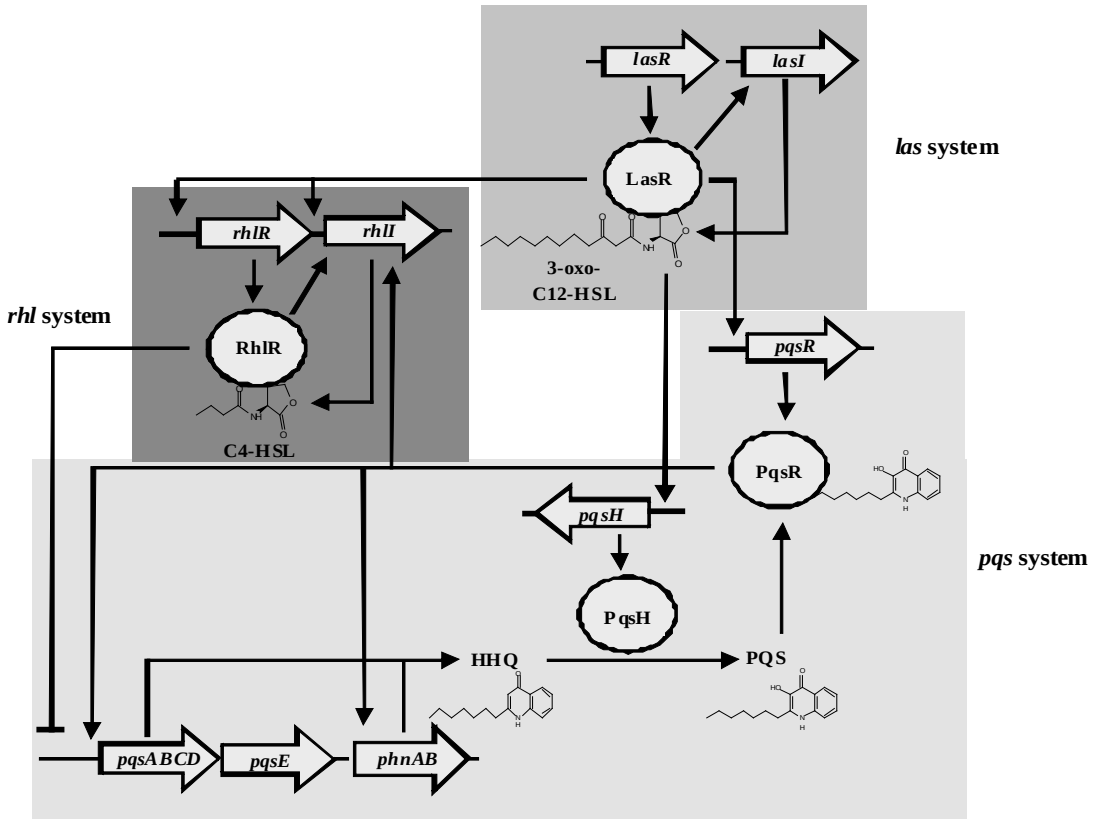

Fig. 1.3: The hierarchical *P. aeruginosa* QS network. Arrows and T-bars indicate positive and negative transcriptional regulation, respectively. The shaded boxes represent the individual QS systems. The gene *lasI* encodes a signal synthase that generates 3-oxo-C12, which binds to the transcriptional activator, LasR. This 3-oxo-C12:LasR complex upregulates both the *rhl* and the *pqs* QS systems. The gene *rhlI* encodes a signal synthase that generates C4-HSL, which binds to RhlR. This C4-HSL:RhlR complex negatively regulates the *pqs* system. Genes *pqsABCDH, phzAB,* and *pqsH* are involved in the generation of the signal PQS, which binds to PqsR. This PQS:PqsR complex up-regulates the *rhl* QS system.

The two systems are highly specific in the sense that the respective signal molecule does not activate the transcriptional regulator protein of the other system (Latifi *et al.*, 1995, Pearson *et al.*, 1997). LasR and RhlR activate gene transcription by binding to palindromic sequences known as *las-rhl* boxes following multimerization,

after binding to their respective signals (Schuster & Greenberg, 2007). Both LasR and RhlR activate expression of their cognate signal synthases (*lasI* and *rhlI*), creating a positive feedback loop which amplifies the signal (Seed *et al.*, 1995). The two systems together regulate expression of more than 300 genes (Hentzer *et al.*, 2003, Schuster *et al.*, 2003, Wagner *et al.*, 2003). A recent global position analysis of LasR by chromatin immunoprecipitation and transcription profiling identified 35 direct targets of LasR (Gilbert *et al.*, 2009).

Non-acyl-HSL QS

Signaling by the PQS system is mediated by 2-alkyl-4-quinolones (AQ) (Pesci *et al*., 1999). It includes the autoinducers 4-hydroxy-2-heptylquinoline (HHQ) and 2-heptyl-3,4-dihydroxyquinoline (PQS), and the cognate receptor PqsR (also termed MvfR) (Fig. 1.3). PqsR is a LysR-type transcriptional regulator that controls expression of *pqsABCDE*, encoding the structural genes responsible for autoinducer synthesis (Deziel *et al.*, 2004, Gallagher *et al.*, 2002). HHQ is synthesized by condensation of precursors, β-keto-fatty acid and anthranilic acid (Bredenbruch *et al.*, 2005, Rampioni *et al.*, 2010). HHQ is converted to PQS by hydroxylation via PqsH, a putative FAD-dependent monoxygenase (Bredenbruch *et al.*, 2005, Deziel *et al*., 2004). Both HHQ and PQS act as ligands for PqsR, binding with low and high affinity, respectively (Wade *et al.*, 2005, Xiao *et al.*, 2006). PQS is hydrophobic and is mainly associated with membrane vesicles (Whiteley *et al.*, 2008). Another gene, *pqsL*,encoding a putative monooxygenase, also influence PQS synthesis as it diverts a fraction of HHQ

precursors away from PQS synthesis. A mutation in the *pqsL* gene causes overproduction of PQS (D'Argenio *et al.*, 2002). The *pqs* system regulates around 141 genes (Rampioni *et al.*, 2010), many of which are dependent on PqsE for expression and some are co-regulated by both the *las* and the *rhl* systems (Deziel *et al.*, 2005). PqsE is a putative metallo-β-lactamase which is thought to be important for the cellular response to PQS (Gallagher *et al*., 2002, Long *et al.*, 2009).

The PQS system serves as the connecting link between the *las* and *rhl* QS systems. The *las* system positively regulates PQS production by activating expression of *pqsR* and *pqsH* (Gallagher *et al.*, 2002, Pesci *et al*., 1999). The *rhl* system negatively regulates PQS production (McGrath *et al.*, 2004), and PqsR in turn induces expression of *rhlI* which encodes C4-HSL (McKnight *et al.*, 2000). A delicate balance between the *las* and the *rhl* system (ratio of 3-oxo-C12-HSL to C4-HSL) controls PQS signaling (McGrath *et al.*, 2004). Under certain growth conditions PQS is produced through LasR-independent activation of the *rhl* system (Diggle *et al.*, 2003, McKnight *et al*., 2000).

The three systems (*las*, *rhl* and *pqs*) allow temporal expression of certain virulence genes, which may be critical for different stages of the infection process. However, it has been shown that the levels of the quorum signals alone are not sufficient for expression of some QS-regulated genes (Diggle *et al.*, 2002, Schuster *et al*., 2003, Whiteley *et al.*, 1999). For example, growth phase and starvation have important roles in co-regulating QS genes.

Additional regulation of *P. aeruginosa* QS

QS is complex and involves other regulatory pathways besides the *las*, *rhl* and *pqs* QS systems. The QS systems themselves and their target genes are subject to additional layers of regulation both at the transcriptional and post-transcriptional level (Venturi, 2006).

QscR is a LuxR homolog which is not genetically linked to a signal synthase (Chugani *et al.*, 2001) but shares the signal 3OC12-HSL with LasR. A transcriptome analysis showed that QscR activates some genes and represses others (Lequette *et al.*, 2006). Activation is mediated by direct binding of QscR:3oxo-C12-HSL to the DNA. QscR represses *lasI* expression at low cell densities possibly through formation of inactive heterodimers with LasR and RhlR (Ledgham *et al.*, 2003, Chugani *et al*., 2001). VqsR positively regulates QS and a mutation in the *vqsR* gene results in abolition of acyl-HSL signal, decreased virulence factor production and reduced pathogenicity (Juhas *et al.*, 2005, Juhas *et al.*, 2004). RsaL negatively regulates *lasI* transcription by competiting with LasR-3OC12-HSL complex for binding to the *lasI* promoter (de Kievit *et al.*, 1999, Rampioni *et al.*, 2006). It prevents early activation of QS-dependent genes. Vfr, the cAMP receptor regulatory protein (Albus *et al.*, 1997) activates expression of *lasR* (Albus *et al.*, 1997) and *rhlR* (Croda-Garcia *et al.*, 2011).

At the post-transcriptional level, DksA controls production of QS-dependent virulence factors by affecting translational efficiency of two QS-regulated genes, namely *lasB*, encoding LasB elastase, and *rhlAB*, responsible for rhamnolipid production (Jude *et al.*, 2003). GacA/GacS, a two-component regulatory system also

influence QS gene expression by activating a small regulatory RNA, RsmZ, which in turn antagonizes the RNA binding protein, RsmA (Lapouge *et al.*, 2008). RsmA represses *lasI* and negatively regulates secondary metabolite production (pyocyanin, hydrogen cyanide), possibly by accessing control to the ribosome binding site and altering mRNA stability (Pessi *et al.*, 2001). QteE and QslA are anti-activator proteins that block QS at low cell densities by protein-protein interactions. QteE, also known as "quorum threshold element" reduces LasR protein stability without affecting transcription or translation (Siehnel *et al.*, 2010) and QslA affects C4-HSL signal concentration and RhlR levels (Seet & Zhang, 2011).

Overall, QS involves a multitude of regulators and is itself integrated into a complex network of global regulation. Several regulators (QscR, QteE, QslA, and RsaL) avoid early activation at low cell densities and ensure the correct timing of the QS response. The complexity and involvement of so many regulators also indicates that QS can be fine-tuned in response to various environmental conditions. Although much has been learned about the pathways that co-regulate QS, a comprehensive analysis, for example through a saturating mutagenesis, to identify factors controlling QS has never been attempted. Identifying some of the unknown regulators and their connection to the known QS circuitry is one of the objectives of this dissertation.

Role of QS in biofilm formation

Biofilms are highly-structured surface-associated microbial communities encased in a gelatinous exopolysaccharide matrix (Flemming & Wingender, 2010). This offers

biofilm cells protection from dessication, predation, and antibiotics. Biofilms are of considerable importance in *P. aeruginosa* pathogenesis as they are formed on the surface of medical devices like catheters, endotracheal tubes, and contact lenses. They also contribute to chronic lung infection in CF patients (Parsek & Singh, 2003). Biofilm development is a coordinated series of events beginning with surface attachment by planktonic bacteria, followed by formation of microcolonies, development of differentiated structures, and finally detachment (Fig. 1.4) (Hall-Stoodley *et al.*, 2004). Biofilm formation and structure depends on both genetic and environmental factors (Sutherland, 2001).

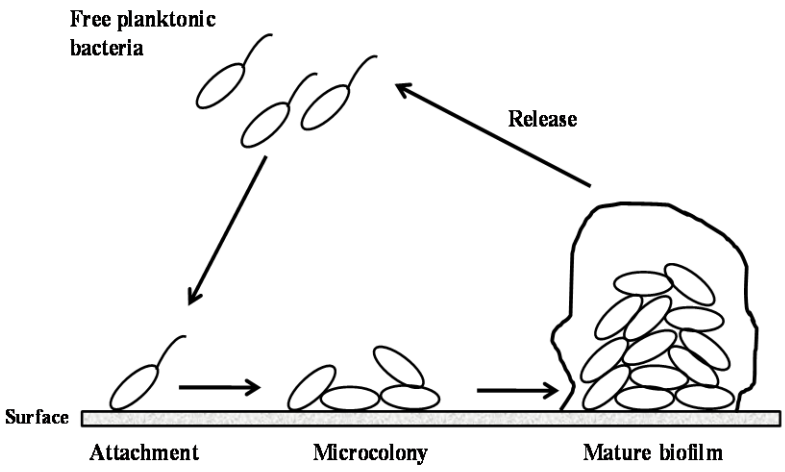

Fig. 1.4: Biofilm formation. It is a multistep process of attachment, microcolony formation, maturation, and release.

Commonly used tools to study biofilms in the laboratory are flow-cells, pellicles, microtiter plates and colonies. The flow-cell system is arguably the gold

standard to study submerged biofilms (Christensen *et al.*, 1999) where biofilms are formed in flow-chambers under continuous nutrient flow and analyzed using confocal laser scanning microscopy (CLSM). Pellicles are floating biofilms that form at the air-liquid interface of standing cultures (Branda *et al.*, 2001, Friedman & Kolter, 2004a). Microtiter biofilms are formed on the surfaces of microtiter dish wells under standing conditions, which can be visualized by a non-specific dye, crystal violet (O'Toole & Kolter, 1998). They are useful for high-throughput screening. The bacterial colonies growing on solid agar surface are an easy, convenient and practical way to study biofilms (Hickman *et al.*, 2005, Sakuragi & Kolter, 2007, Shapiro, 1984). A wrinkled colony is generally indicative of increased extracellular polysaccharide (EPS) and a highly structured biofilm (Friedman & Kolter, 2004a).

The biofilm matrix, an important part of a biofilm, is comprised of proteins, EPS, extracellular DNA (eDNA), and water (Allesen-Holm *et al.*, 2006, Sutherland, 2001). EPS constitutes a major and an important part of biofilm matrix. The three main EPS types in *P. aeruginosa* are alginate, Pel, and Psl. Alginate is an acetylated polymer of β-1,4 linked L-glucoronic and D-mannuronic acids. Alginate production is associated with a mutation in the negative regulator *mucA* and is responsible for the mucoid phenotype often seen in *P. aeruginosa* isolates from CF patients (Martin *et al.*, 1993). Several studies have independently shown that overproduction of alginate results in significant changes in biofilm architecture (Franklin *et al.*, 2001, Hentzer *et al.*, 2001, Mathee *et al.*, 2004). The common laboratory strains PAO1 and PA14 generally form non-mucoid biofilms. These biofilms are composed of Pel and Psl EPS

instead (Wozniak *et al.*, 2003). Psl is a mannose- and galactose-rich EPS while Pel mainly consists of glucose (Friedman & Kolter, 2004a, Jackson *et al.*, 2004, Ma *et al.*, 2007, Matsukawa & Greenberg, 2004). Pel is involved in pellicle formation in *P. aeruginosa* strain PA14 (Friedman & Kolter, 2004a). Both Pel and Psl EPS are involved in early- and late-stages of biofilm development most likely by mediating cell-cell interactions and cell-surface interactions (Friedman & Kolter, 2004b, Jackson *et al*., 2004, Ma *et al.*, 2007, Matsukawa & Greenberg, 2004). Pel and Psl overproduction causes distinct changes in autoaggregative properties, colony morphology, and biofilm architecture (Kirisits *et al.*, 2007, Ma *et al*., 2007).

Several reports have suggested a connection between QS and biofilm formation. The role of QS in biofilm formation was first reported in 1999 by Davies *et al.* who showed that *lasI* mutants formed flat and thin biofilms (Davies *et al.*, 1998) as opposed to the mushroom-shaped biofilms of the wild-type parent (Davies *et al*., 1998). The *rhl* system maintains biofilm archictecture by preventing microbial colonization of open channels surrounding colonies (Davey *et al.*, 2003). The *lasI* mutants are defective in activating *pel* transcription and biofilm formation in *P. aeruginosa* strain, PA14 (Sakuragi & Kolter, 2007). The *lasI rhlI* double mutants produce less eDNA than the wild-type parent indicating compromised biofilm integrity (Allesen-Holm *et al.*, 2006). Recently, Gilbert *et al*. found that LasR binds to the promoter region of the *psl* (Gilbert *et al*., 2009) (Appendix B). In addition studies have shown that QS regulation of biofilm formation is nutritionally conditional (Heydorn *et al.*, 2002, Purevdorj *et al.*, 2002, Stoodley *et al.*, 1999, Shrout *et al.*,

2006) and hence, a clear-cut role of QS in biofilm formation and structure is debatable. In this dissertation, we further investigated the molecular mechanism underlying the connection between QS and biofilm architecture.

Evolution and maintenance of QS in *P. aeruginosa*

QS coordinates several cooperative behaviors in bacterial populations such as production of extracellular factors, also referred to as "public goods" (West *et al.*, 2007). A cooperative behavior is characterized by a fitness consequence to both the actor producing the behavior and the recipient (Diggle, 2010). As QS involves production of costly signals and public goods, it is vulnerable to exploitation by cheaters that do not produce either the signals or QS regulators. Social cheating has been demonstrated in *P. aeruginosa* both in *vitro* (Diggle *et al*., 2007, Sandoz *et al.*, 2007) and *in vivo* (Rumbaugh *et al.*, 2009). Despite the associated cost and social cheating, QS is quite ubiquitous implying that it is evolutionarily stable. Several theories and models have provided explanations for evolution and stability of QS. One explanation is that cooperation provides a direct benefit to the individual that performs the behavior which outweighs the cost of performing the behavior (Sachs *et al.*, 2004). Another explanation is provided by Hamilton's Kin Selection Theory, according to which cooperation provides an indirect benefit by helping close relatives reproduce. This way an individual passes on its genes to the next generation (Hamilton, 1963) in an indirect manner. This can be achieved through kin discrimination where relatives can be distinguished from non-relatives, or by limited dispersal where spatial

proximity keeps the close relatives together. A theoretical kin selection model proposed by Brown and Johnstone (Brown & Johnstone, 2001) also explains how QS is maintained: cooperation increases and conflict decreases with increasing relatedness. Pleiotropy, a phenomenon where a gene has multiple traits also explains QS stability (Foster *et al.*, 2004). For example, a cooperative gene with an additional essential function is less likely to mutate, preventing cheaters from originating. Complementary modeling approach taken by Czárán *et al.* to analyze evolution of QS-regulated cooperation (Czárán & Hoekstra, 2009) suggested that cooperation only evolves under conditions of limited population dispersal. The presence of cooperating strains in a population always selects for the invasion and stabilization by QS. Their model considered the rewards and costs of cooperation, the level of dispersal, and the signal strength required to induce production of public goods. Their cooperative model system also allowed diverse social interactions where cheating and exploitation commonly occurred and were in equilibrium with cooperation.

These various explanations for evolution and stability of QS provide an explanation for the prevalence of QS-deficient strains (primarily *lasR* mutants) in infections and natural environments. These *lasR* mutants do not carry out the costly process of responding to the signal to produce public goods but benefit from their production by others and therefore, behave as social cheaters. The *lasR* cheaters have shown to invade and exploit a QS cooperating population and decrease the overall virulence in mouse burn infections (Rumbaugh *et al.*, 2009). Even in humans, a mixed

infection by QS cheaters and cooperators resulted in a milder infection (Kohler *et al.*, 2009). These studies suggest a potential role of QS cheaters in infection therapy.

Research objectives

QS in *P. aeruginosa* is highly complex and is also integrated with other regulatory circuits. We do not have a complete understanding of all the regulatory links. The objective of this dissertation is to obtain a more complete picture of the QS regulatory network with the goals to identify genes that regulate QS and also the genes that are regulated by QS (Chapters 2 and 3).

Chapter 2

GidA posttranscriptionally regulates *rhl* quorum sensing in *Pseudomonas aeruginosa*

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Abstract

The opportunistic pathogen *Pseudomonas aeruginosa* utilizes two interconnected acyl-homoserine lactone quorum-sensing (acyl-HSL QS) systems, LasRI and RhlRI, to regulate the expression of hundreds of genes. The QS circuitry itself is integrated into a complex network of regulation by other factors. However, our understanding of this network is still unlikely to be complete, as a comprehensive, saturating approach to identifying regulatory components has never been attempted. Here, we utilized a non-redundant *P. aeruginosa* PA14 transposon library to identify additional genes that regulate QS at the level of LasRI/RhlRI. We initially screened all 5,459 mutants for loss-of-function in one QS-controlled trait (skim-milk proteolysis) and then re-screened attenuated candidates for defects in other QS-phenotypes (LasA protease, rhamnolipid, and pyocyanin production) to exclude mutants defective in functions other than QS. We identified several known and novel genes, but only two novel genes, *gidA* and *pcnB*, affected all of the traits assayed. We characterized *gidA*, which exhibited the most striking QS phenotypes, further. This gene is predicted to encode a conserved FAD-binding protein involved in tRNA modification. Inactivation of the gene primarily affected *rhlR*-dependent QS phenotypes such as LasA, pyocyanin, and rhamnolipid production. GidA affected RhlR protein but not transcript levels and also had no impact on LasR and acyl-HSL production. Overexpression of *rhlR* in a *gidA* mutant partially restored QS-dependent phenotypes. Taken together, these results

indicate that GidA selectively controls QS gene expression posttranscriptionally via RhlR-dependent and independent pathways.

Introduction

Pseudomonas aeruginosa is a ubiquitous environmental bacterium commonly found in soil and freshwater. It is also an opportunistic human pathogen that infects immunocompromised individuals, including those suffering from cystic fibrosis. It utilizes a quorum-sensing (QS) mechanism to regulate and coordinate virulence gene expression (Bjarnsholt & Givskov, 2007, Girard & Bloemberg, 2008, Rumbaugh *et al.*, 2000, Smith & Iglewski, 2003). There are two complete acyl-homoserine lactone (acyl-HSL) QS systems in *P. aeruginosa*, the LasR-LasI (*las*) system and the RhlR-RhlI (*rhl*) system. LasI and RhlI produce the signals 3-oxo-dodecanoyl (3OC12) HSL and butanoyl (C4) HSL respectively; these signals bind to and activate their cognate transcriptional regulators LasR and RhlR. There is a third, orphan regulator, QscR, that also responds to 3OC12-HSL (Lee *et al.*, 2006). Under standard growth conditions, the *las* system activates the *rhl* system (Latifi *et al.*, 1996, Pesci *et al.*, 1997), and the two systems together control the expression of hundreds of genes (Hentzer *et al.*, 2003, Schuster *et al.*, 2003, Wagner *et al.*, 2003). A large portion of these QS-controlled genes encode secreted virulence factors such as proteases (LasA protease, LasB elastase, alkaline protease), biosurfactants (rhamnolipid), and secondary metabolites (hydrogen cyanide and pyocyanin).

P. aeruginosa QS is embedded in a complex network of global regulation (Juhas *et al.*, 2005, Schuster & Greenberg, 2006, Venturi, 2006). Most QS-controlled genes are not induced until the stationary phase of growth even when exogenous acyl-HSL signals are present early in growth (Diggle *et al.*, 2002, Schuster et al., 2003, Whiteley *et al.*, 1999, Winzer *et al.*, 2000). Thus, additional factors are required for expression of these QS-controlled genes. Several global regulators and regulatory pathways (e.g. Vfr, GacA/GacS, RsaL, RelA, RpoS, PqsR [MvfR]) have already been identified (Juhas *et al*., 2005, Schuster & Greenberg, 2006). Although much remains to be learned about this complex network of global regulation, it is clear that control of QS is multifaceted and will require an integrative approach to understanding its complexity.

In this study, we employed a saturation mutagenesis approach - making use of a recently constructed non-redundant transposon mutant library of *P. aeruginosa* strain PA14 (Liberati *et al.*, 2006) to identify additional regulators of QS gene expression. We characterized one such novel regulator, GidA, which shares homology with conserved flavin-adenine-dinucleotide-binding proteins (Sha *et al.*, 2004) involved in tRNA modification (Meyer *et al.*, 2008, Yim *et al.*, 2006). Initially isolated as a factor involved in a glucose-inhibited cell division phenotype in *E. coli* (von Meyenburg *et al.*, 1982), more recent studies have established that GidA, together with MnmE, is responsible for the addition of a carboxymethylaminomethyl group to uridine 34 of a subset of tRNAs (Yim *et al.*, 2006). This process is important for the appropriate

decoding of two-family box triplet codons. Despite the potential to indiscriminately affect cellular gene expression, GidA homologs rather specifically regulate virulence gene expression in *Aeromonas hydrophila* (Sha *et al.*, 2004), *Pseudomonas syringae* (Kinscherf & Willis, 2002), *Shigella flexneri* (Durand *et al.*, 2000), and *Streptococcus pyogenes* (Cho & Caparon, 2008), with little to no impact on growth. In *Myxococcus xanthus*, GidA is involved in fruiting body development (White *et al.*, 2001). Our data show that GidA specifically controls *rhl*-dependent QS gene expression in *P. aeruginosa* by modulating RhlR expression posttranscriptionally.

Materials and Methods

Bacterial strains, plasmids and growth conditions

Strains used in this study are listed in Table 2.1.

TABLE 2.1: Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Reference
Strains		
P. aeruginosa		
PA14	Wild-type	(Liberati *et al*., 2006)
PA14 *gidA*7	MAR2xT7 *gidA* mutant derived from PA14 (mutant ID: 44643)	(Liberati *et al*., 2006)
PA14 *lasR*	Tn*phoA lasR* mutant derived from PA14	(Tan *et al.*, 1999)
PA14 *rhlR*	MAR2xT7 *rhlR* mutant derived from PA14 (mutant ID: 37943)	(Liberati *et al*., 2006)
PA14_73400	MAR2xT7 *mnmE* mutant derived from PA14 (mutant ID: 38726)	(Liberati *et al*., 2006)
Additional MAR2xT7 mutants	See Table S2.1 and online: http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi	(Liberati *et al*., 2006)
PA14 Δ*gidA*	Markerless in-frame deletion mutant derived from PA14	This study
E. coli		
DH5α	F^-, Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *deoR recA*1 *endA*1 *hsdR*17 (rk-, mk+) *phoA supE*44 λ- *thi*-1 *gyrA*96 *relA*1	Invitrogen
Plasmids		
pCF430	Broad host-range vector containing *araC-*P_{BAD} promoter (tetracycline-resistant)	(Newman & Fuqua, 1999)
pCF430G	*gidA* ORF and RBS of PA14 in *Hin*dIII/*Xba*I of pCF430	This study
pCF430R	*Nhe*I/*Sac*I fragment containing *rhlR* ORF and RBS of PAO1 subcloned from pJN105.*rhlR*	This study
pEX18Tc	Gene replacement vector with *sacB* counterselectable marker (tetracycline resistant)	(Hoang *et al*., 1998)
pEX18Tc.Δ*gidA*	pEX18Tc containing *gidA* with a markerless in-frame deletion	This study

MAR2xT7 mutants used in the study are taken from a subset of the parental PA14 mariner transposon insertion library (Liberati *et al*., 2006). The mutants were handled and stored as described in the library's user manual (Liberati *et al*., 2006). trains were initially plated from frozen stocks onto LB agar plates and grown for 24 h at 37°C. Unless noted otherwise, *P. aeruginosa* liquid cultures were grown at 37°C with agitation (250 rpm) in Lennox Luria-Bertani (Albus *et al*.) broth buffered with 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7.0. *Escherichia coli* DH5α (Invitrogen, CA) was cultured in LB at 37°C. Where appropriate, the antibiotics gentamicin and tetracycline (10 µg/ml for *E.coli* and 100 µg/ml for *P. aeruginosa*) were added to the growth media.

For high-throughput assays, colonies grown on rectangular LB plates in a 12x8 format were replicate-plated using a 96-pin replicator. Subsequent assays were either plate-based or liquid-based (see below). For the latter, 600 µl of LB-MOPS in 96-deep-well titerblocks were inoculated from plates and grown for 18 h. For liquid-culture assays performed with few individual strains, including LasA protease activity, pyocyanin production, Western blotting, 3OC12-HSL and C4-HSL quantitation, and real-time PCR, experimental *P. aeruginosa* cultures were started by inoculating 25 ml LB-MOPS in 250 ml Erlenmeyer flasks with mid-logarithmic phase bacteria to an optical density (OD_{600}) of 0.02. Culture aliquots were harvested after 7 to 8 h of growth (stationary phase). For real-time PCR analysis, culture aliquots were also harvested after 4 to 5 h of growth (transition from logarithmic to stationary phase).

The culture supernatants were filtered-sterilized (pore size, 0.2 μm) for individual phenotypic assays.

For complementation analysis, LB-MOPS medium containing L-arabinose (50 mM final concentration) and tetracycline was inoculated with a colony from a freshly streaked culture. The cultures were grown for 24 h (stationary phase) prior to analysis.

DNA manipulations

General molecular cloning techniques were based on standard protocols (Sambrook *et al.*, 1989). Chromosomal DNA was isolated using the PUREGENE Core Kit A (Gentra Systems). Plasmids used for complementation were constructed as follows: A 1893 bp DNA fragment containing the *gidA* coding sequence was amplified from PA14 chromosomal DNA using primers 5'-N_6AAGCTTCGCTAAATCCTTATACTGTCCG-3' and 5'-N_6TCTAGAGGTGTTGGGTTACCGCAGAC-3'. The fragment was then digested with *Hin*dIII and *Xba*I (respective restriction enzyme recognition sites are underlined) and ligated to *Hin*dIII/*Xba*I digested pCF430 to generate pCF430G (Table 2.1). Proper construction was verified by DNA sequencing. Plasmid pCF430R (Table 2.1) was generated by subcloning a 700 bp *Nhe*I/*Sac*I fragment from pJN105.*rhlR* (Schuster & Greenberg, 2007).

The location of the transposon (MAR2xT7) insertion in the PA14 *gidA* mutant was confirmed by PCR and subsequent agarose gel electrophoresis. Primers were

designed based on the presumed location of the transposon 1716 bp downstream of the *gidA* translational start (Liberati *et al.*, 2006). Primers 5'-CATTACAGTTTACGAACCGAACAG-3' (forward) and 5'-GCAAGGCAAGGACAGCTGGTG-3' (reverse) amplified a fragment of appropriate size (661 base pairs in length; 179 bp of MAR2xT7 and 482 bp of *gidA*).

Construction of a* gidA *deletion mutant

The gene deletion strategy of Hoang *et al.* was used to construct a markeless *gidA* deletion mutant in *P. aeruginosa* strain PA14 (Schweizer & Hoang, 1995, Hoang *et al.*, 1998). Splicing by overlap extension (SOE)-PCR was used to construct a 981 bp in-frame deletion in *gidA*. The resulting protein contains the first 14 amino acids fused in-frame with the last 289 amino acids. The following primers were used (sequences in 5' to 3' direction): Primer 1, N_6<u>TCTAGA</u>CATGGGATGGATATCCACCTGG; primer 2, ACCGCCGATCACGATTACGTC; primer 3, GACGTAATCGTGATCGGCGGTGCGATCGAGTACGACTTCTTC; and primer 4, N_6<u>AAGCTT</u>CCATTTGATCAGCAGGGCCAAG. Primers 1 and 4 are flanking primers containing engineered *Xba*I and *Hin*dIII restriction sites, respectively (underlined), and primers 2 and 3 are partially overlapping, internal primers to create the in-frame deletion. The fusion product was digested with *Xba*I and *Hin*dIII and ligated to the equally digested allelic exchange plasmid pEX18Tc (Hoang *et al*., 1998). The resulting construct, pEX18Tc.Δ*gidA*, was sequenced prior to

transformation into *E. coli* SM10. Mating with *P. aeruginosa* PA14 and appropriate selection steps yielded a *gidA* deletion mutant. Proper construction was confirmed by PCR amplification of chromosomal DNA.

Growth curves

Growth analysis of each strain listed in Table S2.1 was conducted simultaneously in a 96-well format using a microplate reader (Infinite M200, Tecan). Strains were grown at 37°C with shaking in 200 µl of LB-MOPS medium and sealed with 50 µl of mineral oil. The OD_{600} was determined every 15 min for 8 h, and the doubling time in exponential phase for each strain was calculated with respective data. The presence of mineral oil did not restrict growth during logarithmic phase (data not shown). Growth of PA14 *gidA*::MAR2xT7 and the PA14 wild-type parent was also quantified in standard culture flasks. Twenty-five ml of LB-MOPS medium (in 250 ml flasks) were inoculated with mid-logarithmic phase cells to an OD_{600} of 0.02, and incubated at 37°C with shaking (250 rpm). The OD_{600} was measured every hour for 8 h.

Skim milk proteolysis, rhamnolipid production, and adenosine utilization

Skim milk proteolysis (Sandoz *et al.,* 2007), rhamnolipid production (Kohler *et al.*, 2001) and adenosine utilization (Heurlier *et al.*, 2005, Sandoz *et al.*, 2007) were determined through the use of agar plate assays. Isolated colonies, freshly grown on LB plates, were patched individually with a toothpick or replica-plated onto the

respective agar test plates. Rhamnolipid plates were incubated at 37°C for 24 h followed by 24 h at 4°C or until a blue halo appeared. Relative rhamnolipid production was determined by the size of the blue halo around each colony. Skim milk plates did not only contain 4% (w/v) skim milk but also ¼-strength LB. The addition of LB allowed QS-deficient mutants to grow as well as the wild-type parent.

Pyocyanin production and LasA protease activity

Pyocyanin production was measured in two different ways. For high throughput analysis, 200 µl of culture supernatant were transferred to a 96-well microtiter plate and the OD was determined at 310 nm with a microplate reader (Ohfuji *et al.*, 2004). For accurate quantitation of QS-dependent products from individual cultures grown in flasks, pyocyanin was extracted from liquid-culture supernatant and absorbance was read at 520 nm as described (Essar *et al.*, 1990). LasA protease activity was determined by measuring the rate of *Staphylococcus aureus* cell lysis by *P. aeruginosa* culture supernatant (decrease in OD_{600} per minute) (Diggle *et al*., 2002, Kessler *et al.*, 1993). Twenty microliters of culture supernatant were transferred to microtiter plates containing 180 µl of boiled *S. aureus* suspension (OD_{600} of 0.8) buffered with 10 mM K_2HPO_4 (pH 7.5). OD_{600} readings were taken in a microplate reader every 4 minutes for 1 h.

Assays for 3OC12-HSL, and C4-HSL

Two ml of ethyl acetate-extracted *P. aeruginosa* culture supernatant were used to measure C12-HSL and C4-HSL levels with *E. coli* bioassays as previously described (Passador *et al.*, 1996, Pearson *et al.*, 1995, Pearson *et al.*, 1997). Synthetic 3OC12-HSL and C4-HSL were used to generate standard curves.

Western blot analysis

Western blotting was performed as described previously (Schuster & Greenberg, 2007). Culture aliquots were harvested by centrifugation. Cell pellets were resuspended in LasR protein buffer and sonicated. The resulting lysates were centrifuged to remove insoluble material. Protein concentrations of the soluble fraction were determined by Bradford assay. Equal amounts of protein were separated by 12.5% polyacrylamide gel electrophoresis. The separated proteins were blotted onto a nylon membrane and probed with polyclonal anti-LasR and anti-RhlR rabbit antibodies as described (Schuster & Greenberg, 2007).

Complementation and suppression analysis

Complementation and suppression of the PA14 *gidA* transposon mutant was carried out with the low copy number plasmids pCF430G and pCF430R, which express *gidA* and *rhlR* from the arabinose-inducible *araBAD* promoter. L-arabinose was added to the cultures at a concentration of 50 mM. The *rhlR* allele was from *P. aeruginosa*

PAO1 rather than PA14. Both alleles only differ by a single silent mutation at position 717; however, the affected codon is not in a mixed codon family box and is therefore not recognized by a GidA-modified tRNA.

Real-time RT-PCR

Total RNA isolation and cDNA synthesis with semi-random primers was performed as described (Schuster *et al*., 2003). RNA quality was determined using an Agilent Bioanalyzer 2100. Real-time PCR was performed as described (Schuster & Greenberg, 2007) with a 7300 Real-Time PCR System (Applied Biosystems) using specific primers for *rhlA*, *rhlR*, 1 ng of purified cDNA as measured by Nanodrop (Thermo Scientific), and Power SYBR Green PCR Master Mix reagents (Applied Biosystems). The primers were designed using Primer Express Software (Applied Biosystems). Serially-diluted genomic DNA was used as the template to obtain a relative standard curve. Instead of normalizing transcript levels to those of a calibrator gene, as is commonly done in one-step RT-PCR reactions, we chose a two-step reaction and calibrated the amount of input cDNA prior to PCR (Sandoz *et al*., 2007). Thus, data are normalized to the total amount of cDNA.

Results

High-throughput screening for QS regulators identifies gidA

We used a *P. aeruginosa* PA14 non-redundant transposon insertion library (Liberati *et al*., 2006) to screen for mutants deficient in QS-dependent phenotypes. The library is a collection of 5,459 mutant strains where virtually every strain has a unique gene disabled by transposon insertion. The library allowed us to perform a comprehensive genome-wide search for genes that regulate QS in *P. aeruginosa*. To identify global regulators that affect multiple QS-regulated traits, we employed a two-step screening procedure. Screen 1 involved screening of all mutants for defects in skim milk proteolysis. Screen 2 involved screening proteolysis-deficient mutants identified in Screen 1 for additional QS-controlled phenotypes, namely staphylolytic (LasA) activity, rhamnolipid production, and pyocyanin production. This second, more focused screen allowed us to eliminate mutants from Screen 1 that are defective in processes other than QS, such as protease production or protease secretion. The different QS phenotypes assayed are dependent on the LasRI and/or RhlRI systems to varying degrees (see below).

Screen 1 identified 59 mutants with reduced skim-milk proteolysis (Table S2.1). This number excludes mutants with severely reduced growth on skim-milk plates, and mutants whose doubling time in liquid LB culture, assayed in microtiter-plate format, was >30% that of the PA14 wild-type. We re-discovered several genes known to control QS in *P. aeruginosa* PAO1. These were *vfr*, *gacA*, *rpoN*, *dksA*, and

of course the QS core genes *lasRI* and *rhlRI.* Vfr is a catabolite repressor homolog that directly induces *lasR* transcription (Albus *et al.,* 1997). GacA is part of a regulatory pathway that controls QS post-transcriptionally (Heurlier *et al.,* 2004, Kay *et al.,* 2006). RpoN is an alternative sigma factor that regulates *rhlI* expression (Thompson *et al.,* 2003), and DksA also controls expression of several QS-dependent phenotypes posttranscriptionally (Jude *et al.,* 2003). Several other known QS regulatory genes were not rediscovered (Table S2.1), but were nevertheless included in the second screen for comparison. Screen 2 revealed that many of the 59 mutants were also deficient in additional QS-controlled phenotypes (Table S2.1). However, only three mutants were at least partially deficient in all of the QS phenotypes tested (several others were defective in skim milk proteolysis, LasA activity, and pyocyanin production but did not grow on rhamnolipid minimal media, suggesting a QS-independent pleiotrophic defect such as amino acid auxotrophy). The three mutants harbor transposon insertions in *vfr*, and in two genes not previously known to be involved in QS regulation, *pcnB* and *gidA*. *pcnB* encodes poly(A) polymerase, which is involved in destabilization of mRNA in bacteria, a property that in some cases can directly regulate gene expression (Joanny *et al.,* 2007). In this study, we chose the *gidA* mutant for further characterization as it exhibited the most compelling QS phenotypes, being completely deficient in three of the four traits assayed. We confirmed the location of the transposon insertion in the *gidA* mutant by PCR as described in *Materials and Methods*.

Previous work indicated a role for *gidA* in virulence gene regulation in several bacterial pathogens (Cho & Caparon, 2008, Kinscherf & Willis, 2002, Sha *et al*., 2004, Durand *et al*., 2000). A recent mechanistic study demonstrated its involvement in tRNA modification in association with *mnmE* in *E. coli* (Bregeon *et al.*, 2001). To investigate the contribution of *mnmE* to the observed QS phenotypes in *P. aeruginosa*, we attempted to characterize a PA14 mutant with an insertion in a gene that is 67% identical to *E. coli mnmE*. However, this mutant exhibited severe growth defects in all of the standard media employed, thus not allowing us to investigate QS-specific deficiencies.

A gidA *mutant is defective primarily in* rhl*-dependent QS*

As mentioned above, high-throughput screening revealed that *gidA* causes defects in several QS-dependent phenotypes. To confirm and extend the results from high-throughput screening we repeated assays for the *gidA* mutant, and compared them to the wild-type, a *lasR* mutant and a *rhlR* mutant. We also included a new assay, growth on adenosine, to better determine the relative contribution of *las* and *rhl* QS systems in *gidA*-mediated regulation. Utilization of adenosine as the sole carbon-source requires expression of nucleoside hydrolase (Nuh), which is solely dependent on the *las* system (Heurlier *et al*., 2005, Sandoz *et al*., 2007). We found no adenosine utilization defect in the *gidA* mutant (Fig. 2.1A) indicating that *las* QS is not affected by *gidA*. Consistent with high-throughput data, the *gidA* mutant also only had a modest defect

in skim milk proteolysis (Fig. 2.1C). Skim-milk proteolysis is primarily *las*-dependent (involving *las*-specific alkaline protease and *las* and *rhl*-dependent LasB elastase), because a *rhlR* mutant only exhibited slight proteolytic deficiency. However, the *gidA* mutant was more strongly affected in two different *rhl*-specific QS phenotypes, rhamnolipid and pyocyanin production (Fig. 2.1B and 2.1D). LasA protease expression, which depends on both *las* and *rhl* systems for full activity, was also severely affected in the *gidA* mutant (Fig. 2.1E).

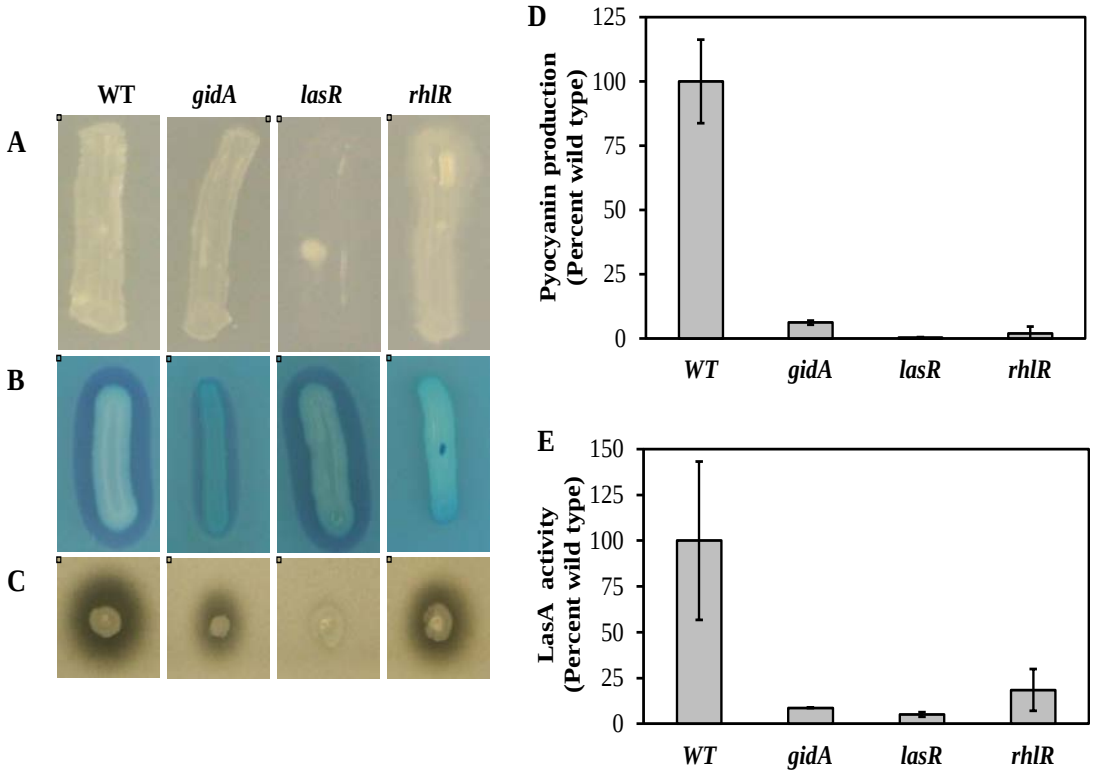

Fig. 2.1: QS-dependent phenotypes of a *P. aeruginosa* PA14 *gidA* mutant. Assays were performed with the wild-type (WT), *gidA*, *lasR*, and *rhlR* mutants. **A.** Growth on adenosine. **B.** Rhamnolipid production. The presence of a blue halo surrounding the bacterial streak indicates rhamnolipid production. **C.** Skim milk proteolysis. Zones of clearance surrounding the bacterial streak indicate proteolytic activity. **D.** Pyocyanin production, expressed as percent of the wild-type. **E.** LasA activity, expressed as percent of the wild-type. Each experiment was performed at least three times. Plate images depict representative experiments. Values shown in graphs are means of three independent biological experiments, normalized to culture density (OD_{600}). Error bars indicate ±1 standard deviation.

We define a phenotype or gene as *las*-specific if it is affected in a *lasR* (or *lasI*) mutant, but not in a *rhlR* (or *rhlI*) mutant. On the other hand, we define a phenotype or gene as *rhl*-specific if it is affected in a *rhlR* (or *rhlI*) mutant, and it is affected to the same degree in a *lasR* (or *lasI*) mutant. Under most growth conditions, a *rhl*-specific trait is equally affected in a *lasR* (or *lasI*) mutant because the *las* system controls expression of the *rhl* system. However, under some conditions, for example those employed for the rhamnolipid assay, the *rhl* system is independent of the *las* system, and a *lasR* (or *lasI*) mutant displays a wild-type phenotype.

Individual growth curves showed that the *gidA* mutant was attenuated in logarithmic growth compared with the wild-type (doubling times of 45 and 36 minutes, respectively), and that it entered stationary phase at a slightly lower density (Fig. 2.2).

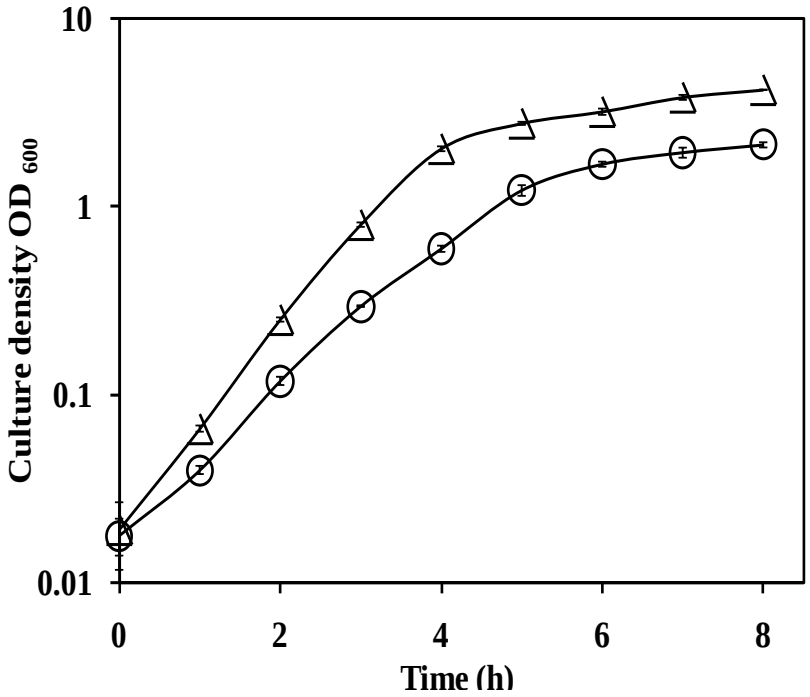

Fig. 2.2: Growth of the *P. aeruginosa* PA14 *gidA* mutant (circles) and the wild-type parent (triangles). Values are means of three biologically independent experiments. Error bars indicate ± 1 standard deviation.

While the *gidA* mutant grew more slowly, this relatively small difference alone is not sufficient to explain the dramatic QS phenotypes observed. In addition, these growth differences were taken into account for all assays performed in liquid culture. Taken together, our phenotypic data indicate that the *gidA* mutant is primarily deficient in *rhl*-dependent QS phenotypes.

Complementation fully restores the wild-type phenotype in a* gidA *mutant

We performed a complementation analysis to exclude the possibility of polar effects of the transposon insertion. This was important because *gidA* is the first gene in a four-gene operon, and because two genes in the operon, *soj* and *spo0J*, are involved in chromosome partitioning and cell division. Introduction of an intact copy of the *gidA* gene in the mutant restored QS-dependent phenotypes to wild-type levels (Fig. 2.3). This indicates that QS-deficient phenotypes conferred by the transposon insertion in the *gidA* gene are solely due to loss of function of *gidA* itself.

Because the transposon insertion was near the 3' end of the *gidA* gene, the resulting protein could have retained residual activity. To test whether this was in fact the case, we constructed a markerless in-frame *gidA* deletion mutant (see *Materials and Methods*). Its QS phenotypes and growth characteristics were indistinguishable from those of the transposon mutant (data not shown). This suggests that the transposon insertion completely inactivated GidA and further confirms that the insertion does not exert any polar effects on downstream genes. As the properties of the deletion and the transposon insertion mutants were identical, we performed further experiments with the transposon insertion mutant.

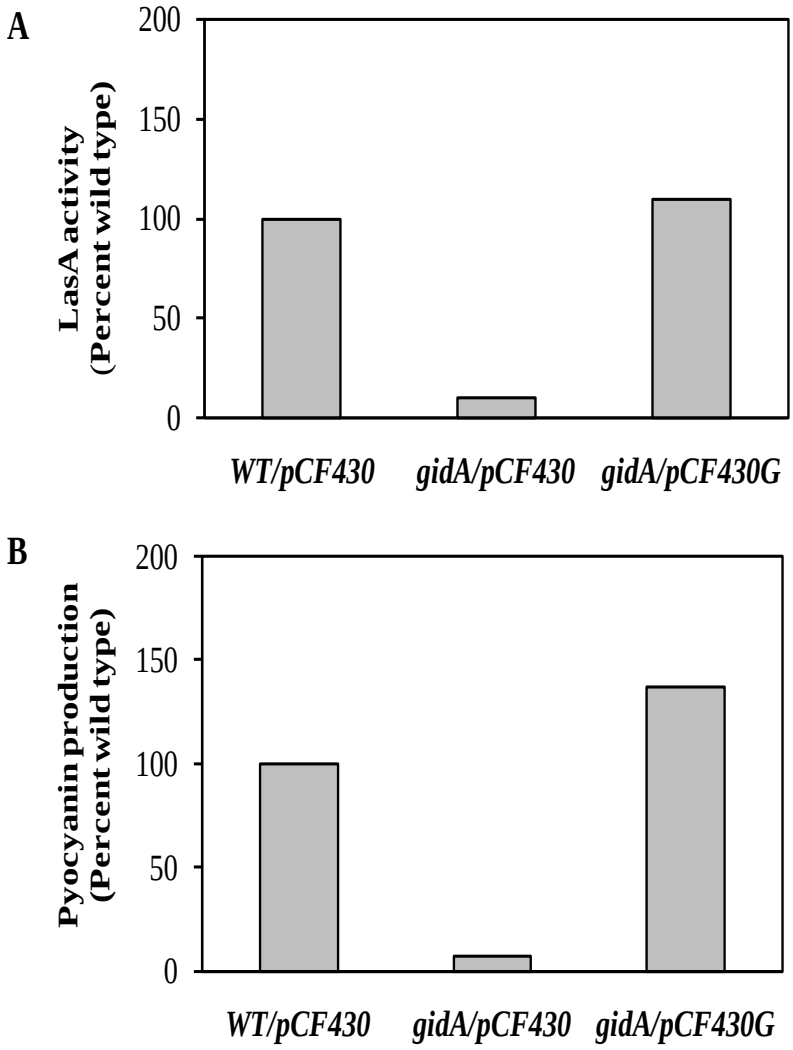

Fig. 2.3: Restoration of the wild-type phenotype in a *P. aeruginosa* PA14 *gidA* mutant. Wild-type (WT) and *gidA* mutant (*gidA*) strains were supplied with a plasmid expressing *gidA* from P_{BAD} (pCF430G), or with empty vector (pCF430). Cultures were grown in the presence of 50 mM arabinose to induce gene expression. **A.** LasA activity. **B.** Pyocyanin production. Values are means of three independent biological experiments, normalized to culture density (OD_{600}), and expressed as percent of the wild-type. Error bars indicate ±1 standard deviation.

GidA controls RhlR expression at the posttranscriptional level

Next, we sought to obtain further insights into how GidA controls QS. Based on the presumed function of GidA, we predicted that it might affect the expression of central QS regulatory proteins posttranscriptionally, in particular RhlI or RhlR. To investigate whether GidA affects acyl-HSL synthase levels, we measured 3OC12-HSL and C4-HSL levels in the wild-type and the *gidA* mutant. We found no differences in the levels of either acyl-HSL (Fig. 2.4A). Next, we evaluated LasR and RhlR protein levels by western blotting. Protein levels of RhlR were greatly reduced in a *gidA* mutant while LasR levels were comparable to the wild-type (Fig. 2.4B). RhlR protein levels were restored to wild-type levels in the *gidA* mutant when complemented with *gidA in trans* (data not shown). These results indicate that GidA influences the expression of RhlR, which is consistent with our phenotypic data.

To distinguish between transcriptional and posttranscriptional control, we measured transcript levels of *rhlR* by reverse transcription real-time PCR (Fig. 2.4C). We also measured transcript levels of the RhlR-dependent gene *rhlA*, which is involved in rhamnolipid biosynthesis (Ochsner *et al.*, 1994a, Ochsner *et al.*, 1994b). We expected down-regulation of the *rhlA* gene in a *gidA* mutant as it has low RhlR protein levels. In the *gidA* mutant, *rhlR* mRNA levels were near wild-type levels whereas *rhlA* mRNA levels, as expected, were significantly decreased, resulting in an elevated wild-type vs. *gidA* mutant transcript ratio (Fig. 2.4C). Taken together, these

results indicate that *gidA* modulates expression of RhlR posttranscriptionally, and that this in turn affects transcription of RhlR-dependent genes.

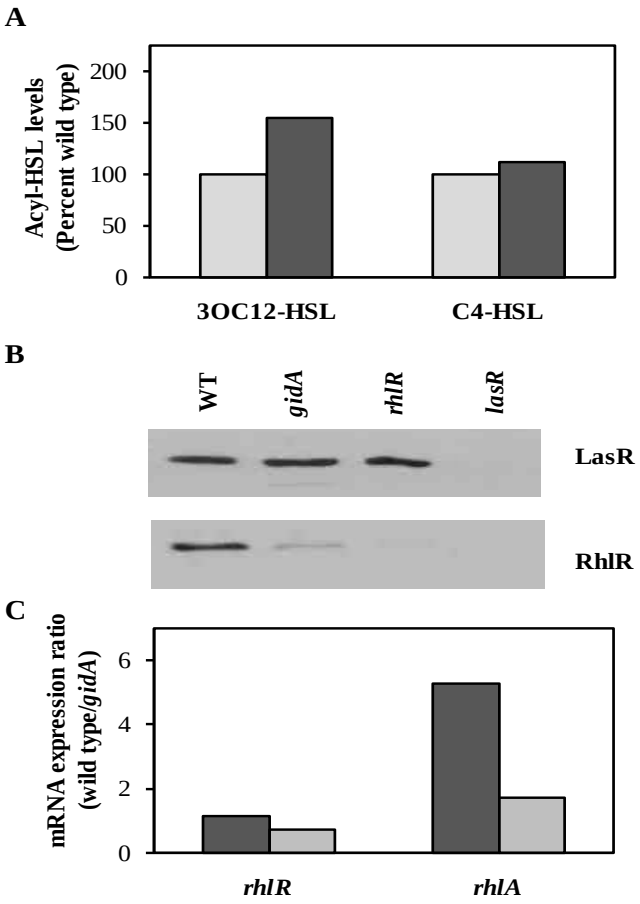

Fig. 2.4: Acyl-HSL levels and QS gene expression in the *P. aeruginosa* PA14 *gidA* mutant and the wild-type parent. A. 3OC12-HSL and C4 HSL levels, expressed as percent of the wild-type. Dark grey bars represent the *gidA* mutant and light grey bars represent the wild-type (WT) parent. **B.** Western blot analysis of LasR (top panel) and RhlR (bottom panel) protein. For comparison, protein levels in the *lasR* and *rhlR* mutants are shown. Equal amounts of clarified lysates were loaded for each strain. Smaller bands, indicative of RhlR degradation products, were not detected. **C.** RT-PCR. mRNA levels of *rhlA* and *rhlR* genes were measured in the *gidA* mutant and the wild-type parent at the transition from logarithmic to stationary phase (4-5 h, dark grey) and in stationary phase (7-8 h, light grey). Values represent transcript level ratios of the wild-type vs. the *gidA* mutant. Equal amounts of cDNA were used for each amplification reaction. Values are means of three independent biological experiments. Error bars indicate ±1 standard deviation.

Induction of **rhlR** *expression partially restores the wild-type phenotype*

Because GidA affected RhlR protein levels, we asked whether RhlR-dependent phenotypes could be rescued by increased expression of RhlR in a *gidA* mutant. We introduced a plasmid carrying *rhlR* under control of an arabinose-inducible promoter into a *gidA* mutant. When induced fully, this strain expressed RhlR protein at levels slightly higher than those of the wild-type, and showed partial restoration of RhlR-dependent phenotypes (Fig. 2.5). This partial suppression suggests that *gidA*, in addition to affecting RhlR expression, also regulates these phenotypes in a RhlR-independent fashion.

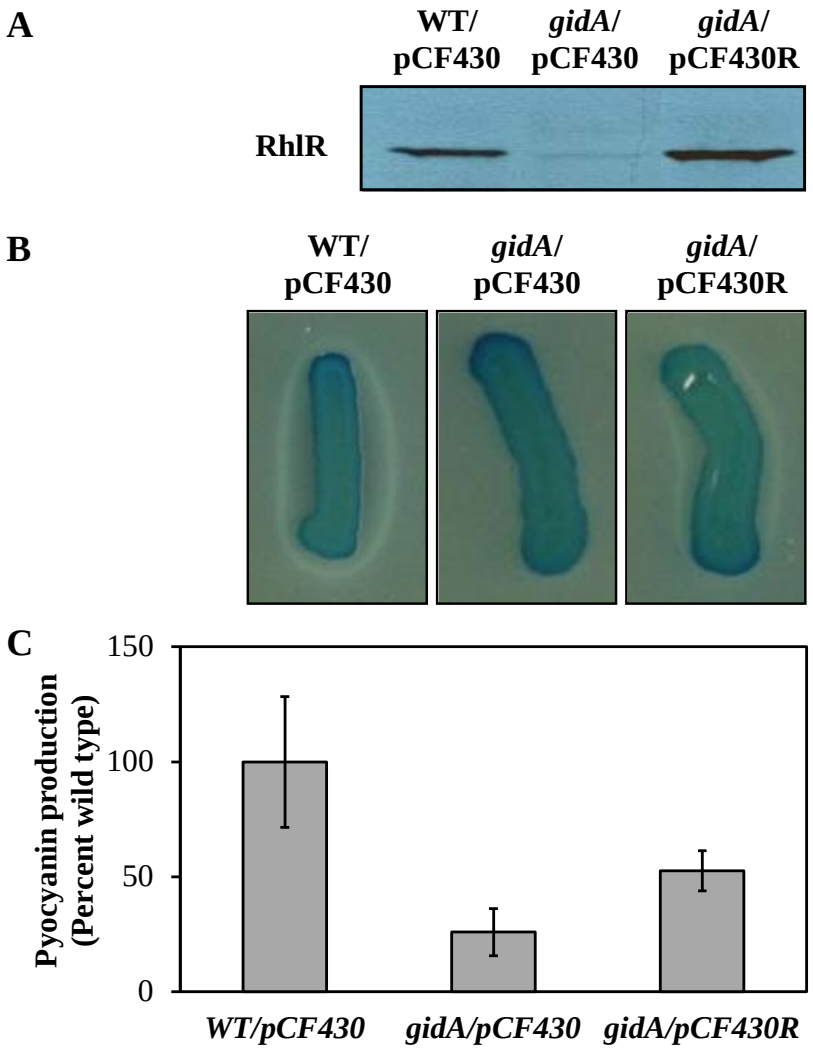

Fig. 2.5: Suppression of *gidA* mutant phenotypes by overexpression of *rhlR*. *P. aeruginosa* PA14 wild-type (WT) and *gidA* mutant (*gidA*) strains were supplied with a plasmid expressing *rhlR* from P_{BAD} (pCF430R), or with empty vector (pCF430). Cultures were grown in the presence of 50 mM arabinose to induce gene expression. **A.** Western blot analysis of RhlR protein. Equal amounts of clarified lysates were loaded for each strain. Smaller bands, indicative of RhlR degradation products, were not detected. **B.** Rhamnolipid production. **C.** Pyocyanin production, expressed as percent of wild-type. Values are means of three independent biological experiments, normalized to culture density (OD_{600}). Error bars indicate ±1 standard deviation.

Discussion

P. aeruginosa QS signaling is highly complex and involves a network of interconnected pathways (Juhas *et al*., 2005, Schuster & Greenberg, 2006, Venturi, 2006). Several factors that regulate QS have been identified, but a comprehensive screen, such as a saturation mutagenesis, has not been attempted. In this study, we utilized a non-redundant *P. aeruginosa* transposon library to identify global regulators of QS gene expression. We rediscovered several known regulators, but missed others (Table S2.1). The fact that *rsaL* was missed is not surprising as it is a repressor, not an activator, of QS (de Kievit *et al.*, 1999). However, it is not clear why other regulators that function as activators were not identified. One reason for this could be strain-specific regulatory differences. We used *P. aeruginosa* PA14 in our study while most of the QS regulators have been identified and characterized in strain PAO1. Overall, the fact that not many new regulatory genes conferring multiple QS deficiencies were discovered suggests that the capacity for global regulation of QS, at the level of LasRI and/or RhlRI, is limited.

One such gene, whose inactivation conferred substantial deficiencies in several QS phenotypes, was *gidA*. A *P. aeruginosa* PA14 *gidA* mutant was primarily deficient in *rhl*-dependent phenotypes (Fig. 2.1). GidA in *P. aeruginosa* is 630 amino acids in length and is 70% identical to the *E. coli* protein. Two forms of the protein exist, a long and a short form. Both forms contain an N-terminal FAD binding domain, but only the long form contains a C-terminal tRNA binding domain required for tRNA

modification (Meyer *et al*., 2008). *P. aeruginosa gidA* is the long form. GidA requires another protein, MnmE, for tRNA modification (Bregeon *et al*., 2001). Unfortunately, we were unable to investigate the effect of a *mnmE* mutation on QS gene expression, thus precluding further evidence for a synergistic role of the two proteins in *P. aeruginosa*. In *E. coli*, both proteins are required for the addition of carboxymethylaminomethyl groups to the C5 carbon of uridine at position 34 of tRNAs that read codons ending with A or G in mixed codon family boxes, including Glu, Gln, Lys, Leu, and Arg codons (Bregeon *et al*., 2001, Yim *et al*., 2006). This modification enhances base-pairing specificity at the wobble position, allowing pairing with G and A, while restricting pairing with C and U. This prevents both misincorporation and +2 frameshifting (Bjork & Hagervall, 2005, Bregeon *et al*., 2001). It has been suggested that tRNA modification could function as a regulatory mechanism to adjust gene expression in response to nutrient deprivation through alteration of pools of cofactors required for the tRNA modification reactions (Persson, 1993).

In principle, GidA-mediated tRNA modification could affect the expression of all proteins in the cell. However, certain proteins will be more susceptible than others, if the respective genes contain a high proportion of codons that require decoding by tRNAs with U34 modifications. This includes many transcriptional regulators that contain clusters of positively charged Lys and Arg residues in their DNA-binding domains. Global, yet specific, regulation is consistent with previous studies. GidA

controls virulence gene expression *A. hydrophila*, *P. syringae, S. flexneri,* and *S. pyogenes* (Cho & Caparon, 2008, Kinscherf & Willis, 2002, Sha *et al*., 2004, Durand *et al*., 2000), and fruiting body development in *M. xanthus* (White *et al*., 2001), with little to no effect on growth. Post-transcriptional regulation has been shown in *A. hydrophila, S. flexneri* and *S. pyogenes*, and involvement of a transcriptional regulator has been demonstrated in *S. flexneri* and *S. pyogenes*.

In this study, we have shown that GidA rather specifically controls RhlR expression in *P. aeruginosa*, also at the posttranscriptional level (see model in Fig. 2.6).

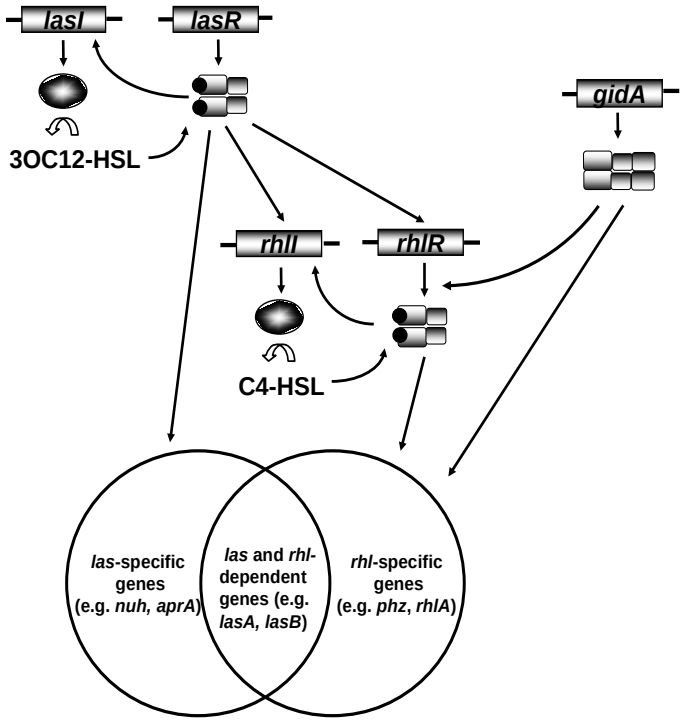

Fig. 2.6: Model depicting the position of GidA in the QS circuitry. See the text for details.

GidA affected RhlR protein but not transcript levels (Fig. 2.4), and introduction of *rhlR* into a *gidA* mutant partially restored *rhl*-dependent phenotypes (Fig. 2.5). Such phenotypic suppression is plausible, because overexpression of *rhlR* mRNA in a *gidA* mutant would result in a higher fraction of correctly translated RhlR, thereby compensating for the decreased level of functional protein translated from native mRNA. In *S. flexneri* and *S. pyogenes*, overexpression of the transcriptional regulators VirF and RopB, respectively, results in full suppression of *gidA* mutant phenotypes (Cho & Caparon, 2008, Durand *et al*., 2000). The fact that there was no full restoration to wild-type levels in *P. aeruginosa* suggests that GidA also affects *rhl*-dependent phenotypes independent of RhlR. It cannot be excluded, however, that a significant portion of the overexpressed RhlR, although soluble, is non-functional due to translation errors in the absence of GidA, resulting in only partial, rather than full, suppression. Despite the modest growth defect (Fig. 2.2), our results argue against a general gene expression defect in the *gidA* mutant: Compared with the wild-type, the *gidA* mutant did not show any deficiency in a *las*-specific phenotype (growth on adenosine as the sole carbon source), in LasR protein levels, and in acyl-HSL production. The observation that C4-HSL levels were not significantly altered in a *gidA* mutant is consistent with *rhlI* expression being primarily *las*-dependent (de Kievit *et al.*, 2002). While RhlR activates *rhlI* expression in the heterologous host *E. coli* (Latifi *et al*., 1996), LasR rather than RhlR is the dominant regulator in *P. aeruginosa* (de Kievit *et al*., 2002).

Interestingly, the coding regions of LasR and RhlR both contain roughly equal numbers of codons (17 and 18, respectively) that require decoding by GidA/MnmE-modified tRNAs, which appears inconsistent with the observation that GidA affected RhlR but not LasR protein levels. However, the frequency of two-family box triplet codons alone does not determine the impact on protein function. Although none of the affected amino acid residues are highly conserved, the contribution of individual, less conserved residues on protein stability and activity may well be different in LasR and RhlR. Both proteins are only 33% identical and function quite differently. While LasR requires its acyl-HSL ligand for proper folding (Schuster *et al.*, 2004), RhlR does not (Medina *et al.*, 2003). In addition, frameshifts, which tend to be much more deleterious than misincorporations, are also strongly dependent on the base composition surrounding the respective codons, although the precise mechanism is not clear (Bregeon *et al*., 2001). It is therefore plausible that GidA-mediated tRNA modification could have a selective effect on RhlR but not LasR protein levels and consequently, impact *rhl*-dependent but not *las*-dependent phenotypes.

Further experiments will be needed to elucidate the precise molecular mechanism of GidA function in *P. aeruginosa*. Taken together, we have identified an additional regulatory component of the QS network in *P. aeruginosa* that affects *rhl*-dependent gene expression through posttranscriptional control of RhlR.

Acknowledgements

We thank Nicole Liberati and Frederick Ausubel, Department of Molecular Biology, Massachusetts General Hospital, for the non-redundant set of *P. aeruginosa* PA14 transposon insertion mutants. This work was supported by start-up funds from Oregon State University, and by NIH grant 1R21AI079454-01 (to MS).

Supplementary Table

Table S2.1: Phenotypic data for *P. aeruginosa* PA14 mutants

Table S2.1: Phenotypic data for *P. aeruginosa* PA14 mutants

Locus[a] (Mutated gene)	Mutant ID	Description	QS-dependent phenotypes[b]			
			Skim-milk proteolysis	Staphylolytic activity	Rhamnolipid production	Pyocyanin production
PA14 wild type			+++	+++	+++	+++
QS core genes						
PA14_19120 *(rhlR)*	37943	Transcriptional regulator	+++,++,++[d]	+	-	-
PA14_19130 *(rhlI)*	33961	Autoinducer synthesis protein RhlI	+++, ++, ++[d]	+	++	-
PA14_39980 *(qscR)*	42798	Transcriptional regulator	+++	+++	+++	++
PA14_45940 *(lasI)*	39292	Autoinducer synthesis protein LasI	+	-	++	-
PA14_45960 *(lasR)*[c]	N/A	Transcriptional regulator	-	-	++	-
Known QS regulatory genes not rediscovered						
PA14_17480 *(rpoS)*	32095	Sigma factor RpoS	+++	++	+++	++
PA14_45950 *(rsaL)*	46524	Regulatory protein RsaL	+++	+++	++	+++
PA14_51340 *(mvfR)*	47015	Transcriptional regulator MvfR	+++	+++	++	+++
PA14_52180 *(relA)*	55087	GTP pyrophosphokinase	+++	+++	++	+++
PA14_52570 *(rsmA)*	44507	RsmA, regulator of secondary metabolites	+++	+++	+++	+++
PA14_56070 *(mvaT)*	34492	Transcriptional regulator MvaT	+++	+++	++	+++
PA14_62530 *(cbrA)*	33836	Two component sensor CbrA	+++	+++	++	+++
Known QS regulatory genes rediscovered						
PA14_08370 *(vfr)*	52692	Cyclic AMP receptor-like protein	++	-, ++, -[d]	++	+
PA14_30650 *(gacA)*	54630	Response regulator GacA	++	-, +++, -[d]	+++	-
PA14_57940 *(rpoN)*	44482	RNA polymerase sigma-54 factor	++	+++	NG	+++

Continued on next page

Table S2.1: Phenotypic data for *P. aeruginosa* PA14 mutants

Locus[a] (Mutated gene)	Mutant ID	Description	QS-dependent phenotypes[b]			
			Skim-milk proteolysis	Staphylolytic activity	Rhamnolipid production	Pyocyanin production
PA14_62490 *(dksA)*	41617	Suppressor protein DksA	++	+++	-	+++
Novel genes						
PA14_05260 *(pyrB)*	52690	Aspartate carbamoyltransferase	++	+++	NG	-
PA14_05380 *(pilK)*	52952	Methyltransferase PilK	++	+++	++	++
PA14_05620 *(sahH)*	56708	S-adenosyl-L-homocysteine hydrolase	+	++	NG	+
PA14_07700 *(apaH)*	36226	Bis(5'-nucleosyl)-tetraphosphatase	-	++	++	+++
PA14_09520 *(mexI)*	43615	Probable RND efflux transporter	++	++	+++	++
PA14_12080 *(sltB1)*	27636	Soluble lytic transglycosylase B	++	+++	++	++
PA14_12490	31830	AMP nucleosidase	+, ++, +++[d]	+++	+++	+++, ++, +[d]
PA14_13220	54206	Possible protein-tyrosine-phosphatase	+++	+++	+++	+++
PA14_14690	34797	Putative rRNA methylase	-	++	NG	+
PA14_14700 *(cysE)*	44806	Serine O-acetyltransferase	-	+++	NG	++
PA14_15600	29758	Conserved hypothetical protein	++	+++	+++	++
PA14_15740 *(purL)*	29716	Phosphoribosylformylglycinamidine synthase	++	+++	NG	+, +++, ++[d]
PA14_16930	25721	Putative cysteine sulfinate desulfinase	++	+++	+++	++
PA14_20010 *(hasR)*	29291	Haem uptake outer membrane receptor HasR precursor	++	++	+++	+++, +++, +[d]
PA14_22020 *(minD)*	52889	Cell division inhibitor MinD	++	+++	++	+++
PA14_22620 *(cyaB)*	52860	Adenylate cyclase CyaB	++	+++, +++, -[d]	++	+++, ++, -[d]

Continued on next page

Table S2.1: Phenotypic data for *P. aeruginosa* PA14 mutants

Locus[a] (Mutated gene)	Mutant ID	Description	QS-dependent phenotypes[b]			
			Skim-milk proteolysis	Staphylolytic activity	Rhamnolipid production	Pyocyanin production
PA14_23920 *(purF)*	23709	Amidophosphoribosyltransferase	++	+++	+	+++
PA14_23970 *(xcpQ)*	43537	General secretion pathway protein D	-	-	+++	++
PA14_23990 *(xcpR)*	48371	General secretion pathway protein E	-	-	+++	+++, +++, +[d]
PA14_24020 *(xcpT)*	34060	Phosphoribosylaminoimidazole-succinocarboxamide synthase	-	-	+++	++
PA14_24060 *(xcpW)*	39963	General secretion pathway protein J	-	-	+++	+++, ++, -[d]
PA14_24070 *(xcpX)*	38700	General secretion pathway protein K	-	-	+++	++
PA14_24100 *(xcpZ)*	41602	General secretion pathway protein M	-	-	+++	++
PA14_25110 *(topA)*	36281	DNA topoisomerase I	-	+++	-	+++
PA14_26890 *(pyrF)*	35555	Amidophosphoribosyltransferase	++	+++	NG	++
PA14_27950	44818	Putative anti-anti-sigma factor	++	+++	++	+++
PA14_41240 *(clpP)*	52957	ATP-dependent Clp protease proteolytic subunit	++	+++	+	+++
PA14_41570 *(oprF)*	23102	Major porin and structural outer membrane porin OprF precursor	++	+++	++	++
PA14_44070 *(gltA)*	34537	Citrate synthase	++	+++	++	+++
PA14_45710	37135	Conserved hypothetical protein	-	+	NG	-
PA14_49010	43220	Hypothetical protein	++	+++	+++	+++
PA14_51240 *(purC)*	34993	Phosphoribosylaminoimidazole-succinocarboxamide synthase	++	+++	NG	+++
PA14_51690	27533	Conserved hypothetical protein	++	+++	++	+++

Continued on next page

Table S2.1: Phenotypic data for *P. aeruginosa* PA14 mutants

Locus[a] (Mutated gene)	Mutant ID	Description	QS-dependent phenotypes[b]			
			Skim-milk proteolysis	Staphylolytic activity	Rhamnolipid production	Pyocyanin production
PA14_51790 *(ruvA)*	30714	Holliday junction DNA helicase RuvA	++	+++	++	++
PA14_52040 *(purM)*	47921	Phosphoribosylaminoimidazole synthetase	++	+++	NG	++
PA14_54370 *(lepA)*	38761	GTP-binding protein LepA	-	-	+++	++
PA14_56300	35583	Putative fumarase	++	+++	++	+++
PA14_58600	27307	Conserved hypothetical protein	++	+++	+++	+++
PA14_60280 *(fimU)*	44085	Type 4 fimbrial biogenesis protein FimU	++	+++, ++, -[d]	++	+++, ++, -[d]
PA14_62560 *(pcnB)*	41679	Poly(A) polymerase	++	++	++	++
PA14_62930 *(carA)*	39940	Carbamoyl-phosphate synthase small chain	-	+++	NG	+
PA14_64220 *(purD)*	29794	Phosphoribosylamine-glycine ligase	++	+++	NG	++
PA14_66110	56681	Putative glycosyl transferase	++	+++	++	+++
PA14_66600 *(aroB)*	38358	3-dehydroquinate synthase	++	++	NG	-
PA14_66940 *(hisI)*	40903	Phosphoribosyl-AMP cyclohydrolase	++	+++	++	++
PA14_67530	42570	Noncatalytic dihydroorotase-like protein	++	+++	++	++
PA14_67560 *(typA)*	37710	GTP-binding protein TypA/BipA	++	+++	+++	++, +++, +[d]
PA14_68370 *(cysQ)*	40356	3'(2'),5'-bisphosphate nucleotidase	++	+++	++	++
PA14_69670 *(lysA)*	27796	Diaminopimelate decarboxylase	++	+++	NG	++
PA14_70370 *(pyrE)*	46326	Orotate phosphoribosyltransferase	-	+++	NG	++

Continued on next page

Table S2.1: Phenotypic data for *P. aeruginosa* PA14 mutants

Locus[a] (Mutated gene)	Mutant ID	Description	QS-dependent phenotypes[b]			
			Skim-milk proteolysis	Staphylolytic activity	Rhamnolipid production	Pyocyanin production
PA14_70370 *(pyrE)*	46326	Orotate phosphoribosyltransferase	-	+++	NG	+
PA14_72490 *(polA)*	31829	DNA polymerase I	+, ++, +++[d]	++	+++	++
PA14_73370 *(gidA)*	44643	Glucose-inhibited division protein A	++	-	-	-

[a] Unless labeled otherwise, all mutants have a MAR2xT7 transposon insertion in the respective ORF.

[b] +++, ++, +, -, and NG indicate a phenotype resembling the wild-type, slightly attenuated, substantially attenuated, absent, and no growth, respectively.

[c] The *lasR* mutant contains a Tn*phoA* insertion in codon 154. The PA14 library does not include a *lasR* mutant.

[d] Due to inconsistent results, phenotypes from each replication are given.

Chapter 3

Quorum sensing modulates colony morphology through alkyl quinolones in *Pseudomonas aeruginosa*

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Abstract

Acyl-homoserine lactone (acyl-HSL) and alkyl quinolone (AQ) based quorum-sensing (QS) systems are important for *Pseudomonas aeruginosa* virulence and biofilm formation. The effect of QS on biofilm formation is influenced by various genetic and environmental factors. Here, we used a colony biofilm assay to study the effect of the central acyl-HSL QS regulator, LasR, on biofilm formation and structure in the representative clinical *P. aeruginosa* isolate ZK2870. A *lasR* mutant exhibited wrinkled colony morphology at 37°C in contrast to the smooth colony morphology of the wild-type. Mutational analysis indicated that wrinkling of the *lasR* mutant is dependent on *pel*, encoding a biofilm matrix exopolysaccharide. Suppressor mutagenesis and complementation analysis implicated the AQ signaling pathway as the link between *las* QS and colony morphology. In this pathway, genes *pqsA-D* are involved in the synthesis of 4-hydroxyalkyl quinolines ("Series A congeners"), which are converted to 3,4-dihydroxyalkyl quinolines ("Series B congeners", including the well-characterized *Pseudomonas* Quinolone Signal, PQS) by the product of the LasR-dependent *pqsH* gene. Measurement of AQ in the wild-type, the *lasR pqsA*::Tn suppressor mutant as well as the defined *lasR*, *pqsH*, and *lasR pqsH* mutants showed a correlation between 4-hydroxyalkyl quinoline levels and the degree of colony wrinkling. Most importantly, the *lasR pqsH* double mutant displayed wrinkly morphology without producing any 3,4-dihydroxyalkyl quinolines. Constitutive expression of *pqsA-D* genes in a *lasR pqsR*::Tn mutant showed that colony wrinkling

does not require the AQ receptor, PqsR. Taken together, these results indicate that the *las* QS system represses Pel and modulates colony morphology through a 4-hydroxyalkyl quinoline in a PqsR-independent manner, ascribing a novel function to an AQ other than PQS in *P. aeruginosa*.

Introduction

Pseudomonas aeruginosa is an important opportunistic human pathogen. It is known for its ability to inhabit diverse habitats ranging from soil to immunocompromised individuals (Kerr & Snelling, 2009). In these environments, it can adopt either a planktonic or a surface-associated biofilm lifestyle. Biofilms, structured surface-associated microbial communities, are of considerable interest as they constitute an important survival strategy in infections (Fux *et al.*, 2005). *P. aeruginosa* forms different types of biofilms depending on the environment. In static liquid culture it forms pellicles at the air-liquid interface, under flow it can form solid surface-associated (SSA) biofilms and on solid agar medium it forms colonies (Branda *et al.*, 2005). Colonial growth is an easy and commonly used assay to study development of multicellular structures like biofilms (Shapiro, 1984, Hickman *et al.*, 2005, Sakuragi & Kolter, 2007).

Biofilms are encased in a matrix composed of exopolysaccharide (EPS), but also extracellular DNA (eDNA), proteins, RNA and ions (Karatan & Watnick, 2009). There are two main EPS in non-mucoid *P. aeruginosa*, Pel (encoded by *pelA-G*) and

Psl (encoded by *pslA-O*) (Fig. 3.1) (Jackson *et al.*, 2004, Matsukawa & Greenberg, 2004, Friedman & Kolter, 2004a). Pel is glucose rich whereas Psl is galactose and mannose rich (Friedman & Kolter, 2004b, Friedman & Kolter, 2004a, Ma *et al.*, 2007). *P. aeruginosa* strain PA14 only contains *pel* while strains PAO1 and ZK2870 contain both *pel* and *psl* (Friedman & Kolter, 2004b, Friedman & Kolter, 2004a). All of these strains are clinical isolates that differ in their aggregative behavior. While strains PA14 and PAO1 are the most commonly used laboratory strains, strain ZK2870 with its autoaggregative phenotype is believed to be the most representative among clinical strains (Friedman & Kolter, 2004b).

Quorum sensing (QS) is a cell density-dependent mechanism of bacterial communication that coordinates other group behaviors. *P. aeruginosa* has two complete acyl-homoserine lactone (acyl-HSL)-based QS systems, *las* and *rhl* (Schuster & Greenberg, 2006, Juhas *et al.*, 2005). They consist of the transcriptional regulators LasR and RhlR and the signal synthases, LasI and RhlI, respectively. LasI and RhlI catalyze the synthesis of N-3-oxododecanoyl-homoserine lactone (3OC12-HSL) and N-butryl-homoserine lactone (C4-HSL), which bind and activate their cognate transcriptional regulator LasR and RhlR, respectively. Both systems are arranged in a hierarchical manner with the *las* system controlling the *rhl* system (Latifi *et al.*, 1996, Pesci *et al.*, 1997). A third QS system in *P. aeruginosa*, *pqs*, is based on alkyl quinolones (AQ) (Diggle *et al.*, 2006a, Heeb *et al.*, 2011). This system connects both the *las* and *rhl* QS systems. It includes the transcriptional regulator PqsR (MvfR),

which positively regulates the expression of the *pqsA-E* operon. PqsA-D enzymes are involved in the synthesis of 4-hydroxyalkyl quinolines (named Series A congeners by Deziel *et al*.) (Deziel *et al.*, 2004). This class of compounds is converted to 3, 4 dihydroxyquinolines (Series B congeners) by a monoxygenase encoded by the *pqsH* gene (Deziel *et al*., 2004). The most prominent Series A congeners are 4-hydroxy-2-heptyl quinoline (HHQ) and 4-hydroxy-2-nonyl quinoline (HNQ), and the most prominent Series B congener is 3,4-dihydroxy-2-heptyl quinoline (PQS), due to their established roles as cell-cell signaling molecules. HHQ/HNQ and PQS bind PqsR with low and high affinity, respectively, and are capable of activating the protein (Xiao *et al.*, 2006b, Wade *et al.*, 2005, Fletcher *et al.*, 2007). LasR positively regulates AQ production by upregulating *pqsR* (Wade *et al*., 2005) and *pqsH* (Gallagher *et al.*, 2002, Deziel *et al*., 2004) transcription, although under certain culture conditions, AQ can also be produced in the absence of a functional *las* system (Diggle *et al.*, 2003). The *rhl* system, in turn, represses *pqsR* and *pqsA-E* expression (McGrath *et al.*, 2004, Xiao *et al*., 2006a, Wade *et al*., 2005). The AQ biosynthetic enzymes enable *P. aeruginosa* to produce more than 50 distinct AQ molecules (Deziel *et al*., 2004, Lepine *et al.*, 2007). Together, the three QS systems, *las*, *rhl*, and *pqs*, regulate >5% of the *P. aeruginosa* genome (Hentzer *et al.*, 2003, Deziel *et al.*, 2005, Schuster *et al.*, 2003, Wagner *et al.*, 2003).

Several studies have investigated the contribution of each QS system to biofilm formation. A functional *las* system is required for formation of highly structured SSA

biofilm communities in *P. aeruginosa* PAO1 (Davies *et al.*, 1998). The *las* system influences biofilm matrix formation and activation of *pel* EPS (Sakuragi & Kolter, 2007). In another study, the *las* system was shown to indirectly inhibit *pel* expression through weak activation of the tyrosine phosphatase TpbA (Ueda & Wood, 2009). The *rhl* QS system contributes to maintenance of biofilm architecture through production of rhamnolipid surfactants (Davey *et al.*, 2003). The *pqs* system in turn is implicated in autolysis (D'Argenio *et al.*, 2002) and maintaining biofilm integrity as a consequence of eDNA release (Allesen-Holm *et al.*, 2006). In addition, the contribution of QS to biofilm formation is modulated by environmental factors such as nutritional cues (Shrout *et al.*, 2006). Taken together, the role of QS in biofilm formation is multifactorial.

Our recent work suggested yet another connection between QS and EPS production. We showed by chromatin immunoprecipitation-microarray analysis (CHIP-chip) and electrophoretic mobility shift assay that LasR binds to the putative promoter region of the Psl EPS operon (Gilbert *et al.*, 2009) (Appendix B) (Fig. 3.1). This finding led us to investigate in more detail how *lasR* mutation affects EPS production and colony biofilm formation. A *lasR* mutant of *P. aeruginosa* strain ZK2870 exhibited a pronounced wrinkled colony morphology at 37°C suggesting a possible link between *las* QS and *psl* expression.

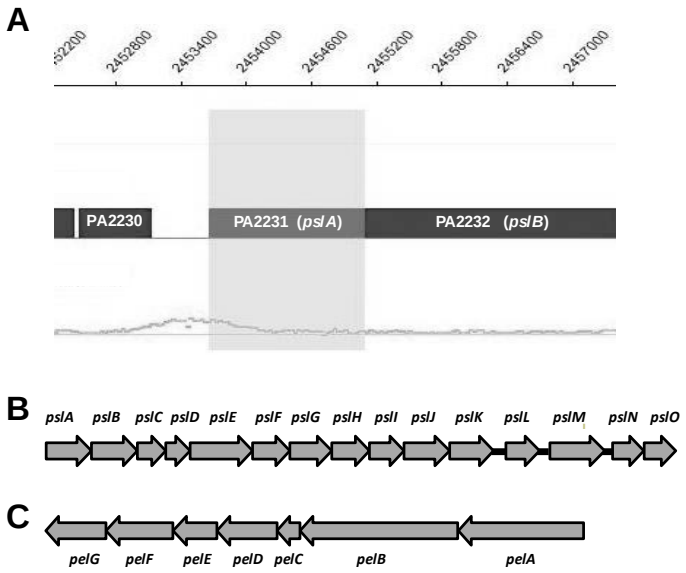

Fig. 3.1: Putative link between LasR and Psl control in *P. aeruginosa* PAO1. A. CHIP-chip analysis performed with LasR-specific antibodies (Gilbert *et al*., 2009). The signal peak near the bottom left corner of the panel indicates enrichment of *psl* promoter fragments and the vertical light grey bar represents the *pslA* gene (PA2231). The data were visualized using SignalMap (Nimblegen). **B.** *psl* EPS locus. **C.** *pel* EPS locus.

However, we found that the wrinkled phenotype is *pel* rather than *psl*-dependent. Subsequent suppressor mutagenesis in the *lasR* mutant background implicated the involvement of the *pqs* pathway. Phenotypic analysis and quantitation of AQ levels by thin-layer chromatography (TLC) of several QS mutants revealed that a Series A congener, likely other than HHQ or HNQ modulates the structural organization of a colony. This study broadens the functional significance of AQ production by *P. aeruginosa*.

Materials and Methods

Bacterial strains and growth conditions

Strains and plasmids are listed in Table 3.1. We used three strains of *P. aeruginosa* used in this study, namely the widely used clinical isolates PAO1 and PA14, and the more recent clinical isolate ZK2870 (herein abbreviated as ZK) (Friedman & Kolter, 2004b). Bacterial cultures were grown at 22ºC and 37ºC as specified. Lennox broth (LB) (Gilbert *et al*., 2009) or tryptone broth were used for routine culturing. Tryptic soy broth (TSB) was used for flow-cell biofilm assays. Where appropriate, antibiotics were added to the growth media as follows: tetracycline and gentamicin,100 µg/ml for *P. aeruginosa* and 10 µg/ml for *Escherichia coli*, carbenicillin 200 µg/ml for *P. aeruginosa*, ampicillin, 100 µg/ml for *E. coli.*

Table 3.1: Strains and plasmids

Strain or plasmid	Relevant property	Reference
Strains		
P. aeruginosa		
PA14	Wild-type	(Rahme *et al.*, 1995)
PAO1	Wild-type	(Holloway *et al.*, 1979)
ZK2870	Wild-type	(Friedman & Kolter, 2004b)
PAO1 *lasR*	Markerless *lasR* mutant derived from PAO1	(Wilder *et al.*, 2011)
PA14 *lasR*	Tn*phoA lasR* mutant derived from PA14	(Liberati *et al.*, 2006)
ZK *lasR*	Markerless in-frame *lasR* deletion in ZK2870	This study
ZK *pelA*	Markerless *pelA* deletion in ZK2870	(Friedman & Kolter, 2004a)
ZK *pslD*	Markerless *pslD* deletion in ZK2870	(Friedman & Kolter, 2004a)
ZK *lasI*	Markerless *lasI* deletion in ZK2870	This study
ZK *pelA lasR*	Markerless *lasR* deletion in a *pelA* mutant of ZK2870	This study
ZK *pslD lasR*	Markerless *lasR* deletion in a *pslD* mutant of ZK2870	This study
ZK *pqsH*	Markerless *pqsH* deletion in ZK2870	This study
ZK *tpbA*	Markerless *pqsH* deletion in ZK2870	This study
ZK *lasR* *pqsA*::Tn	*pqsA* suppressor mutation in a *lasR* mutant of ZK2870	This study
ZK *lasR* *pqsR*::Tn	*pqsR* suppressor mutation in a *lasR* mutant of ZK2870	This study
E. coli		
DH5α	F^- Φ80d*lacZ*ΔM15 Δ (*lacZYA-argF*) U169 *deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Invitrogen
SM10	Mobilizing strain, RP4 *tra* genes integrated in the chromosome, *thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^r	(R. Simon, 1983)
S17-1/λpir	Tp^R Sm^R *recA, thi, pro, hsdR*-M^+RP4: 2-Tc:Mu: Km Tn7 λpir	(R. Simon, 1983)
Plasmids		
mini-CTX-*lacZ*	Chromosomal integration vector, Tet^R	(Becher & Schweizer, 2000)
pEX18 Tc	Allelic exchange suicide vector, Tc^R	(Hoang *et al.*, 1998)
pEX18.Δ*lasR*	Suicide vector with *lasR* in-frame deletion	(Wilder *et al*., 2011)
pEX18.Δ*lasI*	Suicide vector with *lasI* in-frame deletion	(Wilder *et al*., 2011)
pEX18.Δ*tpbA*	Suicide vector containing *tpbA* in-frame deletion	This study
pLM1	Tn*5* delivery vector, Gm^R	(Heeb *et al.*, 2002)
pLG10	*pqsA-E* operon cloned in pUCP18, Ap^R	(Gallagher *et al*., 2002)
pRG10	*pqsA-D* operon cloned under control of P_{lac} of pUCP18, Ap^R	This study
pRG11	Promoter region of *pel* cloned in mini-CTX-*lacZ* vector	This study
pUCP18	Parent vector of pLG10, Ap^R	(Schweizer, 1991)

Strain and plasmid constructions

Deletion mutants were constructed using the strategy of Hoang *et al*. (Hoang *et al*., 1998). ZK *lasR* and *lasI* mutants were generated by introducing the previously constructed allelic exchange plasmids pEX18.Δ*lasR* and pEX18.Δ*lasI,* respectively (Wilder *et al*., 2011), into the parent strain and selecting on LB agar containing nalidixic acid (20 µg/ml) and tetracycline. Double cross-over recombinants were further selected on LB plates supplemented with 5% sucrose (Hoang *et al*., 1998). The *pqsH* and *tbpA* in-frame deletions were constructed using SOE-PCR (Horton *et al.*, 1990). The respective primers are listed in Supplementary Table S3.1. The deletion constructs obtained from SOE-PCR were digested with the appropriate restriction enzymes (see Table S3.1) and ligated into equally digested pEX18 (Hoang *et al*., 1998). The resulting constructs pEX18.Δ*pqsH* and pEX18.Δ*tpbA* were transformed into *E. coli* SM10. Mating with *P. aeruginosa* ZK and appropriate selection as discussed above yielded *pqsH* and *tpbA* deletion mutants. The *pelA lasR* and *pslD lasR* double mutants were constructed by generating an in-frame *lasR* deletion (as described above) in *pelA* and *pslD* mutant backgrounds, respectively. A *lasR pqsH* double mutant was constructed by *pqsH* deletion in a *lasR* mutant background. Proper construction of deletion mutants was confirmed by PCR amplification of chromosomal DNA. The plasmid pRG10 was constructed by amplifying a 5.5 kb region containing the *pqsA-D* genes using appropriate primers (see Table S3.1) and

cloning between the *Pst*I and *Hin*dIII restriction sites of the pUCP18 vector (Schweizer,1991).

Colony biofilm assay

Bacterial cultures were grown overnight in LB at 37°C. The overnight culture was diluted to an optical density (OD_{600}) of 0.0025 in tryptone broth and 10 µl of the diluted culture was spotted onto Congo red plates (Friedman & Kolter, 2004b). The Congo red medium contained tryptone (10 g/l), granulated agar (0.5%), Congo red (40 mg/l), and Coomassie brilliant blue R 250 (20 mg/l). The plates were wrapped with aluminum foil and incubated at 37ºC for 3-5 days. For bacterial strains containing plasmid pLG10 or pRG10, carbenicillin was added to the medium.

Chemical supplementation

3OC12-HSL was added to Congo red plates buffered with 50 µM 3-(N-morpholino) propanesulfonic acid (MOPS) at a final concentration of 10 µM. Pyocyanin was added to Congo red plates at a final concentration of 50 µM. HHQ (a gift from M. Whitelely, University of Texas) and HNQ (a gift from P. Williams, University of Nottingham) were added to MOPS-buffered Congo red plates at a final concentration of 50 µM or directly to the bacterial inoculum at final concentrations of 20, 100 and 500 µM. The respective solvents ethyl acetate, dimethyl sulfoxide (DMSO), and methanol were used as controls.

Pel'-lacZ*-reporter construction and β-galactosidase measurements*

A 555 bp promoter region of the *pel* operon was amplified from the ZK strain using the primers listed in Supplementary Table S3.1 and cloned upstream of the *lacZ* gene in the integration vector mini-CTX-*lacZ* (Becher & Schweizer, 2000). The resulting plasmid pRG11 was then inserted into the chromosome of the wild-type and the *lasR* mutant as described (Becher & Schweizer, 2000). As a control, the mini-CTX-*lacZ* parent vector was also integrated into the genome. The colonies of the ZK wild-type and the *lasR* mutant grown on Congo red plates at 37°C were used to measure β-galactosidase levels. A colony was cut out on the 3rd, 4th, and the 5th day and suspended in 2 ml of 50 mM phosphate buffer, pH 7.4, in a 15 ml conical tube. Cells were lysed by sonication. The total protein was estimated by Bradford assay (Bradford, 1976). The sonicated sample was centrifuged at 4°C for 30 min. The resulting supernatant was used to measure β-galactosidase activity as described previously (Whiteley *et al.*, 1999).

Pellicle biofilm assay

Cultures were inoculated in tryptone broth at an OD_{600} of 0.0025 and incubated at 22°C and 37ºC without shaking (Friedman & Kolter, 2004a). After 24, 48 and 72 h, pellicle formation was observed at the air-liquid interface.

Microtiter plate biofilm assay

Biofilm formation in a microtiter format was assayed as described(Friedman & Kolter, 2004a). Overnight cultures of the wild-type and the *lasR* mutant grown in LB broth at 37°C were diluted 1:100 in tryptone broth. One hundred and fifty µl of the diluted culture was added to 96-well polystyrene microtiter plates (Cellstar-Greiner Bio-one) and incubated at 22°C and 37°C without shaking for 48 and 72 h. Microtiter plates were rinsed in running hot water. Adherent cells were then stained with 1% crystal violet for 20 min. The microtiter plate was again rinsed in running hot water. Ethanol was added to each dry well and the samples were allowed to stand for 20 min. Absorbance was measured at 590 nm.

Flow-cell biofilm assay

Biofilms were grown at 37°C in flow chambers. The system was assembled as described (Coic *et al.*, 2006, Davies *et al*., 1998). The cultures for inoculation were prepared from mid-exponential phase (OD_{600} of 0.4-0.8) TSB cultures grown at 37°C. The cultures were diluted to an OD_{600} of 0.05 in 1:100 diluted TSB medium and injected into the flow cell. Flow was initiated after 1 h. The diluted TSB was supplied at a flow rate of 180 µl/min using a peristaltic pump (Watson Marlow 205S). Images were taken when biofilms had matured (day 3) with an inverted Zeiss LSM Zeta 510 confocal laser scanning microscope (CLSM) using a 63X oil immersion lens. The manufacturer's software and Adobe Photoshop were used for image processing.

Suppressor mutagenesis

For transposon mutagenesis, biparental matings were set up between the *E. coli* donor (S17-1-λpir/pLM1) and the *P. aeruginosa* recipient strain (ZK *lasR* mutant) as described (Fox *et al.*, 2008). The suicide plasmid pLM1 carries a miniTn*5* transposon. The transposon insertion mutants were selected on LB agar plates containing gentamicin (30 µg/ml) and nalidixic acid (20 µg/ml). Colonies were picked manually and patched onto rectangular LB plates containing gentamicin (30 µg/ml) in a 96-well format. Plates were incubated at 37ºC for one day and then replica-plated onto rectangular Congo red plates using a 96-well-pin replicator. The ZK wild-type and the *lasR* mutant were included as controls. These plates were incubated for 3-5 days at 37ºC. Candidate revertants exhibiting a smooth colony morphology identical to the wild-type were streaked for isolated colonies and subjected to a second screen. This screen involved performing the original colony biofilm assay as described earlier. Mutants which again showed a smooth phenotype were considered to be true revertants.

Mapping of transposon insertions

Genomic DNA was isolated from the selected transposon mutants (Qiagen PUREGENE kit) and was digested with *Nco*I. The transposon does not contain an *Nco*I restriction site and has an R6K origin of replication. The digested DNA was self-ligated with T4 DNA ligase (New England Biolabs) and electroporated into

chemically competent *E. coli* S17-1/λpir (R. Simon, 1983). Plasmid DNA was isolated from gentamicin-resistant colonies and was sequenced using the Tn*5* specific primer tnpRL17-1 (Larsen *et al.,* 2002). Transposon insertions were mapped by comparing sequences to a *Pseudomonas* protein database using BlastX.

***Overexpression of* pqsA-E**

The appropriate strains were transformed with plasmid pLG10 (Gallagher *et al*., 2002) and pRG10 carrying the *pqsA-E* operon and *pqsA-D* operon under the control of native and constitutive promoters, respectively, or with pUCP18 (Schweizer, 1991), the parent vector from which pLG10 and pRG10 were derived.

Thin-layer chromatography (TLC)

Samples for TLC analysis were prepared from 3-5 day-old colonies. Two colonies of each strain grown on the same plate were cut out from the agar with minimum possible agar contamination. One colony was used for total protein estimation and the other for AQ extraction. Total protein was estimated by Bradford assay (Bradford, 1976) as described earlier for β-galactosidase measurements. For AQ extraction, a colony was harvested and suspended in 5 ml methanol, homogenized with a tissue tearor, and allowed to stand for 10 min. The suspension was centrifuged for 30 min at 4000 r.p.m. at 4°C. The supernatant was filtered through a 0.2 µM syringe filter and the filtrate was collected in glass vials prewashed with acetone. The samples were then

air-dried, reconstituted in 500 µl of methanol and transferred to 2 ml glass vials. They were again air-dried and finally reconstituted in 100 µl of methanol. TLC plates were prepared and samples were run as described (Fletcher *et al*., 2007). Five µl of the sample (normalized to total protein), 2 µl of the standards-PQS (5 and 10 mM), and HHQ (2.5 and 5 mM,) were used. AQ levels were estimated in the wild-type and the *lasR* mutant by densitometric analysis of relative spot intensities using Imagequant TL software (GE Healthcare) from two independent experiments.

Results

A ZK lasR *mutant forms wrinkly colonies*

We investigated the effect of a *lasR* mutation on colony morphology as an indicator of matrix production (Friedman & Kolter, 2004b, Sakuragi & Kolter, 2007). A wrinkled colony phenotype is generally associated with increased EPS production and biofilm formation. Our agar medium also contained Congo-red, which may stain colonies overproducing EPS (Spiers *et al.*, 2003), but is not always a reliable indicator, especially at 37°C (Hickman *et al*., 2005). We therefore focused on colony wrinkling (rugosity). We grew the wild-type and *lasR* mutants of three *P. aeruginosa* strains, namely widely used strains PAO1 and PA14, and the autoaggregative strain ZK2870 (Friedman & Kolter, 2004b), on agar plates for 5 days at 37°C and at 22°C. Growth conditions are identical to those previously used to investigate EPS-dependent colony morphology (Sakuragi & Kolter, 2007, Friedman & Kolter, 2004b). We did not

observe any significant differences in rugosity between the PAO1 wild-type and *lasR* mutant strains at either temperature (Fig. 3.2A). However, the colonies of the wild-type and the *lasR* mutant of strains PA14 and ZK showed striking differences. A PA14 *lasR* mutant formed a flat, smooth colony as compared to the wrinkled wild-type phenotype at 22°C (Fig. 3.2A). On the contrary, a ZK *lasR* mutant formed a distinctive wrinkled colony at 37°C while the wild-type formed a smooth colony (Fig. 3.2A). At room temperature, the morphological difference between the wild-type and the ZK *lasR* mutant was not as pronounced. A positive regulatory link between *las* QS, *pel* transcription and colony morphology has already been described in strain PA14, which only carries Pel EPS (Sakuragi & Kolter, 2007). The apparently inverse relationship between *las* QS and colony morphology at 37°C in strain ZK, which harbors both Pel and Psl, was intriguing to us and is the focus of this study.

To confirm that the observed phenotype is generally dependent on a non-functional *las* system, we also constructed a ZK *lasI* in-frame deletion mutant. A ZK *lasI* mutant showed a well defined wrinkled colony like the *lasR* mutant at 37°C (Fig. 3.2B). Supplementation of the *lasI* mutation with exogenous 3OC12-HSL signal virtually restored the smooth wild-type phenotype (Fig. 3.2B). This confirms that the *las* system is responsible for the wrinkled colony phenotype. We used the ZK *lasR* mutant for further study.

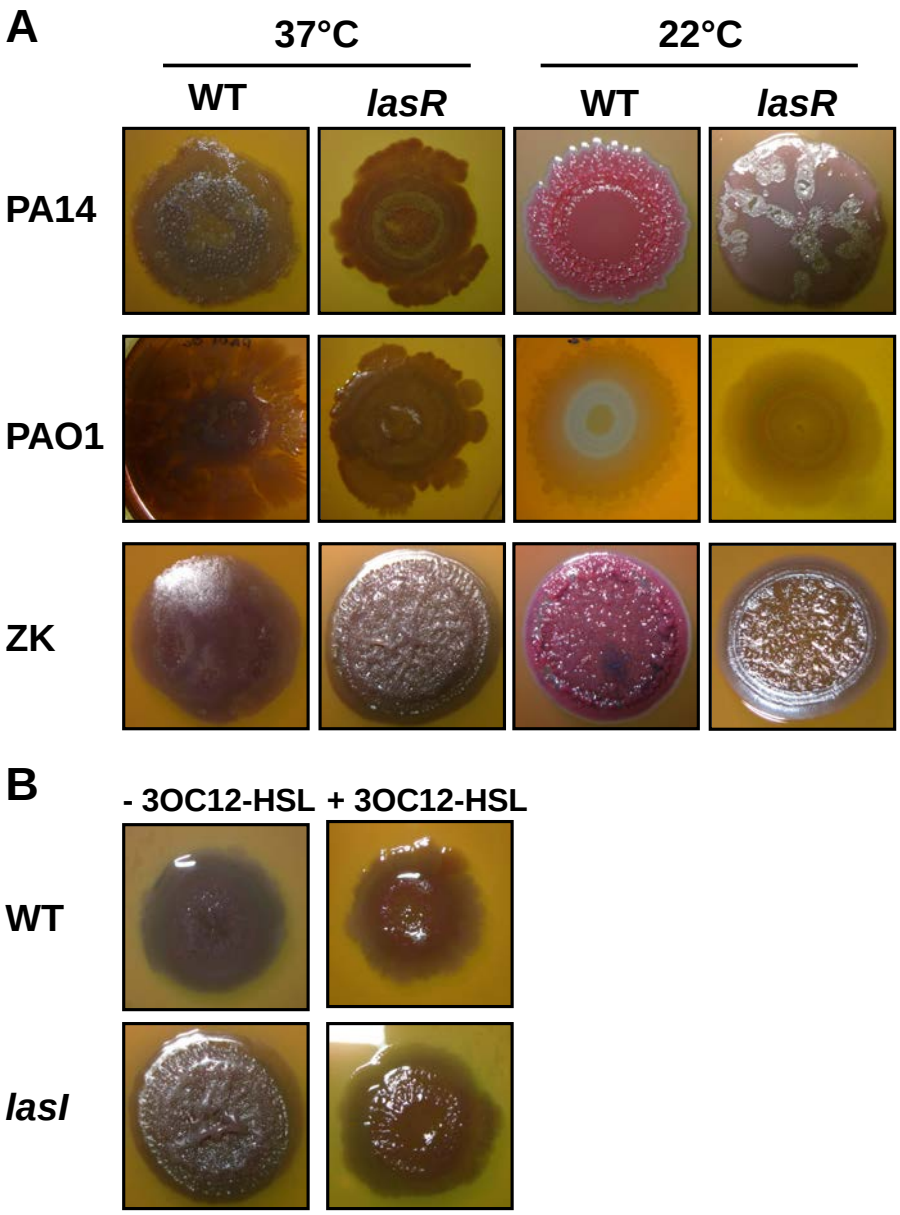

Fig. 3.2: Effect of *las* mutation on colony wrinkling. A. Colony morphology of wild-type (WT) and *lasR* mutant *P. aeruginosa* strains PA14, PAO1 and ZK after 5 days of growth at the indicated temperature. **B.** Colony morphology of the ZK wild-type (WT) and *lasI* mutant in the presence and absence of 10 µM 3OC12-HSL after 5 days at 37 °C.

***Genetic analysis indicates involvement of* pel *rather than* psl**

We performed mutational analysis to investigate whether Pel or Psl EPS might cause wrinkling of the *lasR* mutant. We constructed *pelA lasR* and *pslD lasR* double mutants and compared their colony morphology to that of the *lasR* mutant and the wild-type parent. A *pelA lasR* double mutant showed a nearly smooth colony phenotype while the *pslD lasR* mutant showed a wrinkled phenotype like the *lasR* mutant (Fig. 3.3).

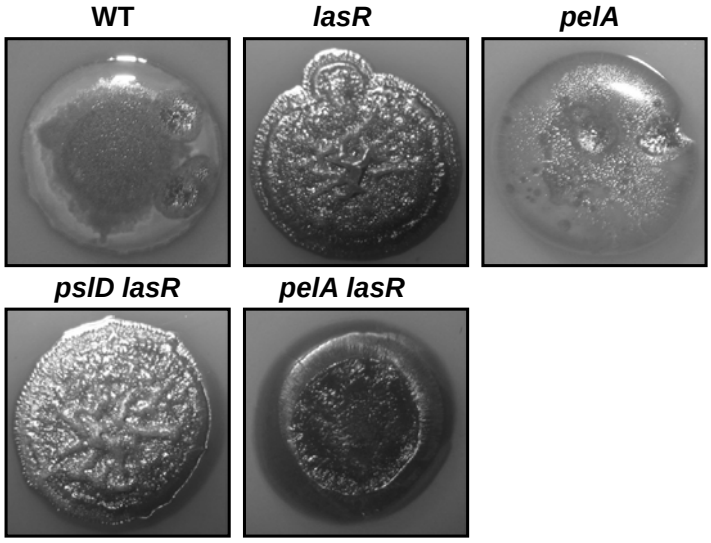

Fig. 3.3: Genetic analysis of *pel* and *psl* involvement. Colony morphology of the ZK wild-type (WT), *lasR* mutant, *pelA* mutant, *pelA lasR* and *pslD lasR* double mutants after 5 days of growth at 37°C.

We evaluated the contribution of *pel* alone by comparing the colony morphology of a *pelA* mutant to the wild-type. The *pelA* colony phenotype was indistinguishable to

that of the wild-type. The partial loss of wrinkles in a *pelA lasR* double mutant therefore indicates inhibition of Pel by LasR.

To determine whether inhibition is at the transcriptional level, we measured *pelA* transcription in the wild-type and the *lasR* mutant using a *pelA'-lacZ* transcriptional fusion. The transcriptional fusion was constructed with a mini-CTX vector (see *Materials and Methods*), which allows site specific integration of DNA sequences upstream of the $tRNA^{Ser}$ transcriptional start site in the bacterial chromosome. We harvested colonies after 3, 4 and 5 days, because a ZK *lasR* mutant begins to show wrinkling at day 3. We found no difference in *pelA* transcription in the wild-type and the *lasR* mutant (Fig. 3.4).

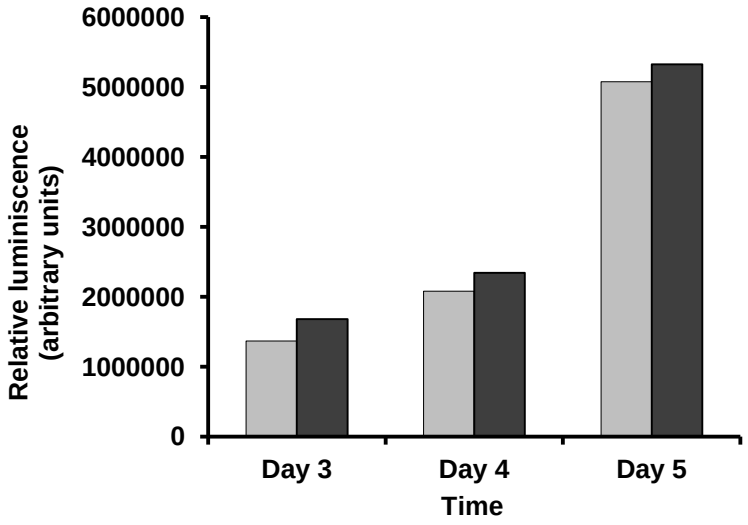

Fig. 3.4: Pel transcription. β-Galactosidase activity of a *pel* promoter-*lacZ* transcriptional fusion in ZK wild-type (light grey bar) and the *lasR* mutant (dark grey bar) colonies grown at 37°C. LacZ activity, expressed as relative luminescence, was normalized to the density of the colony (measured as total protein content). The signal from a promoter-less *lacZ* construct was subtracted.

This indicates that *pel* regulation is at the posttranscriptional level. We attempted to investigate this possibility by quantifying EPS, however, we were unable to perform an EPS composition and linkage analysis because of insufficient amounts of purified EPS extracted from colonies required for such analysis.

Investigation of other factors associated with* pel *and the wrinkled colony phenotype

We investigated the role of phenazines and of the tyrosine phosphatase TpbA in the observed wrinkled phenotype of a ZK *lasR* mutant as both modulate structural organization of *P. aeruginosa* strain PA14 colony biofilms (Dietrich *et al.*, 2008, Ueda & Wood, 2009). We examined the relationship between phenazine deficiency and the wrinkled phenotype through addition of pyocyanin to the agar medium. Pyocyanin supplementation did not result in loss of wrinkles in the *lasR* mutant (Fig. 3.5A). Inhibition of TpbA in strain PA14 has been shown to enhance *pel* expression at 37°C, resulting in a wrinkled colony phenotype (Ueda & Wood, 2009). We therefore constructed a *tpbA* mutant in the ZK background and examined colony morphology. The *tpbA* mutant remained as smooth as the wild-type (Fig. 3.5B). These results indicate neither pyocyanin nor TpbA are responsible for the wrinkled phenotype of the ZK *lasR* mutant.

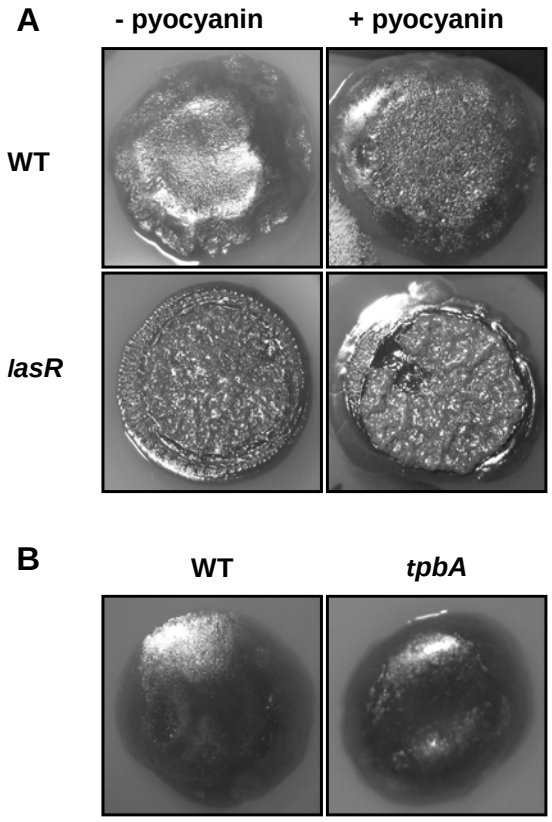

Fig. 3.5: Role of pyocyanin and *tpbA* in the wrinkled colony phenotype. A. Colony morphology of the ZK wild-type (WT) and the *lasR* mutant with and without 50 µM pyocyanin. **B.** Colony morphology of the ZK wild-type (WT) and the *tpbA* mutant. Colonies were grown for 3 days at 37°C.

Hydrated lasR *mutant biofilms do not show altered architecture*

The involvement of *pel* in the wrinkled colony morphology of the ZK *lasR* mutant suggested that it might exhibit generally altered biofilm architecture. We investigated pellicle formation of standing cultures as well as biofilm formation in microtiter plates

and flow-cells. Flow-cell biofilms of the wild-type and the *lasR* mutant after 3 days of growth are shown in Fig. 3.6.

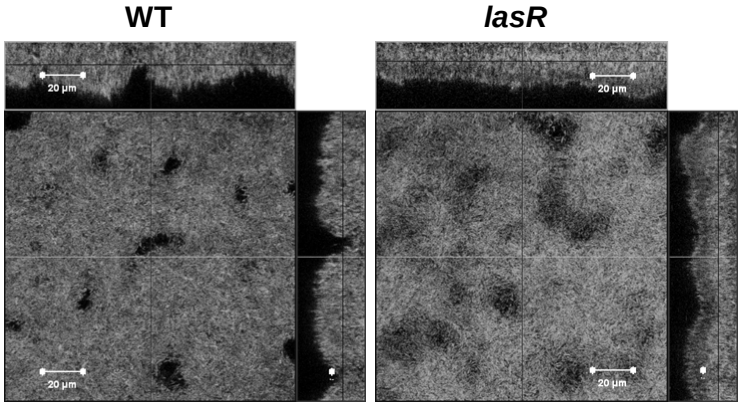

Fig. 3.6: Flow-cell biofilms. CLSM images of flow-cell grown biofilms of the ZK wild-type (WT) and the *lasR* mutant at 37°C after 3 days. The large panel shows the horizontal cross-section and the small panel shows the vertical cross-section of the biofilm. The lines in the panels indicate the planes of the cross-sections.

Neither assay revealed any differences between the two strains. This is consistent with recent results by Colvin *et al.* who also found no defect in attachment or biofilm development for a *pel* mutant of strain PAO1 (Colvin *et al.*, 2011). There is a difference in the degree of hydration in the three biofilm assays we employed. Submerged flow-cell biofilms are fully saturated and hydrated, pellicles and microtiter plate biofilms that form at the air-liquid interface are somewhat less hydrated, whereas

colonies on agar are the least hydrated (Chang & Halverson, 2003). It is possible that the observed phenotype only manifests under conditions of low hydration.

Suppressor mutagenesis implicates the* pqs *pathway

Transposon mutagenesis was performed in the ZK *lasR* mutant background to identify the regulatory link between the *las* QS system and colony morphology. Around 10,000 mutants were screened for reversion to a smooth phenotype. We identified 38 mutants, and mapped transposon insertions in 25 (Supplementary Table S3.2). We found 9 transposon insertions in the *pqsA-D* genes of the AQ biosynthesis operon and one insertion in the gene encoding the transcriptional regulator PqsR that activates *pqsA-E* expression (Fig. 3.7). Given the large fraction of hits (10 out of 25 or 40%), the role of the *pqs* operon was apparent even without mapping the remaining transposon mutants.

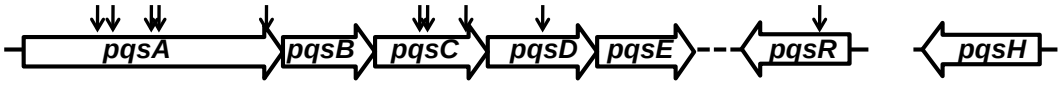

Fig. 3.7: The *pqs* locus and transposon insertions in associated suppressor mutants. Horizontal arrows represent the genes of the *pqsA-E* operon, the *pqsR* transcriptional regulatory gene, and the *pqsH* gene. Small vertical arrows indicate the location of the respective transposon insertions.

We did not identify any insertions in *pqsH*, which promotes the conversion of Series A (4-hydroxyalkyl quinolines) to Series B (3,4 dihydroxyalkyl quinolines) congeners nor

in *pqsE,* which encodes a putative global regulator (Deziel *et al*., 2004, Rampioni *et al.*, 2010). Surprisingly, we also did not identify a transposon insertion in the *pel* operon, although our data in Fig. 3.3 show that the *lasR pel* mutant forms a smooth colony. We found that this mutant displayed very slight wrinkling under the conditions employed for the high throughput screen, in which our primary focus was on the identification of the most obvious smooth revertants.

The wrinkly phenotype of the *lasR pqsA*::Tn suppressor mutant could be restored by introducing plasmid pLG10 (Gallagher *et al*., 2002), which expresses the *pqsA-E* operon from its native promoter (Fig. 3.8A). This verifies that the products of this operon are indeed responsible for the wrinkled phenotype of the *lasR pqsA*::Tn mutant. To investigate whether *pqsA-D* dependent wrinkling of the *lasR* mutant is through PqsR, we introduced plasmid pRG10 into the *lasR pqsR*::Tn mutant. This plasmid constitutively expresses the *pqsA-D* operon from a *lac* promoter. The *lasR pqsR*::Tn mutant colony was as wrinkly as that of the *lasR* mutant indicating that this phenotype is independent of PqsR (Fig. 3.8B).

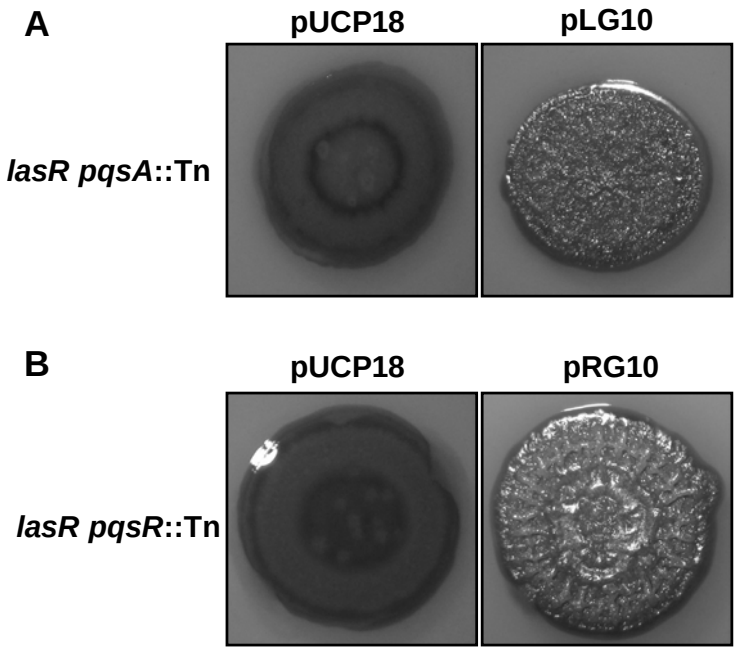

Fig. 3.8: Effect of ectopic *pqsA-E* expression on colony morphology. A. Colony morphology of the ZK *lasR pqsA*::Tn suppressor mutant with plasmid pLG10 expressing *pqsA-E* from the native promoter or control plasmid pUCP18 after 3 days at 37°C. **B.** Colony morphology of the *lasR pqsR*::Tn suppressor mutant with plasmid pRG10 expressing *pqsA-D* from a constitutive *lac* promoter or control plasmid pUCP18 after 4 days at 37°C.

A Series A AQ congener causes the wrinkled phenotype

The previous finding that *lasR* mutants overproduce Series A congeners (D'Argenio *et al.*, 2007, Deziel *et al.*, 2004) and the fact that we did not find any insertion in the *pqsH* gene indicate that Series A congeners rather than Series B congeners are responsible for the wrinkled phenotype. We therefore examined this notion further by

correlating colony morphology and AQ production, as measured by TLC, in a number of mutant strains. TLC allowed us to distinguish between high-abundance Series A and B congeners. This assay was developed and has been optimized to detect PQS and HHQ, owing to their important roles in cell-cell signaling. Compounds within each series, especially C7 and C9 congeners, are not well separated, and low-abundance compounds may not be detectable (Fletcher *et al*., 2007). We included the wild-type, the *lasR* mutant, and the *lasR pqsA*::Tn suppressor mutant in this analysis. In addition, we constructed a *pqsH* single mutant and a *lasR pqsH* double mutant in the ZK background. If a Series A congener caused wrinkling, then a *lasR pqsH* mutant should still be wrinkly, and a *pqsH* mutant would also be wrinkly if a Series A congener accumulates. Indeed, the degree of wrinkling generally correlated well with the amount of Series A congener produced, in the order *lasR-pqsA*::Tn < WT < *pqsH* < *lasR* and *lasR pqsH* (Fig. 3.9A). The wrinkly *lasR* mutant and the *lasR pqsH* double mutant produce the most, while the smooth wild-type produces considerably less (Fig. 3.9B). The fact that the wrinkly *lasR pqsH* mutant does not produce Series B congeners implies a role for a Series A congener. It is not clear why the *pqsH* single mutant does not overproduce Series A congeners as previously shown for strain PA14 (Xiao *et al*., 2006a).

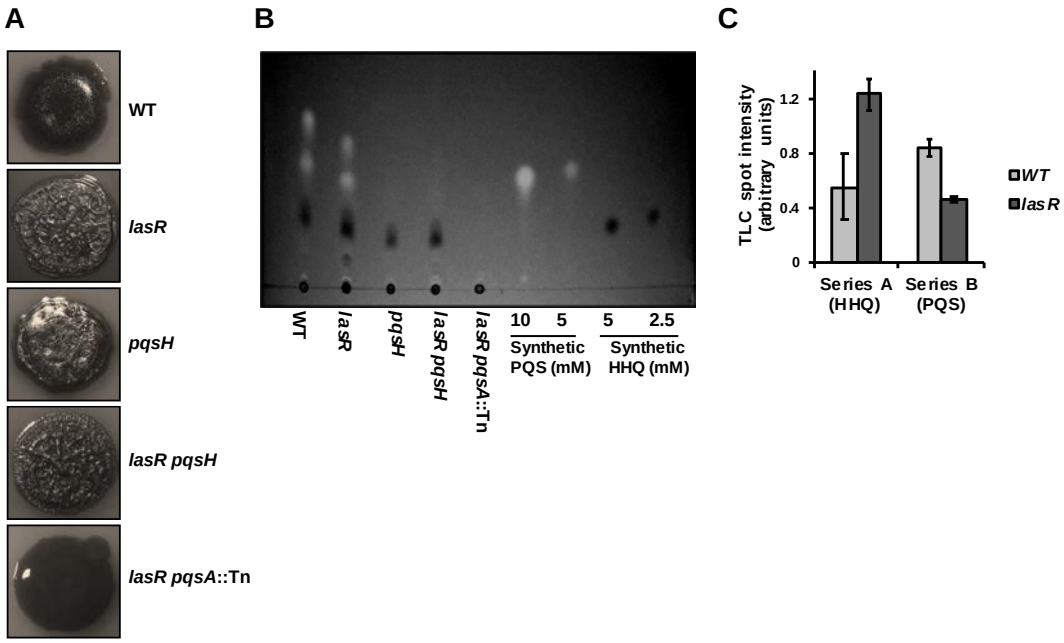

Fig. 3.9: Colony morphology and AQ production of various QS mutants. A. Colony morphology of the ZK wild-type (WT), *lasR*, *pqsH*, *lasR pqsH* double mutant, and *lasR pqsA*::Tn suppressor mutant after 5 days at 37°C. **B.** TLC analysis of AQ production by the respective strains. Approximately 5 µl of each sample (normalized to total amount of protein) was loaded. Note that samples towards the center of the plate ran more slowly than those near the edges. HHQ and PQS, representing Series A and B congeners, respectively, were included as synthetic controls. **C.** Densitometric analysis of TLC spot intensities in the wild-type and the *lasR* mutant from two independent experiments.

It is possible that *pqsA-E* expression is reduced because indirect inhibition by the *las* system via the *rhl* system and the lack of the strong inducer PQS have a larger effect in the ZK background. Regardless, the partially wrinkly phenotype of the *pqsH* mutant indicates that in addition to absolute abundance, the ratio of Series A to B congeners may also be important. Densitometric analysis of wild-type and *lasR* mutant TLC spot

intensities indeed shows that the Series A to Series B ratio is reciprocal in the two strains (Fig. 3.9C).

Two Series A compounds, the PQS precursor HHQ and HNQ, have been shown to be overproduced in a *lasR* mutant (Deziel *et al*., 2004). To examine whether one of these compounds is responsible for the wrinkly morphology of the *lasR* mutant, we added them to the *lasR pqsA* suppressor mutant. Exogenous addition to the agar medium or directly to the bacterial inoculum did not result in any change in colony morphology (data not shown). It is possible that diffusible AQ compounds are unable to enter cells in sufficient quantity, or that another less well-characterized Series A congener is responsible for the observed phenotype. Because exogenous complementation with diffusible AQ has been successful in the past (Ha *et al.*, 2011, Diggle *et al.*, 2006b), we favor the latter.

Discussion

In this study, we investigated the effect of *las* QS on biofilm formation and structure using a colony biofilm approach. This work was motivated by our recent global position analysis of LasR, which showed that this regulator directly binds to the *psl* polysaccharide promoter (Gilbert *et al*., 2009) (Fig. 3.1). While we were unable to demonstrate the significance of this finding in the present study, we established a novel connection between *las* QS and the other major *P. aeruginosa* EPS, Pel. In particular, we provide genetic evidence suggesting that the LasRI system represses

Pel. We do not have any other independent evidence of this regulatory link as EPS composition analysis was unsuccessful. Las QS also only affected colonial morphology and did not affect biofilm formation in other relevant assays, including microtiter plate, pellicle, and flow-cell. It is conceivable that water availability (matric stress) is responsible for the conditionality of the observed phenotype.

It has previously been shown that LasRI induces Pel expression in strain PA14 at room temperature but not at 37°C (Sakuragi & Kolter, 2007). This regulation is probably indirect and mediated via an unknown transcriptional regulator. Our finding that LasRI can also repress Pel expression in strain ZK at 37°C, a temperature relevant to infection, raises the intriguing possibility that QS may trigger dissolution of clinical biofilms. This would be analogous to other bacterial pathogens like *Vibrio cholerae* (Hammer & Bassler, 2003) and *Staphylococcus aureus* (Boles & Horswill, 2008). Results with the particular strain chosen, ZK2870, are significant, because the autoaggregative behavior of this strain under some environmental conditions appears most representative among clinical and environmental isolates of *P. aeruginosa* (Friedman & Kolter, 2004b). The observed differences in the colony phenotype of different *Pseudomonas* strains (Fig. 3.2) might be attributed to the presence or absence of a particular EPS locus or regulatory variability in strains with identical EPS loci.

Our second major finding is that *las* QS mediates colony morphology via AQ signaling. Phenotypic analysis along with AQ signal quantitation by TLC suggested that a Series A congener is involved. PqsA-D produce at least 8 different compounds

within this series (Lepine *et al.*, 2004). Of these, HHQ and HNQ have been shown to accumulate in a PAO1 *lasR* mutant (Deziel *et al.*, 2004). Other prominent AQs, 2,4-dihydroxyquinoline (DHQ) and 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO), that require some of the enzymes encoded by *pqsA-D*, but are not PqsH substrates, show reduced levels in a *lasR* mutant compared with the wild-type (Deziel *et al.*, 2004). Our chemical supplementation experiments indicate that neither HHQ nor HNQ modulate wrinkling. This implies that one of the other less-well characterized Series A congeners have a role in this process, further expanding the various cellular functions of AQs in *P. aeruginosa*. A detailed investigation utilizing liquid chromatography/mass spectrometry along with chemical synthesis would be able to identify the compound in question. PqsE, a putative regulator encoded by the *pqsA-E* operon whose precise function is not known, is unlikely to have a role in AQ-mediated colony wrinkling, because *pqsA-D* expression in a *lasR pqsR* mutant that does not express *pqsE* was sufficient to induce wrinkling (Fig. 3.8B).

Interestingly, in *Burkholderia pseudomallei* the lack rather than the overproduction of the Series A congener HHQ results in a wrinkly colony phenotype (Diggle *et al.*, 2006b). In addition, AQ signal overproduction has previously been shown to induce autolysis in *P. aeruginosa* populations, forming plaques that result in characteristic translucent zones in colonies (D'Argenio *et al.*, 2002), different from those we observed. Autolysis appears to be mediated by PQS rather than a Series A congener (Haussler & Becker, 2008).

Taken together, our data can be rationalized as follows: In the wild-type, both Series A and Series B congeners are produced as LasR activates *pqsR* and *pqsH*. In the *lasR* mutant, Series A congeners accumulate and the Series A to Series B ratio increases because of (1) reduced *pqsH* expression and (2) presumably *lasR*-independent expression of *pqsR* (Diggle *et al.*, 2003) resulting in continued activation of *pqsA-E*. In this case, one or more Series A congeners activate Pel production postranscriptionally in a PqsR-independent fashion, manifesting in a wrinkled phenotype. Colony morphology would be affected by a combination of *pel*-dependent and independent mechanisms, as *lasR*-mediated wrinkling was only partially *pel*-dependent (Fig. 3.3). The particular AQ compound could alter colony morphology by binding to a novel receptor protein or through membrane interactions. While both PQS and HHQ have been shown to associate with outer membrane LPS, only PQS induces vesicle formation (Mashburn-Warren *et al.*, 2008). Such distinct interactions might have direct macroscopic effects on colony morphology, but might also alter the periplasmic environment in a way that affects the signaling status of receptor proteins in the cytoplasmic membrane. Posttranscriptional regulation of Pel could be mediated via a transmembrane signaling pathway that involves the LadS/RetS/GacS/GacA two-component system, the RNA-binding protein RsmA and the small RNA RsmZ (Ventre *et al.*, 2006). Pel translation has been shown to be repressed by the RNA-binding protein RsmA (Brencic & Lory, 2009).

Author's contribution

MS and RG designed and RG performed the experiments. RG and MS analyzed and interpreted the results. RG drafted the manuscript and MS critically revised it. All authors read and approved the final manuscript.

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Supplementary Tables

Table S3.1: Oligonucleotides for deletion, overexpression, and reporter fusion constructs.

Primer name	Forward/reverse	Primer sequence 5' to 3' [a]
pqsH deletion		
pqsH-del-1	Forward	N_6**CTGCAG**TTGACAGGAGCGGGGTC (*Pst*I)
pqsH-del-2	Reverse	ACTGGAAGGCATCGACATCAG
pqsH-del-3	Forward	CTGATGTCGATGCCTTCCAGTCCGAATGCCAGTCGCAGGC
pqsH-del-4	Reverse	N_6**AAGCTT**AGGACTTCAGCGCCAGTTGC (*Hin*dIII)
tpbA deletion		
PA3885-del-1	Forward	N_6**GAGCTC**GCGGCATCCCAGGACAATC (*Sac*I)
PA3885-del-2	Reverse	CAGGAAGGCGCCGAGCACG
PA3885-del-3	Forward	CGTGCTCGGCGCCTTCCTGCGCTTCGCCGTCTGCCATG
PA3885-del-4	Reverse	N_6**AAGCTT**AGTCGCCGACGAACAGGAT (*Hin*dIII)
pqsA-D constitutive expression		
pRG10-1	Forward	N_6**CTGCAG**TCTCCTGATCCGGATGCATATC (*Pst*I)
pRG10-2	Reverse	N_6**AAGCTT**CAGGCACAGGTCATCATCCAG (*Hin*dIII)
P_{pel}-*lacZ*-transcriptional fusion		
pRG11-1	Forward	N_6**AAGCTT**CCTCGGTGTGGCTGGTGCG (*Hin*dIII)
pRG11-2	Reverse	N_6**CTGCAG**ATGTTACGGCGGGACGGCAG (*Pst*I)

[a] Bold letters indicate the recognition sequence of the restriction enzyme in parentheses

Table S3.2: List of insertion mutants with the location of the transposon insertion

Candidate No. [a]	PA number	Gene	Gene function	Percent identity [c]	Location of insertion (bp)	Direction of insertion [e]
1	PA0396	*pilU*	Twitching motility protein PilU	100	634	R
2	PA0420	*bioA*	Adenosylmethionine-8-amino-7-oxononanoate aminotransferase	99.6	1160	R
3	PA0441	*dht*	Dihydropyrimidinase	100	451[d]	F
4	PA0500	*bioB*	Biotin synthase	100	569	F
5	**PA0996**	***pqsA***	**Probable coenzyme A ligase**	**100**	**700**	**R**
6	**PA0996**	***pqsA***	**Probable coenzyme A ligase**	**100**	**801**	**F**
7	**PA0996**	***pqsA***	**Probable coenzyme A ligase**	**100**	**1211**	**R**
8	**PA0996**	***pqsA***	**Probable coenzyme A ligase**	**100**	**1294**	**R**
9	**PA0997**	***pqsA***	**Probable coenzyme A ligase**	**100**	**228**	**R**
10	**PA0998**	***pqsC***	**Beta-keto-acyl-acyl-carrier protein synthase**	**99.5**	**863**	**R**
11	**PA0998**	***pqsC***	**Beta-keto-acyl-acyl-carrier protein synthase**	**100**	**474**	**F**
12	**PA0998**	***pqsC***	**Beta-keto-acyl-acyl-carrier protein synthase**	**100**	**434**	**F**
13	**PA0999**	***pqsD***	**3-oxoacyl-[acyl-carrier-protein] synthase III**	**98.8**	**502**	**R**
14	**PA1003**	***pqsR***	**Transcriptional regulator PqsR, also known as MvfR**	**100**	**404**	**R**
15	PA1614	*gpsA*	Glycerol-3-phosphate dehydrogenase	98.2	326	R
16	PA3763	*purL*	Phosphoribosylformylglycinamidine synthase	100	2725	F
17	PA3763	*purL*	Phosphoribosylformylglycinamidine synthase	100	2725	F

Continued on next page

Table S3.2: List of insertion mutants with the location of the transposon insertion

Candidate No. [a]	PA number	Gene	Gene function	Percent identity [c]	Location of insertion (bp)	Direction of insertion [e]
18	PA4488	hypothetical	Hypothetical protein	100	713	F
19	PA4760	*dnaJ*	DnaJ protein	100	175	R
20	PA4760	*dnaJ*	DnaJ protein	100	813	R
21	PA4762	*grpE*	Heat shock protein GrpE	100	259	R
22	PA5013	*ivlE*	Branched-chain amino acid transferase	100	81	R
23	PA5304	*dadA*	D-amino acid dehydrogenase, small subunit	100	712	F
24	N[b]	N[b]	N[b]	N[b]	N[b]	N[b]
25	N[b]	N[b]	N[b]	N[b]	N[b]	N[b]

[a] Bold represents candidates with an insertion in the *pqs* pathway

[b] N, no significant match found

[c] Percent identity of BLASTX match

[d] Insertion not in the gene but rather 451 bp upstream

[e] F, forward direction, R, reverse direction

Chapter 4

Conclusion

P. aeruginosa has a large genome (6.3 Mb) with a great proportion of genes (10%) dedicated to regulatory functions (Stover *et al.*, 2000). QS is one such regulatory mechanism that controls expression of several genes involved in production of extracellular factors, biofilm development and other virulence-associated traits as a function of cell-density. It involves three well-studied transcriptional regulators, LasR, RhlR and PqsR, which bind to cognate autoinducers to regulate expression of around 6-10% of genes (Hentzer *et al.*, 2003, Rampioni *et al.*, 2010, Schuster *et al.*, 2003, Wagner *et al.*, 2003). Some of the QS-regulated genes possess LasR and RhlR DNA binding regions known as *las-rhl* boxes (Schuster & Greenberg, 2007, Gilbert *et al.*, 2009) upstream of their translational start sites, while most other QS genes do not possess these boxes and are most likely regulated indirectly. We recently identified additional direct targets of LasR by performing a global CHIP-chip analysis. One such target is the promoter region of the *psl* operon encoding a biofilm matrix EPS (Gilbert *et al*., 2009) (Appendix B).

Many QS-controlled genes are not expressed until the stationary phase of growth, even though autoinducers have already accumulated, indicating that additional regulators are involved. Several of these influence *las* and *rhl* mediated QS either

transcriptionally or posttranscriptionally and have already been identified. However, no one has ever attempted a comprehensive screening approach to identify such QS regulators. We employed a saturation mutagenesis that utilized a non-redundant transposon mutant library to identify global activators of QS gene expression (Chapter 2). We identified a novel regulator, GidA, also known as glucose-inhibited cell division protein, that regulates QS at the level of the *rhl* system (Chapter 2) (Gupta *et al.*, 2009). We found that several representative *rhl*-dependent genes, *phz, lasA, and rhlA*, which are involved in synthesis of pyocyanin, protease, and rhamnolipid, respectively, were positively regulated. Rhamnolipids are important for their antimicrobial activity, swarming motility, and biofilm maintenance in *P. aeruginosa* (Reis *et al.*, 2011). Pyocyanin and LasA protease inhibit another opportunistic pathogen, *Staphylococcus aureus* that often coexists with *P. aeruginosa* in the lungs of cystic fibrosis patients. Pyocyanin is a redox active secondary metabolite that kills bacterial cells by generating oxidative intermediates.

GidA also appeared to regulate some genes independent of RhlR as we only observed partial restoration of pyocyanin and rhamnolipid production to wild-type levels after overexpression of RhlR in the *gidA* mutant. GidA affected RhlR protein but not transcript levels. This post-transcriptional regulation of RhlR by GidA is predicted to be through tRNA modification. GidA together with another protein MnmE, modifies tRNA by adding a carboxymethylaminomethyl (cmnm) group at the

wobble position (U^{34}). This is important for mRNA decoding and hence crucial for accuracy and efficiency of protein synthesis (Bregeon *et al.*, 2001).

The *gidA* gene is pleiotropic as it affects diverse phenotypic traits. GidA is reported to affect antibiotic production, swarming, and most notably virulence gene expression. It affects virulence factor production in several pathogens including *P. syringae, Aeromonas hydrophila,* and *Streptococcus pyogenes* (Cho & Caparon, 2008, Sha *et al., 200*4). Why would GidA control all these traits including QS-dependent virulence factors in *P. aeruginosa*? What would GidA sense? GidA has a FAD binding site, so it is possible that it senses the intracellular oxidation state. FAD-binding proteins are generally required for oxidation-reduction reactions for energy production. It is possible that the oxidation state together with the quorum co-regulate gene expression. Co-regulation of genes allow *P. aeruginosa* to integrate multitude of signals for better adaptability to constantly changing environmental conditions.

In addition to QS activators, anti-activators also influence QS and form an important part of its regulatory network. QscR and the recently identified 'quorum threshold element', QteE, function by affecting LasR and RhlR levels through protein-protein interactions (Chugani *et al.*, 2001, Siehnel *et al.*, 2010). These anti-activators ensure that QS is not triggered unless a critical threshold of the signal is reached.

QS regulators (LasR, RhlR and PqsR) regulate expression of several downstream genes which together constitute a QS regulon. Our recent CHIP-chip analysis suggested regulation of Psl EPS by LasR in the *P. aeruginosa* strain PAO1

(Gilbert *et al*., 2009) (Appendix B). The genes involved in biofilm formation and structure are not completely understood, particularly their connection with QS. We investigated the link between QS and biofilm matrix gene expression (*pel* and *psl*) in Chapter 3 (Gupta & Schuster, 2012). This study showed that LasR inhibits Pel, and therefore, the loss of LasR results in a wrinkled colony, a phenotype indicative of high EPS production. We did not find a correlation between between *psl* and LasR as suggested by the ChIP-chip analysis. Nevertheless, the finding of repression of Pel EPS might be significant in that it could be a mechanism for *P. aeruginosa* to disperse from biofilms as planktonic cells in search for new environments. Analysis of biofilm formation under various conditions indicated that LasR does not affect the initial attachment stage of biofilms but rather plays a role in maturation in non-saturated environments such as colonies on an agar plate. Biofilms are of great clinical relevance as they are implicated in persistent pathogenic infections (Parsek & Greenberg, 2005). This study is a step towards understanding biofilm formation, which might contribute to the development of new treatments for chronic infections.

We found that the LasR-dependent modulation of colony biofilm architecture was mediated through 4-hydroxyalkyl quinolines in a PqsR-independent manner. We do not know the identity of the involved alkyl quinoline nor exactly how it contributes to a wrinkled colony phenotype through Pel-dependent and –independent pathways. HHQ, a prominent member of this class of akylquinolines are known to associate with outer membrane LPS. Their association with either the membrane or with some novel

receptor protein potentially regulates Pel and colony phenotype. Pel expression is controlled by the small RNA binding protein RsmA through the LadS/RetS/GacS/GacA transmembrane signaling pathway (Ventre *et al.*, 2006, Brencic & Lory, 2009). Further investigation can be carried out to confirm the identity of the involved 4-hydroxy alkyl quinoline. This can be accomplished by a combination of liquid chromatography and mass spectrometry techniques. Taken together, this study showed for the first time an interconnection between *las* and *pqs* QS systems in the regulation of Pel EPS for the modulation of biofilm architecture in the clinical *P. aeruginosa* isolate ZK2870 (Fig. 4.1).

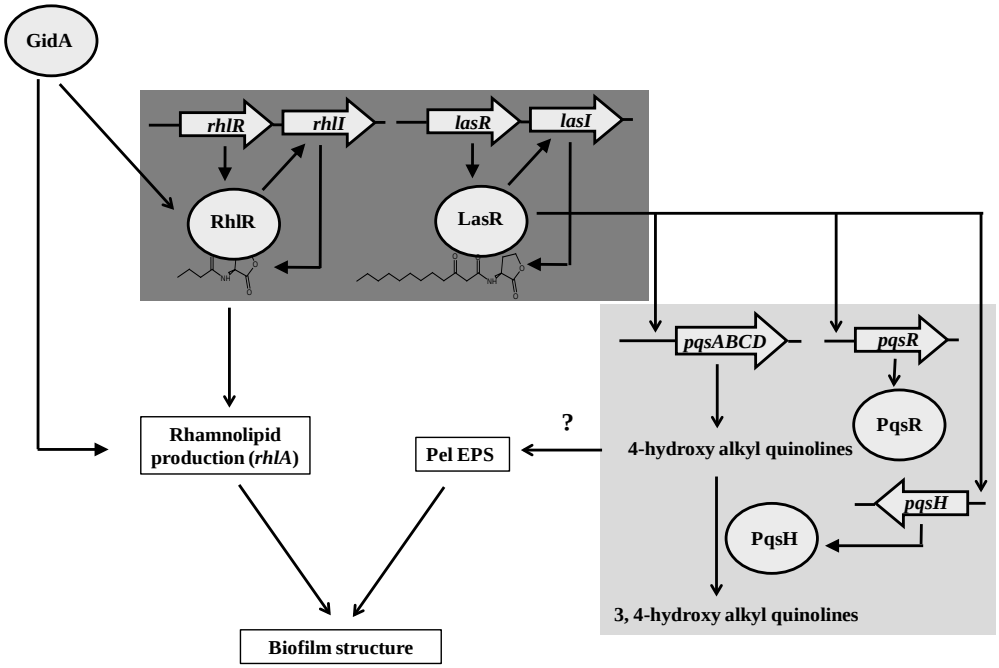

Fig. 4.1: Understanding QS regulation at the regulator and the downstream level. GidA, the newly identified QS regulator, regulates RhlR posttranscriptionally and also activates transcription of target genes, such as *rhlA*, which is involved in rhamnolipid synthesis. LasR modulates colony biofilm architecture by negatively regulating Pel EPS production through 4-hydroxy alkylquinolines by an unknown mechanism. Both Pel and rhamnolipids are important for biofilm development. The light grey colored box represents AQ based QS while the dark colored box represents acyl-HSL based QS.

Outlook

Taken together, the study investigated the regulatory aspects of QS in *P. aeruginosa* and highlighted its complex nature. QS is integrated with other global regulatory systems to fine-tune the expression of genes in response to fluctuating environmental conditions. Identification of all the regulatory components of QS and the genes

regulated by QS will provide a better understanding of the QS regulatory network, which will help develop QS-targeted antivirulence drugs. Further research will be needed to fully understand the complexity of QS and also its significance.

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Appendices

Appendix A

Insights into the ecological role of quorum sensing through constitutive expression of target genes in *Pseudomonas aeruginosa*

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Unpublished

Preliminary, requires more work

Abstract

Pseudomonas aeruginosa is an opportunistic human pathogen that regulates expression of hundreds of genes through quorum sensing (QS). QS is a cell–density dependent gene regulation mechanism that coordinates expression of other, generally more costly cooperative behaviors that are vulnerable to cheating from an evolutionary perspective. Thus QS may have evolved to optimize cooperative behaviors. In this study, we experimentally tested this prediction by utilizing strains that express QS target genes virtually constitutively (constitutive cooperation). These strains independently harbor mutations in the QS repressor genes *qscR* or *qteE*. We compared their intra- and interspecific fitness to strains with normal QS (conditional cooperation, wild-type) and no QS (no cooperation, *lasR* receptor gene mutants) in single and co-cultures. We employed media with either caseinate or casamino acids as the sole carbon source, representing growth conditions that require and that do not require cooperation, respectively. The *qscR* and *qteE* mutants grew faster than the wild-type and the *lasR* mutant in caseinate medium and slower in casamino acids medium, indicating an advantage from constitutive QS gene expression when cooperation is favored and a metabolic burden when cooperation is not favored. As expected, in the interspecies competition between *P. aeruginosa* and another opportunistic pathogen, *Staphylococcus aureus,* the constitutively cooperating strains of *P. aeruginosa* showed higher fitness than the conditionally cooperating strain. On the other hand, constitutively cooperating populations are vulnerable to cheating, and

are invaded by strains with conditional cooperation. This study suggests that QS is an evolutionary stable strategy for bacteria that frequently alter between conditions that favor and that do not favor cooperation.

Introduction

Pseudomonas aeruginosa is an opportunistic human pathogen that infects immunocompromised individuals such as those suffering from cystic fibrosis and burns. It coordinates gene expression through a cell density-dependent regulation method known as quorum sensing (QS). It possesses two complete acyl-homoserine lactone (acyl-HSL) based QS systems, namely the *las* and the *rhl* systems (Juhas *et al.*, 2005, Schuster & Greenberg, 2006). The *las* system consists of a transcriptional regulator, LasR, and a signal synthase, LasI, that synthesizes 3-oxo-dodecanoyl-HSL (3OC12-HSL). The *rhl* system consists of a transcriptional regulator, RhlR, and the signal synthase, RhlI, that synthesizes butanoyl-HSL (C4-HSL). LasR and RhlR bind their respective signal molecules and this signal-receptor complex regulates expression of various genes. The *las* system controls the *rhl* system and is at the top of the hierarchy (Latifi *et al.*, 1996, Pesci *et al.*, 1997b). The two systems together control expression of over 300 genes (Schuster *et al.*, 2003, Wagner *et al.*, 2003, Hentzer *et al.*, 2003).

The expression of QS-controlled products is suppressed before the quorum is reached by various QS regulators, including QscR and QteE. QscR is an orphan LuxR-

type protein that shares the signal 3OC12-HSL with LasR (Chugani *et al.*, 2001). It negatively regulates expression of LasR-dependent and RhlR-dependent genes including *lasI* and *rhlI* possibly by forming inactive heterodimers with LasR and RhlR. (Chugani *et al.*, 2001, Ledgham *et al.*, 2003). Similarly, QteE also modulates the quorum threshold by blocking QS at low cell density by interacting with LasR (Siehnel *et al.*, 2010). QteE, known as "quorum threshold element" reduces LasR protein stability without affecting transcription or translation. It also blocks RhlR protein accumulation independent of action on LasR. Inhibition of expression of various virulence factors at low cell density presumably is a conservation strategy that benefits the bacterial cell.

QS regulates several cooperative behaviors like production of extracellular proteases, siderophores, exopolysaccharides, antibiotics and biosurfactants (West *et al.*, 2007a). The production of these "public goods" is subject to cheating from an evolutionary perspective. This has been demonstrated both *in vitro* (Diggle *et al.*, 2007, Sandoz *et al.*, 2007) and *in vivo* (Rumbaugh *et al.*, 2009). Thus, explaining the evolution of cooperative behaviors has been challenging. An explanation for the evolution of cooperation is kin selection, according to which the cooperative genes are favored if they increase the reproductive potential of related individuals carrying the same gene. In microbes, kin selection is thought to be achieved through limited dispersal, which keeps related individuals together. Pleiotropy, prudent regulation of cooperative genes, punishment of cheaters and rewarding cooperators can also explain

the stability of cooperation (Foster *et al.*, 2004, West *et al.*, 2007b, Xavier & Foster, 2007).

We hypothesize that QS evolved to optimize group beahvior. In this study, we tested this prediction by comparing the fitness benefits of social interactions of constitutively cooperative strains (*qscR, qteE*) with those of conditionally cooperating (wild-type) and non-cooperating (*lasR*) strains in individual and mixed cultures. In addition, we also investigated interspecies interactions between *P. aeruginosa* and another opportunistic pathogen, *Staphylococcus aureus.* These interspecific interactions are important in understanding community interactions in mixed microbial infections. We showed that constitutive cooperation increases the population fitness under conditions that require cooperation but makes the population vulnerable to invasion by cheaters.

Materials and Methods

Bacterial strains and plasmids

Bacterial strains used in this study are listed in Table A.1.

Table A.1: Strains and plasmids

Strain or plasmid	Relevant property	Reference
Strains		
P. aeruginosa		
PAO1	Wild-type, PAO1 UW library strain	(Jacobs *et al*., 2003)
PW3598	*lasR-C01*::IS*lacZ*/hah, UW strain, Tc^R	(Jacobs *et al*., 2003)
PW4325	*qscR-B10*::IS*lacZ*/hah, UW strain, Tc^R	(Jacobs *et al*., 2003)
PW5355	*qteE-C10*::IS*lacZ*/hah, UW strain, Tc^R	(Jacobs *et al*., 2003)
PAO1-Cb	Wild-type tagged with the carbenicillin resistance gene, Cb^R	This study
PAO1 *lasR*-Cb	PW3598 tagged with the carbenicillin resistance gene, Cb^R	This study
S. aureus	Wild-type, Newman strain	(Duthie & Lorenz, 1952)
E. coli		
DH5α	F^- φ80d*lacZ*ΔM15 Δ (*lacZYA-argF*) U169 *deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Invitrogen
SM10	Mobilizing strain, RP4 *tra* genes integrated in the chromosome, *thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^r	
Plasmids		
pminiCTX3a	Allelic exchange suicide vector, Tc^R	(Hoang *et al*., 1998)
pRG13	1200 bp ampicillin/carbenicillin resistance gene cloned with its native promoter in pminiCTX3a	This study
pProbeAT	Broad-host-range vector with a promoter less *gfp*	(Miller *et al*., 2000)
pRG12	240 bp *lasB* promoter cloned into pProbeAT	This study

The mutant strains of *P. aeruginosa* were obtained from a non-redundant transposon library from the University of Washington (Jacobs *et al*., 2003). Luria-Bertani broth (LB) was used for routine culturing unless specified. Antibiotics tetracycline (50 µg/ml), and carbenicillin (200 µg/ml) for *P. aeruginosa* and ampicillin (100 µg/ml) for *E. coli* were used where appropriate.

Tagging of the strains

Wild-type and *lasR* mutant strains were tagged with a carbenicillin resistance gene using site-specific integration (Becher & Schweizer, 2000). A 1200 bp ampicillin resistance gene with its promoter was PCR-amplified from pUCP18 (Schweizer, 1991) and digested with *Hin*dIII and *Xho*I. This fragment was ligated into the equally digested miniCTX3a vector. The resulting construct, pRG13, was confirmed by sequencing. Plasmid pRG13 was introduced into the wild-type and the *lasR* mutant by electroporation and selected for integration into the respective *P. aeruginosa* genome as described (Hoang *et al.*, 2000).

Growth curve

Individual cultures of wild-type, *qscR*, *qteE*, and *lasR* strains were grown in minimal medium with 1% (w/v) sodium caseinate (caseinate) and 0.5% (w/v) casamino acids (CAA) as the sole carbon source representing conditions that do and do not require cooperation, respectively (Sandoz *et al.*, 2007, Wilder *et al.*, 2011). An overnight culture inoculated from an isolated colony of a freshly streaked culture in 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7.0 buffered LB was used as the inoculum. The starting optical density (OD_{600}) of the culture was 0.05. The culture was grown in an incubator/shaker at 37°C. For growth curve analysis in CAA, 200 µl of the medium was inoculated with the cultures in transparent 96 well plates (Greiner bio-one, Cat. No. 655185). Absorbance at OD_{600} was measured after every 15 min for

5 h. The growth of the tagged wild-type and the *lasR* mutant was compared to their respective untagged parents in a similar manner. The growth curve in the caseinate medium was performed by inoculating 4 ml of the medium in glass tubes with the appropriate strains. At 0, 12, 24, 36, and 48 hr an aliquot was removed and appropriate dilutions were plated onto LB plates to determine colony forming units (CFU)/ml.

Construction of a lasB-gfp *transcriptional fusion*

A 240 bp *lasB* promoter region was PCR-amplified from the PAO1 genome using the primers pRG12-F (5'N_6AAGCTTGGCCTACAAGCTCGACGTCA 3', *Hin*dIII) and pRG12-R (5'N_6 GAACTTGTTTTCGACGGTGCTTTCGT 3', *Eco*RI). The PCR product was digested with the indicated restriction enzymes (in parentheses) and ligated with an equally digested promoter probe vector, pProbeAT (Miller *et al.*, 2000). The resulting construct pRG12 was confirmed by sequencing.

lasB *expression*

Expression levels of a QS regulated gene, *lasB,* were measured in the wild-type, *lasR*, *qscR* and *qteE* strains transformed with plasmid pRG12 through monitoring of promoter activity. Experimental cultures were started from overnight MOPS-buffered LB cultures that had been inoculated with a freshly grown single colony. The cultures were washed once in M9 salts and inoculated into fresh LB at an OD_{600} of 0.002. The cultures were grown to an OD_{600} of 0.2, washed again and finally diluted to an OD_{600}

of 0.01 in CAA. This washing and culturing scheme was done to reduce basal expression levels of GFP protein. Samples were taken at early logarithmic (OD_{600} of 0.5-0.6) and stationary phase (OD_{600} of 3.0) during culture growth, and GFP fluorescence was measured using a Tecan plate reader (Infinite M200, excitation and emission wavelengths of 480 nm and 535 nm, respectively).

Co-culture experiments

The experimental cultures for inter-species and intra-species competition experiments were started from overnight (18 h) MOPS-buffered LB cultures that had been inoculated with a freshly grown single colony of wild-type, *lasR, qscR* and *qteE* strains. The starting OD_{600} of the cultures was 0.05. Strains were inoculated at the appropriate ratios. At the indicated times, culture aliquots were removed, appropriately diluted and plated to determine CFU/ml.

Intra-species co-culture experiments were carried out with two or three strains in caseinate medium. For 2-way competition experiments, strains in the co-culture were distinguished by plating onto LB plates with and without the appropriate antibiotic. A 3-way competition was carried out with the wild-type, the *lasR* mutant, and the *qteE* mutant at a ratio of 100:1:1. The strains were distinguished by plating onto LB agar with or without tetracycline. The *lasR* and the *qteE* mutant populations were distinguished based on their colony size on LB plates containing tetracycline. The *qteE* mutant forms larger colonies than the *lasR* mutant on these plates. Percent

enrichment was calculated by dividing the final ratio by the initial ratio. The statistical significance was determined by a two-tailed unpaired *t*-test.

For inter-species competition, *S. aureus* was inoculated at 50% and 90% with either the *P. aeruginosa* wild-type, the *qscR* mutant or the *qteE* mutant in LB-MOPS medium. *P. aeruginosa* and *S. aureus* colonies were distinguished by their colony size and color. *S. aureus* forms small, white colonies whereas *P. aeruginosa* forms large, off-white colonies. The initial and the final percentage of *S. aureus* at time 0 and 12 h was calculated by dividing the number of *S. aureus* colonies by the total number of colonies. A total of approximately 600 colonies were counted to determine the final *S. aureus* percentage.

Results

Individual qscR *and* qteE *mutant cultures are fit under cooperative conditions but not under non-cooperative conditions*

We assessed the fitness levels of wild-type, *lasR, qscR* and *qteE* strains individually by comparing their single culture growth in a minimal medium with either caseinate or CAA as the sole carbon source. Caseinate utilization requires QS-dependent extracellular proteases (Iglewski & Van Delden, 1998) whereas CAA can be taken up and metabolized without QS. We observed that both the *qscR* and *qteE* mutants grew faster than the wild-type and the *lasR* mutant in caseinate medium. This was expected because the *qscR* and *qteE* mutants express QS genes virtually constitutively without

an apparent quorum threshold and therefore are more likely to efficiently utilize caseinate than the wild-type (Fig. A.1A). Conversely, *qscR* and *qteE* mutants grew more slowly in CAA (Fig. A.1B and Table A.2) than the wild-type and the *lasR* mutant. This slow growth of the *qscR* and the *qteE* mutants in CAA reflects their high investment in the production of costly QS-dependent factors. To confirm this, we quantified QS-regulated gene expression levels by measuring *lasB* (elastase) promoter activity with a GFP transcriptional reporter (pRG12) in the wild-type, *lasR*, *qscR* and *qteE* strains. Elastase, a QS-dependent virulence factor, is the predominant casein protease in *P. aeruginosa*. The *qscR* and *qteE* mutants showed increased elastase production in early logarithmic phase as well as in stationary phase as compared to the wild-type and the *lasR* mutant (Fig. A.1C). Increased *lasB* levels are indicative of the high metabolic burden imposed due to early QS gene expression and thus can explain the slow growth of the *qscR* and *qteE* mutants. Constitutively cooperating *qscR* and *qteE* mutants showed higher fitness under conditions that require cooperation but not under conditions that do not favor cooperation.

Fig. A.1: *P. aeruginosa* growth and *lasB* expression in individual cultures. A. Culture density **(**CFU/ml) of wild-type (open squares), *lasR* (stars), *qscR* (crosses) and *qteE* (open circles) strains grown in caseinate medium. **B.** Culture density (OD_{600}) of wild-type (open squares), *lasR* (stars), *qscR* (crosses) and *qteE* (open circles) strains grown in CAA**. C.** Relative GFP fluorescence was measured in the wild-type (open squares), *lasR* (stars), *qscR* (crosses) and *qteE* (open circles) strains carrying the transcriptional reporter of *lasB* at logarithmic phase (OD_{600} of 0.6, light grey bar) and at stationary phase (OD_{600} of 3.0, dark grey bar). The error bars indicate standard deviation of the mean of three replicates from three independent experiments. The asterisk indicates statistically significant difference compared to the wild-type ($P< 0.05$).

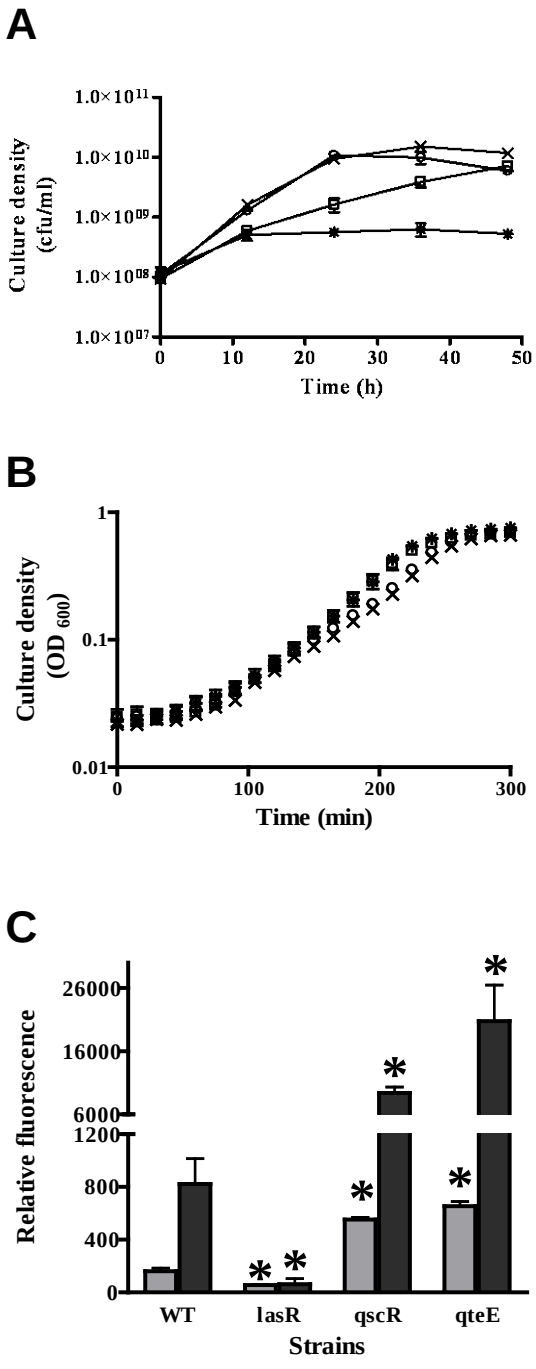

Fig. A.1: *P. aeruginosa* growth and *lasB* expression in individual cultures.

Table A.2: Growth in CAA medium

Strain	Doubling time (min)	*P* versus wild-type
Wild-type	35.0 ± 1.0	N/A
lasR	37.3 ± 4.2	0.43
qscR	44.4 ± 1.2	0.00046
qteE	48.7 ± 6.4	0.021

In mixed cultures,* qteE *and* qscR *mutants are invaded by* lasR *mutants under cooperation-requiring conditions

We co-cultured the tagged *lasR* mutant with either the wild-type, the *qscR* mutant or the *qteE* mutant at a ratio of 1:100 in caseinate medium to assess how *lasR* cheaters would benefit from varied levels of cooperation. In individual culture, the *qscR* and *qteE* mutants reached stationary phase after 24 h while the wild-type took 48 h (Fig. A.2). Therefore, *lasR* mutant enrichment was assessed after 24 and 48 h. After 24 h, *lasR* mutant enrichment was significantly higher in *qscR* or *qteE* mutant co-culture than in wild-type co-culture (Fig. A.2). In contrast, after 48 h the enrichment of the *lasR* mutant was higher when co-cultured with the wild-type than when co-cultured with the *qscR* and *qteE* mutants (Fig. A.2). This is not too surprising given that the *lasR* mutants had more time to actively grow and exploit the slow-growing wild-type than the *qscR* and *qteE* mutants.

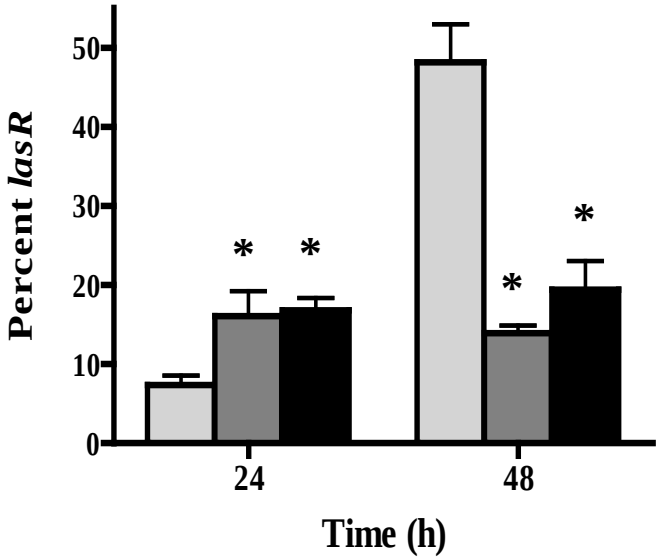

Fig. A.2: Invasion of *qscR* and *qteE* mutant cultures by the *lasR* mutant. Co-culturing of the *lasR* mutant with wild-type (light grey bars), *qscR* (dark hrey bars) and *qteE* (black bars) strains at a ratio of 1:100 in caseinate medium. The error bars indicate standard deviation of the mean of three replicates from three independent experiments. The asterisk indicates statistically significant difference from the wild-type ($P< 0.05$). In all cases, enrichment was significantly above the initial (1%).

In mixed cultures, the wild-type can invade* qscR *and* qteE *mutants under cooperation-requiring conditions but* qscR *and* qteE *mutants cannot invade the wild-type

To investigate whether the conditionally cooperating wild-type strain can also exploit the QS factors produced by constitutively cooperating *qscR* and *qteE* strains, we co-cultured the wild-type with either the *qscR* or the *qteE* mutant in caseinate medium at a ratio of 1:100 for 24 h. The wild-type enriched to around 7.5% (Fig. A.3A), suggesting that it behaved like a cheater.

Fig. A.3: Invasion of *qscR* and *qteE* mutant cultures by the wild-type. A. Co-cultures of wild-type with *qscR* and *qteE* mutants at a ratio of 1:100 ratio in caseinate medium. Percent enrichment of the wild-type after 24 h is shown. **B.** Co-cultures of *qscR* or *qteE* mutants with the wild–type at a ratio of 1:100 in caseinate medium. Percent enrichment of the mutants after 24 h is shown. **C**. A 3-way competition between the *qteE* mutant, wild-type (WT), and the *lasR* mutant (100:1:1) in caseinate medium. Percent enrichment of the individual strain after 24 h is shown. The error bars indicate standard deviation of the mean of three replicates from three independent experiments. In all cases, enrichment was significantly above the initial (1%).

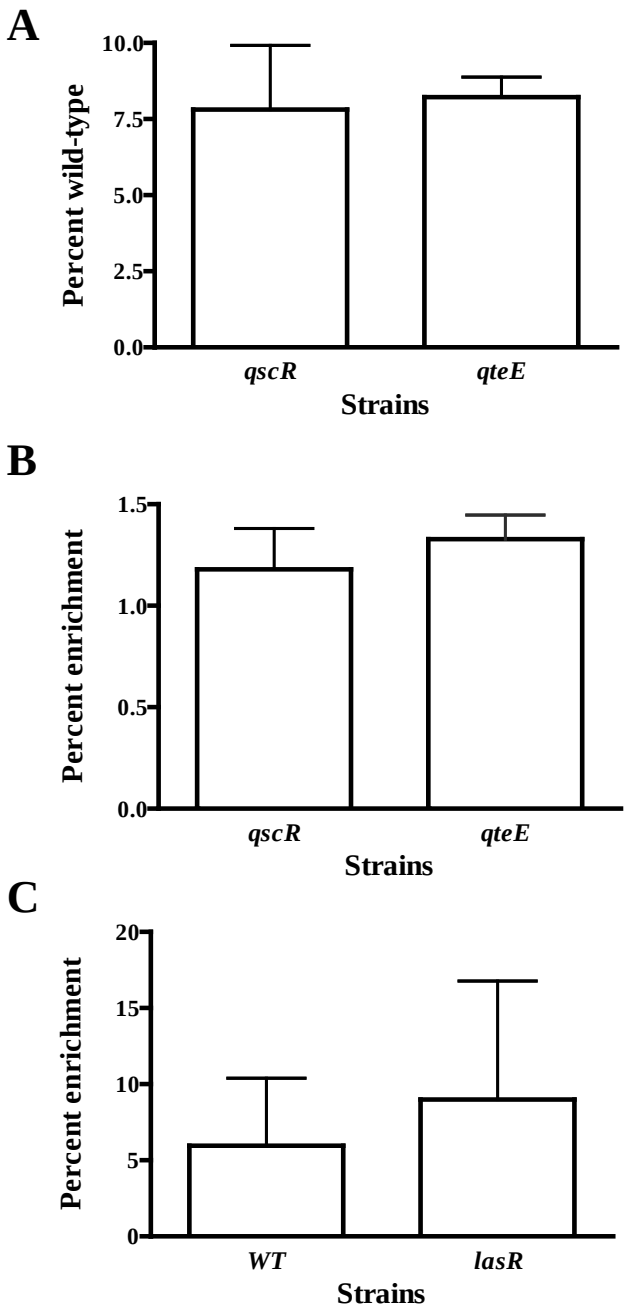

Fig. A.3: Invasion of *qscR* and *qteE* mutant cultures by the wild-type.

Conversely, when either the *qscR* or *qteE* mutants were inoculated at 1% in a wild-type culture, the mutants did not enrich (Fig. A.3B). These results indicate that conditional cooperation can invade constitutive cooperation. As both the *lasR* mutant and the wild-type can enrich in *qscR* and *qteE* mutant caseinate co-cultures, we performed a 3-way competition to evaluate the relative enrichment of the wild-type and the *lasR* mutant. The *lasR* mutant and the wild-type were inoculated at 1% in a *qteE* mutant culture, and enrichment was calculated after 24 h. The wild-type and the *lasR* mutant enriched to 7% and 9%, respectively (Fig. A.3C), which is in accordance with the 2-way co-cultures (Fig. A.2).

qteE *and* qscR *mutations are beneficial in inter-species competition*

Next, we assessed the benefit of constitutive cooperation in interspecific competition with another opportunistic human pathogen, *S. aureus* that often coexists with *P. aeruginosa* in the lungs of cystic fibrosis patients. We co-cultured *S. aureus* with either the wild-type, or *lasR*, *qscR*, or *qteE* strains at two different ratios, 50 % and 90 % initial frequency in MOPS-buffered LB medium for 12 h and determined the final *S. aureus* percentage. Individually, *P. aeruginosa* and *S. aureus* grew to similar culture densities in this medium. The final percentage of *S. aureus* was significantly lower when co-cultured with *qscR* and *qteE* mutants than when co-cultured with the wild-type or the *lasR* mutant (Fig. A.4). This is most likely due to the lack of QS-dependent extracellular factors such as LasA, an extracellular protease with staphylolytic activity, hydrogen cyanide, pyocyanin and 2-hydroxy-4-heptyl-quinolone-N-oxide (HQNO).

The *qscR* and the *qteE* mutants inhibited *S. aureus* more strongly than the wild-type at 90% than at 50% initial *S. aureus* frequency (Fig. A.4). This indicates that constitutively cooperating *P. aeruginosa* strains have a strong advantage over the conditionally cooperating strain, most likely due to early and high expression of QS-dependent factors.

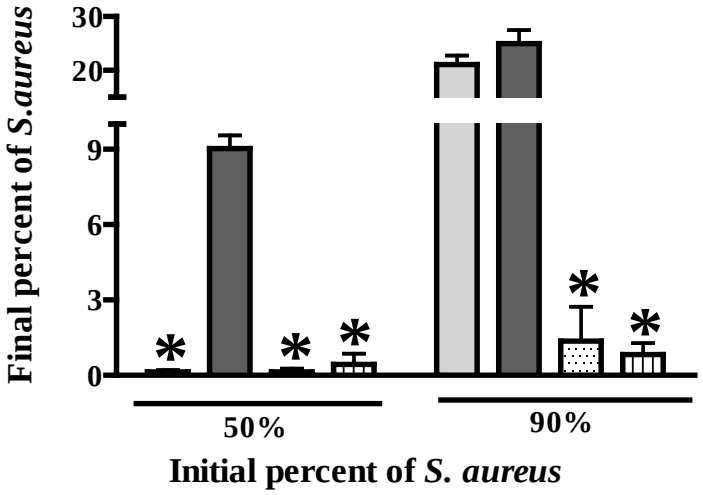

Fig. A.4: Interspecies competition. *S. aureus* was co-cultured with either the wild-type (light grey), or *lasR* (dark grey), *qscR* (dotted), and *qteE* (vertical lines) mutants in MOPS-buffered LB at a starting OD of 0.05 in MOPS buffered LB for 12 h. The final *S. aureus* percentage is shown. The data is from three replicates from three independent experiments and the error bars indicate the standard deviation of the mean. The asterisk indicates statistically significant difference from the *lasR* mutant ($P < 0.05$).

Discussion

P. aeruginosa QS is a cooperative behavior that regulates other generally more costly cooperative behaviors. It is generally difficult to explain the maintenance of costly cooperative behaviors over time as they are vulnerable to exploitation by cheaters. In this study, we tested the notion that QS evolved to optimize cooperative behaviors. We reasoned that if there was no QS control over cooperative traits, they would be expressed constitutively, which would be wasteful under conditions that do not favor QS. We tested this idea by using *qscR* and *qteE* mutants as examples of constitutive, unconditional cooperation. These mutants show advanced QS gene expression without the apparent quorum required for QS gene induction and therefore, have virtually constitutive production of QS factors (Siehnel *et al.*, 2010, Chugani *et al*., 2001).

Our data suggest that conditional expression of QS genes constitutes a trade off between high absolute (individual) and low relative fitness under conditions that favor cooperation. Constitutively cooperating strains showed higher fitness than the conditionally cooperating and the non-cooperating strain in individual caseinate cultures. On the other hand, however, they were exploited by both conditionally and non-cooperating strains (Fig. A.3A and A.2). Conditional cooperators were also exploited but by non-cooperators only. The conditionally cooperating wild-type strain showed resistance to invasion by *qscR* and *qteE* mutants (Fig. A.3B). Constitutive cooperation was also disadvantageous under conditions that do not favor cooperation. As expected, the *qscR* and *qteE* mutants had decreased fitness compared with the wild-type and the *lasR* mutant in individual casamino acids cultures, presumably

because of the metabolic burden from advanced QS gene expression (Fig. A.1B and A.1C). We further predict that these constitutively cooperating populations would be invaded by the conditionally cooperating and the non-cooperating strain in a co-culture under conditions that do not favor QS.

Conversely, we predicted that conditional cooperation might be disadvantageous to bacteria in interspecies competition. We demonstrated this with co-cultures of *P. aeruginosa* and *S. aureus*. Constitutively cooperating *P. aeruginosa qscR* and *qteE* mutants inhibited *S. aureus* more strongly than the conditionally cooperating wild-type (Fig. A.4). There was a significant decline in the number of *S. aureus* cells from the initial level indicative of their killing by *P. aeruginosa*. Notable *P. aeruginosa* QS-dependent factors involved in this process are LasA protease and HQNO. LasA causes lysis of *S. aureus* cells (Kessler *et al*., 1993), and HQNO impairs *S. aureus* growth by inhibiting respiration (Machan *et al.*, 1992). We observed small colonies of *S. aureus* during the co-culturing of the two and especially when co-cultured with constitutively cooperating *qteE* and *qscR* mutants. These small colony variants of *S. aureus* are known to occur in the presence of HQNOs produced by *P. aeruginosa* (Hoffman *et al.*, 2006). Other QS-controlled factors involved in killing are likely hydrogen cyanide and pyocyanin (Voggu *et al.*, 2006).

The absolute and relative fitness benefits of conditional cooperation, constitutive cooperation and no cooperation under social and non-social conditions are summarized in Fig. A.5.

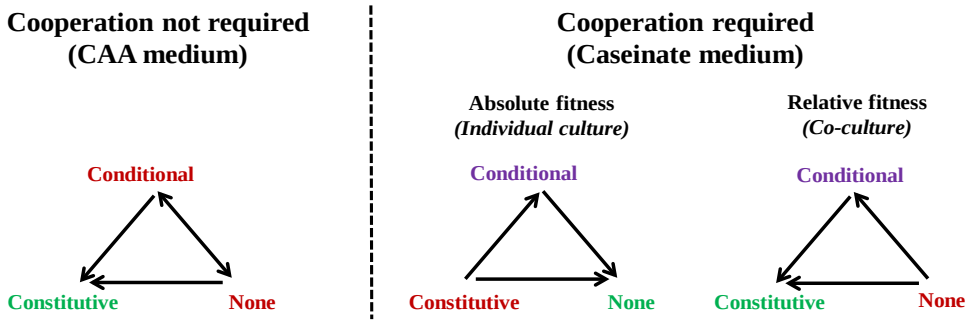

Fig. A.5: Evolutionary game between different QS populations. The color indicates which of the populations are favored in a particular condition. Red: favored/wins, green: loses, purple: intermediate.

Conditional cooperation appears as a compromise between constitutive cooperation and no cooperation that is most beneficial under frequently changing environmental conditions. This is consistent with the observation that QS-regulated control of virulence genes is less common in obligate intracellular pathogens than in facultative pathogens like *P. aeruginosa* (Duerkop *et al*., 2009). Facultative, opportunistic pathogens generally experience more dramatic life-style changes than obligate pathogens, transitioning between host-associated and free-living states. The selective pressures acting upon QS under laboratory growth conditions that favor cooperation are likely similar to those encountered in QS-dependent infections. Taken together, our results provide support for the idea that QS evolved as a strategy to optimize more costly cooperative behaviors.

Acknowledgements

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Appendix B

Global position analysis of the *Pseudomonas aeruginosa* quorum-sensing transcription factor LasR

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Abstract

In *Pseudomonas aeruginosa* quorum sensing (QS), the transcriptional regulator LasR controls the expression of more than 300 genes. Several of these genes are activated indirectly via a second, subordinate QS regulator, RhlR. Conserved sequence elements upstream of individual other genes have been shown to bind LasR *in vitro*. To comprehensively identify all regions that are bound by LasR *in vivo*, we employed chromatin immunoprecipitation in conjunction with microarray analysis. We identified 35 putative promoter regions that direct the expression of up to 74 genes. *In vitro* DNA binding studies allowed us to distinguish between cooperative and non-cooperative LasR binding sites, and allowed us to build consensus sequences according to the mode of binding. Five promoter regions were not previously recognized as QS-controlled. Two of the associated transcript units encode proteins involved in the cold-shock response and in Psl exopolysaccharide synthesis, respectively. The LasR regulon includes seven genes encoding transcriptional regulators, while secreted factors and secretion machinery are the most overrepresented functional categories overall. This supports the notion that the core function of LasR is to coordinate the production of extracellular factors, although many of its effects on global gene expression are likely mediated indirectly by regulatory genes under its control.

Introduction

Quorum sensing (QS) allows populations of bacteria to communicate via the exchange of chemical signals, resulting in coordinated gene expression in response to cell density (Bassler, 2002, Taga & Bassler, 2003, Williams, 2007). This process has been extensively studied in *Pseudomonas aeruginosa*, a Gram-negative environmental bacterium capable of causing acute and chronic infections in immunocompromised individuals. In this organism, QS controls the expression of numerous virulence factors including extracellular enzymes and toxins (Bjarnsholt & Givskov, 2007, Girard & Bloemberg, 2008, Rumbaugh *et al.*, 2000, Smith & Iglewski, 2003).

The *P. aeruginosa* QS circuitry is comprised of two complete systems, LasR-LasI and RhlR-RhlI. LasI and RhlI are acyl-homoserine lactone (acyl-HSL) synthases, producing 3-oxo-dodecanoyl (3OC12) HSL and butanoyl (C4) HSL respectively, while LasR and RhlR are the transcriptional regulators which bind to their cognate signals to activate target gene expression (Fuqua & Greenberg, 2002, Whitehead *et al.*, 2001). Both systems are connected in a hierachical fashion, as LasR, under standard culture conditions, controls the expression of *rhlI* and *rhlR* (Latifi *et al.*, 1996, Medina *et al.*, 2003a, Pesci *et al.*, 1997). Target genes respond to each system with varying degrees of specificity (Schuster *et al.*, 2003, Whiteley & Greenberg, 2001, Whiteley *et al.*, 1999). LasR and RhlR have been shown to recognize conserved palindromic sequences, *las*-*rhl* box sequences, of individual QS-controlled promoters, and more such sites have been predicted upstream of other QS-controlled genes (Anderson *et al.*, 1999, Pessi & Haas, 2000, Rust *et al.*, 1996, Schuster *et al.*, 2003, Wagner *et al.*,

2003, Whiteley & Greenberg, 2001, Whiteley *et al*., 1999). *In vitro* binding studies with purified LasR showed that some promoters bind LasR cooperatively, while others bind LasR non-cooperatively (Schuster *et al.*, 2004b). Microarray analyses revealed that *P. aeruginosa* QS constitutes a global regulatory system in which the LasRI and RhlRI systems govern the expression of hundreds of target genes (Hentzer *et al.*, 2003, Schuster *et al*., 2003, Wagner *et al*., 2003), many of which encode central metabolic functions. In addition, acyl-HSL QS is highly interconnected with other cellular regulatory pathways, resulting in a complex network with the potential to integrate and respond to a multitude of environmental signals (Juhas *et al.*, 2005, Schuster & Greenberg, 2006, Venturi, 2006).

The purpose of this study was to obtain further insights into the complexity of the QS network by separating direct from indirect regulatory effects. Such information is desirable to fully appreciate the mechanism of action of novel anti-virulence strategies that target QS. Here we report the genome-wide identification of direct targets of the central QS regulator LasR using chromatin immunoprecipitation in conjunction with DNA microarray analysis (ChIP-chip) (Aparicio *et al.*, 2005, Buck & Lieb, 2004). LasR-target promoter interactions were independently confirmed by electrophoretic mobility shift assays (EMSA) and transcriptional reporter fusions. EMSA also allowed us to sort promoters according to the mode of binding, and to define putative LasR binding sites. Our experiments confirmed known members of the LasR regulon, and identified five novel LasR-regulated promoters.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains used in this study include the *Pseudomonas aeruginosa* PAO1 wild-type, the *P. aeruginosa* mutants *lasR*::Gm^R, *rhlR*::Gm^R, *lasR*:: Tc^R *rhlR*::Gm^R (Rahim *et al.*, 2001, Schuster *et al.*, 2003), and *Escherichia coli* DH5α (Invitrogen, CA). All strains were cultured in Lennox LB medium at 37°C. Where indicated, LB was buffered with 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7.0. Antibiotics, when required, were used as follows: *P. aeruginosa*, 200-300 μg/ml carbenicillin; *E. coli*, 100 μg/ml ampicillin, 10 μg/ml gentamicin.

Chromatin immunoprecipitation

A chromatin immunoprecipitation protocol was developed for *P. aeruginosa* based on published procedures (Horak & Snyder, 2002, Molle *et al.*, 2003a, Molle *et al.*, 2003b), and a protocol available from Roche Nimblegen Technical Services (www.nimblegen.com). Details are as follows: Bacteria were grown in 40 ml of buffered LB to early stationary phase (OD_{600} = 2.0). Cells were inoculated from mid-logarithmic phase cultures to initial optical densities of 0.01. The experimental strain was *P. aeruginosa* PAO1, and the non-specific control strain was an isogenic *lasR*, *rhlR* double mutant. Ten ml aliquots were cross-linked by the addition of formaldehyde to a final concentration of 1% and incubation at room temperature for 15 min with gentle agitation. The cross-linking reaction was quenched by the addition of fresh glycine to a final concentration of 125 mM and incubation at room

temperature for 10 min. Next, the cells were washed twice in 10 ml of cold 1xTBS (20 mM Tris-HCL, pH 7.5, 150 mM NaCl) and the pellets were frozen at -80°C. After thawing, cells were resuspended in 1 ml immunoprecipitation (Diggle *et al.*) buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholic acid) supplemented with 250x diluted Protease Inhibitor Cocktail (Set III, Novagen) and 1 mg/ml lysozyme. The suspension was incubated at 37°C for 15 minutes. Samples were chilled on ice and sonicated three times for 10 seconds each using a Branson microtip sonicator at an output of 0.4 with no pulse. A 25 μl aliquot from the cleared lysate was set aside as the total DNA control.

A 20 μl aliquot was retained for size analysis by agarose gel electrophoresis to ensure that fragments were in the 300 – 1000 bp range. To prepare samples, they were treated with SDS, Proteinase K, RNaseA, extracted with phenol-chloroform, and precipitated with ethanol as described for IP samples below.

The remaining supernatant was pre-cleared by incubation with one-tenth volume of a 50% protein A Sepharose slurry (nProtein A 4 Fast Flow, Amersham Biosciences) for 1 hour at 4°C. LasR-DNA complexes were precipitated by the addition of 6 μg of affinity-purified LasR rabbit polyclonal antibody to 850 μl of the supernatant and incubation on ice overnight. Then, 50 μl of the 50% slurry were added and incubated at 4°C for 1 h. Subsequently, the complexes were washed five times for 10 min each: twice with IP buffer, and once with each of Buffer II (50mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% deoxycholic acid), Buffer III (10 mM Tris-HCl pH 8.0, 250 mM LiCl, 1 mM EDTA, 0.5% deoxycholic

acid, 0.5% Nonidet P-40), and 1xTBS. Finally, the slurry was resuspended in 100 μl of elution buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% SDS) and incubated at 65°C for 15 min to elute the antibody-protein-DNA complexes from the beads. The supernatant was transferred to a new microcentrifuge tube and the beads were washed with another 100 μl of elution buffer, which was added to the eluate. Cross-links between the protein and DNA were reversed by incubating the eluate at 65°C overnight. The total DNA control sample set aside earlier was also incubated with 175 μl of elution buffer overnight at 65°C. To further remove the protein, 200 μl TE supplemented with 100 ug proteinase K and 20 μg glycogen were added to each sample and incubated at 37°C for 2 h. DNA was purified using phenol-chloroform-isoamyalcohol extraction and ethanol precipitation. All samples were resuspended in 30 μl TE containing 0.33 μg/μl RNase A and incubated at 37°C for 2 h. The DNA was then purified using the QIAquick PCR Purification Kit (QIAGEN) and eluted in 50 μl of elution buffer. Blunting of DNA ends, ligation of linkers, and first-round linker-mediated PCR, was performed according to the Roche NimbleGen Chromatin Immunoprecipitation and Amplification protocol, Sections 8 – 10.

Microarray processing and data analysis

P. aeruginosa whole-genome microarrays, including all intergenic regions, were custom-made by Nimblegen and consisted of 60-mers tiled with a spacing of 30 bp. The ChIP DNA and control input DNA were differentially labeled, hybridized to one array, and scanned. Separate arrays were used for wild type (specific) and *lasR rhlR* mutant (non-specific) samples. These steps were performed by Nimblegen. The raw data were treated as follows (Kim *et al.*, 2005). Intensity data (signals) were normalized within each array and also across all arrays, using Loess normalization. This procedure resulted in comparable array signals for the entire data set in terms of mean signal and variance. After this two-step normalization, replicate probes were fit and averaged using a linear model and Bayes Statistics. A Bayes statistical approach uses a weighted t-statistics that also considers within-treatment variation, which is particularly suitable for a low number of replicates. The non-specific signals were then subtracted from the specific signals to eliminate non-specific effects. This signal difference represents a net-enrichment resulting from specific binding of the respective transcription factor. The so-processed data were viewed in Nimblegen's SignalMap software, which provides a graphical representation of the hybridization data and allows straight-forward identification of candidate transcription factor binding sites. To assign confidence values to these differentially enriched sites, we performed a two-tailed Student's *t*-test of five consecutive probes between specific and non-specific signals. We considered those sites as significantly enriched that showed $p<0.0001$ and an enrichment ratio >2-fold.

As mentioned above, we used a *lasR, rhlR* double mutant as a non-specific control. We chose this mutant instead of a *lasR* single mutant because our initial goal was to identify genes directly controlled by LasR and RhlR. The *lasR, rhlR* mutant was intended as a single, economical control of non-specific enrichment for ChIP with either LasR-specific antibody or RhlR-specific antibody. We decided to not pursue the RhlR-ChIP further at this time, because we have not been able to purify soluble, active RhlR for confirmation of ChIP-chip results by EMSA. Regardless, the use of a *lasR* single mutant as a control is expected to yield results identical to those of a double mutant, because under standard growth conditions such as those used for ChIP-chip, the *las* system strictly controls expression of the *rhl* system (Latifi *et al*., 1996, Medina *et al*., 2003a, Pesci *et al*., 1997). A potential problem with the use of any negative control that does not express *rhlR* is that it would not be able to detect any non-specific binding of the LasR-ChIP antibody to RhlR, if it occurred. Importantly, however, our affinity-purified antibody did not cross-react with RhlR (see below), and LasR-ChIP did not enrich known RhlR-specific promoter fragments, eliminating such concerns. The same is true for QscR, the third LuxR-type regulator in *P. aeruginosa*.

Quantitative PCR

ChIP replicates were also analyzed by quantitative real-time PCR using the Applied Biosystems 7300 sequencing system as described (Sandoz *et al.*, 2007, Schuster & Greenberg, 2007). Eight promoter fragments (*rsaL*, PA0026, PA2069, PA2305, PA2763, PA4117, PA4305/6, PA5184), and fragments internal to the *lasB*, PA0996,

PA1914, and PA4677 genes were selected for analysis by qPCR. The latter served as background controls. Primers were designed such that the amplified sequence overlapped with the peak ChIP-chip signal. Experimental conditions for culture growth, immunoprecipitation, and further sample processing were identical to those described above. Specific enrichment was calculated as the difference in the critical threshold (ΔCt) of immunoprecipitated DNA from the wild type and the *lasR rhlR* mutant, normalized to the ΔCt of total, non-immunoprecipitated DNA. This approach assumed a direct correlation between Ct and DNA template abundance, which was validated by quantifying the relative abundance of individual templates at defined dilutions using the standard curve method. A baseline was established by calculating the average enrichment of four control fragments, which was subtracted from the specific enrichment. Enrichment was considered significant if the resulting value was > 2-fold, and if the resulting value was > 2 standard deviations above the average enrichment of the background control fragments (Kim *et al.*, 2007). All qPCR reactions were performed in duplicate for two independent ChIP samples.

Affinity purification of antibody

LasR polyclonal rabbit antiserum was purified by pre-adsorption and affinity chromatography. First, clarified antiserum was pre-adsorbed by incubation with an equal volume of the soluble, filtered fraction (20 x concentrate) of a stationary-phase culture of PAO *lasR*::Gm^R. After centrifugation, the supernatant was applied to a Sulfolink column (Pierce) to which purified LasR (Schuster *et al.*, 2004b) had been

cross-linked. Column preparation, washing, and elution were according to manufacturer's instructions. The quality and specificity of the purified antibody was assessed by western blotting (data not shown). A single band (LasR) was detected in whole-cell lysates of PAO1 wild type cultures, and there was no cross-reactivity with RhlR or with QscR.

Electrophoretic mobility shift assays

Gel-shifts were performed as previously described (Schuster *et al.*, 2004b) with the following modifications. Each reaction contained 25 pM of either a specific or a non-specific DNA probe. In total, 17 promoters were PCR-amplified with fragment sizes ranging from 269 bp to 301 bp. The 146 bp *lasB* intergenic fragment was used as a non-specific control. The location of all fragments (excluding controls) was chosen such that it overlapped with the peak ChIP-chip signal. Promoters of categories II and III, on which the calculation of a false-positive rate is based, were randomly chosen for EMSA analysis.

Promoter fusion construction and measurements

LacZ reporter fusions were constructed as follows. Upstream regulatory regions of genes PA1159 and PA2231 were PCR-amplified from PAO1 genomic DNA, with primer pairs containing engineered *Nco*I/*Hind*III restrictions sites. PCR fragments were cloned into the broad-host-range, low copy-number plasmid pQF50 (Farinha & Kropinski, 1990) using identical restriction sites. This plasmid contains a promoterless

lacZ gene, allowing for the construction of transcriptional reporter fusions. Constructs were verified by DNA sequencing, and were transformed into the respective *P. aeruginosa* strains as described (Chuanchuen *et al.*, 2002). An *rsaL-lacZ* fusion in pQF50 (pMW312) had been constructed previously (Whiteley & Greenberg, 2001).

Mid-logarithmic phase cultures of the respective strains grown in MOPS-buffered Lennox LB were diluted to an OD_{600} of 0.05 in the same medium and grown for 8 h, shaking, at 37ºC. After 8 h, culture aliquots were withdrawn and β-galactosidase activity measured in a microplate reader (Infiniti M200, Tecan) using the Galacton Plus reagent (Applied Biosystems) as described (Whiteley *et al.*, 1999). Reported values are based on three independent biological replicate experiments. Statistical significance was determined by a two-tailed Student's *t*-test. Prior to averaging, background expression from a strain containing pQF50 without a promoter was subtracted from the individual expression values obtained with each promoter fusion construct.

A two-plasmid system was used to assess heterologous gene expression in *E. coli* DH5α as described (Lee *et al.*, 2006).

Identification of conserved sequence motifs

The program CONSENSUS (Hertz & Stormo, 1999) was used to identify a LasR consensus sequence within the sets of ChIP-chip sites indicated in Fig. B.4. The entire intergenic region was used as input sequence for CONSENSUS if the precise binding site had not been mapped by footprinting. In those cases where footprinting data were

available, the mapped sequence was chosen. To search for a consensus sequence within cooperatively bound sites, we seeded with the consensus identified for all ChIP-chip sites, because no seeding only yielded AT-rich regions of low complexity. Consensus motifs were visualized by WEBLOGO v.3 (Crooks *et al.*, 2004). The program PATSER (Hertz & Stormo, 1999) was used to assign statistical significance to the sequences identified by CONSENSUS. The weight matrix generated with CONSENSUS was used as input.

Results

Identification of direct LasR targets in vivo

While results from microarray studies indicated that at least 6% of the *P. aeruginosa* genome is regulated by LasRI and RhlRI QS (Schuster et al., 2003, Wagner et al., 2003), many may be indirect targets. We employed ChIP-chip to identify direct targets of LasR *in vivo*. We used early-stationary phase *P. aeruginosa* cultures grown in buffered Lennox LB. These conditions are identical to our previous transcriptome analysis, allowing direct comparison of data sets, and they result in high-level induction of QS target genes (Schuster *et al.*, 2003). We chose the wild type strain expressing *lasR* in its native context to capture protein-DNA interactions under physiologically relevant conditions. The results from two independent ChIP-chip experiments are averaged and presented in Table B.1. In total, 35 sites of the *P. aeruginosa* genome had enrichment values considered significant (average enrichment > 2-fold; p-value <0.0001). All of these sites were in intergenic regions upstream of

open reading frames, consistent with LasR's function as a transcriptional activator. Thus, these sites constitute putative LasR-dependent promoters.

Table B.1: The LasR regulon

Region detected[a]	Enrich. factor[b]	Transcript data[c] +C12 / +C12&C4	EMSA pattern[d]	New LasR binding sequence[e]	Independent evidence[f]	Function[g]
Category I – previously identified as QS-controlled and previously known to be directly regulated by LasR						
PA1003* (*mvfR*)	2.4	8.1 / 6.6	-	CTAACAAAAGACATAG (-534 to -519; TS1 -248.5)	*lacZ* fusion, mutagenesis (Xiao *et al.*, 2006)	Transcriptional regulator
PA1431* (*rsaL*) →	7.8	350 / 340	NC	CTAGCAAATGAGATAG (-74 to -59; TS *lasI* -40.5)	EMSA (Schuster *et al.*, 2004b)	Regulatory protein
PA1432* (*lasI*)		-				Autoinducer synthesis protein
PA2591* (*vqsR*) →	3	21 / 25	NC	CTACCAGAACTGGTAG (-112 to -97; TS -54.5)	EMSA (Li *et al.*, 2007)	Probable transcriptional regulator
PA2592* (*potF*5) - PA2593		5.6 / 8.7				Probable periplasmic spermidine//putrescine-binding protein
PA3104* (*xcpP*) - PA3105	6.4	3.1 / 3.2	-	CCGTCAGTATTGTTAG (-157 to -142)	H-H system (Chapon-Herve *et al.*, 1997)	Secretion protein

Continued on next page

Table B. 1: The LasR regulon

Region detected[a]	Enrich. factor[b]	Transcript data[c] +C12 / +C12&C4	EMSA pattern[d]	New LasR binding sequence[e]	Independent evidence[f]	Function[g]
PA3477 (*rhlR*)	7.8	8.5 / 9.6	NC	CTGGCATAACAGATAG [i] (-118 to -103; TS1 -86.5)	H-H system (Pesci *et al.*, 1997)	Transcriptional regulator
PA3479 (*rhlA*) - PA3478	3.6	10 / 120	N	CTGTGAAATCTGGCAG (-278 to -263; TS -40.5)	H-H system (Pearson *et al.*, 1997)	Rhamnosyl-transferase chain A
PA3724 (*lasB*)	3.2	110 / 180	C	CCTGCTTTTCTGCTAG (OP2 -252 to -237; TS -102.5)	EMSA (Schuster *et al.*, 2004b)	Elastase
PA3904-PA3908	8.5	140 / 130	NC	TGAGCAGTTCAGATAG (-75 to -60)	EMSA (Schuster *et al.*, 2004b)	Hypothetical protein
Category II – previously identified as QS-controlled but not previously known to be directly regulated by LasR						
PA0026 (*plcB*) - PA0028	6.9	5.8 / 7.5	N	CCATCTGACATGTAGG [j] (-69 to -54)	-	Phospholipase
PA0122	2.4	13 / 36	-	CTACCAGATCTGGCAG (-163 to -148)	-	Conserved hypothetical protein
PA0143 (*nuh)*	3.8	4.7 / 4.7	-	CTGTCGGTTCGCATAG (-73 to -58)	-	Nonspecific ribonucleoside hydrolase
PA0144	5.7	1.5 / 19	-	CTGCCCATAATCCCCG (-67 to -52)	-	Hypothetical protein

Continued on next page

Table B. 1: The LasR regulon

Region detected[a]	Enrich. factor[b]	Transcript data[c] +C12 / +C12&C4	EMSA pattern[d]	New LasR binding sequence[e]	Independent evidence[f]	Function[g]
PA0855*	4.1	2.4 / 2.5	-	CTATCAGTTCTGTAGT (-81 to -66)	-	Hypothetical protein
PA0996* (*pqsA*) - PA1000	2.4	220 / 90	N	CTGTGAGATTTGGGAG (-389 to -374; TS -310.5)	-	Probable coenzyme A Ligase
PA1245 (*aprX*) - PA1248	3.8	8.6 / 10	NC	CTGGCAGAACTGACAG- (-337 to -322)		Hypothetical protein
PA1656-PA1659	2.2	5.9 / 15	-	CTACCTGTTTTGGTAG (-301 to -286)	-	Hypothetical protein
PA1914 (*hvn*)	3.2	42 / 190	NC	CTACCCACCCTGTTCG [k] (-289 to -274)	-	Conserved hypothetical protein
PA2081* (*kynB*) – PA2080	12	3.3 / 4.3	-	CTATCAGAATGAATAG (-123 to -108)	-	Kynurenine formamidase
PA2305-PA2302	6.6	52 / 51	C	CTATCAATTGTATTAG (-83 to -68)	-	Probable non-ribosomal peptide synthetase
PA2426* (*pvdS*)	4.3	NDE[h]	-	CTATCCATTCTGATGG (-428 to -413)	-	Sigma factor PvdS
PA2587* (*pqsH*) →	7	12/12	NC	CTCGATCTTCTGATAG (-123 to -108)	-	Probable FAD-depend. mono-oxygenase

Continued on next page

Table B. 1: The LasR regulon

Region detected[a]	Enrich. factor[b]	Transcript data[c] +C12 / +C12&C4	EMSA pattern[d]	New LasR binding sequence[e]	Independent evidence[f]	Function[g]
PA2588*		15 / 22				Probable transcriptional regulator
PA2763*	3.5	NDE[h]	N	-[i]	-	Hypothetical protein
PA2939* (*pepB*)	7.5	38 / 42	-	CCGACAAATCTGTGAC (-344 to -328)	-	Probable amino-peptidase
PA3326*	5.6	6.6 / 20	C	CTTACAAATCTGTTAG (-238 to -223)	-	Probable Clp-family ATP-dependent protease
PA3535* (*eprS*)	6.5	7.5 / 8.1	-	CTGGCCGATGGGCGCG (-150 to -135)	-	Probable serine protease
PA4117	2.7	5.3 / 5.6	N	CCTGGAGACCAGGCTG[j] (-24 to -9)	-	Probable bacteriophytochrome
PA4306* (*flp*)	2.7	10/16	N	CTATGCGTCCGGACAG (-396 to -381)	-	Type IVb pilin
PA4778 (*ybbI*)	3.2	5.4 / 4.9	C	CTTCGCAACCTCCCAG[k] (-25 to -10)	-	Probable transcriptional regulator
PA5232 (*yhiI*)	4	NDE[h]	-	CTATCCGTTCTGTCCC (-200 to -185)	-	Conserved hypothetical protein
Category III – newly identified						
PA0805* →	2.6	NDE	-	GTACCTGAACTGCCAG (-149 to -134)	-	Hypothetical protein
PA0806*		NDE				Hypothetical protein

Continued on next page

Table B. 1: The LasR regulon

Region detected[a]	Enrich. factor[b]	Transcript data[c] +C12 / +C12&C4	EMSA pattern[d]	New LasR binding sequence[e]	Independent evidence[f]	Function[g]
PA1159	5.3	NDE	NC	TTTTCAGTTGGACTCG [j,k] (-257 to -242)	-	Probable cold-shock protein
PA2231 (*pslA*) – PA2242	2.1	NDE	C	CGCGTCAGATTTCCTCG[k] (−162 to −147)-	-	Probable glycosyl transferase
PA3384* (*phnC*) →	3.9	NDE	-	CTATCTGAAATGCGAG (−199 to −184)	-	ATP-binding component of ABC phosphonate transporter
PA3385* (*amrZ*)		NDE				Alginate and motility regulator
PA5184	6.6	NDE	N	CAATCAGAAATGTCCC [j] (-114 to -99)	-	Hypothetical protein

a. LasR-ChIP enriched regions are indicated by the PA number of the associated gene or operon. The respective gene name (first gene only if in an operon) is indicated in parentheses. Enriched regions which are between divergently expressed genes are indicated by an asterisk (*); for Categories I and II, only the direction that shows differential expression by transcriptomics is shown. If both directions are differently expressed, then an arrow (→) indicates that the subsequent, indented gene is associated with the same enriched region.

b. Average fold-enrichment of wild type vs. *lasR rhlR* mutant ChIP DNA from two independent experiments, *p*-value <0.0001.

c. Maximum fold-change in gene expression in the *lasI, rhlI* double mutant with the addition of either 3OC12-HSL or both 3OC12-HSL and C4-HSL *vs.* no acyl-HSL, taken from Schuster *et al*., 2003.

d. Modes of binding in EMSA are cooperative binding (C), non-cooperative binding (NC), and no binding (N). A "-" indicates that no evidence is available.

e. *las*-box sequences as identified by CONSENSUS (see *Experimental Procedures*). Start and stop positions of each sequence are indicated in parentheses relative to the translational start site. In addition, the centered position of the sequence is given relative to the transcriptional start (TS), where available. In cases where multiple TS sites have been identified, only the most relevant site is indicated. Information on TS sites is based on the following references: PA0996 (McGrath *et al.*, 2004), PA1003 (Wade *et al.*, 2005), PA1432 (Seed *et al.*, 1995), PA2591 (Li *et al.*, 2007), PA3477 (Medina *et al.*, 2003a), PA3479 (Pearson *et al.*, 1997), PA3724 (Rust *et al.*, 1996).

f. Independent experimental evidence for direct interaction of LasR with target promoters shown by promoter fusions in heterologous host (H-H) systems, mutagenesis of predicted binding sites, or EMSA.

g. The annotated function of ChIP-chip genes was taken from *www.pseudomonas.com*. For promoters upstream of operons, only the function of the first gene is indicated.

h. NDE: Not differentially expressed in Schuster *et al.*, 2003, but identified in other studies: PA2426 (Whiteley *et al.*, 1999), PA2763 and PA5232 (Yarwood *et al.*, 2005).

i. Sequence identified does not match the previously published *las-rhl* box sequence for this region.

j. Sequence identified was not considered significant according to PATSER (weight score <5, p-value >0.0005).

k. CONSENSUS analysis of only cooperative/non-cooperative binding regions identified different sequences: PA1159, TAGCGAAAACATATCG (-257 to -242); PA1914, CACCCTGTTCGTATCT (-284 to -269); PA2231, CTAAGATAGCTATCAC (-162 to -147); PA4778, GAAAGGGATAGGCTAG (-44 to -29). All sequence except for PA2231 were considered significant by PATSER.

l. This sequence was not included in CONSENSUS analysis as it was determined to be a false-positive.

Thirty ChIP-chip enriched regions are associated with genes that transcription studies identified as QS-controlled (Hentzer et al., 2003, Schuster et al., 2003, Wagner et al., 2003, Whiteley et al., 1999, Yarwood *et al.*, 2005). We divided these regions into two categories, based on whether independent evidence for direct regulation by LasR is available (Category I) or is not available (Category II). Several ChIP-chip regions are upstream of divergently expressed genes or operons. In some cases only one direction of transcription is QS-controlled, while in other cases both directions are QS-controlled (Table B.1). Overall, the 30 ChIP-chip regions are associated with 56 QS-controlled genes. The most overrepresented functional classes are, as expected, *secreted factors* and *protein secretion/export apparatus*, although *biosynthesis of cofactors, prosthetic groups and carriers, adaptation and protection*, and *transcriptional regulators* are also overrepresented (Fig. B.1).

Fig. B.1: Functional classification of genes directly regulated by LasR. Included are those genes which are associated with ChIP-chip enriched promoters and which have been previously identified as QS-controlled. Values represent the percentage of genes enriched by LasR-ChIP within the respective class. The open bar shows the percentage of all 56 ChIP-enriched genes. Thus, classes with percentage values higher than the open bar are over-represented within the genes directly controlled by LasR. The most over-represented classes are shown in black.

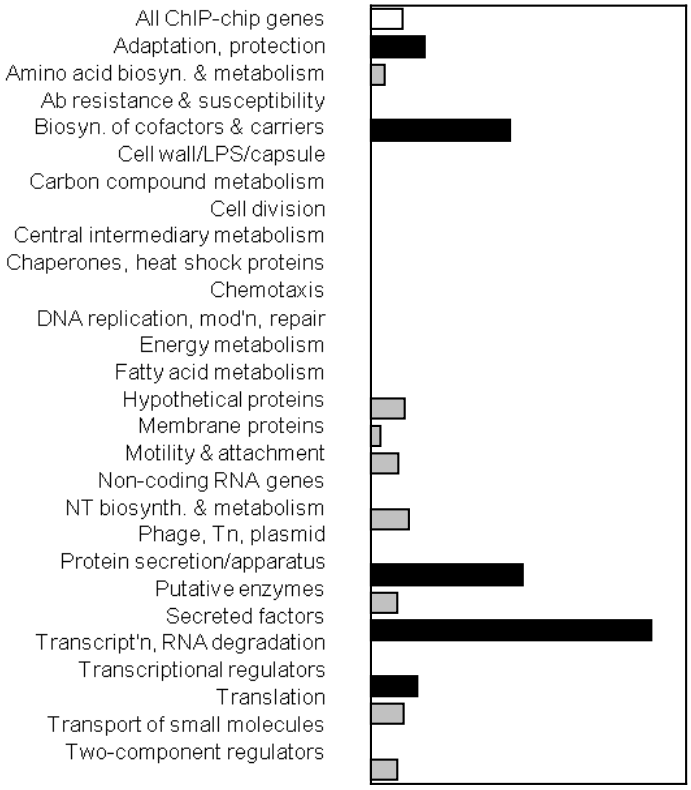

Fig. B.1: Functional classification of genes directly regulated by LasR.

In order to estimate the fraction of *las*-activated promoters that are directly regulated by LasR, we re-interrogated our transcriptome data (Schuster *et al.*, 2003). Among the set of 315 QS-activated genes, we identified 195 as *las*-activated. This includes genes that only respond to LasR-3OC12-HSL, and those that respond to LasR-3OC12-HSL, but respond better to both LasR-3C12-HSL and RhlR-C4-HSL. We predicted that these genes are organized into 114 transcript units, which are controlled by 104 promoters (10 bi-directional promoters), and we identified conserved *las-rhl* box sequences in 29 of those promoters (Schuster *et al.*, 2003).

Twenty-five out of the 30 ChIP-chip regions, then, are associated with *las*-activated promoters. Among the five remaining promoters, two (PA0144 and PA0855) also respond to LasR-3OC12-HSL, albeit below the cut-off chosen in the transcriptome study (≥2.5-fold induction), and three (PA2426, PA2763, and PA5232) were only identified in other studies (Table B.1). Therefore, 24% (25 out of 104) of all *las*-activated promoters identified in our transcriptome study appear to be regulated by LasR directly, and 10 contain a *las-rhl* box sequence as defined previously.

A small set of the QS target genes identified by microarray analysis have been previously shown to be regulated by LasR directly. The respective promoters should therefore be among the sites enriched by ChIP-chip. Thirteen promoter regions, controlling 43 genes, have been previously identified as direct targets of LasR either by mutagenesis of predicted promoter binding sites, heterologous expression, or biochemically using purified protein. These promoters include *hcnABC*, *lasB*, *lasI/rsaL*, *mvfR (pqsR)*, *rhlI*, *rhlR*, *rhlAB*, *qsc117* (PA1869), *vqsR*, *xcpP/xcpR*, PA0572, PA3904, and PA4677 (de Kievit *et al.*, 2002, Latifi *et al*., 1996, Li *et al.*, 2007, Pearson *et al.*, 1997, Pesci *et al.*, 1997, Pessi & Haas, 2000, Schuster *et al.*, 2004b, Whiteley & Greenberg, 2001, Xiao *et al.*, 2006). The primarily *rhl*-responsive promoter *rhlAB*, which does not bind LasR *in vitro* (Schuster *et al.*, 2004b), was included here because it shows significant activation by LasR-3OC12-HSL not only in *P. aeruginosa* (Schuster *et al.*, 2003), but also in a heterologous host system (Medina *et al.*, 2003c, Pearson *et al.*, 1997). We identified nine of the 13 regions by ChIP-chip (Table B.1, Category I). The promoter region of *las*-specific gene PA0572, which

binds purified LasR *in vitro* (Schuster *et al.*, 2004b), showed enrichment but at levels below the cut-off (p-value <0.0001 but enrichment only 1.9-fold) . The promoter region of PA1869 showed no appreciable enrichment, but it is in fact primarily *rhl*-responsive and is only mildly activated by LasR-3OC12-HSL (Schuster *et al.*, 2003). Curiously, the *rhlI* and *hcnABC* promoters also did not show any enrichment. These genes show substantial activation by LasR-3OC12-HSL alone although they require both LasR-3OC12-HSL and RhlR-C4-HSL for full activation (de Kievit *et al*., 2002, Pessi & Haas, 2000, Schuster *et al*., 2003).

Analysis of the ChIP-chip data also revealed five promoters for which there is no previous evidence of QS-dependent regulation (Category III). These five regions are upstream of 18 putative novel quorum-sensing genes. One of these promoter regions, PA2231, is upstream of the *psl* gene cluster, which has been shown to play a role in biofilm formation (Friedman & Kolter, 2004, Jackson *et al.*, 2004, Matsukawa & Greenberg, 2004). Additionally, as two regions are upstream of divergently expressed genes, it is possible that one or both directions are under the control of LasR.

Analysis of binding characteristics in vitro

To further confirm the ChIP-chip results, a subset of promoter regions was selected for *in vitro* DNA binding analysis using purified LasR. Fourteen ChIP-chip enriched promoters from Categories I, II, and III were chosen. In addition, we included the *lasI/rsaL* promoter, which had previously been shown to bind LasR *in vitro*, and the

PA2069 promoter, which has been shown to be responsive to RhlR but not LasR (Schuster *et al.*, 2003), and was also not detected by ChIP-chip. A fragment internal to the *lasB* gene was included as a control for non-specific binding. Nine of the 14 promoters bound LasR *in vitro* (Fig. B.2).

Fig. B.2: Electrophoretic mobility gel shift analysis of ChIP-chip enriched sites. Regions of interest were PCR amplified, radiolabeled, incubated with purified LasR protein and evaluated on a native polyacrylamide gel (see *Experimental procedures*). LasR concentration is shown in nM. ChIP-chip categories from Table 1 are indicated by a superscript I, II, or III.

A. Single-shift complexes, indicative of non-cooperative binding. The positive control, *rsaL*, is included.

B. Multiple-shift complexes, indicative of cooperative binding.

C. No binding. The non-specific control internal to *lasB* and the RhlR-specific control, PA2069, are included.

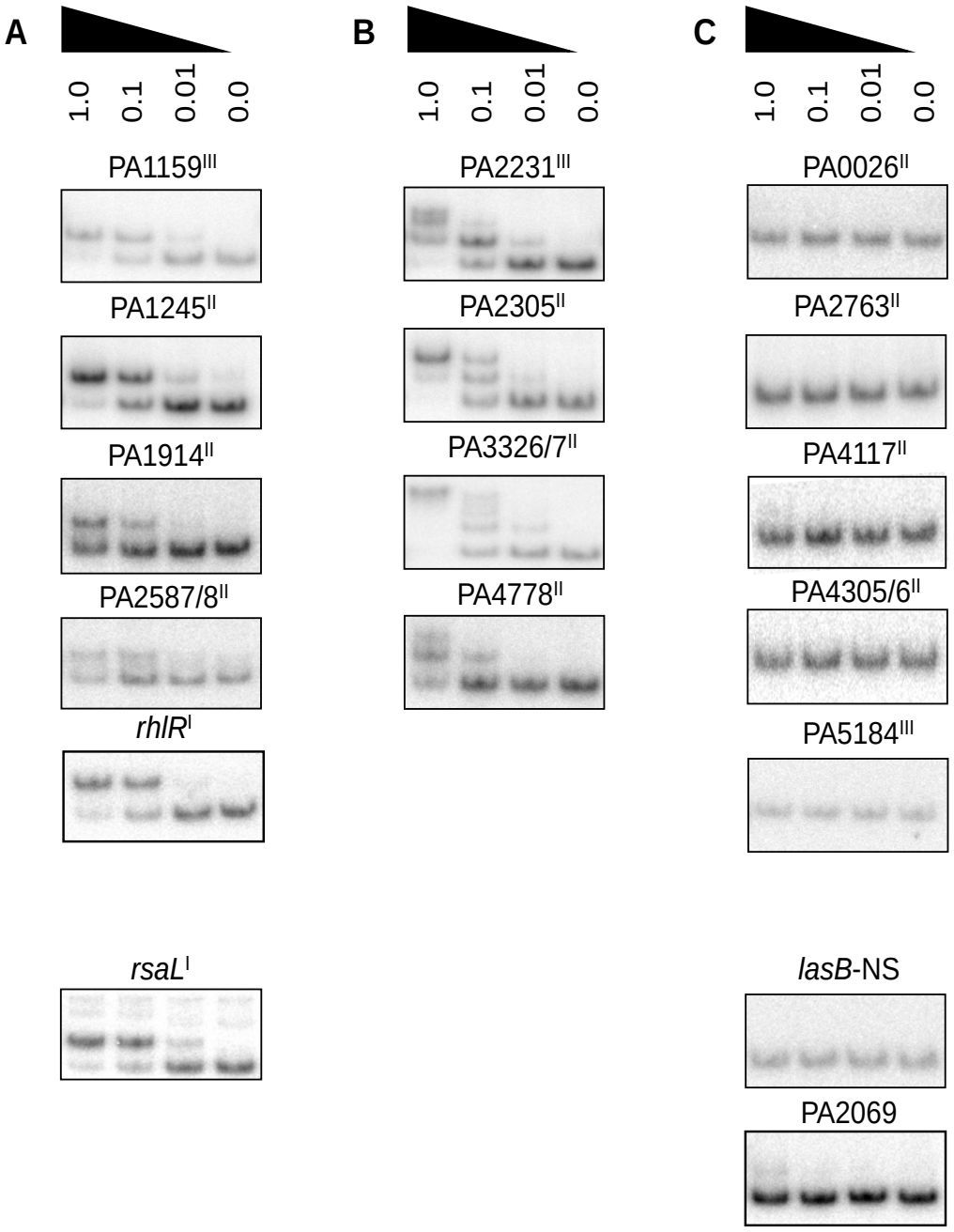

Fig. B.2: Electrophoretic mobility gel shift analysis of ChIP-chip enriched sites

Two distinct binding patterns were observed. Five promoters (plus *rsaL*) bound LasR as single-shift complex, while four promoters bound LasR as a multiple-shift complex. These binding patterns were virtually identical to those observed in our previous study for the *rsaL* promoter and other promoter fragments (Schuster *et al.*, 2004b). In that study, a careful Hill-plot analysis revealed that the formation of single-shift and multiple-shift complexes corresponds to non-cooperative and cooperative LasR binding, respectively. As expected, the RhlR-specific fragment PA2069, and the non-specific intergenic control fragment did not bind LasR specifically. Non-specific binding was generally observed as a smear at concentrations at or above 10 nM. Unexpectedly, however, five promoter fragments identified by ChIP-chip also did not bind LasR *in vitro*. It is possible that binding *in vivo* requires additional factors not present *in vitro*, or that these sites simply represent false-positives. To distinguish between these possibilities, we performed a second ChIP. This time, enrichment by LasR was evaluated using qPCR (Table B.2).

Table B.2: ChIP-qPCR of EMSA-negative promoters.

Probe	Average enrichment[a]
rsaL (positive control)	11*
PA2305 (positive control)	7.3*
PA0026	9.6*
PA2763	1.0
PA4117	3.1*
PA4305/6	2.7*
PA5184	5.5*
PA2069 (RhlR-specific negative control)	0.85

a. Values indicate ratios of wild type vs. *lasR rhlR* mutant ChIP-DNA, averaged from two independent experiments. Significant enrichment is indicated by an asterisk. Enrichment was considered significant if the average ratio was >2, and if it was >2 standard deviations above the average enrichment of the background control fragments.

The promoters upstream of *rsaL* and PA2305 were included as positive controls, while four intergenic fragments were used to calculate background levels. Four of the five fragments, which were negative in the EMSA experiment, showed significant enrichment using qPCR, suggesting that these sites are true positives. We conclude that LasR binding *in vivo* is complex and cannot always be reproduced accurately *in vitro*.

Based on confirmatory EMSA and qPCR data, we calculate a false-positive rate for our ChIP-chip experiment of 8% (only one out of 13 ChIP-chip enriched sites, excluding known Category I targets, was not confirmed by EMSA or qPCR). Typically, the false-positive rate of a successful ChIP-chip analysis ranges from 2 to 10% (Kim *et al.*, 2007).

Promoter fusion studies

Next, we tested the prediction that ChIP-chip enriched fragments not previously associated with QS-dependent genes can function as promoters and drive transcription *in vivo*. Two putative promoter regions from Category III (PA1159, encoding a predicted cold-shock protein, and PA2231, encoding an enzyme involved in *psl* exopolysaccharide synthesis) were selected for gene expression analysis. The activity of plasmid-borne transcriptional *lacZ* reporter fusions was evaluated in the *P. aeruginosa* PAO1 wild type and an isogenic *lasR* mutant. The *rsaL* promoter was included as a positive control. The two Category III promoters showed modestly decreased, but statistically significant, expression in the *lasR* mutant strain as compared to the wild-type (Fig. B.3).

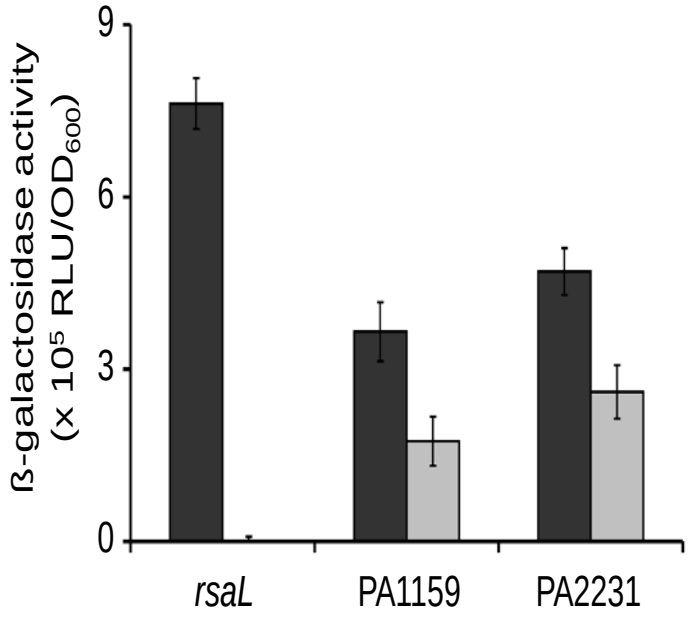

Fig. B.3: Activity of promoter::*lacZ* fusions carried on plasmid pQF50 in the PAO1 wild-type (dark bars) and a *lasR*::Gm^R mutant (light bars). Data presented are from three independent replicates. β-galactosidase levels were normalized to culture density (OD_{600}). In all cases, gene expression was significantly different between wild type and mutant (p<0.05).

Re-interrogation of our microarray data, which were obtained under identical culture conditions, revealed that the expression of both genes was modestly QS-dependent, but this difference was below the threshold chosen (Schuster *et al.*, 2003). It is possible that the contribution of *lasR* to the regulation of these genes is stronger under different growth conditions. Previous studies have shown that PA1159 is up-regulated during biofilm formation (Waite *et al.*, 2006), conceivably in a QS-dependent fashion. Consistent with the notion that additional transcription factors mediate activation, the two promoter fragments were unable to drive LasR-3OC12-

HSL-dependent expression of a *lacZ* reporter in the heterologous host *E. coli* (data not shown).

Identification of a LasR-specific sequence motif

The program CONSENSUS (Hertz & Stormo, 1999) was used to identify a common DNA sequence upstream of the ChIP-chip positive promoters. Conserved binding sites, so-called *las-rhl* box sequences, have been previously identified upstream of several *las* and *rhl*-responsive genes (Schuster *et al*., 2003, Wagner *et al*., 2003), including those that have been shown to directly bind LasR *in vitro* (Li *et al*., 2007, Schuster *et al*., 2004b). Our ChIP-chip data set provided us with the opportunity to identify a consensus sequence specific to LasR (herein designated *las*-box) among a much larger set of binding sites. Thirty-four ChIP-chip enriched sites (excluding the false-positive PA2763) plus PA0572 were used as input to generate a LasR consensus sequence (Fig. B.4A). The resulting sequence contains the expected CT-(N_{12})-AG motif, consistent with previous alignments (Schuster *et al*., 2003, Schuster *et al*., 2004b, Wagner *et al*., 2003, Whiteley & Greenberg, 2001, Whiteley *et al*., 1999). Almost the entire sequence exhibits dyad symmetry, although conservation at some positions is weak. For all regions in Category I, except PA3477 (*rhlR*), the identified *las*-box sequences match published sequences. Because CONSENSUS identifies a sequence pattern in every input sequence without assigning significance, we used PATSER to scan all input sequences for those that closely match the consensus

sequence. All but four sequences identified by CONSENSUS were re-identified and considered significant by PATSER (weight score >5, p-value <0.0005) (Table B.1).

Next, we analyzed sequences according to their mode of binding as determined by EMSA. We used CONSENSUS to identify separate consensus sequences for all nine cooperatively bound and all six non-cooperatively bound sites. The promoter regions that bind LasR in a non-cooperative manner include five fragments from this study (Fig. B.2A), as well as *rsaL* and three others previously shown to bind LasR *in vitro*, PA0572, PA2591, and PA3904 (Li *et al.*, 2007, Schuster *et al.*, 2004b). The promoter fragments that bind LasR in a cooperative manner include four regions identified in this study (Fig. B. 2B) as well as two sequences identified previously, *lasB* OP2 and PA4677 (Schuster *et al.*, 2004b). In both cases, a 16 bp consensus sequence was identified (Fig. B.4B, C).

Both contain the conserved CT-(N_{12})-AG motif. Differences at individual positions that would distinguish cooperative from non-cooperative binding sites are not readily apparent. Because cooperatively bound LasR footprints a region of more than 40 bp in length (Schuster *et al.*, 2004b), we re-interrogated all cooperatively bound sites for the presence of two adjacent *las*-box sequences. However, a second sequence was not identified, suggesting that, if present, it is too degenerate to be recognized by CONSENSUS.

Fig. B.4: LasR consensus sequences. The size of each letter indicates the relative abundance at the respective position in the consensus matrix generated with CONSENSUS.

A. Consensus sequence for all ChIP-chip regions (excluding PA2673), plus PA0572.

B. Consensus sequence for 9 promoters that bind LasR non-cooperatively.

C. Consensus sequence for 6 promoters that bind LasR cooperatively.

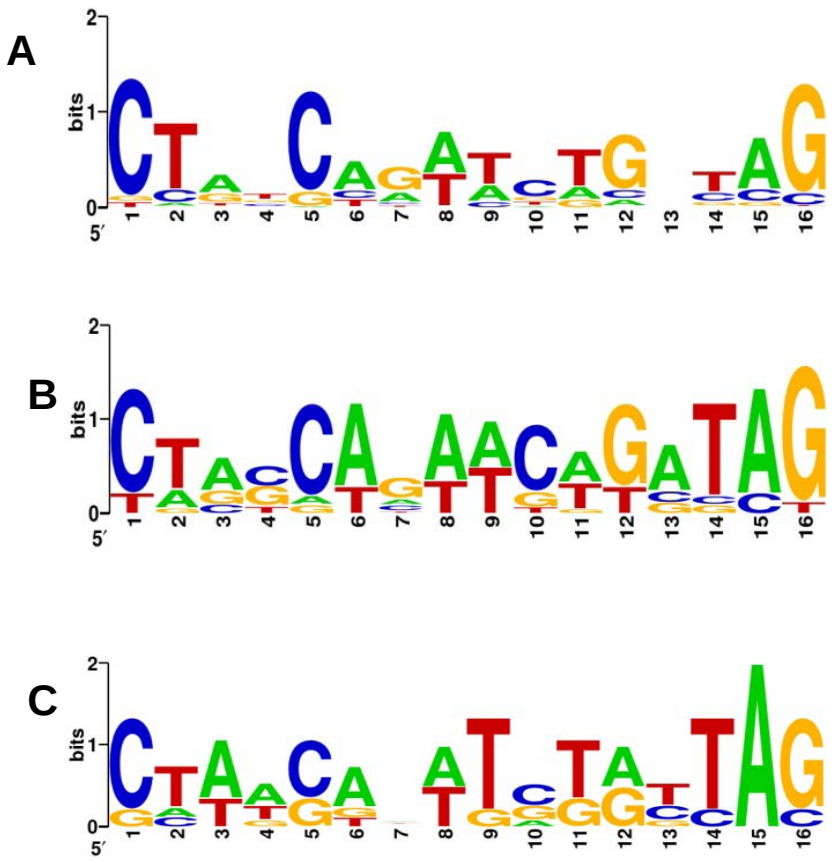

Fig. B.4: LasR consensus sequences

Discussion

Microarray technology has identified more than 300 genes responsive to acyl-HSL QS in *P. aeruginosa*, including those regulated by LasR (Hentzer *et al*., 2003, Schuster *et al*., 2003, Wagner *et al*., 2003). However, many of these genes are predicted to be further downstream in the QS hierarchy and as such would not be directly regulated by LasR. To identify the direct targets of this DNA-binding protein, ChIP-chip in conjunction with EMSA, qPCR and promoter fusions, was performed. ChIP-chip

technology identified 35 regions as direct targets of LasR *in vivo*. Thirty of these promoters are associated with previously identified QS-controlled genes.

We identified many, but not all, of the promoters previously shown to interact with LasR directly. The only *las*-specific promoter region we did not identify was PA0572, which bound LasR *in vitro* (Schuster *et al.*, 2004b). It showed enrichment but at levels below the cut-off chosen. The other promoters, *hcnABC*, *rhlI*, and PA1869, all respond to both LasR-3OC12-HSL as well as RhlR-C4-HSL (Schuster *et al.*, 2003). Another promoter that belongs in this category is *lasA*. It was not identified by ChIP-chip, although the presence of a *las-rhl* box sequence suggests direct regulation (Schuster *et al.*, 2003). In fact, only 10 of 29 *las*-responsive promoters with a *las-rhl* box sequence - as predicted previously - were isolated by ChIP-chip. A closer analysis of the transcriptome data reveals that most of these 19 regions not identified by ChIP-chip respond much better to LasR-3OC12 and RhlR-C4-HSL than LasR-3OC12-HSL alone. In many cases, only one *las-rhl* box sequence was identified in each of the corresponding promoter regions. This could mean that several of these promoters are directly regulated by RhlR, and only indirectly by LasR. It could also mean that several promoters are directly regulated by both transcription factors, which bind to the same sequence. In the case of *hcnABC*, the latter has in fact been shown experimentally using a heterologous host system (Pessi & Haas, 2000). Thus it is conceivable that in a stationary-phase wild type cell, in which both LasR and RhlR are expressed at high levels, RhlR displaces LasR at these “single-occupancy” binding

sites. Consequently, the respective promoters would not be recognized by ChIP using a LasR-specific antibody. This would be in contrast to *lasB*, another *las/rhl* responsive promoter that contains two *las-rhl* box sequences, one of which is exclusively bound by LasR (Anderson *et al.*, 1999), and that was indeed identified by ChIP-chip. An alternative explanation might be that LasR and RhlR bind to individual sites as heterodimers (as suggested by Pessi and Haas, 2000) or higher-order multimers, which might also preclude enrichment by ChIP using LasR-specific antibodies. Taken together, the available data favor a mechanistic, biological explanation and do not suggest a technical deficiency with ChIP-chip.

Our criteria chosen to filter significantly enriched promoters strike a balance between false-positive and false-negative discovery rates. As mentioned above, we estimated the false-positive rate to be 8%. A false-negative rate can be calculated by considering only *las*-specific promoters (those that respond to LasR-3OC12-HSL but not RhlR-C4-HSL) and those that bind LasR at distinct sites (i.e. *lasB*). Taken together, there are nine such Category I promoters for which independent evidence for direct regulation is available. Only one of those, PA0572, was not identified by LasR-ChIP, giving a false-negative discovery rate of 11%. For this calculation, we did not consider promoters that also respond to RhlR-C4-HSL as these may not be recognized by LasR-ChIP for biological reasons, namely binding site competition or oligomerization *in vivo*, and therefore should not be considered “false-negatives”.

Promoters that have been shown to be directly and exclusively activated by RhlR-C4-HSL or QscR-3OC12-HSL were not identified here, confirming that the immunoprecipitation procedure was highly specific for LasR. This includes *lecA* (PA2570) and PA1897 (Lee *et al.*, 2006, Winzer *et al.*, 2000). PA1897 is directly regulated by QscR rather than LasR, as had been suggested initially (Lee *et al*., 2006, Schuster *et al*., 2004b, Whiteley & Greenberg, 2001).

Furthermore, we identified five novel QS regulated promoters. Two of these, PA1159 and PA2231, were of particular interest to us as they suggested potential involvement of QS in previously unrecognized functions, the cold-shock response and Psl exopolysaccharide synthesis. QS has been shown to regulate the production of another *P. aeruginosa* EPS, Pel, in strain PA14 (Sakuragi & Kolter, 2007). Other studies have demonstrated that three of these novel QS promoters, PA0805, PA1159 and PA3384, respond to other stimuli, including osmotic stress (Aspedon *et al.*, 2006), tributyltin (Dubey *et al.*, 2006), biofilm formation (Hentzer *et al.*, 2005) and aminoglycosides (Marr *et al.*, 2007).

The ChIP-chip data were also evaluated for the presence of promoters targeted for down-regulation by LasR. The transcript data for down-regulated genes from Schuster *et al*. (2003) and Wagner *et al*. (2001) are different in size, but suggest that upwards of 200 genes may be down-regulated in a QS-dependent manner. Because none of the genes listed in either study were identified by ChIP-chip, we conclude that LasR does not function as a transcriptional repressor. Rather, LasR appears to mediate

repression indirectly by activating a transcriptional repressor. Seven genes annotated as transcriptional regulators were identified by ChIP-chip, including the well characterized MvfR, RhlR, RsaL, and VqsR (for one additional regulator in Category III, AmrZ, QS-dependent transcription has yet to be verified experimentally). RhlR functions as a repressor of *rhlAB* in the absence of C4-HSL (Medina *et al.*, 2003b). VsqR controls the expression of 580 transcripts, 476 of which are repressed (Wagner *et al.*, 2007), and RsaL controls the expression of 130 transcripts, 120 of which are repressed (Rampioni *et al.*, 2007). RhlR, RsaL, and VqsR may therefore mediate the repression of many QS-repressed genes.

EMSA analysis not only confirmed binding to many ChIP-chip enriched sites, but also provided mechanistic insights into LasR binding to target promoters. We noted previously that LasR exhibits two modes of binding (Schuster *et al.*, 2004b). LasR bound several promoter cooperatively, and a few others non-cooperatively. Cooperative binding may be the result of LasR multimerization. Here we show that these distinct binding patterns extend to many other LasR-controlled promoters. However, six ChIP-chip enriched promoters did not demonstrate any specific binding to LasR, including the four promoters from this study (excluding one false-positive; Fig. B.2), and two promoters, *rhlAB* and *pqsA*, from previous studies (Schuster *et al*., 2004b, Wade *et al.*, 2005). This finding is not unique to our work. In another ChIP-chip study, for example, 15% of promoters identified by ChIP-chip also did not bind the purified transcription factor by EMSA (Molle *et al.*, 2003a). Such inconsistencies

are not unexpected as EMSA detects binding *in vitro* while ChIP-chip detects binding *in vivo*. It is intriguing to speculate that additional transcription factors aid in the recruitment and/or binding of LasR *in vivo*. RhlR, the stationary phase sigma-factor RpoS (σ^S), and the LysR-type regulator MvfR may be such factors. Three out of the six ChIP promoters that did not bind LasR *in vitro* (PA0026, PA4117, and PA4306) show RpoS-dependent activation (Schuster *et al.*, 2004a), two promoters, *rhlAB* and PA4306, depend on RhlR-C4-HSL for full activation (Table B.1), and one promoter, PA0996 (*pqsA*), binds MvfR *in vitro* (Wade *et al.*, 2005). Cooperative interactions between these transcription factors and LasR *in vivo* could explain why purified LasR did not shift several ChIP-chip enriched promoter fragments by EMSA. Taken together, interactions between LasR and RhlR might be particularly complex, involving either antagonism (as described above), synergy, or no interaction at all, depending on the specific promoter architecture.

Previous studies have identified conserved sequences upstream of QS genes, termed *las*-*rhl* box sequences, which included *las*-responsive and *rhl*-reponsive genes (Schuster *et al*., 2003, Wagner *et al*., 2003, Whiteley & Greenberg, 2001). LasR binding sites identified by ChIP-chip in this study provided an excellent source for a more refined characterization of conserved recognition sequences. We performed a bioinformatics analysis that treated promoter sites separately, according to their mode of binding, in addition to an overall analysis of all ChIP-chip enriched sites. All three analyses revealed the general CT-N_{12}-AG motif, but did not confirm the notion from

our previous study that cooperative sites are different from non-cooperative sites (Schuster *et al.*, 2004b). For cooperative promoters, we predicted the presence of an elongated sequence or the presence of additional, adjacent binding sequences, but we were only able to identify the standard 16 bp motif. It is possible that a second, more degenerate, sequence is present, which is bound by additional copies of LasR, once the strong consensus sequence has been bound. There appears to be no correlation between conservation of the binding site (particularly the CT-N_{12}-AG motif), binding strength of LasR *in vivo* (as revealed by the ChIP-chip enrichment factor), and binding strength of LasR *in vitro*. Interestingly, a *las*-box sequence with low similarity to the consensus was found in only 14% (2 of 14) of regions that bound LasR *in vitro* versus 50% (3 of 6) of regions that did not bind LasR *in vitro*. Thus, LasR recognition sites in promoters that did not bind LasR *in vitro* may be more degenerate and presumably mediate weaker binding, which is consistent with the notion that LasR requires additional factors for binding to these promoters *in vivo*.

The location of the LasR binding sequence with respect to the transcriptional start site in individual promoters, as determined previously (Table B.1), suggests multiple modes of transcription activation. A proximal location in some promoters suggests that LasR could function as a class II-type activator by making multiple contacts with RNA polymerase (Nasser & Reverchon, 2007). A more distal location in other promoters suggests that LasR could also function as a class I-type activator by contacting only the α-subunit carboxy-terminal domain, or even as an enhancer similar

to σ^{54}-dependent promoters (Rappas *et al.*, 2007). As for other LuxR-type transcription factors, it is known that *Vibrio fischeri* LuxR functions as a class II-type activator (Finney *et al.*, 2002, Johnson *et al.*, 2003), while *Agrobacterium tumefaciens* TraR functions both as a class I and class II-type activator (White & Winans, 2005).

Taken together, our ChIP-chip data presented here, combined with recent transcriptome data (Schuster *et al.*, 2003), suggest that a principal function of LasR is to directly regulate expression of extracellular factors (Fig. B.1). We propose that other functions controlled by LasR, many likely indirectly, help reprogram cellular metabolism for the production of these secreted factors. Only 24% of all *las*-activated QS promoters showed significant enrichment by LasR-ChIP. This probably underestimates the total number of genes directly controlled by LasR, primarily because several *las/rhl*-responsive promoters escaped enrichment. If RhlR indeed replaced LasR at "single-occupancy" binding sites, as proposed above, then a second ChIP-chip experiment with a *rhlR* mutant could potentially reveal additional targets. Nevertheless, the fact that seven directly controlled genes encode transcriptional regulators supports the notion that many genes are regulated by LasR indirectly. Such hierarchical regulatory control, together with co-regulation at the target promoter level - characterized as "dense-overlapping regulon" in a previous study (Schuster & Greenberg, 2007) - allows for a high level of signal integration. We suggest that the resulting network architecture is responsible for the exceptional adaptability of the QS response to different environmental conditions.

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