AN ABSTRACT OF THE DISSERTATION OF

Brett L. Mellbye for the degree of Doctor of Philosophy in Molecular and Cellular Biology presented on November 15, 2013.

Title: Quorum-sensing-controlled Public Goods in *Pseudomonas aeruginosa*: Regulation and Application.

Abstract approved:

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Martin Schuster

Cell-to-cell communication by chemical signals, termed quorum sensing (QS), is a common regulatory scheme in the microbial world. *Pseudomonas aeruginosa*¸ an opportunistic pathogen of burn wounds and cystic fibrosis lungs, uses QS to control the expression of hundreds of genes, particularly those necessary for population level benefits such as biofilm formation and secretion of extracellular virulence factors (so-called public goods). *P. aeruginosa* has two QS systems, *las* and *rhl*, that use diffusible acyl-homoserine lactone signals (acyl-HSL). Each system is comprised of a signal synthase (LasI and RhlI) and a cognate receptor transcription factor (LasR and RhlR). Under certain conditions, the *las* system regulates the *rhl* system. The circuitry is subject to additional regulation as accumulation of signal is necessary, but not sufficient to activate most QS-controlled genes. From a social evolution perspective, *P. aeruginosa* QS is considered a cooperative behavior that can be exploited by *lasR* mutant cheaters that do not contribute public goods. Here, we answer two questions: how social cheating influences the evolution of quorum-sensing inhibitor (QSI) resistance, and what nutritional cues promote QS gene expression.

We designed a proof-of-concept experiment to understand how bacterial social interactions affect the evolution of resistance to QSI antivirulence. We cultured QS-deficient mutants with small proportions of QS-proficient wild-type to mimic QSI-sensitive and QSI-resistant cells, respectively. We employed two different carbon sources

that are degraded by QS-controlled extracellular, secreted (public) or cell-associated (private) enzymes. We found that QSI-sensitive mimics (QS-deficient cells) behave as social cheaters that delayed population growth and prevented enrichment of QSI-resistant mimics (QS-proficient cells) only when nutrient acquisition was public, suggesting that QSI resistance would not spread.

To answer the second question, we used minimal medium batch and chemostat cultures to demonstrate that specific macronutrient starvation coupled with growth rate reduction induces expression of secreted factors directly controlled by the *las* and *rhl* QS systems. The *rhl* system was more responsive to starvation and growth rate reduction as the transcriptional regulator RhlR and its cognate acyl-HSL were strongly induced. Our results also showed that a slow growth rate inverted the *las*-to-*rhl* acyl-HSL signal ratio, previously considered a distinguishing characteristic between planktonic and biofilm lifestyles. Importantly, expression level depended on the elemental composition of the secreted product and increased only when the limiting nutrient was not also a building block. Such supply-driven regulation is metabolically prudent as it reduces the costs associated with public goods production, which in turn can help limit the metabolic advantage of non-secreting social cheaters. Our results define the physiological basis for the co-regulation of QS-controlled genes by stress responses. They have implications for the evolutionary stability of microbial cooperation as well as for the efficacy of antivirulence drugs and the emergence of resistance to these drugs.

Quorum-sensing-controlled Public Goods in *Pseudomonas aeruginosa*: Regulation and Application.

by

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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.

Brett L. Mellbye, Author

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Dr. Martin Schuster and Brett L. Mellbye were involved in scientific discussions, literature review, and writing of Chapter 2. Dr. Martin Schuster and Brett L. Mellbye were involved in experimental design, scientific discussions, data interpretation, and writing of Chapters 3 and 4.

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my family and friends

Quorum-sensing-controlled Public Goods in *Pseudomonas aeruginosa*: Regulation and Application.

Chapter 1

General introduction

Bacterial cell-cell communication, or quorum sensing (QS) via acyl-homoserine lactones (acyl-HSL) is an important field of study. *Pseudomonas aeruginosa*, a ubiquitous, opportunistic pathogen with a well-characterized QS system, is an ideal model organism to study QS (Schuster *et al.*, 2013). This organism has two acyl-HSL systems, *las* and *rhl*, each composed of a signal synthase and response regulator (LasI/R and RhlI/R) (Mellbye & Schuster, 2011a). These two systems generally exist in a hierarchy with the *las* system regulating the *rhl* system, but they are also regulated by various environmental factors (Mellbye & Schuster, 2011a) (Chapter 2; Fig. 1.1). QS in *P. aeruginosa* controls the expression of hundreds of genes, particularly those that encode secreted factors such as exoenzymes, metabolites, and toxins whose benefits are shared among the population (Schuster *et al.*, 2003, West *et al.*, 2006) (Fig. 1.1). In this dissertation, we present our investigation of the genetic regulation of QS-controlled secreted factors and the social consequences of sharing.

QS-controlled, secreted factors (also referred to as products) in *P. aeruginosa* are important for several reasons: Many secreted factors are also virulence factors important in human disease, secretion of shared factors is considered a cooperative behavior, and they are considered "public goods" shared within the entire population (Waters & Bassler, 2005, West *et al.*, 2006). Previous studies have shown that bacterial cells can experience social conflict due to "cheaters", cells that do not contribute public goods (Fiegna & Velicer, 2003, Sandoz *et al.*, 2007, Diggle *et al.*, 2007b). In *P. aeruginosa*, these mutants can arise due to mutations in the QS master regulator *lasR* demonstrating that the QS regulon is intimately tied to the regulation of these public goods (Schuster *et al.*, 2013). In fact, while 1% of *P. aeruginosa*'s genes encode secreted factors, at least 12% of the known QS regulon is associated with secreted factor production, and 20% of all secreted factors are QS-controlled (Stover *et al.*, 2000, Wagner *et al.*, 2003, Schuster

& Greenberg, 2006). While previous work shows that the regulation of public goods by QS optimizes the costs and benefits of their production (Darch *et al.*, 2012, Pai *et al.*, 2012), the purpose of their co-regulation by numerous other pathways and environmental conditions are less apparent.

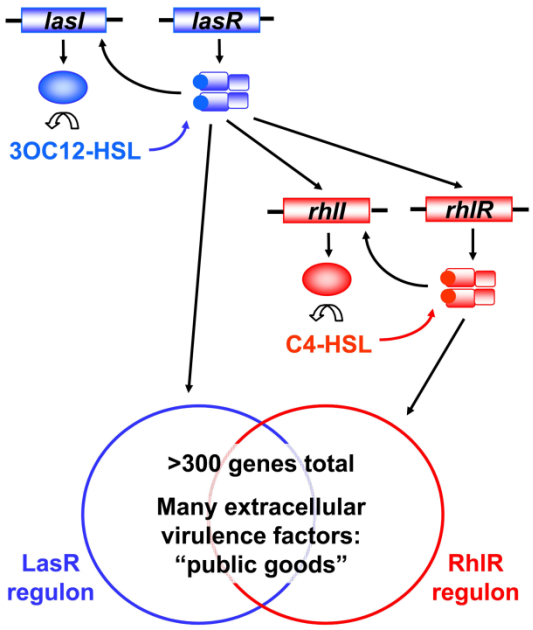

Figure 1.1. Acyl-HSL QS in *P. aeruginosa*. Acyl-HSL QS in *P. aeruginosa* consists of the *las* system and the *rhl* system. The *las* system is composed of a signal synthase, LasI, that synthesizes N-3-oxo-dodecanoyl-homoserine lactone (3OC12-HSL), and a signal receptor, LasR, that binds the signal and regulates transcription. The *rhl* system is composed of a signal synthase, RhlI, that synthesizes N-butanoyl-homoserine lactone (C4-HSL), and a signal receptor, RhlR, that binds the signal and regulates transcription. Together, these two systems control hundreds of genes, many which encode secreted, virulence factors that also function as public goods that are shared among the population of bacteria.

Since *P. aeruginosa* is commonly resistant to antibiotics during infection, inhibition of QS, particularly QS-controlled public goods, was suggested as an antivirulence therapy alternative to traditional antibiotics (Bjarnsholt & Givskov, 2007a). Antivirulence therapy prevents pathogens from producing factors that may harm the host, but does not directly harm the pathogen (Clatworthy *et al.*, 2007). QS inhibitors (QSI) are an antivirulence treatment that would inhibit the production of QS-controlled, secreted virulence factors by manipulating the acyl-HSL QS systems (*las* and *rhl*) (Bjarnsholt &

Givskov, 2007a). There are many small molecule QSIs, and many of them are acyl-HSL analogs that cause rapid signal receptor turnover (Bjarnsholt & Givskov, 2007a) (Fig. 1.2).

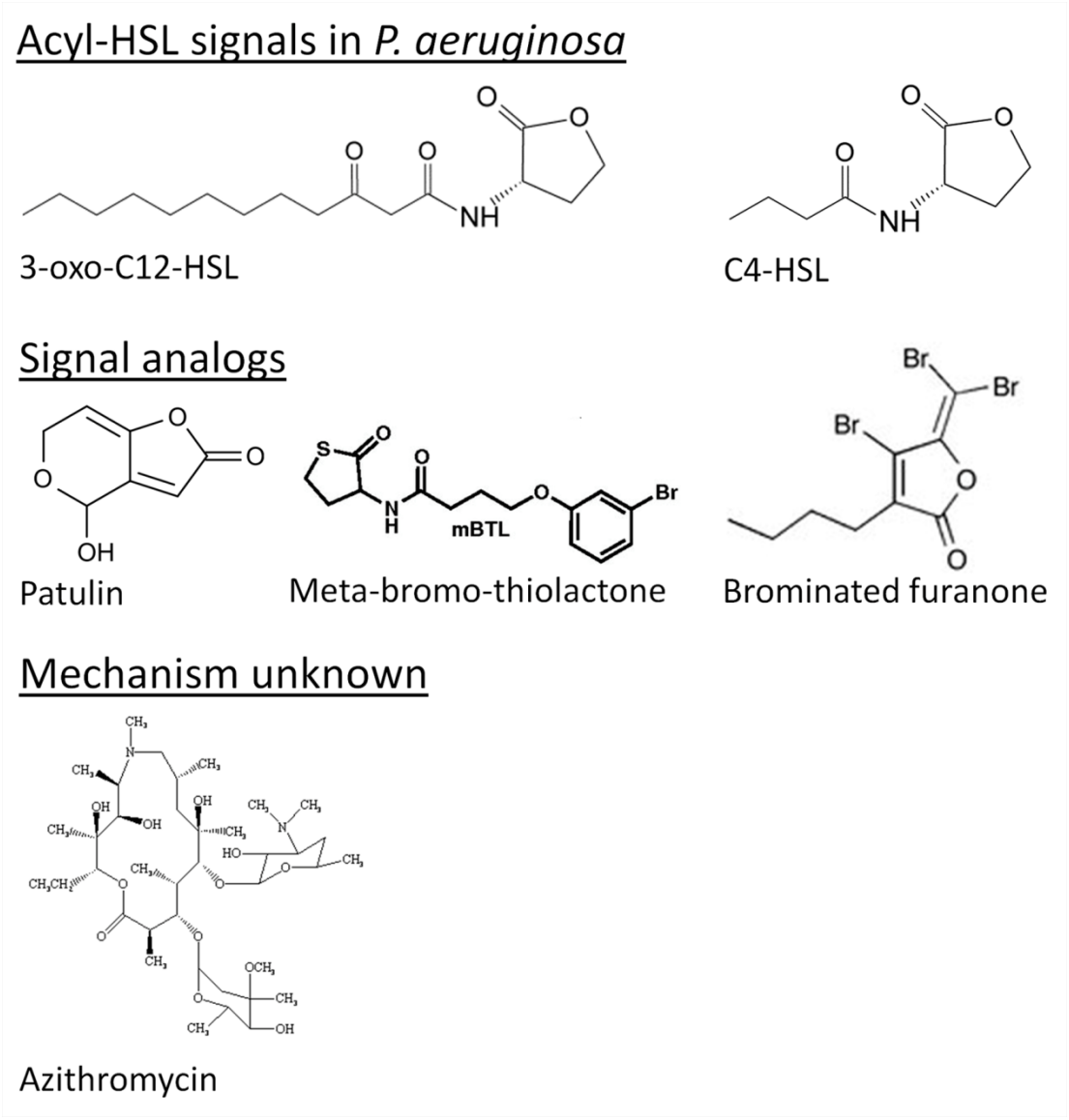

Figure 1.2. Examples of QSIs. Many QSIs are small molecule analogs of acyl-HSL signals that diffuse into the cell, bind to the QS signal receptor, and cause rapid turnover of the bound protein. Macrolide antibiotics, particularly azithromycin, have been reported to inhibit QS. The mechanism for macrolide inhibition of QS is unknown, but it is likely do to non-specific interactions with the ribosome.

Since these treatments do not directly kill the bacteria, they are proposed to prevent or delay development of resistance, but previously this hypothesis was not directly tested. We hypothesized that, if growth depended on QS-controlled public goods, mutants resistant to QSI would be QS-proficient cells in a population of QS-deficient social cheaters, and that these social interactions would suppress resistance (Fig. 1.3). However, if growth depended on an unshared or private QS-controlled product, resistant mutants would have an advantage and enrich (Fig. 1.3). We designed a proof-of-concept experiment to simulate the potential development of resistance to quorum sensing inhibitors. The results of this experiment are presented in Chapter 3 (Mellbye & Schuster, 2011b).

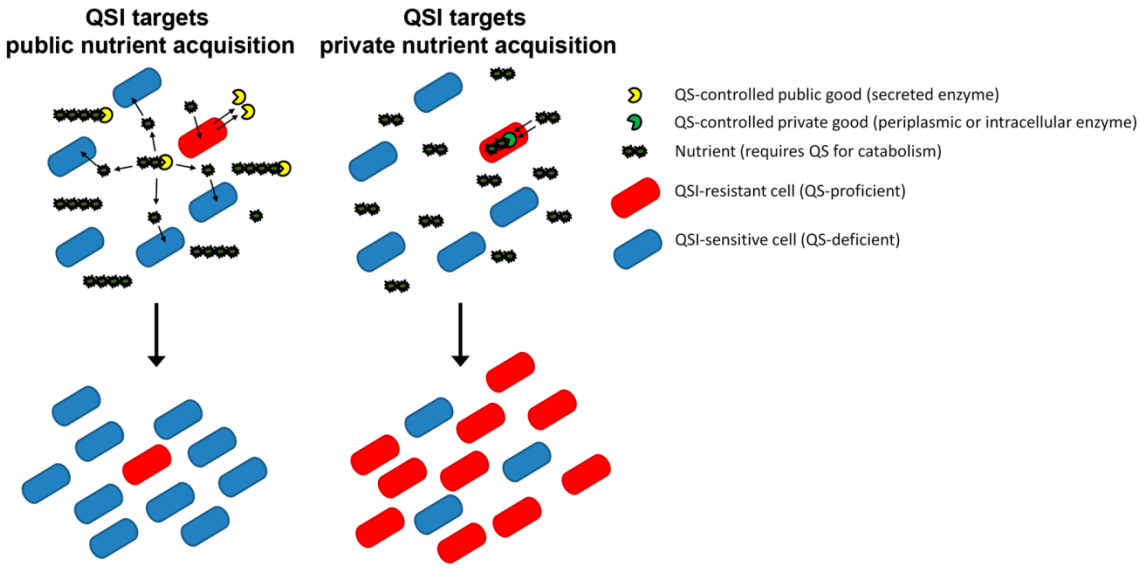

Figure 1.3. QSI resistance development: public versus private nutrient acquisition. When cells depend on production of a QS-controlled public good (secreted enzyme) to breakdown an extracellular nutrient source, QSI-resistant cells would be unable to enrich due to social cheating by QSI-sensitive cells. When a nutrient source is acquired by a QS-controlled private good (e.g. periplasmic enzyme), then QSI-resistant cells would have a growth advantage.

Despite their vulnerability to cheating in lab culture and experimental infection, QS and related cooperative behaviors are evolutionarily stable. Mechanisms must therefore exist to stabilize these behaviors in the natural environment. It is known that

QS gene expression is subject to regulation by other cellular pathways, including starvation and stress responses (Mellbye & Schuster, 2011a) (Chapter 2; Fig. 1.3). Previous work suggests that some environmental conditions help maintain cooperative behaviors and suppress cheaters (Xavier *et al.*, 2011, Dandekar *et al.*, 2012), which in turn may have consequences for the successful use of QSIs.

In Chapter 4, we investigated which environmental cues influence production of public goods. We hypothesized that macronutrient starvation and slow growth increase QS-controlled gene expression (Fig. 1.3). Previous studies with undefined medium (LB) observed that QS genes are induced as cultures reach stationary phase, but were unable to determine whether this effect is due to nutrient starvation or a decrease in growth rate (Schuster *et al.*, 2003, Schuster *et al.*, 2013). We aimed to determine how and why QS-controlled public goods are regulated by macronutrient starvation and changes in growth rate using defined minimal medium batch cultures and a chemostat continuous culture system.

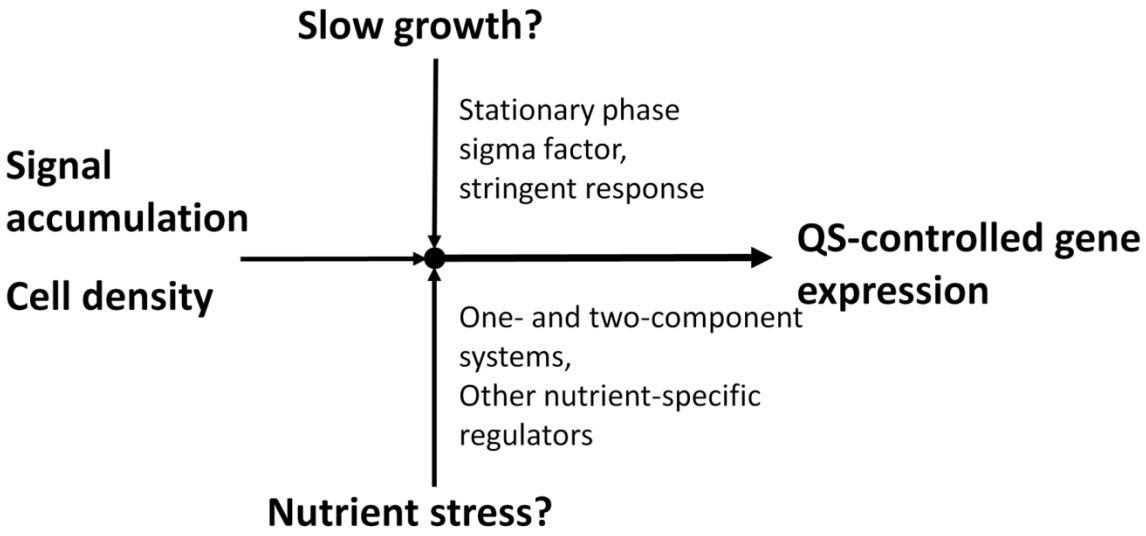

Figure 1.4. QS and integration of environmental stress signals. Previous studies have demonstrated that many QS-controlled genes are co-regulated by stress responses related to nutrient starvation and slow growth (Mellbye & Schuster, 2011a).

Chapter 2

Literature review

More than just a quorum: Integration of stress and other environmental cues in acyl-homoserine lactone signaling

Brett Mellbye and Martin Schuster

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Abstract

Cell-cell communication by chemical signals is prevalent in the microbial world. Bacteria use this form of signaling, termed quorum sensing (QS), to coordinate other behaviors that generally involve population-level benefits such as biofilm formation or secretion of extracellular factors. In this chapter, we review mechanistic and evolutionary aspects of acyl-homoserine lactone (acyl-HSL) QS in Gram-negative bacteria. We emphasize the integration of acyl-HSL signaling with stress responses and other environmental cues. The interconnection of regulatory pathways is particularly well understood in the opportunistic pathogen *Pseudomonas aeruginos*a, but is also apparent in other host-associated bacteria. A benefit of integrating quorum and starvation responses is most apparent for bacteria that acquire nutrients via the secretion of digestive enzymes.

Introduction

Many bacteria employ chemical communication to coordinate group behaviors, a process that has been termed 'quorum sensing' (QS). While many different classes of bacteria use QS, the mechanism that has emerged as common in Gram-negative proteobacteria is based on acyl homoserine lactone (acyl-HSLs) signal molecules. QS is an important field of study due to its important regulatory roles in pathogenic and environmental bacteria. Acyl-HSL QS regulates virulence in many plant and animal pathogens such as *Pseudomonas aeruginosa* (Bjarnsholt & Givskov, 2007b, Girard & Bloemberg, 2008), *Burkholderia cepacia* (Venturi *et al.*, 2004), and *Agrobacterium tumefaciens* (White & Winans, 2007). Interspecies communities of bacteria in soils use signal molecules to regulate complex interactions in the rhizosphere such as symbiosis and nitrogen fixation (Sanchez-Contreras *et al.*, 2007).

In its simplest definition, QS refers to the ability of bacteria to monitor their cell density, and express genes in a coordinated manner, via the production and detection of small signaling compounds (often called autoinducers) (Fuqua *et al.*, 1994). This simple definition has been extended by the concept of 'efficiency sensing', which recognizes

that effective autoinducer concentration in the natural environment is likely not only dependent on cell density, but also mass transfer (diffusion) and spatial distribution of signal-producing cells (Hense *et al.*, 2007). In the following, we will use the term "quorum" to mean a "threshold concentration of autoinducer" regardless of how this threshold was reached. It has become increasingly apparent, however, that a "quorum" by itself is often not enough to initiate certain group behaviors. In bacteria such as *P. aeruginosa*, a threshold signal concentration is necessary, but not sufficient to induce expression of many QS genes (Whiteley *et al.*, 1999, Diggle *et al.*, 2002, Schuster *et al.*, 2003, Schuster & Greenberg, 2006). This is because QS is embedded in a network of global regulation. Specific environmental conditions, such as nutrient deprivation, can exert control over QS genes through one and two-component regulatory systems and post-transcriptional regulation (Boyer & Wisniewski-Dye, 2009, Williams & Camara, 2009, Gooderham & Hancock, 2009). This chapter focuses on the integration of acyl-HSL QS and such stress responses.

The basic acyl-HSL QS circuitry

Acyl-HSL QS was originally discovered in *Vibrio fischeri*, a marine bacterium that acts as a symbiont in the light organs of fish and squid (Nealson *et al.*, 1970, Eberhard, 1972, Nealson & Hastings, 1979, Eberhard *et al.*, 1981). The eukaryotic host provides the bacteria with ample nutrients to grow to particularly high cell densities (10^{10} CFU/mL) and the bacteria produce light which is used by the host animal for behaviors like obscuring its shadow to avoid predation (Nealson & Hastings, 1979). *V. fischeri* bacteria only produce light when at high cell density, for example within the light organ of a squid host, but not when at low density in sea water (Nealson & Hastings, 1979, Engebrecht *et al.*, 1983, Ruby & McFall-Ngai, 1992).

The gene cluster responsible for light production in *V. fischeri* is the luciferase (*luxICDABE*) operon. The proteins involved in the regulation of luciferase expression are LuxI and LuxR (Nealson & Hastings, 1979, Engebrecht *et al.*, 1983, Engebrecht & Silverman, 1984). LuxI is an acyl-HSL autoinducer synthase that produces N-3-

oxohexanoyl homoserine lactone (3OC6-HSL; Fig. 2.1) (Nealson & Hastings, 1979, Kaplan & Greenberg, 1985). This synthesis is accomplished by the formation of an amide bond between *S*-adenosylmethionine and an acyl moiety of a cognate acyl carrier protein (Hanzelka & Greenberg, 1996, More *et al.*, 1996). The synthesized acyl-HSL freely diffuses across the cell envelope into the surrounding environment until a critical concentration is reached (Nealson & Hastings, 1979, Kaplan & Greenberg, 1985). LuxR is a cytoplasmic autoinducer receptor and DNA-binding transcription factor that specifically binds the 3OC6-HSL produced by LuxI (Kaplan & Greenberg, 1985). The complex of LuxR:3OC6-HSL activates transcription of the *lux* operon by binding to a palindromic *cis*-regulatory element, a so-called *lux* box, localized in the promoter region (Kaplan & Greenberg, 1985, Devine *et al.*, 1989, Urbanowski *et al.*, 2004). In addition to activating the structural proteins necessary for luminescence, LuxR:3OC6-HSL also activates transcription of LuxI resulting in a positive feedback loop that amplifies production of 3OC6-HSL leading to a rapid rise in gene expression in the entire population (Kaplan & Greenberg, 1985).

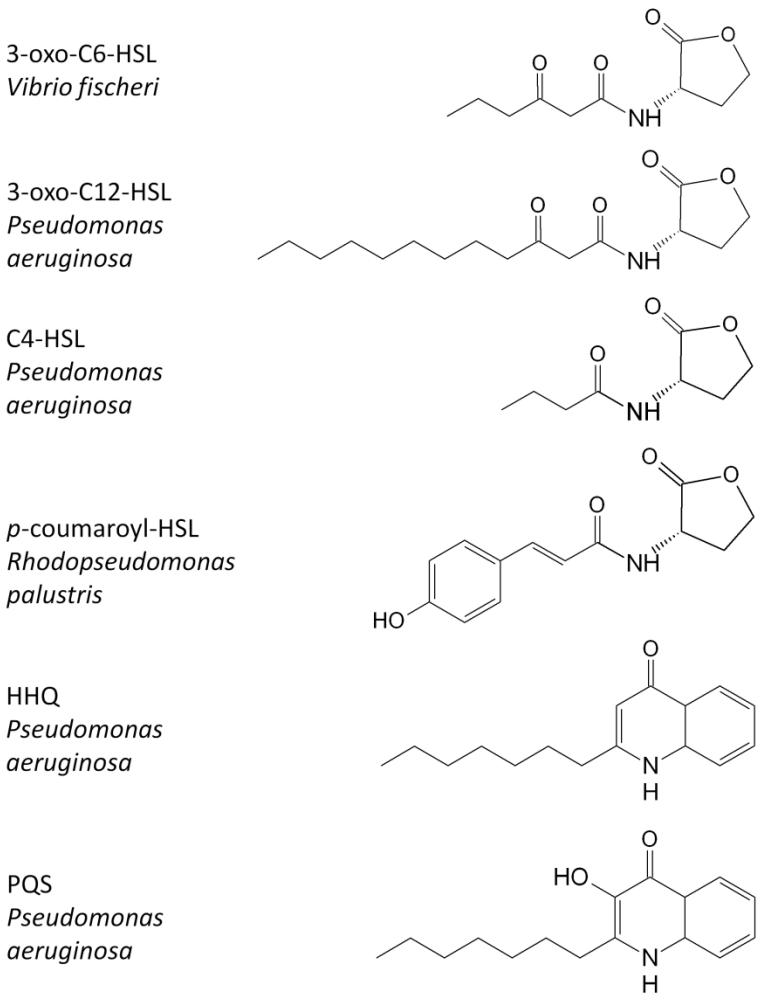

Figure 2.1: Structures of selected homoserine lactone and alkylquinolone signals.

LuxI and LuxR homologs have now been discovered in over 70 genera belonging to the proteobacteria (Boyer & Wisniewski-Dye, 2009). LuxI homologs produce unique acyl-HSLs by linking variable acyl side chains to a homoserine lactone moiety (Fuqua & Greenberg, 2002). Many LuxI homologues can produce multiple acyl-HSLs due to their ability to accept multiple acyl carrier proteins (Boyer & Wisniewski-Dye, 2009). Excretion of acyl-HSLs with longer tailed side chains is assisted by efflux pumps (Pearson *et al.*, 1999). LuxR homologues are surprisingly divergent with relatively little sequence similarity and few conserved amino acid residues (Whitehead *et al.*, 2001). They do contain a conserved tertiary structure including an N-terminal acyl-HSL binding

domain, a C-terminal helix-turn-helix DNA-binding domain, and a central region involved in oligomerization of the protein (Stevens & Greenberg, 1997, Zhu & Winans, 1999, Fuqua & Greenberg, 2002). LuxR homologues are mostly specific to acyl-HSLs produced by a cognate LuxI homologue from the same species, although recognition of signals from other species can occur (see later text) (Venturi & Subramoni, 2009). Responses mediated by acyl-HSL signaling include, but are not limited to, increases in motility, plasmid transfer, nodulation, biofilm formation, and production of extracellular enzymes, antibiotics, and redox-active compounds (Fuqua & Greenberg, 2002, Williams *et al.*, 2007) (Table 2.1).

Table 2.1: Examples of signal integration through acyl-HSL QS systems.

Organism	LuxR-LuxI homolog	Acyl-HSL	Environmental cue/ regulatory pathway	Target function
Agrobacterium tumefaciens	TraR-TraI	3-oxo-C8-HSL	Opines/AccR	Plasmid conjugation
Burkholderia cenocepacia	CepR-CepI	C8-HSL, C6-HSL	Growth conditions/ unknown, RpoS	Exoenzymes, motility, biofilm, virulence
Erwinia carotovora	CarI-CarR, CarI-VirR, ExpR	3-oxo-C6-HSL	Carbon source, oxygen/unknown	Carbapenem synthesis, exoenzymes, virulence
Pseudomonas aeruginosa	LasR-LasI, RhlR-RhlI, QscR	3-oxo-C12-HSL, C4-HSL	Starvation/stringent response (RelA, SpoT), phosphate/PhoR-PhoB, iron/Fur, calcium-cAMP/adenylate cyclases-Vfr, stationary phase/RpoS, oxygen/ANR	Exoenzymes, secretion, motility, biofilm, virulence
Sinorhizobium meliloti	SinR-SinI, ExpR, TraR	C8-HSL, C12-HSL, 3-oxo-C14-HSL, 3-oxo-C16:1-HSL, C16:1-HSL, C18-HSL	Phosphate/PhoR-PhoB	Nodulation/symbiosis
Vibrio fischeri	LuxR-LuxI	3-oxo-C6-HSL	cAMP, glucose, iron, oxygen/CRP, unknown	Luminescence

Pseudomonas aeruginosa QS and signal integration

A well-studied example of a ubiquitous bacterium with a QS system that integrates multiple environmental signals is *Pseudomonas aeruginosa*. *P. aeruginosa* is a Gram-negative bacterium with a dynamic genome specializing in adaptation to diverse environmental niches, including a range of eukaryotic hosts (Kulasekara & Lory, 2004). *P. aeruginosa* is also an opportunistic human pathogen that infects immunocompromised individuals. Cystic fibrosis (CF) patients are particularly susceptible to both chronic and acute infections of the lungs by *P. aeruginosa* (Moreau-Marquis *et al.*, 2008, Wagner & Iglewski, 2008). The level of gene regulation required for such efficient adaptation can be attributed to the fact that over 10% of the genome is devoted to regulatory elements including sophisticated QS circuitry (Stover *et al.*, 2000, Kulasekara & Lory, 2004).

In *P. aeruginosa*, QS controls the expression of numerous virulence factors such as extracellular enzymes (LasB elastase, LasA protease, alkaline protease), secondary metabolites (pyocyanin, hydrogen cyanide, pyoverdin), and toxins (exotoxin A) (Rumbaugh *et al.*, 2000, Whitehead *et al.*, 2001, Smith & Iglewski, 2003). In several animal models of acute and chronic infection, QS mutants show decreased colonization and reduced or no pathology compared to the respective parent strains (Rumbaugh *et al.*, 1999, Pearson *et al.*, 2000, Wu *et al.*, 2001, Lesprit *et al.*, 2003). QS also influences biofilm development (Davies *et al.*, 1998), although its involvement is environmentally conditional (Kirisits & Parsek, 2006, de Kievit, 2009). Biofilm bacteria are up to a thousand times more resistant to antibiotics than their planktonic counterparts, and they are thought to play a significant role in many persistent infections (Hall-Stoodley *et al.*, 2004). Under some *in vitro* conditions *P. aeruginosa* biofilms form structured groups with stalked mushroom-shaped aggregates more than 100 μm in thickness. The formation of these biofilms involves QS, as QS-deficient mutants form thin and unstructured biofilms, and are more susceptible to antibiotic treatment than fully differentiated biofilms (Hentzer *et al.*, 2003).

The *P. aeruginosa* QS circuit is composed of two acyl-HSL signaling systems, *las* and *rhl*, in a sequential regulatory circuit (Fig. 2.2). Both systems consist of the LuxR-

LuxI homologs LasR-LasI and RhlR-RhlI. The signal synthase LasI produces N-3-oxo-dodecanoyl-homoserine lactone (3OC12-HSL; Fig. 2.1) that is bound by a cognate receptor, LasR, to form a complex that activates the transcription of specific genes (Gambello & Iglewski, 1991, Passador *et al.*, 1993, Pearson *et al.*, 1994). Transcription activation is dependent on multimerization of LasR following binding of 3OC12-HSL (Kiratisin *et al.*, 2002). The *rhl* system is activated by the *las* system when LasR:3OC12-HSL initiates transcription of *rhlI* and *rhlR* (Ochsner *et al.*, 1994b, Ochsner & Reiser, 1995, Pearson *et al.*, 1995, Latifi *et al.*, 1996, Pesci *et al.*, 1997, de Kievit *et al.*, 2002, Medina *et al.*, 2003a). The second autoinducer synthase, RhlI, produces N-butanoyl-homoserine lactone (C4-HSL; Fig. 2.1) (Pearson *et al.*, 1995); which, together with its cognate receptor RhlR, induces specific genes (Ochsner *et al.*, 1994a, Winson *et al.*, 1995, Brint & Ohman, 1995, Latifi *et al.*, 1995, Pearson *et al.*, 1995). In the absence of C4-HSL, RhlR can also function as a repressor while binding the same promoter (Medina *et al.*, 2003c). Both LasR and RhlR activate transcription of their cognate synthase genes, generating a positive feedback loop to amplify signal production (Seed *et al.*, 1995, Latifi *et al.*, 1996). The hierarchy of the *las* and *rhl* system does not hold true under all conditions. In late stationary phase or under specific starvation conditions, *rhl*-dependent genes are expressed even in the absence of a functional *las* system (van Delden *et al.*, 1998, Diggle *et al.*, 2003, Medina *et al.*, 2003a, Dekimpe & Deziel, 2009).

Microarray studies revealed that the *las* and *rhl* systems together control the expression of more than 300 genes (Wagner *et al.*, 2003, Schuster *et al.*, 2003, Hentzer *et al.*, 2003). A recent global position analysis of LasR by chromatin immunoprecipitation and microarray profiling identified 35 promoters directly bound by this transcription factor (24% of all *las*-activated promoters) (Gilbert *et al.*, 2009). The majority of the associated genes are predicted to encode secreted factors and secretion machinery, confirming the notion that the core function of *las* QS is to control the expression of extracellular factors.

Another QS system in *P. aeruginosa*, which utilizes 2-alkyl-4-quinolones (AQ) instead of acyl-HSLs, forms a link between the *las* and *rhl* systems (Fig. 2.1). The

system, first described by Pesci *et al.* (Pesci *et al.*, 1999), consists of the secondary metabolites 2-heptyl-4 quinolone (HHQ) and 2-heptyl-3-hydroxy-4 quinolone (Pseudomonas quinolone signal, PQS) (Fig. 2.1) and a LysR-type regulator PqsR (MvfR) (Dubern & Diggle, 2008). PqsR controls the expression of AQ biosynthesis genes, including the *pqsABCDE* operon and *phnAB*, encoding anthranilate synthase (Gallagher *et al.*, 2002, Deziel *et al.*, 2004). HHQ is synthesized from anthranilate by the products of *pqsABCD* (Deziel *et al.*, 2004, Bredenbruch *et al.*, 2005). HHQ is converted to PQS by a putative mono-oxygenase encoded by another gene, *pqsH* (Deziel *et al.*, 2004). PQS and HHQ function as co-inducers of PqsR as binding of PqsR to the *pqsABCDE* promoter increases dramatically in the presence of both compounds (Wade *et al.*, 2005, Xiao *et al.*, 2006). Transcriptome analysis indicates that the PqsR regulon comprises a subset of *las* and *rhl*-dependent QS genes (Deziel *et al.*, 2005). Many genes in the PqsR regulon appear to be regulated indirectly via PqsE, a putative hydrolase (Farrow *et al.*, 2008, Yu *et al.*, 2009). PqsR also positively regulates the *rhl* system (McKnight *et al.*, 2000). RhlR, in turn, represses *pqsA* and *pqsR* (McGrath *et al.*, 2004), while LasR activates *pqsR* and *pqsH* (Wade *et al.*, 2005).

The three major QS systems in *P. aeruginosa* (*las*, *rhl*, and the PQS system) constitute biological regulatory circuit. This circuit is essentially a feedforward loop that coordinates the expression of hundreds of genes during cellular growth. Microarray analysis of QS-controlled genes in *P. aeruginosa* has indentified genes that respond to one autoinducer, either autoinducer, or multiple autoinducers (Hentzer *et al.*, 2003, Schuster *et al.*, 2003, Wagner *et al.*, 2003, Wagner *et al.*, 2004). In addition, analysis showed that many genes were expressed at specific times during growth independent of specific autoinducer concentration (Schuster *et al.*, 2003). These results indicate that the QS regulatory network may have evolved to produce a temporally ordered sequence of gene expression specific for growth conditions encountered in the environment (Whiteley *et al.*, 1999, Schuster *et al.*, 2003).

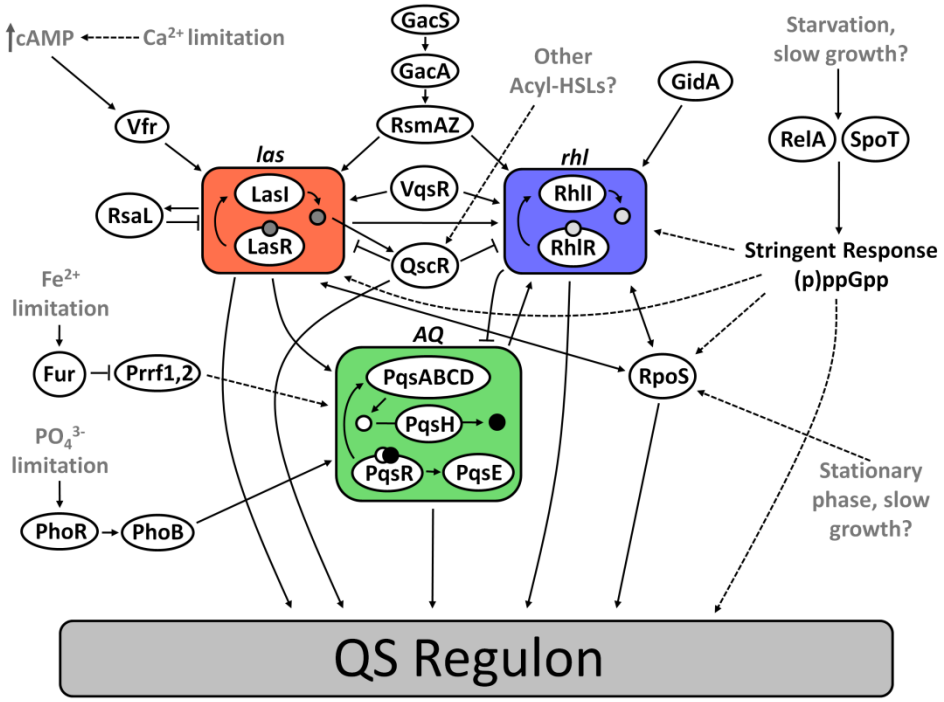

Figure 2.2: Signal integration in the acyl-HSL and AQ-QS systems of *P. aeruginosa*. GacA activates *lasI* expression to generate 3OC12-HSL that is bound by LasR, a LuxR homologue. RsaL is activated by LasR-3OC12-HSL and acts to maintain 3OC12-HSL homeostasis by negatively regulating *lasI*. LasR-3OC12-HSL activates a second acyl-HSL QS system, *rhl*, the AQ-dependent QS system, and many other target genes. The LuxR homologue RhlR binds C4-HSL generated by RhlI and activates further *rhlI* expression and negatively regulates the AQ-dependent QS system. Both acyl-HSL QS systems are controlled at both the transcriptional and post-transcriptional level by regulators including VqsR, the regulatory RNA RsmA, and GidA. Vfr, a cAMP-binding protein, responds to increased cAMP production during calcium limitation and positively regulates LasR. An orphan LuxR homologue, QscR, negatively regulates acyl-HSL QS and controls other genes in response to endogenous and perhaps exogenous acyl-HSLs. LasR-3OC12-HSL drives the AQ-dependent QS system by positively regulating *pqsH* and *pqsR* that code for a putative mono-oxygenase that converts HHQ to PQS and a LysR-type regulator that responds to HHQ and PQS. AQ biosynthesis is positively regulated by PqsR. The response regulator PhoB co-regulates transcription of *pqsR* during phosphate limiting conditions. Under iron limiting conditions Fur indirectly regulates PQS production through regulation of degradation of its precursor anthranilite. Starvation and stress leads to induction of the stringent response and RpoS that directly and indirectly control QS gene expression. Solid arrows indicate positive regulation, solid T-bars, negative regulation, dotted lines, indirect regulation. Red, *las* QS system; blue, *rhl* QS system; green, AQ-QS system. Circles refer to signal molecules: red, 3OC12-HSL; blue, C4-HSL; dark green, HHQ; light green, PQS.

In *P. aeruginosa*, numerous transcriptional and post-transcriptional regulators influence QS, both directly and indirectly, in response to environmental conditions (Juhas *et al.*, 2005, Schuster & Greenberg, 2006, Venturi, 2006, Boyer & Wisniewski-Dye, 2009, Williams & Camara, 2009) (Fig. 2.2). Previous studies have shown that most quorum-controlled genes exhibit a delayed response to exogenously added acyl-HSL autoinducer signals in complex medium until cells reach the stationary phase of growth (Whiteley *et al.*, 1999, Winzer *et al.*, 2000, Diggle *et al.*, 2002, Schuster *et al.*, 2003). These observations suggest that additional factors besides accumulation of signal are important for QS gene expression. Duan and Surette offered some support for this theory by testing the effect of over 40 different growth conditions on the expression of *lasI, lasR, rhlI,* and *rhlR* in batch culture (Duan & Surette, 2007). Their study found that QS genes were more highly expressed in diluted and minimal media, and under different nutrient conditions, such as iron limitation and in the presence of CF sputum. A study by Yarwood *et al.* further indicated that complex medium (Luria Broth, LB) contained an inhibitory component that is metabolized during growth and contributes to the delay of QS-dependent gene expression (Yarwood *et al.*, 2005). For some QS target genes, the expression levels of the QS regulators themselves appear limiting. When induction of *lasR* and *rhlR* was advanced from stationary to early logarithmic phase, a subset of QS target genes showed significant induction (Schuster & Greenberg, 2007). This suggested that other regulatory pathways intersect with QS gene expression at the level of LasR and RhlR, as well as at the level of QS target promoters. Several regulatory systems that are intimately tied to QS are described in the following. We place an emphasis on those pathways for which an environmental stimulus is known or at least seems apparent.

Microaerobic and anaerobic conditions. The transcriptional regulator ANR controls expression of *P. aeruginosa* genes under anaerobic and low oxygen conditions (Ray & Williams, 1997, Comolli & Donohue, 2004). Quorum-controlled hydrogen cyanide biosynthetic genes *hcnABC* are co-regulated by ANR and both QS systems (Pessi & Haas, 2000). QS gene expression, including *lasI* and *rhlI*, is enhanced during growth

under low oxygen, but the role of ANR in this process has not been explored (Alvarez-Ortega & Harwood, 2007).

Calcium limitation. *P. aeruginosa* possesses a global regulatory network based on production of 3', 5'-cyclic AMP (cAMP) by several adenylate cyclases (Smith *et al.*, 2004). Calcium limiting conditions, which may be encountered during contact with eukaryotic cells, trigger cAMP synthesis (Wolfgang *et al.*, 2003). Vfr, a global regulator of *P. aeruginosa* virulence gene expression, is a cAMP receptor that responds to increased levels of cAMP in the cell (Albus *et al.*, 1997, Kanack *et al.*, 2006). Besides activating multiple virulence genes, such as secretion systems and secreted virulence factors, Vfr positively regulates expression of *lasR* and *rhlR* and may negatively regulate the PQS signaling system (Albus *et al.*, 1997, Medina *et al.*, 2003a, Whitchurch *et al.*, 2005, Kanack *et al.*, 2006).

Phosphate limitation. Under phosphate-limiting conditions, expression of certain QS-regulated virulence factors, including the secondary metabolite pyocyanin, increases significantly (Medina *et al.*, 2003a, Bazire *et al.*, 2005, Zaborin *et al.*, 2009). This effect is mediated by the PhoR-PhoB two-component-regulatory system (Jensen *et al.*, 2006). *P. aeruginosa* PhoR-PhoB is thought to function similarly to *E. coli* and other enteric bacteria with the sensor kinase, PhoR, phosphorylating the response regulator, PhoB, in response to low phosphate and other unknown environmental conditions (Anba *et al.*, 1990, Lamarche *et al.*, 2008). During low phosphate conditions, PhoB positively regulates *rhlR*, the PQS system, and phenazine production (Jensen *et al.*, 2006). PhoB binding sites (PHO box promoter elements) have been predicted upstream of over 400 genes in the *P. aeruginosa* genome, including *lasR, rhlR, rhlI, pqsR*, and phenazine biosynthesis genes (Jensen *et al.*, 2006).

Iron limitation. The potential for iron toxicity under replete conditions and starvation under limiting conditions requires *P. aeruginosa* to carefully regulate iron homeostasis. A link between QS and iron has been suggested by many (Whiteley *et al.*, 1999, Bollinger *et al.*, 2001, Juhas *et al.*, 2004, Kim *et al.*, 2005), but a mechanistic basis - involving the ferric uptake regulator, Fur, and AQ signaling - has not been elucidated

until recently (Oglesby *et al.*, 2008). Fur mediates iron homeostasis by repressing and derepressing transcription of target genes under iron-replete and iron-limiting conditions, respectively (Cornelis *et al.*, 2009). Target genes include a number of virulence genes and genes involved in iron uptake (Leoni *et al.*, 2000, Ochsner *et al.*, 2002, Hunt *et al.*, 2002, Vasil, 2003). Fur can indirectly activate genes by repressing two small regulatory RNAs, PrrF1 and PrrF2 (Wilderman *et al.*, 2004). These small RNAs provide a regulatory link between iron and quorum sensing in *P. aeruginosa*. PrrF1 and PrrF2 repress genes involved in degradation of anthranilate, a precursor of PQS (Oglesby *et al.*, 2008). Thus, under iron-limiting conditions, anthranilate is spared from degradation to be available for PQS production. Levels of PQS, in turn, influence expression of AQ and *rhl*-dependent genes (Diggle *et al.*, 2003, Oglesby *et al.*, 2008). Interestingly, in addition to its function as a signaling molecule, PQS also has an iron chelating activity, providing an immediate explanation for its link to iron homeostasis (Diggle *et al.*, 2007c, Bredenbruch *et al.*, 2006).

The stringent response. Gram-negative bacteria respond to prolonged periods of starvation through the stringent response. The stringent response is controlled by the alarmone guanosine tetraphosphate, (p)ppGpp. Production and degradation of ppGpp is mediated by the ppGpp synthase RelA and the pyrophosphohydrolase SpoT, respectively. In *E. coli*, SpoT also has ppGpp synthase activity (Gentry & Cashel, 1996). RelA induces the stringent response upon amino acid starvation, while SpoT induces the stringent response upon carbon and phosphate starvation (Spira *et al.*, 1995, Braeken *et al.*, 2006, Potrykus & Cashel, 2008). Induction of the stringent response in *P. aeruginosa* inhibits most stable RNA synthesis and other processes related to growth, but also induces certain genes such as those involved in amino acid biosynthesis and transport, the stationary sigma factor RpoS, and a variety of virulence factors (van Delden *et al.*, 2001). The stringent response may play an important role in QS, but research has been contradictory with different groups publishing different results (van Delden *et al.*, 2001, Erickson *et al.*, 2004). Initially, it was found that overexpression of *E. coli* RelA in *P. aeruginosa* enhanced production of 3OC12-HSL, C4-HSL, and LasB elastase (van

Delden *et al.*, 2001). These results could only partially be reproduced in a later study that used *P. aeruginosa* instead of *E. coli relA* (Erickson *et al.*, 2004). RelA had a positive effect on 3OC12-HSL and elastase production, but a negative effect on PQS and pyocyanin levels, and these effects were only observed under conditions of low magnesium. However, despite using the more relevant *relA* allele, the problem with the latter study is that the overexpression construct only partially complemented a *relA* mutation, and that different culture conditions were used that may not have elicited a stringent response in the wild type.

Stationary phase sigma factor RpoS. Bacterial sigma factors direct the expression of specific subsets of genes by binding to promoter sequences and recruiting core RNA polymerase (Wosten, 1998). The stationary phase sigma factor RpoS serves a pivotal role in *E. coli* in the response to a variety of stresses including starvation associated with entry into stationary phase, hyperosmotic stress, acid stress, and low temperature (Hengge, 2008). In *P. aeruginosa*, RpoS is induced as cultures enter stationary phase (Fujita *et al.*, 1994), but the physiological role is less defined. The increased susceptibility of *rpoS* mutants to carbon starvation and other stresses is less pronounced than in *E. coli* (Jorgensen *et al.*, 1999, Suh *et al.*, 1999). RpoS regulates the production of several exoproducts associated with virulence (Suh *et al.*, 1999). QS in *P. aeruginosa* has been shown to have a small effect on *rpoS* expression (Latifi *et al.*, 1996, Wagner *et al.*, 2003, Schuster *et al.*, 2004) and several studies have identified QS-controlled genes being activated by RpoS (Winzer *et al.*, 2000, Medina *et al.*, 2003b, Schuster *et al.*, 2004). In batch cultures grown in rich medium, RpoS is induced at the onset of stationary phase and controls over 700 genes, including over 40% of all QS genes, 53 of which were shown to be repressed by RpoS (Schuster *et al.*, 2004). Schuster *et al.* used a matrix-based pattern search to identify the *Pseudomonas aeruginosa* putative RpoS binding site consensus sequence CTATACT (Schuster *et al.*, 2004). This sequence is very similar to the proposed -10 consensus sequence for *E. coli*, TCTATACTTAA (Typas *et al.*, 2007). The induction of QS genes by RpoS is likely both direct, because

many QS genes possess putative RpoS binding sites, and indirect by activation of *lasR* and *rhlR*. Gene repression by RpoS is indirect (Schuster *et al.*, 2004).

Other regulatory pathways. Several other global regulatory pathways in *P. aeruginosa* play an important role in QS, but the signal for the activation of these pathways is unknown (Gooderham & Hancock, 2009). Two examples of global regulatory pathways are GacAS/RsmAZ and GidA.

The GacA/GacS two-component signaling pathway affects QS gene expression and virulence postranscriptionally through the small regulatory RNA RsmZ and the RNA-binding protein RsmA (Reimmann *et al.*, 1997, Pessi *et al.*, 2001, Heurlier *et al.*, 2004). GacA activates RsmZ which antagonizes the activity of RsmA. RsmA represses the synthesis of acyl-HSLs in the absence of RsmZ, and also regulates the production of many quorum-controlled virulence factors. Despite playing an integral role in QS gene regulation, the identity and structures of signals that activate the GacA/GacS two-component system have yet to be elucidated (Lapouge *et al.*, 2008).

GidA, a tRNA modification protein, controls RhlR post-transcriptionally, but any signal that may control its expression is unknown (Gupta *et al.*, 2009).

Acyl-HSL QS and signal integration in other bacteria

Signal integration by QS is not unique to *P. aeruginosa* (Table 2.1). It has also been observed in other proteobacteria with acyl-HSL signaling systems, including *Agrobacterium tumefaciens, Burkholderia cenocepacia, Erwinia carotovora, Vibrio fischeri,* and *Sinorhizobium meliloti*. The plant pathogen *A. tumefaciens* contains a QS system, TraR-TraI, which mediates conjugal transfer of the Tumor-inducing (Ti) plasmid to recipient bacteria (White & Winans, 2007). AccR, a transcriptional repressor, relieves repression of the LuxR homolog TraR after detection of opine compounds produced by the crown gall tumor of the plant host (White & Winans, 2007, von Bodman *et al.*, 1992).

Bacterial species belonging to the *Burkholderia cepacia* complex cause problematic opportunistic infections in CF and other patients (Eberl, 2006). This pathogen contains a QS system, CepR-CepI, that controls virulence factor production and

biofilm formation (Eberl, 2006). Three regulatory genes, *yciR*, *suhB* and *yciL*, affect activity of CepI/CepR, probably by interfering with post-transcriptional control of CepR expression (Huber *et al.*, 2002), although the mechanism and any environmental signals that influence expression are unknown. Interestingly, CepR-C8-HSL itself represses the stationary phase sigma factor RpoS (Aguilar *et al.*, 2003).

E. carotovora, a plant pathogen that causes soft rot, contains a QS system, CarR-VirR-CarI, that regulates antibiotic production and exoenzyme synthesis important for virulence (Barnard *et al.*, 2007). The presence of plant-derived carbon sources, such as sucrose, and anaerobic conditions stimulates QS and expression of virulence factors through unknown regulators (Barnard *et al.*, 2007).

The *lux* operon of *V. fischeri* is under the control of LuxR, but luminescence also depends on levels of cAMP, glucose, iron, and oxygen (Dunlap & Greenberg, 1988, Dunlap, 1992, Dunlap & Kuo, 1992). Regulation of cAMP levels allows repression of luminescence in the presence of glucose, under certain conditions (Dunlap & Greenberg, 1985). *V. fischeri* responds to these environmental signals through integration of the LuxR-LuxI QS system, a cAMP receptor protein (CRP) similar to *P. aeruginosa* Vfr, and other unknown factors (Dunlap & Greenberg, 1988, Dunlap & Kuo, 1992, Dunlap, 1992, Albus *et al.*, 1997).

The Sin QS system in the plant symbiont *S. meliloti* is one of several signals that regulate the transition from a free-living state to a nitrogen-fixing symbiotic state with leguminous plants (Sanchez-Contreras *et al.*, 2007). The two component regulatory system, PhoR-PhoB, activates transcription of *sinR* under phosphate limiting conditions (McIntosh *et al.*, 2009). This response ensures QS gene expression and symbiosis at lower population numbers during phosphate restricted growth (McIntosh *et al.*, 2009). As indicated above, acyl-HSL QS systems exist in many other Gram-negative bacteria, but co-regulation by environmental factors has yet to be explored (Williams *et al.*, 2007).

Orphan LuxR-type regulators

Many Gram-negative bacteria possess a LuxR homolog with no obvious cognate LuxI homolog (Subramoni & Venturi, 2009). LuxR-type transcriptional regulators with conserved DNA-binding and autoinducer-binding domains are considered LuxR 'orphans' or 'solos' (Patankar & Gonzalez, 2009, Subramoni & Venturi, 2009). LuxR orphans are found both in bacteria with no other QS systems and those that possess other complete acyl-HSL QS systems (Subramoni & Venturi, 2009).

Orphan LuxR homologs in bacteria with complete QS systems may function to extend the QS regulon to other genes. Alternatively, these regulators may interact with other signals in the environment, such as other bacterial QS autoinducers or eukaryotic compounds (Patankar & Gonzalez, 2009). *P. aeruginosa* is an excellent example of a bacterium that possesses two complete QS circuits, *las* and *rhl,* and one LuxR-type homolog, QscR (Chugani *et al.,* 2001). Mutation of *qscR* results in hypervirulence, presumably due to the early production of several QS-controlled virulence factors (Chugani *et al.,* 2001). Besides controlling the timing of QS genes through repression, QscR also functions as an activator of gene expression together with the *P. aeruginosa* LasI-generated signal, 3OC12-HSL (Lee *et al.*, 2006). QscR controls a regulon distinct from the *las* and *rhl* systems and extends acyl-HSL QS regulation to other gene targets (Lequette *et al.,* 2006). Non-conserved amino acids in its acyl-HSL-binding pocket may allow QscR to respond to other bacterial or environmental signals, particularly closely related *Pseudomonas* and *Burkholderia* spp. (Case *et al.*, 2008). This is also the presumed function for LuxR orphans in bacteria with no complete QS systems.

LuxR orphans are found in almost 20% of Proteobacteria that do not produce acyl-HSLs (Subramoni & Venturi, 2009). Three of the genes in this class have been well studied: SdiA from *Salmonella*, *Escherichia coli*, and *Klebsiella* and OryR and XccR from *Xanthomonas*. *Salmonella* SdiA may function to detect the intestinal environment of animal hosts via the acyl-HSL production of other numerous gut flora (Ahmer, 2004, Smith *et al.,* 2008). This hypothesis was supported by the finding that SdiA is activated during transit of *Salmonella enterica* through the intestinal tract of turtles (Smith *et al.,*

2008). *Xanthomonas* OryR and XccR function as regulators of plant virulence (Zhang *et al.*, 2007, Ferluga & Venturi, 2009). These LuxR homologs lack two key amino acids in their acyl-HSL-binding domain and do not bind acyl-HSLs (Zhang *et al.*, 2007, Ferluga & Venturi, 2009). Rather, OryR and XccR are hypothesized to perceive specific molecules produced by the plant host (Subramoni & Venturi, 2009). Orthologs of OryR and XccR may serve a similar purpose in other bacteria that have QS systems, but future research is needed to confirm this hypothesis (Zhang *et al.*, 2007).

Aryl-homoserine lactones: a new class of HSL QS signals

The discovery of a new class of autoinducers, aryl-HSLs, has opened up a new mechanism for QS to detect environmental cues. *Rhodopseudomonas palustris*, a phototrophic soil bacterium, uses an acyl-HSL synthase to produce a novel autoinducer, *p*-coumaroyl-homoserine lactone (Schaefer *et al.*, 2008). The *p*-coumaroyl moiety, an aromatic monomer of plant lignin, is derived from the environment instead of cellular fatty acid pools (Schaefer *et al.*, 2008). The discovery of a non-fatty acyl-HSL (aryl-HSL) autoinducer expands the range of possible signals to almost any organic acid in the environment. In addition, the use of an environmental substrate to produce a QS autoinducer allows the bacteria to integrate sensing population density and availability of an exogenous substrate or eukaryotic host into one signal (Schaefer *et al.*, 2008). This ability may be important in bacteria that use QS to control functions under a specific set of conditions.

The evolution of QS and the potential for conflict

Much has been learned about QS on the mechanistic level, but little attention has thus far been given to the evolutionary implications of QS. Microbiologists commonly assume that QS is selected for because it benefits the population as a whole. However, evolutionary theory suggests that cooperation and communication can only evolve under very restricted conditions. Why should an individual perform a costly behaviour for the benefit of the group? Indeed, cheating is considered a major problem in the evolution of

cooperation (Keller & Surette, 2006, West *et al.*, 2006, Foster *et al.*, 2007). A cheater is an individual that reaps the benefit of a social trait (for example, the production of 'public goods') while contributing less than average to the cost. Cheaters have been shown to arise in several microbial systems (Velicer *et al.*, 2000, Ennis *et al.*, 2000, Greig & Travisano, 2004). In the spore-forming bacterium *Myxococcus xanthus*, asocially evolved mutant lines that are by themselves deficient in sporulation outcompete the developmentally proficient wild-type in mixed cultures by sporulating more efficiently (Velicer *et al.*, 2000). In *P. aeruginosa*, cheating has been demonstrated in the context of siderophore production (Griffin *et al.*, 2004). In mixed populations, strains of *P. aeruginosa* that do not produce these extracellular, iron-scavenging molecules outcompete the wild-type when starved for iron.

In the case of QS, a double evolutionary problem arises if we assume that QS involves cooperative, honest communication to coordinate other cooperative behaviors (Diggle *et al.*, 2007a). As indicated above, such behaviors include the formation of biofilms and the production of common goods, for example extracellular enzymes. Within-species communication by acyl-HSLs is considered true signalling between cells as signals produced by the sender appear to have evolved specifically due to their effect on the receiver, which in turn has evolved in response to the signal (Diggle *et al.*, 2007a). In principle, QS populations are at risk of invasion by cheaters which either do not produce signals, which overproduce signals to coerce others into cooperating, or which do not produce costly extracellular factors. For *P. aeruginosa*, the metabolic cost associated with acyl-HSL signal production is predicted to be small compared to the cost for responding to the signal, because approximately 6% of the *P. aeruginosa* genome is regulated by QS (Hentzer *et al.*, 2003, Schuster *et al.*, 2003, Wagner *et al.*, 2003, Heurlier *et al.*, 2006). Social conflict should be of particular significance in a localized, long-term infection such as CF, which according to evolutionary theory, would result in increased competition and selection for cheaters (West *et al.*, 2006, Diggle *et al.*, 2007b).

Recent experimental work by us and others confirmed these predictions (Diggle *et al.*, 2007b, Sandoz *et al.*, 2007). Under growth conditions that require QS, *lasR* mutants,

but not *lasI* mutants, emerge during *in vitro* evolution (Sandoz *et al.*, 2007), and their fitness in wild type co-culture is frequency-dependent (Diggle *et al.*, 2007b). These are "social" conditions and as such likely reflect the selective forces that *P. aeruginosa* encounters in its natural environment at least temporarily, and that help shape the natural evolution of QS. Under conditions that favor QS, the response to QS imposes a metabolic burden on the bacterial cell, because QS genes not normally expressed until cessation of growth are highly expressed early in growth (Sandoz *et al.*, 2007). *lasR* mutants also increase in frequency during acute *P. aeruginosa* infection of mechanically ventilated patients (Kohler *et al.*, 2009), and during experimental co-infection in a mouse burn wound model (Rumbaugh *et al.*, 2009).

Despite the propensity for conflict, QS is ubiquitous in bacteria. Its evolutionary stability must therefore be explained. The basis for an evolutionary explanation of cooperation, including QS, is provided by Hamilton's kin selection theory. Kin selection is defined as a process by which certain traits are favored because of their beneficial effects on the fitness of relatives (Keller & Surette, 2006, West *et al.*, 2006). Diggle *et al.* provided experimental evidence suggesting that kin selection may be a mechanism by which QS is stably maintained within a population (Diggle *et al.*, 2007b). QS was favored when *P. aeruginosa* was cultured under conditions of high relatedness (i.e. *lasR* mutant and parent strains grown separately) compared to conditions of low relatedness (i.e. *lasR* mutant and parent grown in co-culture). The form of kin selection that is relevant to bacteria appears to involve limited dispersal as clonal growth tends to keep related bacteria together (Keller & Surette, 2006, West *et al.*, 2006). Recent modeling of the evolution and stability of QS in bacterial populations also found that QS is stable even when the primary benefit is attained from a cooperating group (Czaran & Hoekstra, 2009). A major prerequisite is the limited dispersal of bacterial cells, maintaining a high level of relatedness between neighboring cells, keeping cooperators in and cheaters out. Another prerequisite is that the cost of QS signaling is cheap compared to the cost of cooperation (i.e. the production of common goods). The result of this simulation was a

dynamic equilibrium between cooperating and cheating subpopulations (Czaran & Hoekstra, 2009).

To avoid an explanation for the evolution of cooperation altogether, an alternative concept to density sensing - as QS was initially understood by most - has been put forth. This concept has been termed “diffusion sensing” (Redfield, 2002). Here, autoinducer levels simply reflect the diffusion properties of the environment surrounding a single cell, independent of cell density. The diffusion sensing hypothesis therefore suggests that sensing evolved because of a direct benefit for the individual. In contrast to density sensing, there is no need to postulate a group-level benefit. The opposing concepts of density sensing and diffusion sensing have recently been reconciled by the unifying theory of “efficiency sensing”. Efficiency sensing includes cell-density, mass transfer (diffusion), and spatial distribution as possible environmental parameters that cells might sense (Hense *et al.*, 2007). Thus, efficiency sensing would evolve because of both direct fitness benefits for the individual, as well as because of group benefits of cooperation. Here, too, simulation studies predict that efficiency sensing is stable when cooperating cells grow in clonal clusters, so-called microcolonies, which is further enhanced by a typical feature of autoinducer sensing, namely the positive feedback in autoinducer production.

These experimental findings, simulations, and social evolution theory can help explain both the evolutionary stability of QS as well as the prevalence of QS-deficient variants in natural populations. QS mutants have been isolated from various habitats (Heurlier *et al.*, 2006), including acute and chronic infections, soils, rivers, and public swimming pools. The vast majority of the isolates indeed possess mutations in the central regulatory gene *lasR*. In one study, 12 out of 66 clinical and environmental *P. aeruginosa* isolates had insertion, missense, or nonsense mutations in *lasR* (Cabrol *et al.*, 2003). In addition to social cheating, alternative explanations for the emergence of *lasR* mutants have also been put forth. First, *P. aeruginosa lasR* mutants can have a selective advantage after cessation of growth (Heurlier *et al.*, 2005). When cultured in unbuffered, complex medium, *lasR* mutants of strain PAO1 are more resistant to cell lysis and death

than the wild-type in stationary phase at high cell densities and alkaline pH. Second, several clinical *lasR*-deficient isolates and a defined *lasR* mutant of *P. aeruginosa* strain PA14, but not PAO1, showed an increased growth yield on certain carbon and nitrogen sources (D'Argenio *et al.*, 2007). Both mechanisms may contribute to the selection of *lasR* mutants in certain, nutrient-rich environments. Indeed, a recent population analysis of concurrently isolated *P. aeruginosa* from individual cystic fibrosis lung infections revealed great heterogeneity of QS phenotypes and genotypes within as well as among patients (Wilder *et al.*, 2009). This finding suggests that a single selective mechanism, whether of social or non-social nature, is unlikely to explain the emergence of QS variants during CF infection. Multiple selective forces appear to shape evolution of QS during chronic persistence, which is consistent with the heterogeneity of the lung environment and patient-to-patient variation.

Outlook

The current view of QS is that it evolved as a system that coordinates group behaviors. The concentration of diffusible quorum signals perceived by the cell serves as a proxy for engaging in such behaviors. This is energetically favorable as the QS components themselves are relatively cheap to produce compared to the many costly functions controlled by QS, such as light and exoenzyme production. In addition, one could argue that QS itself is a stress response as it may represent adaptation to a "crowded" environment; a response to crowding might anticipate nutrient deprivation. However, as we have seen above, high cell density per se is not essential to achieve a quorum, and a crowded situation may manifest differently to a bacterial population, depending on other conditions. Crowding may, but does not have to mean nutrient deprivation. It probably means accumulation of waste products, but these will depend on the particular nutrients metabolized. Therefore, considering the available evidence presented in this chapter, we conclude that QS is not a stress response *per se*, but that distinct stress and starvation signals are often integrated in a quorum response.

Why would this be beneficial for the bacteria? QS often coordinates the production of common goods, for example secreted enzymes that break down complex food sources into smaller units that can be taken up by the cell. As such, QS can be seen as a "nutrient acquisition response". A quorum alone would not justify production of these common goods unless the bacteria were also starved. In the laboratory, when *P. aeruginosa* is grown planktonically in rich medium, QS target genes are not induced until the culture reaches high density and growth slows in stationary phase. It appears unlikely that such conditions reflect what would happen in the natural environment. Upon colonizing a new site, or upon establishing an infection within a host, bacteria presumably often immediately experience starvation at fairly low density. Thus, it seems important to be able to activate common goods production at low population size, when needed for growth. For the bacteria, this would constitute a serious balancing act between starvation and wasteful common goods production. In certain confined niches, such as within the phagosome of a eukaryotic cell, QS gene induction can indeed occur at very low density, presumably because diffusion of the QS signal is limited (Shompole *et al.*, 2003).

From a mechanistic point of view, regulation of QS gene expression by other factors occurs at two different levels (Fig. 2.2): Either QS target promoters are co-regulated or central QS circuitry genes (e.g. *lasRI* and *rhlRI* in *P. aeruginosa*) are "super"-regulated. While the former allows integration of parallel signalling pathways, the latter allows modulation of the quorum itself. For example, activation of *lasR* and *lasI* expression by the stringent response would result in a QS-response at a smaller population size, which would be beneficial under the nutrient-limiting conditions described above.

A major emphasis for future research will be to further integrate mechanistic and evolutionary approaches, as well as experimental and *in silico* modelling studies, to better understand the complexities and selective forces that shape QS. It will also be important to shift focus from batch cultures and mono-species biofilms to ecologically more relevant conditions, such as spatially structured multi-species communities. Given the

importance of bacterial communication and cooperation in pathogenic and beneficial host-microbe interactions, better understanding of these processes can help devise treatment strategies in infectious disease and improve agricultural productivity.

Chapter 3

The sociomicrobiology of antivirulence drug resistance: A proof of concept

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Abstract

Antivirulence drugs disarm rather than kill pathogens and are thought to alleviate the problem of resistance, although there is no evidence to support this notion. Quorum sensing (QS) often controls cooperative virulence factor production and is therefore an attractive antivirulence target for which inhibitors (QSI) have been developed. We designed a proof-of-principle experiment to investigate the impact of social interactions on the evolution of QSI-resistance. We co-cultured *Pseudomonas aeruginosa* QS-deficient mutants with small proportions of the QS-proficient wild-type, which in the absence of QSI mimic QSI-sensitive and resistant variants, respectively. We employed two different QS-dependent nutrients that are degraded by extracellular (public) and cell-associated (private) enzymes. QS-mutants behaved as cheaters that delayed population growth and prevented enrichment of wild-type cooperators only when nutrient acquisition was public, suggesting that QSI-resistance would not spread. This highlights the potential for antivirulence strategies that target cooperative behaviors and provides a conceptual framework for future studies.

Introduction

Infectious diseases are the second-leading cause of death worldwide and cause significant morbidity. A contributing factor to the prevalence of infectious disease has been the development and spread of resistance to current antibiotics (Clatworthy *et al.*, 2007). Despite this alarming trend, research into the discovery of new antibiotics by large pharmaceutical companies has dwindled (Projan & Shlaes, 2004). Traditionally, antibiotics have been classified for their ability to either kill bacteria (bacteriocidal) or inhibit bacterial growth (bacteriostatic) by targeting functions essential to bacterial viability. While historically effective, this approach imposes selective pressure that results in the evolution of resistant strains (Clatworthy *et al.*, 2007). An alternative approach is to develop "antivirulence" drugs that disarm pathogens within their host (Clatworthy *et al.*, 2007, Rasko & Sperandio, 2010). These new compounds would target specific factors essential for successful infection such as toxin function, toxin delivery,

virulence gene regulation, or cell adhesion. The benefits of this approach may be two-fold: reduction in selective pressure for resistance and preservation of the host microflora. Quorum sensing (QS) is one important target for antivirulence therapy because it controls virulence gene expression in many bacterial pathogens (Waters & Bassler, 2005). In the opportunistic pathogen *Pseudomonas aeruginosa*, QS is mediated by acyl-homoserine lactone (acyl-HSL) signals (Williams & Camara, 2009). Two interconnected pairs of signal synthase and cognate regulator (LasI-LasR and RhlI-RhlR) control the transcription of more than 300 genes, many of which encode virulence factors, including extracellular enzymes, toxins, and secondary metabolites (Hentzer *et al.*, 2003, Schuster *et al.*, 2003, Wagner *et al.*, 2003). A number of QS inhibitors (QSIs) have been developed with efficacy against *P. aeruginosa* QS *in vitro* and *in vivo* (Rasmussen & Givskov, 2006). In particular, acyl-HSL analogs such as halogenated furanones have been studied in great detail. However, to this date, there have been no experimental data on the evolution of resistance to antivirulence drugs. A recent review by Defoirdt *et al.* suggested that QS-proficiency and hence resistance would be selected for *in vivo* during infection, whenever QS promotes colonization, systemic spread, or immune evasion (Defoirdt *et al.*, 2010). However, this opinion does not consider social interactions that take place during QS.

P. aeruginosa QS is a cooperative behavior that controls the production of many important extracellular factors that are "public goods" for the population (West *et al.*, 2006). Mutants that do not produce these goods, but benefit from them, are considered social cheaters (West *et al.*, 2006, Foster *et al.*, 2007). Under culture conditions that favor QS, such cheaters emerge in the form of signal-blind *lasR* mutants (Sandoz *et al.*, 2007). They invade wild-type populations with negative frequency-dependence (Diggle *et al.*, 2007b, Rumbaugh *et al.*, 2009, Wilder *et al.*, 2011). As their proportion increases in a population, their relative fitness decreases as there are less cooperators to exploit. In *P. aeruginosa*, signal-blind mutants are favored over signal-negative mutants because common goods production is much more costly than signal production (Wilder *et al.*, 2011). These social interactions have generally been investigated with low proportions

of cheaters, but the situation is expected to be reverse in the emergence of QSI resistance: If strains evolved resistance and retained infectivity, they would likely become QS-proficient cells in a population of QS-deficient social cheaters. We predict that exploitation of QSI-resistant clones by the QSI-sensitive majority would greatly slow the development of resistance and prevent the enrichment of a QSI-resistant population. We tested this prediction in the present study.

Results and Discussion

Experimental rationale. As a proof of principle, we designed an experiment that simulates the development of a QSI-resistant population of *P. aeruginosa*. We employed strains that, in the absence of QSI, mimic the phenotypes of QSI-resistant and -sensitive cells. QSI-resistant variants are represented by the *P. aeruginosa* wild-type and QSI-sensitive variants are represented by signal-blind *lasR rhlR* double mutants. We reasoned that a desirable, potent QS inhibitor would completely block acyl-HSL signal reception in *P. aeruginosa*. We determined the fitness of these strains, individually and mixed, *in vitro* under growth conditions that require QS. In this respect, selective pressures are akin to those encountered by *P. aeruginosa* in QS-dependent infections. We used a minimal medium that contains either bovine serum albumin (BSA) or adenosine as the sole carbon (C-) source. We considered BSA a "publicly acquired" and adenosine a "privately acquired" C-source. While both C-sources require QS-controlled enzymes, the former is degraded extracellularly by proteases (Diggle *et al.*, 2007b), benefitting the entire population, and the latter is metabolized in the periplasm by a nucleoside hydrolase (Heurlier *et al.*, 2005), only benefitting the individual producing cell. The importance of extracellular proteases such as elastase in *P. aeruginosa* pathogenesis is well established (Liu, 1974), whereas the significance of adenosine utilization and its regulation by QS is not clear. Regardless, the distinction between public and private goods allows us to make general inferences about the effect of antivirulence drugs that target extracellular (secreted) vs. cell-associated virulence factors. We predict that social conflict should only have a role when QSIs target the production of public, extracellular factors.

Co-culturing experiments. To assess whether social conflict could affect the emergence of QSI-resistant variants, we initiated QS mutant cultures with varying proportions (50, 10 and 1%) of wild-type cells, and measured the duration of growth to stationary phase. As controls, we grew wild-type-only cultures containing the same wild-type inoculum as that used for the co-cultures. A decrease in wild-type inoculum size itself delayed growth, presumably because cell numbers are reduced, and because acyl-HSL signals and extracellular enzymes present in the inoculum are diluted. Importantly, however, in BSA medium we observed a significant delay in the growth to stationary phase for the co-cultures with 50 and 10 % wild-type compared with the respective wild-type-only controls (Fig. 3.1A). Thus, the presence of mutant cells incurs a significant cost to population growth when nutrient acquisition is public. The wild-type-only control for the 1% co-culture failed to reach high density within the duration of the experiment (Fig. 3.1A), likely because the cells were unable to reach the quorum threshold necessary to utilize the BSA in the media. Not surprisingly, the mixed culture with 1% wild-type also did not reach saturation.

Figure 3.1. The role of social conflict revealed through co-culturing.
Assays were performed with a *P. aeruginosa lasR rhlR* mutant (M) and its wild-type (WT) parent. **(A and B)** Duration of culture growth to stationary phase in minimal medium with BSA **(A)** or adenosine **(B)** as the sole C-source. The relative amounts of WT and M inocula (normalized for between-culture comparison) as well as the WT percentage in the respective single and co-cultures are indicated. Arrows indicate that the culture failed to reach stationary phase within the duration of the experiment (15 days). Error bars indicate standard deviation of the mean of four replicates. Statistically significant differences between pairs (WT and M alone; WT+M co-culture and the corresponding WT-only culture) were determined using one-way ANOVA and Bonferroni's post-hoc multiple comparison. Brackets with '*' indicate *P*-values < 0.05. **(C)** Enrichment of the wild-type in co-culture. Initial and final frequency of WT inoculated at approximately 1% (circles), 10% (triangles), and 50% (squares) in BSA and adenosine medium (open and filled symbols, respectively). Error bars indicate standard deviation of the mean of four replicates. Statistical significance was determined by a two-tailed unpaired *t*-test. All changes in the mean of the WT frequency were found to be significant ($P<0.05$) except the BSA co-culture inoculated with 1% WT. **(D)** Relative fitness (v) of the wild-type in BSA (open circles) and adenosine (filled circles) culture. Relative fitness was calculated as the comparison of the initial and final WT frequencies. Differences in the mean relative fitness between BSA cultures inoculated at approximately 10% and 50% were not significant ($F(1,6) = 0.151$, $P>0.05$) and differences in the mean relative fitness between all adenosine cultures were significant ($F(2,9) = 11.5$, $P<0.05$), as determined by one-way ANOVA.

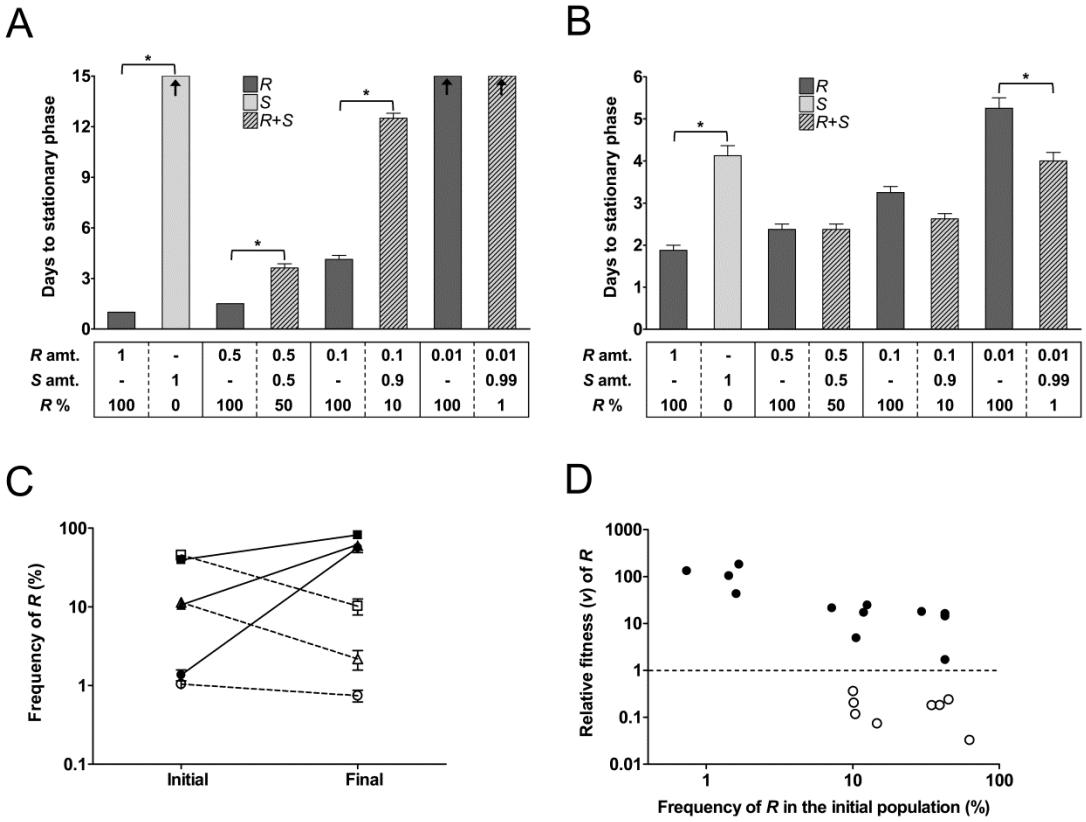

Figure 3.1. The role of social conflict revealed through co-culturing.

Growth characteristics were different when adenosine was the sole C-source. While the QS mutant grew significantly slower than the wild-type in single culture, the presence of the mutant in co-culture did not slow population growth (Fig. 3.1B). In fact, co-cultures at 10 and 1% initial wild-type frequency reached saturation somewhat faster (Fig. 3.1B). This result is likely due to the ability of the mutant cells to grow slowly on adenosine and contribute to total population growth in co-cultures. As shown earlier, growth on adenosine is not as stringently controlled by QS as is growth on BSA (Heurlier *et al.*, 2005).

To investigate the relative fitness of the wild-type in co-culture, we quantified the proportion of wild-type cells at the beginning and the end of growth. In BSA medium, wild-type cells showed no enrichment at either inoculation ratio (Fig. 3.1C). In fact, there was a statistically significant decrease in enrichment when inoculated at 50% and 10%. Consequently, the relative fitness of the wild-type is ≤ 1 in all cases (Fig. 3.1D; see *Materials and Methods* for the calculation of relative fitness). On the other hand, there is a significant increase in the frequency of wild-type cells in adenosine co-culture, and the relative fitness is >1 in all cases (Fig. 3.1C and D).

We were also able to discern trends regarding the frequency-dependence of relative fitness, although this is typically done with cultures grown for equal amounts of time (Diggle *et al.*, 2007b, Ross-Gillespie *et al.*, 2007, Wilder *et al.*, 2011). In our experiment, the duration of competition is different for each initial frequency, because cultures reached stationary phase at different times. The result may be frequency-independence when social conflict predominates, as is the case in BSA medium: Cultures with lower proportions of wild-type cooperators grow more slowly and are exploited by cheaters less efficiently, but cheaters also have more time to exploit cooperators. Indeed, there is no difference in relative fitness of the wild-type with 50% and 10% initial frequency (Fig. 3.1D). Relative fitness is higher at 1% initial frequency, but in this case the culture did not reach saturation and can therefore not be directly compared with the other two. In adenosine medium, where social conflict is insignificant, relative fitness appears negative frequency-dependent, presumably because

wild-type cells, when inoculated at low frequency, have more time to grow and outcompete QS mutant cells.

Implications. Our findings provide an idea about how microbial social interactions might affect the evolution of antivirulence drug resistance during infection. With respect to QSI, the results obtained with BSA appear more clinically relevant than those obtained with adenosine, as it is the QS-controlled production of extracellular (public) virulence factors that contributes to infection. These data suggest that social cheating would play an important role in reducing the development of resistance to QSI and - perhaps even more significantly - also suggest that QSI-resistant mutants would be unable to enrich during infection. This would be completely different from the expansion of clones that are resistant to traditional antibiotics. In terms of tangible implications for experimental research, our results suggest that QSI-resistant mutants are best identified in the absence of social conflict, e.g. in adenosine media. More generally, a comparison of the effects of "public" and "private" nutrient acquisition on fitness indicates that using small molecules to target production of shared virulence factors, such as extracellular enzymes or toxins, may be more effective than targeting private virulence factors, such as cell-associated adhesins.

In addition to the impact of social conflict, the role of the infective dose needs to be considered in the emergence of QSI-resistance. QSI-resistant cells initially likely comprise a very small fraction of the infecting population, which may be so low that it does not constitute a quorum in the first place and may not be able to express virulence factors when treated with a QSI early. Although not the primary focus of our experiment, decreasing inocula of the wild-type emulated this effect to a certain degree.

Our reductionist proof-of-concept experiment relies on two assumptions: the existence of a potent QSI and population mixing. We assume that next-generation QSIs would effectively inhibit a range of LuxR homologs, including LasR and RhlR. Previous *in vitro* evolution experiments have shown that *lasR* single mutants can re-gain the ability to produce certain QS-controlled factors including exoproteases, possibly through compensation by the *rhl* system (van Delden *et al.*, 1998, Sandoz *et al.*, 2007). A potent

QSI would be needed to avoid the rapid development of resistance through this mechanism. These re-evolved cooperators appear to be resistant to exploitation by cheaters, similar to the "Phoenix" variant of the fruiting bacterium *Myxococcus xanthus* (Fiegna *et al.*, 2006), but it is not known whether they are also fully virulent. Our second assumption is that the bacterial population would be exposed to a certain degree of mixing during infection, similar to *in vitro* culturing. *P. aeruginosa* is known to form biofilm structures *in vivo*, and such limited dispersal of the population could increase the fitness of QSI-resistant clones (Diggle *et al.*, 2007b, Kummerli *et al.*, 2009). However, limited dispersal is unlikely to completely eliminate social conflict as *P. aeruginosa lasR* mutants are able to exploit wild type populations during experimental infection of mice (Rumbaugh *et al.*, 2009).

Finally, one last unknown in the development of resistance to QSI is the contribution of the immune system during infection. QS has been shown to protect *P. aeruginosa* from polymorphonuclear leukocytes (Bjarnsholt *et al.*, 2005). It is therefore conceivable that immune cells would preferentially target and remove the QS-deficient sub-population, essentially enriching for QSI-resistant cells. Appropriately designed experiments with animal infection and cell culture models should be able to address these complexities. Our work provides a conceptual basis for such studies.

Materials and Methods

Bacterial strains, culture conditions, and growth assays. Bacterial strains were the *P. aeruginosa* PAO1 wild-type chromosomally tagged with a trimethoprim resistance marker (Wilder *et al.*, 2011) from pUC18T-mini-Tn7TTp (Choi & Schweizer, 2006), and a PAO1 Δ*lasR*::Tc^R Δ*rhlR*::Gm^R double mutant (Rahim *et al.*, 2001). As determined previously, the presence of the respective tag or antibiotic resistance cassette did not affect growth (Schuster *et al.*, 2003, Wilder *et al.*, 2011). Bacteria were routinely cultured on Lennox LB agar plates or in Lennox LB broth buffered with 50 mM MOPS (3-(N-morpholino)-propanesulfonic acid, pH 7.0). For QS growth experiments, bacteria were cultured in M9 minimal medium (Sandoz *et al.*, 2007, Wilder *et al.*, 2011)

containing either 1% BSA (Sigma) (Diggle *et al.*, 2007b) or 0.1% adenosine (Sigma) (Heurlier *et al.*, 2005). Growth experiments were carried out in an incubator/shaker at 37 °C in glass culture tubes containing 4 ml of media.

QS growth experiments were conducted in principle as described (Diggle *et al.*, 2007b, Sandoz *et al.*, 2007, Wilder *et al.*, 2011). Experimental cultures were started from overnight (18 hour) LB-MOPS cultures that had been inoculated with a freshly-grown single colony of the respective *P. aeruginosa* strain. M9-BSA and M9-adenosine cultures were inoculated to an optical density (OD_{600}) of 0.3 and 0.05, respectively, with wild-type and/or mutant at specific frequencies based on OD_{600} (Fig. 3.1). Cells were not washed prior to inoculation as washing greatly delayed growth and reduced reproducibility. To control for differences in growth caused solely by varying inoculum sizes, wild-type-only cultures were inoculated with the same wild-type inoculum as that used for the co-cultures (Fig. 3.1). Appropriately diluted aliquots of these cultures were plated to determine the initial CFU/ml. Every 12 h, culture aliquots were removed to determine OD_{600}. Time span was recorded and aliquots were removed for dilution plating when cultures reached a threshold density indicative of entry into stationary phase ($OD_{600} \geq 2.5$ for M9-BSA and $OD_{600} \geq 0.45$ for M9-adenosine). Cultures that failed to reach the respective threshold OD_{600} were plated after 15 days. In co-cultures, wild-type and mutant subpopulations were distinguished by plating on LB agar supplemented with either trimethoprim at a concentration of 100 μg/ml or tetracycline at a concentration of 50 μg/ml, respectively.

The relative fitness (v) of the wild-type in each co-culture was determined by comparing its initial and final frequencies during growth, with $v=x_1(1-x_0)/x_0(1-x_1)$, where x_0 and x_1 are the initial and final wild-type frequencies, respectively (Diggle *et al.*, 2007b, Ross-Gillespie *et al.*, 2007, Wilder *et al.*, 2011). The value v signifies whether the wild-type population increases in frequency ($v>1$), decreases in frequency ($v<1$), or remains at the same frequency ($v=1$) over the duration of the experiment.

Acknowledgements

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Chapter 4

A physiological framework for the regulation of quorum-sensing-dependent secretions in *Pseudomonas aeruginosa*.

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Abstract

Many bacteria possess cell density-dependent quorum sensing (QS) systems that often regulate cooperative secretions involved in host-microbe or microbe-microbe interactions. These secretions or "public goods" are frequently co-regulated by stress and starvation responses. Here we provide a physiological rationale for such regulatory complexity in the opportunistic pathogen Pseudomonas aeruginosa. Using minimal medium batch and chemostat cultures, we identified specific growth-rate limiting macronutrients as key triggers for the expression of extracellular enzymes and metabolites directly controlled by the las and rhl QS systems. Expression was unrelated to cell density, depended on the secreted product's elemental composition, and was induced only when the limiting nutrient was not also a building block of the product; rhl-dependent products showed the strongest response, caused by the largely las-independent induction of the regulator RhlR and its cognate signal. Consistent with the prominent role of the rhl system, slow growth inverted the las-to-rhl signal ratio, previously considered a distinguishing characteristic between planktonic and biofilm lifestyles. Our results highlight a supply-driven, metabolically prudent regulation of public goods that minimizes production costs and thereby helps stabilize cooperative behavior. Such regulation would be beneficial for QS-dependent public goods that act broadly and non-specifically, and whose need cannot always be accurately assessed by the producing cell. Clear differences in the capacity of the las and rhl systems to integrate starvation signals provide an explanation for the existence of multiple acyl-HSL QS systems in one cell.

Introduction

In a process termed quorum sensing (QS) bacteria regulate gene expression in response to self-produced diffusible signal molecules (Waters & Bassler, 2005, Williams *et al.*, 2007). As bacterial cell density increases, accumulation of signals to a critical threshold triggers the activation of target genes. These genes often encode so-called "public goods", secreted factors implicated in virulence, biofilm formation, nutrient acquisition, and microbial competition. Public goods represent a form of cooperation; they are costly

to produce, provide a density-dependent benefit, and are shared among the population. (West *et al.*, 2006, Schuster *et al.*, 2013). In many Gram-negative proteobacteria, QS is mediated by acyl-homoserine lactone (acyl-HSL) signals (Waters & Bassler, 2005, Williams *et al.*, 2007, Schuster *et al.*, 2013). These signals are produced by a synthase enzyme of the LuxI family and bound by a cognate receptor of the LuxR family that regulates transcription.

The opportunistic pathogen *Pseudomonas aeruginosa* serves as a model to study acyl-HSL QS (Jimenez *et al.*, 2012, Williams & Camara, 2009, Schuster & Greenberg, 2006). This organism has the metabolic versatility and regulatory capacity to adapt to diverse environments including soil, freshwater, and the human host. There are two complete acyl-HSL systems in *P. aeruginosa*, LasR-LasI (*las*) and RhlR-RhlI (*rhl*). LasI and RhlI produce the autoinducers 3-oxo-dodecanoyl (3OC12)-HSL and butanoyl (C4)-HSL, respectively. LasR and RhlR bind their cognate signals to activate the transcription of overlapping regulons. The *las* system also activates the *rhl* system (Latifi *et al.*, 1996, Pesci *et al.*, 1997), although this regulatory hierarchy is conditional (Medina *et al.*, 2003a, Dekimpe & Deziel, 2009). There is also an orphan LuxR-type regulator, QscR, and a 2-alkyl-4-quinolone-based QS system (PQS system) (Lee *et al.*, 2006, Dubern & Diggle, 2008). A large fraction of the QS-controlled genes in *P. aeruginosa* encode secreted factors such as exoproteases (e.g. LasA and LasB elastase, aminopeptidase PaAP) and secondary metabolites (e.g. hydrogen cyanide, pyocyanin, rhamnolipid) implicated in virulence and other group behaviors (Girard & Bloemberg, 2008, Schuster & Greenberg, 2006). Most of these secreted factors are at least partially regulated by the *rhl* system (Schuster & Greenberg, 2006). A potential function for multiple QS systems within one organism is to produce a temporally ordered sequence of gene expression (Whiteley *et al.*, 1999, Schuster *et al.*, 2003, Long *et al.*, 2009).

While signal accumulation and cell density are important triggers for QS, a threshold concentration is necessary, but not sufficient to induce most QS-regulated genes. During growth in rich medium batch culture, most QS-controlled genes are not expressed until stationary phase, even with the addition of exogenous acyl-HSL signals

(Diggle *et al.*, 2002, Schuster *et al.*, 2003, Whiteley *et al.*, 1999). QS is embedded in a network of global regulation and specific environmental conditions can co-regulate QS (Mellbye & Schuster, 2011a, Williams & Camara, 2009). Starvation has an important role in the induction of QS. For example, the stringent response, the alternative sigma factors RpoS and RpoN, phosphate signaling via PhoRB, and iron regulation via Fur all directly or indirectly contribute to the expression of QS-controlled genes (van Delden *et al.*, 2001, Schuster *et al.*, 2004, Oglesby *et al.*, 2008, Jensen *et al.*, 2006, Medina *et al.*, 2003c). Such signal integration has also been observed in the QS systems of other bacterial species (Mellbye & Schuster, 2011a).

Early biotechnological studies conducted to optimize the production of certain *P. aeruginosa* secreted factors such as exoproteases and rhamnolipid, that later turned out to be quorum-controlled, showed that the limitation of specific nutrients enhances expression (Whooley *et al.*, 1983, Guerra-Santos *et al.*, 1984). To date, investigations on quorum-controlled gene expression have almost exclusively involved batch cultures, in which the effects of cell density, nutrient starvation, and slow growth cannot be clearly separated. Here, we employed a defined minimal medium and a continuous culture system to identify specific macronutrient limitation and slow growth as key conditions that induce QS-controlled public good genes. We found that *rhl*-dependent genes are more strongly affected than *las*-dependent genes, and that the limiting nutrient induces expression of the secreted product only when it is not a building block of that product, thus minimizing the burden on individual fitness. Our data suggest a functional split between the *las* and *rhl* QS systems with the *rhl* system integrating stress and starvation signals. Taken together, our results provide a physiological framework for the complex regulation of QS-controlled secretions by stress and starvation responses.

Materials and Methods

Bacterial strains, plasmids, and routine culture conditions. *P. aeruginosa* and *Escherichia coli* strains used in this study are listed in Table 4.1. Transcriptional fusion strains of *P. aeruginosa* were constructed by PCR amplification of promoter regions (Table S1), cloning into mini-CTX-*lacZ*, and integration into the PAO1 chromosome at the *attB* site as previously described by Hoang *et al.* (Hoang *et al.*, 2000). Routine liquid cultures were grown at 37 °C in Lennox Luria-Bertani (LB) broth buffered with 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS), pH 7.0.

Batch culture experiments. Nutrient-limiting batch culture experiments were carried out in MOPS minimal medium described by Neidhardt *et al.*(Neidhardt *et al.*, 1974), with minor modifications. The medium contains 50 mM MOPS (pH 7.0), 4 mM tricine (pH 7.0), 50 mM NaCl, 15 mM NH_4Cl, 4 mM K_2HPO_4, 1 mM K_2SO_4, 0.05 mM $MgCl_2$, 0.01 mM $CaCl_2$, 0.005 mM $FeCl_2$, trace elements, and a carbon source (25 mM monosodium glutamate, 25 mM disodium succinate, or 25 mM D-glucose). Specific nutrients were diluted to yield C-, N-, P-, S-, or Fe-limiting media. Nutrient dilution cultures were inoculated to an optical density at 600 nm (OD_{600}) of 0.005 from mid-exponential-phase cultures containing the same undiluted medium, and they were grown for 16 h in 96 deep-well plates (VWR) at 37 °C with agitation (250 rpm). Samples were taken to measure OD_{600} and β-galactosidase activity. For these experiments, OD_{600} values were read by a microplate reader (Infiniti M200, Tecan) and are reported without conversion to a 1 cm path length.

To measure gene expression in different growth phases by quantitative real-time polymerase chain reaction (qPCR), batch cultures were grown in glass tubes at 37 °C with agitation, inoculated as described above for the nutrient dilution experiments. The minimal medium was used at full strength. Exponential phase, early stationary phase, and late stationary phase samples were taken at OD_{600} values (1 cm path length) of 0.15, 1.5, and 2.5, respectively.

Table 4.1. Bacterial strains and plasmids.

Strain or plasmid	Relevant genotype or phenotype	Reference or source
Strains		
P. aeruginosa		
PAO1	Wild type	(Holloway *et al.*, 1979)
PAO1-*pepB'-lacZ*	Markerless transcriptional fusion of *pepB* promoter to *lacZ* in chromosomal *attB* site, derived from PAO1	This study
PAO1-*lasB'-lacZ*	Markerless transcriptional fusion of *lasB* promoter to *lacZ* in chromosomal *attB* site, derived from PAO1	This study
PAO1-*phzA1'-lacZ*	Markerless transcriptional fusion of *phzA1* promoter to *lacZ* in chromosomal *attB* site, derived from PAO1	This study
PAO1-*rhlA'-lacZ*	Markerless transcriptional fusion of *rhlA* promoter to *lacZ* in chromosomal *attB* site, derived from PAO1	This study
PAO1 Δ*lasR*::Tet^r	*lasR* mutant via insertion of tetracycline resistance cassette, derived from PAO1	(Rahim *et al.*, 2001)
PAO1 Δ*rhlR*::Gm^r	*rhlR* mutant via insertion of gentamicin resistance cassette, derived from PAO1	(Rahim *et al.*, 2001)
PAO1 Δ*lasR*::Tet^r Δ*rhlR*::Gm^r	*lasR, rhlR* double mutant via insertion of tetracycline and gentamicin resistance cassettes, respectively, derived from PAO1	(Rahim *et al.*, 2001)
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*	Life Technologies
Plasmids		
mini-CTX-*lacZ*	Tet^r, integration-proficient vector for chromosomal insertion at the *attB* site	(Becher & Schweizer, 2000)
pFLP2	Amp^r, *flp* recombinase plasmid	(Hoang *et al.*, 2000)

Chemostat experiments. Continuous culture experiments were carried out in glass vessels containing 100 mL of MOPS minimal medium, as described by Whiteley *et al.*(Whiteley *et al.*, 2001) with minor modifications. The medium contained 75 mM MOPS, 93.5 mM NH_4Cl, 42.8 mM NaCl, 4 mM KH_2PO_4, 1 mM $MgSO_4$, 0.0075 mM $FeSO_4$, and trace elements. *P. aeruginosa* strains were grown at 37 °C with aeration, in either C-limited medium (4 mM KH_2PO_4 and 3 mM monosodium glutamate) or P-limited medium (0.11 mM KH_2PO_4 and 10 mM monosodium glutamate). Chemostat vessels were routinely treated with Sigmacote (Sigma-Aldrich) to minimize biofilm formation. Chemostat cultures were inoculated to an OD_{600} of 0.01 from an overnight culture and grown to an OD_{600} of approximately 0.1 before flow of media was initiated. All chemostat experiments began at the fastest dilution rate (D) of 0.5 h^{-1} and were incrementally reduced to the slowest D of 0.125 h^{-1}. Bacteria were allowed to adjust to steady-state for at least three doublings before samples were taken to measure OD_{600}, gene expression, acyl-HSL, pyocyanin, and protein levels. At steady-state, D is equal to the specific growth rate (μ).

β-galactosidase assay. β-galactosidase activity in batch and chemostat cultures was measured in a Tecan microplate reader using a Galacto-Light Plus kit (Applied Biosystems) as previously described (Whiteley *et al.*, 1999). Batch culture results are expressed as fold change of photons per OD_{600} between undiluted and diluted medium. To visualize these data in a heat map, the pheatmap package in R 2.15.3 was used (http://CRAN.R-project.org/package=pheatmap).

Quantitative real-time PCR. Transcript levels in batch and chemostat cultures were analyzed by a two-step reverse transcription qPCR procedure using the Applied Biosystems 7300 sequencing system as described (Sandoz *et al.*, 2007, Schuster & Greenberg, 2007). Total RNA isolation and cDNA synthesis with semi-random primers was performed as previously described (Schuster *et al.*, 2003). An Agilent Bioanalyzer 2100 was used to determine RNA quality. The 25 µL qPCR reaction mixture contained forward and reverse primers (300 nM each, listed in Table S1), 1 ng of purified cDNA, and Power SYBR green PCR master mix reagents (Applied Biosystems). Equal amounts

of cDNA were used for each qPCR reaction. Relative expression levels were determined using a genomic DNA standard curve.

Acyl-HSL detection. *E. coli* reporter strain bioassays were used to quantify 3OC12-HSL and C4-HSL levels from ethyl acetate extracts of chemostat culture supernatants as previously described (Pearson *et al.*, 1997, Pearson *et al.*, 1994).

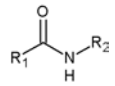
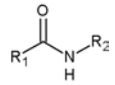
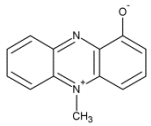
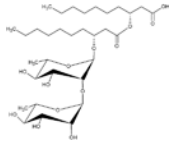
Pyocyanin production. Pyocyanin was extracted from chemostat culture supernatants and measured as described by Essar *et al.* (Essar *et al.*, 1990). Briefly, pyocyanin was extracted from 5 mL of culture supernatant with 3 mL of chloroform. 1 mL of 0.2 M HCl was used to extract the pyocyanin from chloroform. Absorbance was measured at 520 nm and converted to µg/mL via a previously reported conversion factor of 17.072 (Essar *et al.*, 1990).

Western blot analysis. Western blotting was performed as previously described (Schuster & Greenberg, 2007). Chemostat culture aliquots were harvested by centrifugation and pellets were resuspended in lysis buffer. Cells were lysed by sonication and the lysates were centrifuged to remove insoluble material. The protein concentration of the soluble fraction was determined by Bradford assay. Equal amounts of soluble protein were separated by polyacrylamide gel electrophoresis. Separated proteins were blotted onto a nitrocellulose membrane and probed with polyclonal anti-LasR and anti-RhlR antibodies (Schuster & Greenberg, 2007).

Results

Serial dilution of macronutrients in batch culture yields distinct expression profiles for QS-controlled genes. We began our study by investigating the responses of individual QS-controlled genes to the limitation of specific macronutrients during batch culture growth in defined medium. We selected four QS-controlled genes, *pepB, lasB, phzA1,* and *rhlA,* associated with public goods production (Table 4.2). Our previous transcriptome analysis in rich medium showed that these genes exhibit varying specificities to the *las* and *rhl* QS systems, and that their expression increases at the transition to stationary phase, regardless of signal concentration (Fig. S4.1) (Schuster *et al.*, 2003). Expression is directly controlled by LasR, RhlR, or both regulators (Schuster *et al.*, 2003, Gilbert *et al.*, 2009). To quantify the expression of these genes, we fused a *lacZ* reporter, encoding β-galactosidase, to the respective promoter regions, and inserted the resulting constructs into a neutral site on the *P. aeruginosa* chromosome. We grew the reporter strains in a minimal medium with the synthetic organic buffer MOPS (Neidhardt *et al.*, 1974). In this medium, each macronutrient is present at a sufficiently high level to meet the nutritional needs of the bacterial cell. We separately and progressively diluted five macronutrients: carbon (C), nitrogen (N), phosphorous (P), sulfur (S), and iron (Fe). We also tested three C- and energy sources: succinate, glutamate, and glucose. *P. aeruginosa*'s preferences for these C-sources vary and can influence QS-controlled phenotypes (Shrout *et al.*, 2006). Organic acids such as TCA-cycle intermediates are generally preferred, as they repress the utilization of sugars and other substrates through carbon catabolite repression (Rojo, 2010).

Table 4.2. QS-controlled genes and their properties.[a]

Gene	QS specificity[b]	Gene function	Relevant product and localization	Structure of the product[c]	Composition of the product	Limiting, inducing macronutrient
pepB	*las*	Aminopeptidase (PaAP)	Extracellular enzyme		C, N	P, Fe[d]
lasB	*las*, *rhl*	LasB elastase	Extracellular enzyme		C, N	P, Fe
phzA1	*rhl*	Phenazine biosynthesis protein	Extracellular antibiotic (pyocyanin)		C, N	P, Fe
rhlA	*rhl*	Rhamnosyl transferase subunit	Extracellular surfactant (rhamnolipid)		C	N, P, Fe

[a]Includes data from previous studies and from this work.
[b]In the main text, we primarily distinguish between a "*las*-specific" gene *pepB* that only responds to the *las* system, and "*rhl*-dependent" genes *lasB*, *phzA1*, and *rhlA*, that respond to either only the *rhl* system or both systems.
[c]The shown peptide bonds symbolize proteins.
[d]Effect on gene expression was small.

First, we used the *lasB-lacZ* reporter strain to test progressive 2-fold dilutions of essential nutrients (examples of C- and P-limitation in Fig. 4.1; complete data in Figs. S4.2-S4.4, *lasB* column). Limitation of all macronutrients except S substantially decreased the growth yield of the cultures. Despite this reduction in cell density, P- and Fe-limitation stimulated *lasB* expression. C- and N-limitation, on the other hand, reduced *lasB* expression. The expression of *lasB* was highest when succinate was the sole C-source. The C-source glutamate can also serve as an N-source so N is not limiting in glutamate medium. S dilution in these experiments only had a small effect on growth yield with no appreciable impact on gene expression. It is possible that *P. aeruginosa* can utilize MOPS as an S-source, as has been shown for some strains of *E. coli* (Neidhardt *et al.*, 1974, van Der Ploeg *et al.*, 1999).

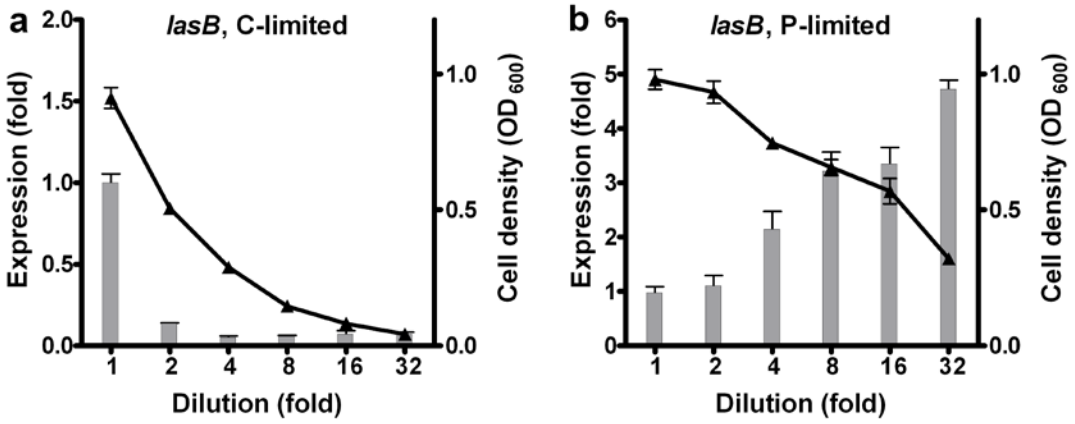


Figure 4.1. Examples of nutrient dilution series with the *P. aeruginosa lasB* reporter strain. Progressive 2-fold dilutions of **(a)** C and **(b)** P were carried out in minimal medium batch cultures with glutamate as the sole C-source. Bars indicate the fold change in β-galactosidase expression compared to undiluted medium (left *y*-axis). Triangles indicate optical density at 600 nm (OD_{600}; right *y*-axis). Fold-change values were normalized to OD_{600}. Values are the mean of three independent biological replicates. Error bars indicate the standard deviation of the mean.

For the analysis of the remaining reporter fusions, *pepB*, *phzA1*, and *rhlA*, we limited ourselves to the 8- and 32-fold nutrient dilutions (Figs. S2-S4, *pepB*, *phzA1*, and *rhlA* columns; Fig. 4.2 for a summary of all gene expression data). We found that *pepB*, *phzA1*, and *rhlA*, like *lasB*, are induced by P- and Fe-limitation and suppressed by C-limitation. In contrast, *rhlA* was induced by N-limitation, whereas the other genes were either suppressed or showed no response. Also, *pepB* was not as highly induced ($\leq$ 2-fold) as *lasB*, *phzA1*, and *rhlA*. In some cases, expression decreased after further dilution of an inducing macronutrient. Induction patterns were generally qualitatively similar for all three C-sources (excluding N dilutions in glutamate medium).

Our results suggest that nutrient stress is a major trigger of QS-controlled public good expression and that individual QS-controlled genes respond differently to the limitation of specific nutrients. The extent of regulation appears to correlate with *las* vs. *rhl* promoter specificity (Table 4.2, Fig. 4.2), because *rhl*-dependent genes are more responsive to nutrient starvation than a *las*-specific gene. Most intriguingly, and as we will discuss in more detail below, responses to individual nutrients appear to be a function of the molecular composition of the regulated secreted product (Table 4.2). Limitation of a particular macronutrient enhances expression only if it is not a building block of the product.

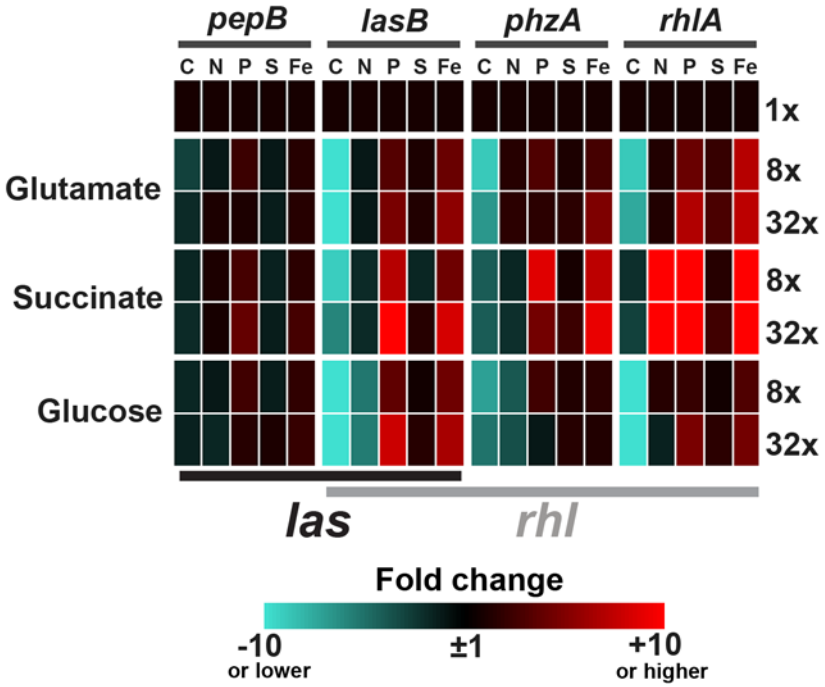


Figure 4.2. QS-controlled gene expression of *P. aeruginosa* during specific macronutrient limitation in batch culture. To compare the expression of QS-controlled genes (*pepB*, *lasB*, *phzA1*, and *rhlA*) across many nutrient conditions, fold-change data from Figs. S2-S4 are visualized in a heat map. Numbers on the right indicate fold-dilution of the limiting macronutrient (either C, N, P, S, or Fe). Labels on the left indicate carbon source and labels on the top indicate QS-controlled gene and limiting nutrient. Labels on the bottom indicate *lasR* (black) or *rhlR* (grey) promoter specificity. In the text, genes regulated exclusively by the *las* system are referred to as *las*-specific (*pepB*) and genes dependent on regulation by the *rhl* system are referred to as *rhl*-dependent (*lasB*, *phzA1*, *rhlA*).

Slow growth induces QS-controlled gene expression in continuous culture when specific nutrients are limiting. The batch culture experiments conducted above indicate that QS-controlled public good expression is triggered by specific nutrient limitation rather than cell density. To further investigate the role of nutrient-limited slow growth independent of cell density, we employed a chemostat culture system. In a chemostat, cell density is determined by the concentration of the growth-limiting nutrient in the medium reservoir. The specific growth rate (μ) on the other hand, is determined by the flow rate, and hence the dilution rate (D) of the medium. As D decreases, the actual concentration of the growth-limiting substrate in the chemostat decreases and

consequently reduces μ without affecting density. As cells reach a steady-state, the μ equals the *D*. This way, cells can be maintained in balanced growth at rates similar to those transiently experienced by batch cultures during entry into stationary phase when many QS genes are induced. We measured QS-controlled gene expression at four different μ of 0.5 h^{-1}, 0.33 h^{-1}, 0.25 h^{-1}, and 0.13 h^{-1} using qPCR. The range of μ was dictated, at least in part, by culturing constraints. A $\mu > 0.5$ h^{-1} approaches the maximum possible growth rate in the medium ($\mu_{max} = 0.83 \pm 0.15$ h^{-1}), resulting in a decrease in culture density and eventual wash-out. A $\mu < 0.13$ h^{-1} causes biofilm formation on the walls of the chemostat vessel. The cell density, measured as OD_{600}, was kept constant at approximately 0.15. This density is below the quorum-threshold in standard batch culture (Fig. S4.1 and see below). We used P as an example of a nutrient that induces QS when limiting, and C as a nutrient that does not induce QS when limiting. We chose glutamate as the C-source.

We found that P-limited but not C-limited slow growth induces QS-controlled gene expression, and that *rhl*-dependent genes are more strongly induced than a *las*-specific gene (Fig. 4.3, left panels, wild-type section). Between a μ of 0.5 h^{-1} and of 0.13 h^{-1}, the expression of the *las*-specific gene *pepB* increased by $\leq$ 2-fold, whereas the expression of the *rhl*-dependent genes *lasB*, *phzA1*, and *rhlA* increased by 7.5, 28, and 36-fold, respectively (Fig. 4.3, left panels, wild-type section). The induction of *phzA1* correlated with a striking blue-green pigmentation of the chemostat culture indicative of high-level pyocyanin secretion (Fig. S4.5), demonstrating that transcription correlates with actual public good production.

Figure 4.3. Transcript levels of QS-controlled genes in chemostat and batch culture. Relative abundances of **(a)** *pepB*, **(b)** *lasB*, **(c)** *phzA1*, and **(d)** *rhlA* mRNA were measured by qPCR. The *P. aeruginosa* wild-type was cultured in glutamate minimal medium in a P- or C-limited chemostat (left panels) and in batch culture (right panels). A *lasR* mutant, a *rhlR* mutant, and a *lasR rhlR* double mutant (indicated as "both") were cultured in a P-limited chemostat for comparison (left panels). Chemostat cultures were at a steady-state OD600 of 0.15. Batch cultures were sampled during exponential (E), early stationary (ES), and late stationary (LS) phase, at the indicated densities. Values are the mean of three independent biological replicates. Error bars indicate the standard deviation of the mean.

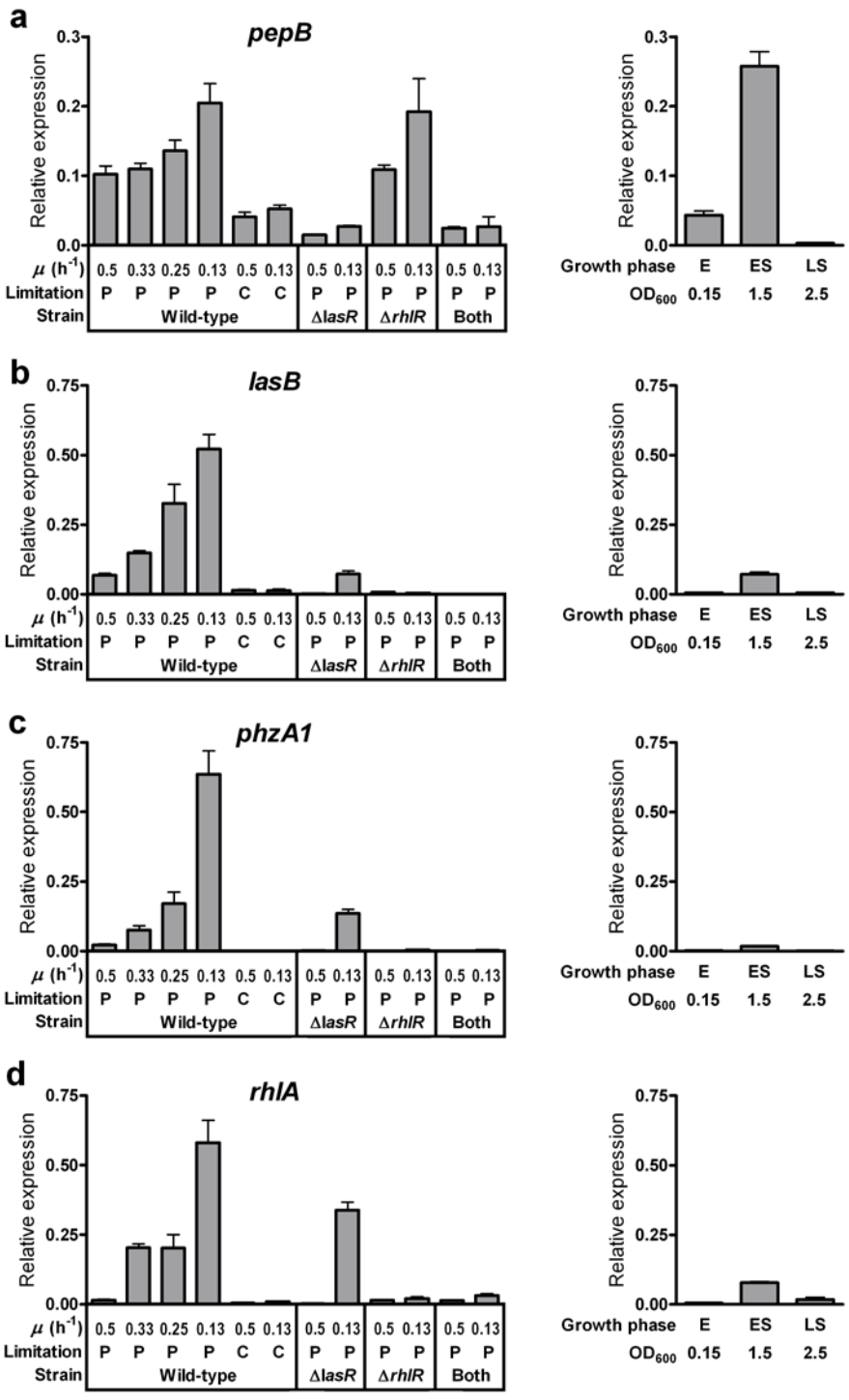


Figure 4.3. Transcript levels of QS-controlled genes in chemostat and batch culture.

To relate the chemostat data to those from more familiar batch cultures, we also measured gene expression during batch-culture growth (Fig. 4.3, right panels), acknowledging that direct comparison is somewhat limited by the fact that chemostat cultures are at steady-state, whereas batch cultures are not. The minimal medium for the batch cultures was used at full-strength, with P and C concentrations higher than those used for chemostat cultures. This allowed us to establish baseline gene expression at μ_{max} during exponential growth, at a density identical to that of the chemostat. In batch cultures, the expression of all QS-controlled genes was lowest during exponential phase, increased in early stationary phase, and decreased again in late stationary phase (Fig. 4.3, right panels), similar to the expression pattern in rich medium (Fig. S4.1). When glutamate is the C-source, the growth yield in the full-strength minimal medium is C-limited, although other macronutrients are also approaching depletion (Fig. S4.2). Interestingly, gene expression of *rhl*-dependent genes in P-limited chemostats was higher than that in batch cultures at comparatively higher cell density. Taken together, these data show that a reduction in growth rate under specific nutrient limitation induces the expression of secreted products, independent of cell density.

To confirm the chemostat expression data obtained with qPCR, we independently assessed QS gene expression via our transcriptional *lacZ* reporter fusions (Fig. S4.6). Both approaches yielded very similar results. The reporter data also confirm the notion that the starvation-dependent regulation of QS transcripts is at the level of transcription initiation.

Slow growth primarily induces the *rhl* system in continuous culture when specific nutrients are limiting. We hypothesized that the preferential induction of *rhl*-dependent genes during P-limited slow growth in chemostat culture is reflected in the differential activation of the *las* vs. the *rhl* system. To test this, we compared the transcript levels of *lasR*, *rhlR*, *lasI*, and *rhlI* by qPCR (Fig. 4.4a, b). We further examined acyl-HSL levels by bioassay and LasR and RhlR protein levels by Western blot (Fig. 4.4c, d). Growth rate reduction had a much more pronounced effect under P-limiting than C-limiting conditions, and affected the *rhl* system more than the *las* system, in support of

our hypothesis. The increase in *rhlR* and *rhlI* transcript levels under P-limited slow growth correlated with the increase in RhlR protein and C4-HSL levels, respectively (Fig. 4.4). On the other hand, while *lasR* showed a modest increase, *lasI* transcript levels showed little to no increase during P-limited slow growth (Fig. 4.4a, b). This corresponded to virtually unchanged LasR protein levels, and an actual decrease in 3OC12-HSL levels (Fig. 4.4c, d). Interestingly, the ratios of the two acyl-HSL signals were dramatically affected by growth rate (Fig. 4.4c). During faster growth (μ = 0.5 h^{-1}), the concentration of 3OC12-HSL was much higher than that of C4-HSL. During slow growth (μ = 0.13 h^{-1}), in contrast, the concentration of C4-HSL was much higher than that of 3OC12-HSL.

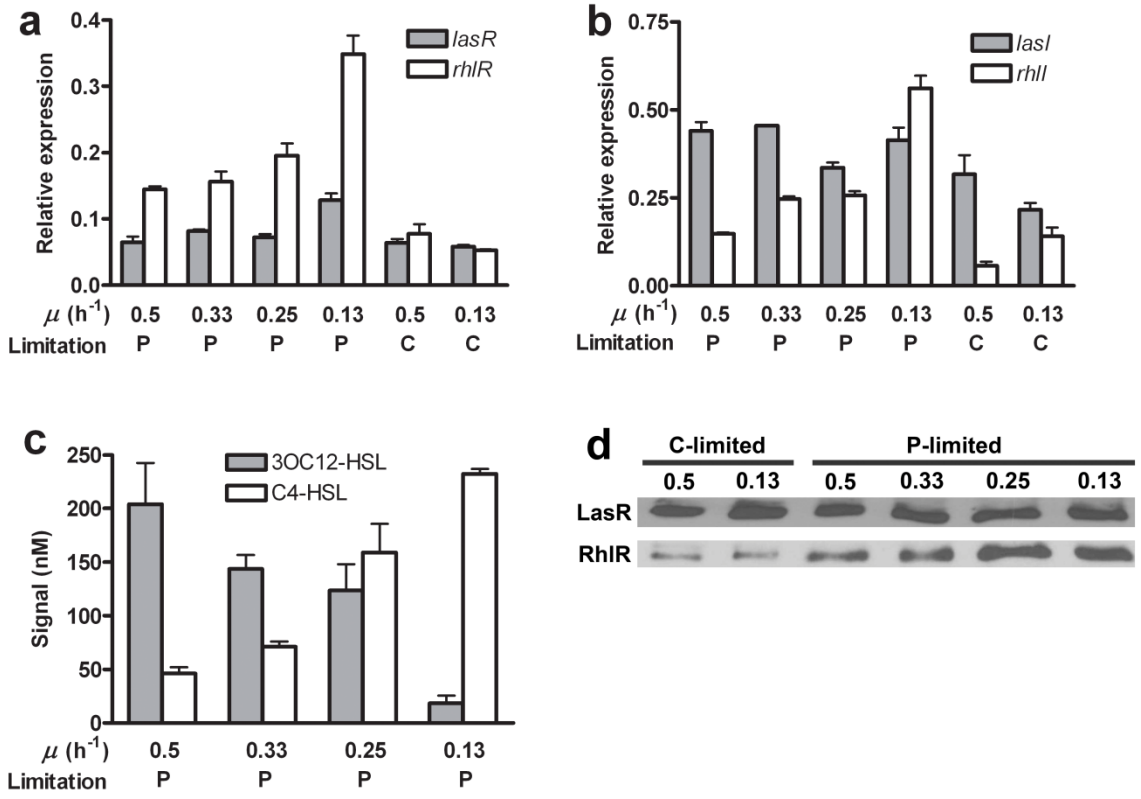


Figure 4.4. Expression levels of *las* and *rhl* system components in chemostat culture. The *P. aeruginosa* wild-type was cultured in P- or C-limited glutamate minimal medium as indicated. Chemostat cultures were at a steady-state OD_{600} of 0.15. **(a, b)** Relative transcript abundances of **(a)** *lasR* and *rhlR*, and **(b)** *lasI* and *rhlI* measured by qPCR. **(c)** Acyl-HSL concentrations measured by bioassay. **(d)** LasR and RhlR protein levels determined by Western blot analysis. Equal amounts of total protein were loaded for each condition. Values in panels a, b, and c are the mean of three independent biological replicates. Error bars indicate the standard deviation of the mean.

To finally demonstrate that preferential induction of the *rhl* system by P-limited slow growth actually causes activation of target genes, we measured the expression of QS-controlled public good genes in a *lasR* mutant, a *rhlR* mutant, and a *lasR rhlR* double mutant. We found that *lasB, phzA1*, and *rhlA* expression substantially increases during slow growth in a *lasR* mutant but not in a *rhlR* mutant (Fig. 3, left panels). However, expression in the *lasR* mutant was significantly lower than in the wild-type ($p \leq 0.049$ at $\mu = 0.5$ $h^{-1}$and $\mu = 0.13$ $h^{-1}$by two-tailed *t*-test), indicating that *lasR* is still required for full induction, presumably as an epistatic regulator of the *rhl*-system, and also as a co-

regulator in the case of *lasB*. The expression of *pepB* was greatly reduced in a *lasR* mutant, but was not significantly different from wild-type in a *rhlR* mutant ($p \geq 0.60$ at $\mu = 0.5$ $h^{-1}$and $\mu = 0.13$ h^{-1} by two-tailed *t*-test), confirming that this gene is *las*-specific (Fig. 3, left panels). As expected, all genes exhibited little to no expression in the *lasR rhlR* double mutant control (Fig. 3, left panels). Taken together, these data suggest that the *rhl* system is mainly responsible for increased QS-controlled gene expression during P-limited slow growth and that the *las* system plays a minor role.

Discussion

Our work consolidates a large body of literature on the complex regulation of secreted products in *P. aeruginosa*, and provides insights into the evolutionary and ecological purpose of such regulation. While research shows that the regulation of public goods by QS optimizes the costs and benefits of their production (Darch *et al.*, 2012, Pai *et al.*, 2012), the purpose of their co-regulation by numerous other pathways and environmental conditions have been less apparent. Our approach was to comprehensively identify specific nutrient conditions that induce QS-controlled public good genes and to determine whether these genes are controlled in a growth-rate-dependent manner. We utilized a defined minimal medium that permits limitation of each macronutrient, and a continuous culture system that separates the effects of growth rate and cell density. We found that QS-controlled genes are differentially regulated under different nutrient conditions and during slow growth. Successive reduction in growth rate through specific nutrient limitation augmented induction (see Fig. 4.5 for a schematic model, additional details of which are discussed below).

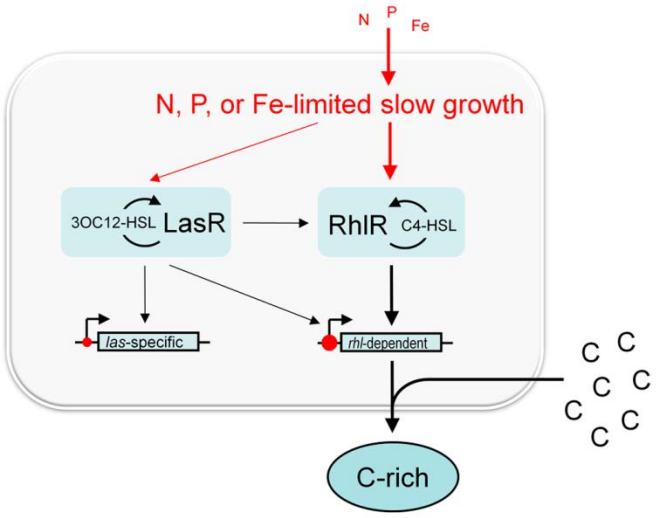


Figure 4.5. Supply-driven regulation of QS-dependent public goods in *P. aeruginosa*. Specific macronutrient limitation and concomitant reduction in growth rate induce the expression of secreted products, primarily those under direct control of the *rhl* system (indicated by thicker arrows). Nutrient limitation induces RhlR and C4-HSL expression, but likely also directly regulates target gene expression at the promoter level (indicated as red circles; larger circle indicates stronger effect). In the depicted example, the secretion of a C-rich molecule is induced by N, P, or Fe starvation in the presence of excess C. In another example from this study, the secretion of C- and N-rich molecules (e.g. extracellular enzymes) is induced by P or Fe starvation in the presence of excess C and N.

Our data indicate that central factors in governing QS gene expression are regulation by either the *las* or the *rhl* system, and the molecular composition of the relevant secreted product. A *las*-controlled gene showed little response to nutrient starvation compared to genes directly controlled by the *rhl* system, and the observed expression pattern correlated with, and was caused by, the preferential induction of the *rhl* system. Secondly, products were generally highly expressed when their molecular building blocks were in excess and other nutrients were limiting, but suppressed when their molecular building blocks became limiting (Fig. 4.2, Table 4.2). A simple statistical thought experiment indicates that this association between molecular composition and macronutrient regulation is strong. The probability of the particular expression pattern observed for all genes, namely that they are induced by P and Fe (not building blocks) but not C (a building block) is 1 in 2^3 possible permutations, and the probability that only

rhlA and none of the three other genes are induced by N (rhamnolipids are the only product devoid of N) is 1 in 2^4 possible permutations. Thus, the combined probability for the observed expression pattern, which is the only pattern consistent with our conclusion, is 1 in 8 (0.125) x 1 in 16 (0.0625) = 0.0078.

Our results attribute general validity to the 'metabolically prudent' regulation of secretions that minimizes the costs of their production (Xavier *et al.*, 2011). Metabolic prudence was introduced by Xavier *et al.* to describe the nutritionally conditional regulation of rhamnolipid secretion in *P. aeruginosa* (Xavier *et al.*, 2011). Expression of this C-rich molecule is induced only when another nutrient, N, is limiting, when there is excess carbon, and when the cells approach the stationary phase of growth. Metabolic prudence minimizes the fitness costs of cooperation through efficient use of available resources because cells do not secrete molecular building blocks that could instead be used for growth. This regulatory strategy therefore has the potential to stabilize cooperative behavior according to inclusive fitness theory (Hamilton, 1964, West *et al.*, 2006), and to limit the spread of non-cooperating mutants or 'cheaters', as previously demonstrated (Xavier *et al.*, 2011).

What might be the ecological significance of such supply-driven regulation, seemingly unrelated to the function of the public good? As we have pointed out previously (Gupta & Schuster, 2013), QS-dependent public goods are secreted irrespective of whether they provide a direct fitness benefit (i.e. promote growth of clonal populations) or not. *P. aeruginosa* and other opportunistic pathogens frequently reside in non-parasitic environments, and it is possible that secreted products primarily provide an indirect fitness benefit in this context. Many of the QS-controlled secretions have the potential to harm other cells. In light of the recently proposed "competition sensing" hypothesis (Cornforth & Foster, 2013), such secretions can be viewed as a broad, non-specific microbial counter-attack to ecological competition inferred from nutrient starvation. If the presence of competition cannot be sensed directly and reliably, then the production of secretions appears prudent whenever the cost of their production is low.

In our work, successive limitation of C, and in some cases N, during batch culture growth reduced public goods expression. Even though none of the macronutrients in the full-strength minimal medium were in great excess, we can infer from the individual nutrient dilution experiments that the medium was C- and N-limited when succinate and glucose were the C-sources, and C-limited when glutamate was the C-source (Figs. S2-S4). Even under C-limited conditions there was some induction of QS-controlled public good genes in glutamate medium batch culture in stationary phase (Fig. 4.4). This is consistent with previous work. Individual QS-controlled genes are induced during C-limited slow growth in casein medium (Sandoz *et al.*, 2007), and exoprotease activity is induced during C-limited chemostat culture at very low growth rates ($\mu \geq 0.05$ h^{-1}, attained at reduced temperature with a different *P. aeruginosa* strain and an anti-foaming agent) (Whooley *et al.*, 1983). However, we also showed that QS gene expression decreases in batch cultures when C-limitation becomes more pronounced through successive dilution. This decrease was specific to certain macronutrients and target genes and is thus unlikely to be caused solely by a concomitant reduction in cell density. These data point to the ratio of macronutrients as a key determinant in regulating secretions (Guerra-Santos *et al.*, 1984). For example, gene expression decreases with decreasing C-to-N or C-to-P ratios. The inclusion of C levels as a regulatory cue is equally 'prudent' as all QS-controlled secreted products in *P. aeruginosa* are rich in C (Schuster & Greenberg, 2007).

Additionally, we found that QS gene expression in batch cultures decreases when cells enter late stationary phase in C-limited medium (Fig. 4.3), and in some cases when an inducing macronutrient is highly diluted (Figs. S4.2-S4.4). This pattern may be interpreted as a complete "shutdown" of cells in response to severe starvation.

To address the mechanism of the preferential regulation of *rhl*-dependent public goods, we measured the expression of central QS components in the wild-type, and of QS target genes in signal receptor mutants, during P- and C-limited chemostat growth. Quantitation of transcript, acyl-HSL, and regulator protein levels indicate that the *rhl* system is greatly induced by P-limited slow growth, and that this induction is largely

responsible for the observed expression pattern in QS target genes. Consistent with the predominant role of the *rhl* system, we found that progressively slower growth rates invert the 3OC12-HSL/C4-HSL ratio in favor of C4-HSL. Lower dilution rates could conceivably have an impact on the steady-state concentrations of each signal due to their different chemical stabilities. However, if this was the case, the effect would be opposite of what we observed because long-chain acyl-HSL signals are more resistant to lactonolysis than short-chain signals (Yates *et al.*, 2002). Thus, signal ratios likely represent real physiological changes rather than an artifact of the culturing method. Signal ratios have been proposed as a diagnostic marker for the growth of *P. aeruginosa* as a biofilm in cystic fibrosis (CF) lung infections (Singh *et al.*, 2000). The signal ratio, as measured by radiometry, was the same in laboratory biofilms and in CF sputum, but was opposite to that in planktonic culture (Singh *et al.*, 2000). Our results suggest more broadly that the signal ratio is primarily an indicator of growth rate and nutrient status, irrespective of the mode of growth.

Previous studies identified mechanisms that allow *P. aeruginosa* to respond to low N, P, and Fe during batch culture growth, and they provide the link between QS and the physiological cues identified in this study. The two-component regulatory system PhoRB induces expression of *rhlR* when growth becomes P-limited (Jensen *et al.*, 2006). Regulatory control appears to be direct as *rhlR* possesses a PhoB binding site in its promoter region (Jensen *et al.*, 2006), but may also be indirect through a newly identified non-acyl-HSL QS signal, termed IQS (for 'integrating QS and stress response') (Lee *et al.*, 2013). Fe depletion enhances *rhlR* and *rhlI* expression via ferric uptake regulator protein and the PQS system (Jensen *et al.*, 2006, Oglesby *et al.*, 2008), whereas N starvation enhances *rhlR* transcription via the alternative sigma factor RpoN (Medina *et al.*, 2003a). However, taken together, regulatory control at the level of *rhlR* and/or *rhlI* is unlikely to be sufficient to produce the diverse responses to macronutrient limitation that we observed. While induction by P and Fe limitation was universal, induction by N limitation was specific to only one secreted product, rhamnolipid. Further control must therefore exist at the target promoter level of the individual QS-controlled genes. This

conclusion is consistent with the finding that combined overexpression of *rhlR* and addition of exogenous signal are not sufficient to induce *rhlA* expression (Medina *et al.*, 2003b).

The stringent response can activate the *las* and *rhl* QS systems in *P. aeruginosa* during amino acid starvation (van Delden *et al.*, 2001). Because the minimal media employed in this study lack amino acids and require de-novo synthesis, the amino-acid dependent stringent response likely contributed to a basal level of target gene induction. It is conceivable that N, P, or Fe-limited slow growth resulted in further activation of the stringent response, in addition to their activation of specific starvation pathways as described above. For example, in *E. coli* the stringent response is also activated upon P limitation in addition to amino acid limitation (Spira *et al.*, 1995).

Macronutrient ratios may be integrated at the level of intracellular metabolite fluxes. This is perhaps best exemplified by the signal transduction pathway that regulates nitrogen assimilation. It consists of the two component system NtrBC and the signal protein PII, which senses nitrogen availability via glutamate (Leigh & Dodsworth, 2007). However, PII is also responsive to cellular carbon levels and energy charge through direct binding of α-ketoglutarate and ATP (Leigh & Dodsworth, 2007).

Our results finally help explain why there is a need for multiple QS systems in one organism. While two QS systems may serve to temporally scale gene induction (Whiteley *et al.*, 1999, Schuster *et al.*, 2003, Long *et al.*, 2009), distinct stress signal integration in *P. aeruginosa* QS suggests an additional role. The *las* system appears to predominately respond to autoinducer accumulation whereas the *rhl* system integrates nutritional cues. Thus, multiple QS systems can provide a functional split between gene products that should be prudently regulated during starvation and gene products that should be expressed whenever the cell density is adequate for their efficient use. Interestingly, the *rhl* system was acquired later in the evolutionary history of *P. aeruginosa* via horizontal gene transfer (Lerat & Moran, 2004). It is intriguing to speculate that this event facilitated the integration of quorum and stress responses.

Acknowledgements

We thank Rashmi Gupta for cloning the *lasB* and *rsaL* promoters, and Herbert P. Schweizer for the mini-CTX-*lacZ* construct. We also thank Cara Wilder, Rashmi Gupta, D. Joseph Sexton, Kyle Asfahl, and Erin Bredeweg for their helpful discussions. This work was supported by National Institutes of Health grant AI079454, and by National Science Foundation grant 1158553 (both to M.S.). B.M. was supported in part by the P.F. and Nellie Buck Yerex Graduate fellowship.

Supplemental Material

Table S4.1. Primers used in this study.

Construct or gene	Primer name	Primer sequence (5'-3')[a]
Plasmid		
mini-CTX-*pepB'-lacZ*	PA2939-*Eco*RI-F	N_6GAATTCGGAGGACGTCGTTTTCATGG
	PA2939-*Bam*HI-R	N_6GGATCCGAGACTCCGTTCCTTGTGAG
mini-CTX-*rsaL'-lacZ*	PA1431-*Hind*III-F	N_6AAGCTTGAAGAATTTATGCAAATTTCATAA
	PA1431-*Eco*RI-R	N_6GAATTCTCTTTTCGGACGTTTCTTCG
mini-CTX-*lasB'-lacZ*	PA3724-*Hind*III-F	N_6AAGCTTGGCCTACAA GCTCGACGTCA
	PA3724-*Eco*RI-R	N_6GAATTCCTTCTTCATCTTTTCAGTTCTCC
mini-CTX-*phzA1'-lacZ*	PA4210-*Xho*I-F	N_6CTCGAGCCAGAGCCTTTTCCTGCGTA
	PA4210-*Bam*HI-R	N_6GGATCCCTCGCGGCATCGGTTATTC
Real-time qPCR		
pepB	PA2939-qPCR-F	CGGAAGCGCAACAGTTCAC
	PA2939-qPCR-R	CAACGGCGATTTGCAGATC
rsaL	PA1431-qPCR-F	GACGTTTCTTCGAGCCTAGCA
	PA1431-qPCR-R	CAAATAGGAAGCTGAAGAATTTATGCA
lasB	PA3724-qPCR-F	CCAGGCCAAGAGCCTGAAG
	PA3724-qPCR-R	CGGATCACCAGTTCCACTTTG
phzA1	PA4210-qPCR-F	CCACTACATCCATTCCTTCGAACT
	PA4210-qPCR-R	AATTTCTGCATCGGGTTCATG
rhlA	PA3479-qPCR-F	GGCGCGAAAGTCTGTTGGT
	PA3479-qPCR-R	CCAACGCGCTCGACATG
lasR	*lasR*-qPCR-F	AGCCGGGAGAAGGAAGTGTT
	lasR-qPCR-R	GAGCAGTTGCAGATAACCGATATC
rhlR	*rhlR*-qPCR-F	ACCGCGAGATCCTGCAATG
	rhlR-qPCR-R	TCAGGATGATGGCGATTTCC
lasI	*lasI*-qPCR-F	GCCCCTACATGCTGAAGAACA
	lasI-qPCR-R	CGAGCAAGGCGCTTCCT
rhlI	*rhlI*-qPCR-F	GCAGCTGGCGATGAAGATATT
	rhlI-qPCR-R	TGGCGCCCAGGTACCA

[a]Restriction sites are underlined.

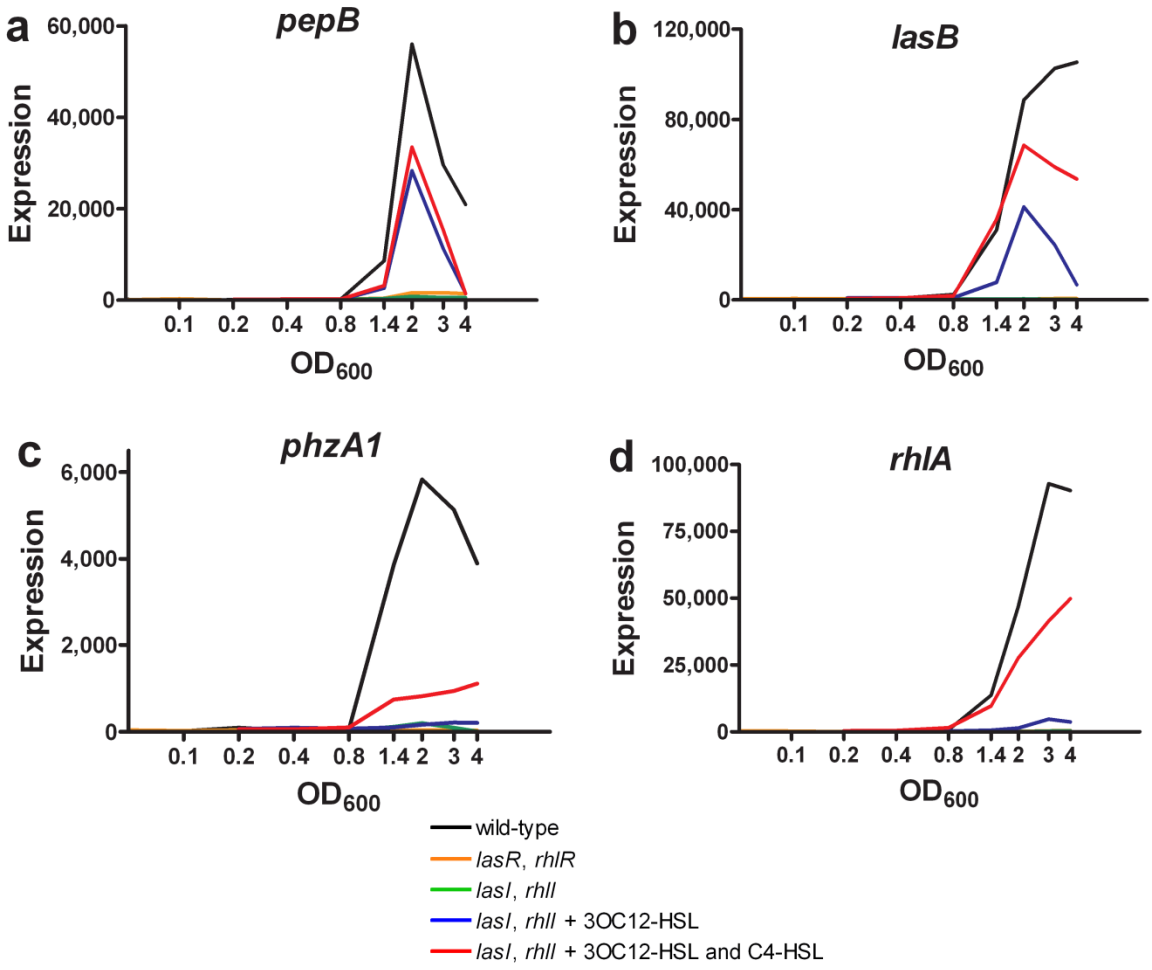


Figure S4.1. QS-controlled gene expression in complex medium. Graphical representation of microarray expression data from Schuster *et al.*(Schuster *et al.*, 2003) for QS-controlled genes investigated in this study. Expression of **(a)** *pepB*, **(b)** *lasB*, **(c)** *phzA1*, and **(d)** *rhlA* in the *P. aeruginosa* PAO1 wild-type (black line), an isogenic *lasR*, *rhlR* receptor mutant (orange line), and a non-isogenic *lasI*, *rhlI* signal generation mutant without added acyl-HSL (green line), with 3OC12-HSL (blue line), and with C4-HSL and 3OC12-HSL (red line). Strains were cultured in LB medium from early exponential to stationary phase and transcript levels were determined at the indicated culture densities (OD_{600}). An $OD_{600} \geq 1.4$ signifies stationary phase. The values on the *y*-axis represent transcript abundance as determined by the array software. The absence of green and yellow lines in some panels indicates baseline gene expression too low to be visible.

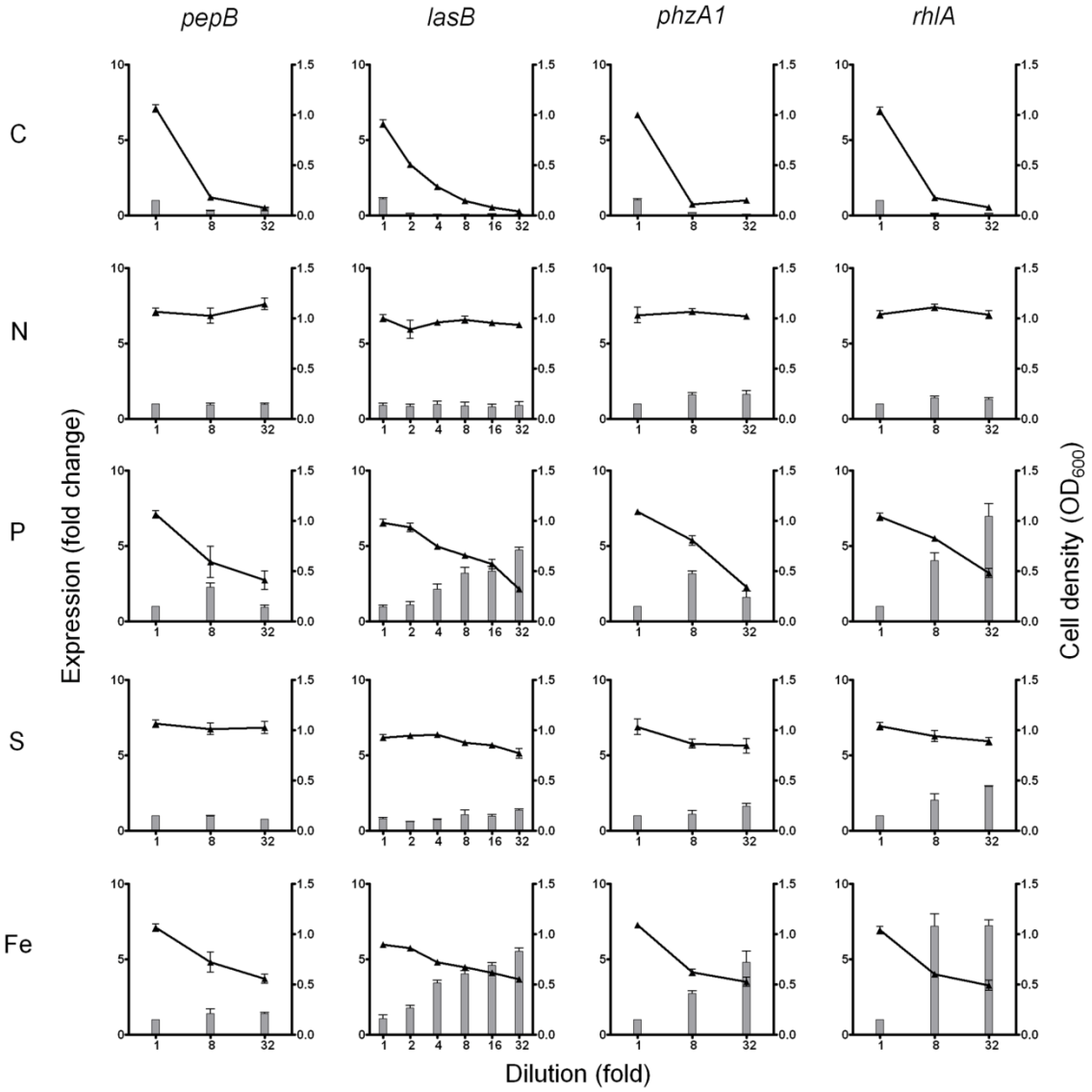


Figure S4.2. Nutrient dilution in glutamate minimal medium. Progressive dilution of carbon (C), nitrogen (N), phosphorous (P), sulfur (S), and iron (Fe) was carried out in MOPS minimal medium batch cultures with glutamate as the sole C-source and with *P. aeruginosa pepB*, *lasB*, *phzA1*, and *rhlA* reporter strains. Bars indicate the fold change in β-galactosidase expression compared to undiluted medium (left *y*-axis). Triangles indicate culture density (OD_{600}; right *y*-axis). Fold change values shown in graphs are means from three independent biological replicates, normalized to OD_{600}. Error bars indicate standard deviations of the mean.

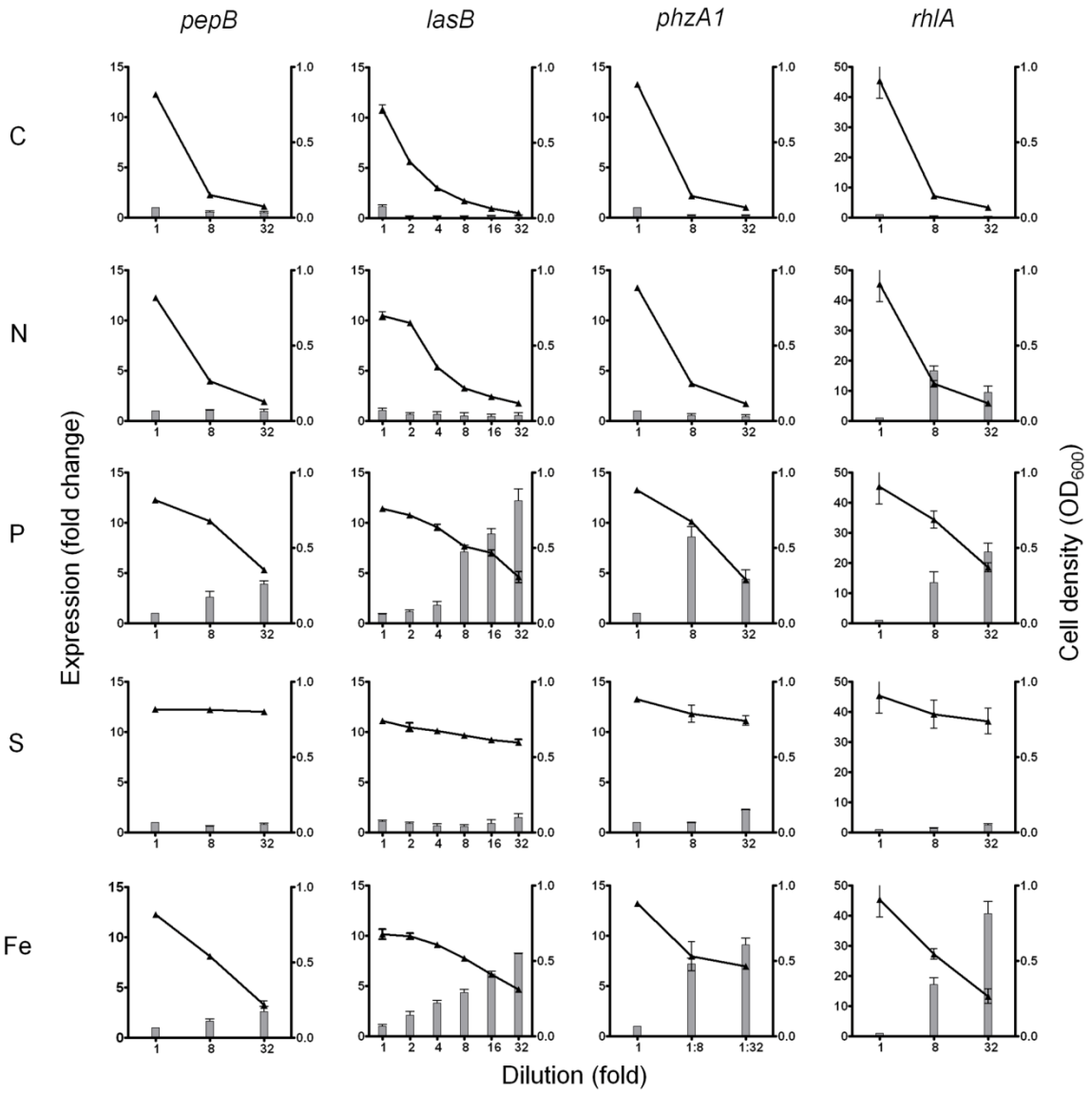


Figure S4.3. Nutrient dilution in succinate minimal medium. Progressive dilution of C, N, P, S, and Fe was carried out in MOPS minimal medium batch cultures with succinate as the sole carbon source and with *P. aeruginosa pepB*, *lasB*, *phzA1*, and *rhlA* reporter strains. Bars indicate the fold change in β-galactosidase expression compared to undiluted medium (left *y*-axis). Triangles indicate culture density (OD_{600}; right *y*-axis). Fold change values shown in graphs are means from three independent biological replicates, normalized to OD_{600}. Error bars indicate standard deviations of the mean.

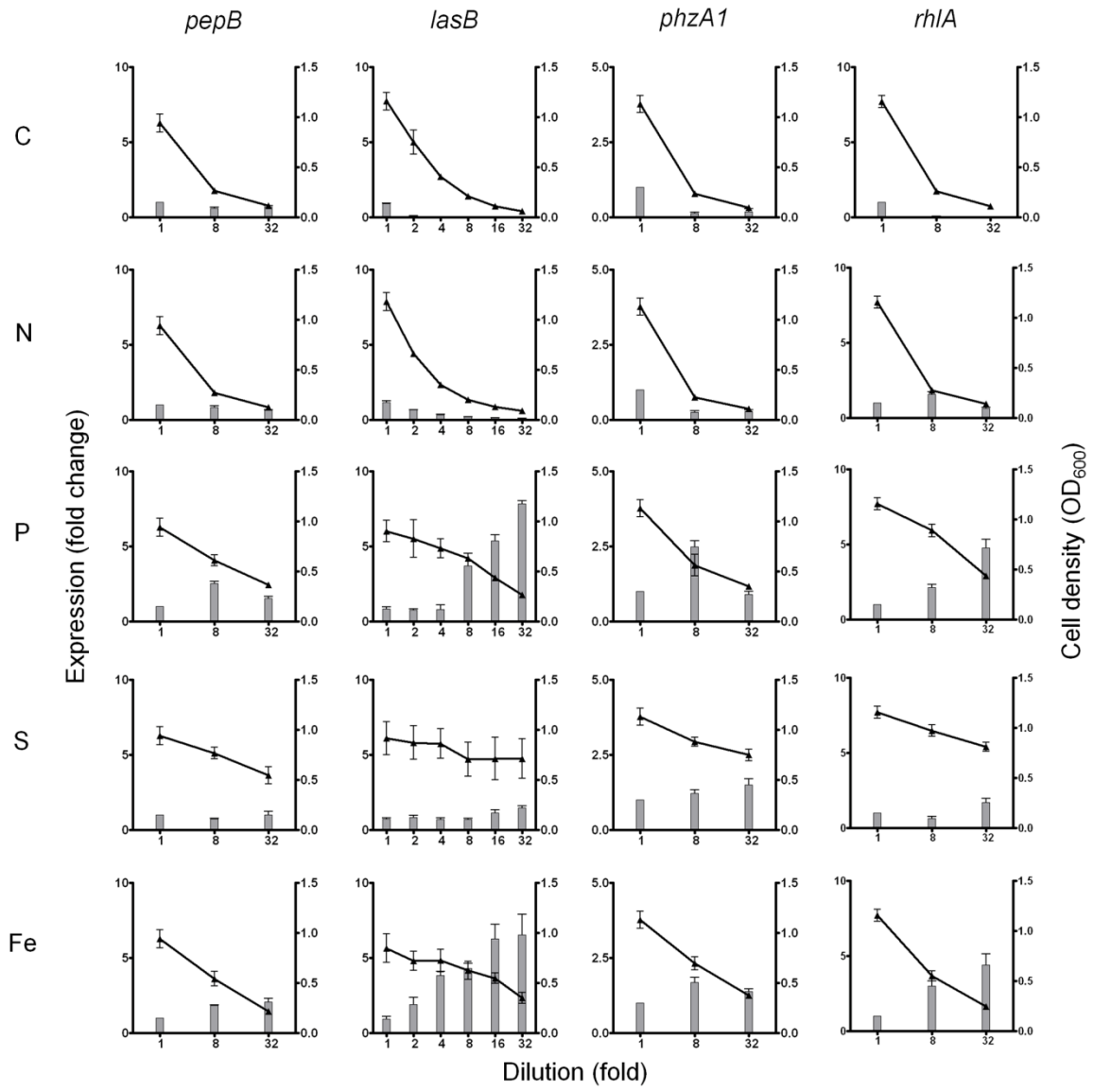


Figure S4.4. Nutrient dilution in glucose minimal medium. Progressive dilution of C, N, P, S, and Fe was carried out in MOPS minimal medium batch cultures with glucose as the sole C-source and with *P. aeruginosa pepB, lasB, phzA1*, and *rhlA* reporter strains. Bars indicate the fold change in β-galactosidase expression compared to undiluted medium (left *y*-axis). Triangles indicate culture density (OD_{600}; right *y*-axis). Fold change values shown in graphs are means from three independent biological replicates, normalized to OD_{600}. Error bars indicate standard deviations of the mean.

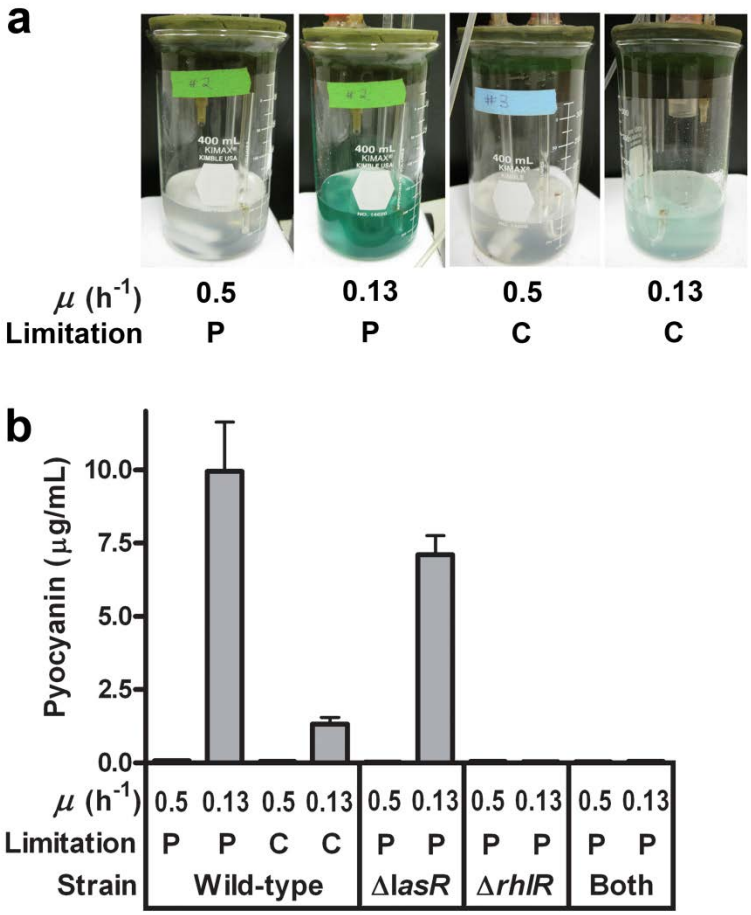


Figure S4.5. Influence of growth rate and limiting nutrients on pyocyanin production in chemostat culture. *P. aeruginosa* was grown in P-limited or C-limited glutamate minimal medium. **(a)** Images of *P. aeruginosa* wild-type cultures. The blue-green pigmentation is characteristic of pyocyanin, a secreted, redox-active, phenazine antibiotic. **(b)** Quantitation of pyocyanin production of the *P. aeruginosa* wild-type, a *lasR* mutant, a *rhlR* mutant, and a *lasR rhlR* double mutant (indicated as "both"). Pyocyanin concentrations are the means of three independent biological replicates. Error bars indicate standard deviations of the mean.

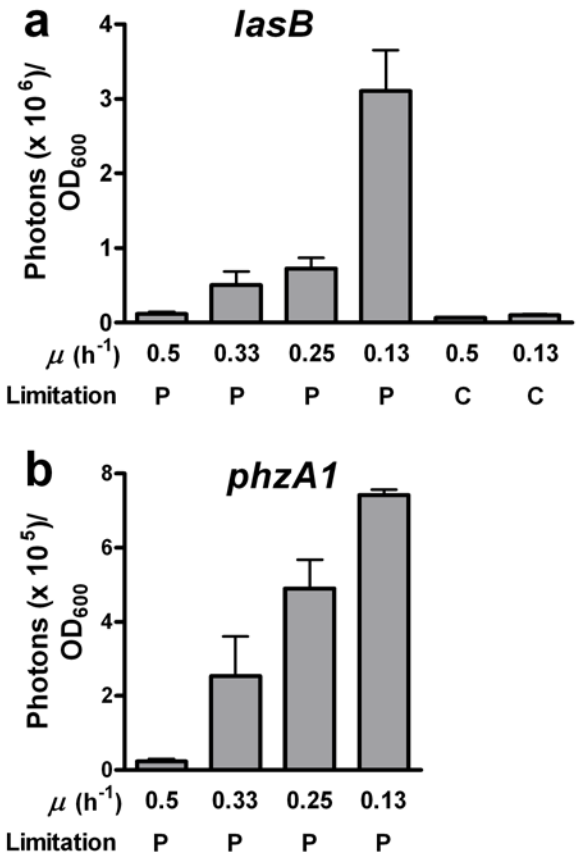


Figure S4.6. QS-controlled gene expression during chemostat culture measured by *lacZ* reporter fusions. Expression of **(a)** *lasB* and **(b)** *phzA1* in *P. aeruginosa* reporter strains during P-limited or C-limited growth in glutamate minimal medium, as indicated. Bars indicate β-galactosidase expression (in photons) normalized to culture density (OD_{600}). Values shown in the graph are the means of three independent biological experiments. Error bars indicate standard deviations of the means of three independent biological replicates.

Chapter 5

Conclusion

Pseudomonas aeruginosa is a metabolically versatile organism, well-adapted to many environments due to its large genome (6.3 Mb) (Stover *et al.*, 2000). *P. aeruginosa* may be found in soil, water, or as an opportunistic pathogen of burn wounds and cystic fibrosis lungs (Kulasekara & Lory, 2004, Moreau-Marquis *et al.*, 2008, Wagner & Iglewski, 2008). The organism's ability to effectively change gene expression between environments may be due to the large proportion (about 10%) of the genome devoted to regulatory functions (Stover *et al.*, 2000). One such regulatory system in *P. aeruginosa* that we studied is cell-to-cell communication or quorum sensing (QS). QS controls the expression of hundreds of genes, particularly those associated with secreted factors (public goods) such as toxic metabolites and enzymes (Stover *et al.*, 2000, Wagner *et al.*, 2003, Schuster & Greenberg, 2006). QS regulates many shared public goods and is considered a cooperative behavior (Schuster *et al.*, 2013). In this dissertation, we investigated the effect of social cheating on the development of QS inhibitor (QSI) resistance and the capability of supply-driven regulation to maintain cooperation.

Many QS-controlled public goods function as virulence factors during human infection (Schuster *et al.*, 2013). This observation makes QS an attractive target for small-molecule inhibitors as a so-called antivirulence therapy (Bjarnsholt & Givskov, 2007a). Antivirulence drug therapy disarms rather than kills pathogens and was proposed as a solution to the development of resistance to antibiotics (Clatworthy *et al.*, 2007, Rasko & Sperandio, 2010). Resistance to traditional antibacterial drugs develops and spreads because susceptible cells are destroyed and resistant cells are able to take over the population (Clatworthy *et al.*, 2007). Antivirulence drugs do not directly kill bacteria and offer several advantages including less selective pressure towards resistance, preservation of normal microflora, and the addition of new targets for small-molecule inhibitors

(Schuster *et al.*, 2013). However, the hypothesis that antivirulence drugs would reduce development of resistance had never been previously tested. While several small molecules have shown modest selective inhibition of acyl-HSL QS systems (Bjarnsholt & Givskov, 2007a), there are currently no known QSI in drug development. This drought in drug development may be due to a lack of understanding of the development of resistance to QSI (Projan & Shlaes, 2004). We sought to apply our knowledge of secreted factors to test if QSI-resistant mutants could enrich in a population of cells that no longer produce QS-controlled goods.

We reasoned that extracellular virulence factors would function as public goods for the population, while cell-associated virulence factors would offer a private benefit for the individual cell. For example, shared, public virulence factors would typically be enzymes, toxins, or metabolites secreted into the environment, and private virulence factors may be adhesins or periplasmic enzymes (Mellbye & Schuster, 2011b, Schuster *et al.*, 2013) (also see Fig 1.2). Public goods are considered to be regulated by a social behavior (e.g. QS), costly to produce, and have the potential to be exploited by cheaters (Schuster *et al.*, 2013).

We designed a proof-of-concept model to test whether competition for public goods would suppress the enrichment of QSI-resistant mutants (Mellbye & Schuster, 2011b) (Chapter 3). We found that QSI-resistant mutants may not enrich if conditions require public goods expression (Mellbye & Schuster, 2011b) (Chapter 3; also see Fig. 1.1). Thus, antivirulence drug therapy targeted against shared virulence factors may be more resistant to invasion by drug resistant mutants (Mellbye & Schuster, 2011b) (Chapter 3). Further experiments are needed to apply our *in vitro* model to the development and use of antivirulence drugs *in vivo* (Mellbye & Schuster, 2011b) (Chapter 3).

While social conflict has been demonstrated *in vitro* and in infection models, and has the potential to suppress the development of QSI resistance, cheating has not eliminated cooperation in *P. aeruginosa*. Previous work suggests that some environmental conditions maintain cooperative behaviors and suppress cheaters (Xavier

et al., 2011, Dandekar *et al.*, 2012). Cooperation may be favored by integrating QS and environmental stress to either suppress or increase public goods production (Schuster *et al.*, 2003, Schuster *et al.*, 2013). Results from Chapter 3 provide insights in this regard, and have implications for the efficacy of QSIs (Schuster *et al.*, 2013).

Many previous studies on quorum sensing have investigated how gene expression is regulated (Mellbye & Schuster, 2011a) (Chapter 2). In Chapter 4, we sought to understand both why and when QS-controlled secreted factors are expressed, and how signal integration of QS and nutrient stress may help stabilize cooperation. Previous studies have almost exclusively focused on batch culturing in rich medium so we employed defined minimal medium to control macronutrient concentration (Mellbye & Schuster, 2011a) (Chapter 2). In addition, we used a chemostat continuous culture system to test the effect of growth rate on public good expression. We used minimal medium batch cultures to characterize the gene expression of QS-controlled genes associated with secreted factor production (LasB elastase, aminopeptidase PaAP, rhamnolipid surfactant, pyocyanin toxin) in response to progressive macronutrient dilution. These four secreted factors were chosen because of their direct control by either the *las*, *rhl*, or both acyl-HSL QS systems (Schuster *et al.*, 2003, Wagner *et al.*, 2003, Gilbert *et al.*, 2009). In addition, the genes *pepB, lasB, rhlA*, and *phzA1* are well-studied in the QS field and can serve as public good production reporters (Mavrodi *et al.*, 2001, Bleves *et al.*, 2010, Abdel-Mawgoud *et al.*, 2010) (Chapter 2). Finally, to directly test the effects of growth rate, we tested QS-controlled gene expression using a minimal medium chemostat culturing system.

The central factors determining the cell's response to their environmental conditions were regulation by either the *las* or *rhl* system and the molecular composition of the public good (Chapter 2). The effect on public good expression was independent of the secreted products function (Chapter 2). Instead, expression depended on the goods' elemental composition and was initiated only if the limiting nutrient is not also a building block of the good (Chapter 2). This "supply-driven" method of regulation provides the physiological basis for the co-regulation of QS-controlled genes by stress responses

(Chapter 2). As previously demonstrated, QS-controlled public good expression is not restricted to environments that require QS and requires negative regulation to maintain cooperation (Gupta & Schuster, 2013). Our results attribute general validity to the "metabolically prudent regulation of secretion" hypothesis initially formulated for the control of rhamnolipid biosynthesis in *P. aeruginosa*: cells secrete public goods only when the cost of their production and impact on individual fitness is low (Xavier *et al.*, 2011) (Chapter 2). This regulatory strategy minimizes the cost of production, thereby reducing the evolutionary advantage of non-secreting 'cheaters' (Xavier *et al.*, 2011). Metabolic prudence might first have evolved to optimize growth or in response to cheaters and may be a general mechanism to stabilize cooperation (Xavier *et al.*, 2011).

Our role-play experiment in Chapter 3 suggests that cooperative behaviors may be a promising target for antivirulence drug therapy. However, Chapter 4 suggests that environmental conditions can reduce the individual fitness cost of public good production to zero. In this case, QS-proficient cells would not be at a growth disadvantage, but they would not carry any advantage over QS-deficient cells either. In this case, a QSI-resistant cell would still be unable to enrich and there would be a population-wide cost of reduced public good production. Our hypothesis is that a QS-deficient cell could still function as a cheater and burden on the population when nutrient acquisition is public even if its fitness is equal to 1. Further experiments are needed to support this hypothesis.

Recent work on the potential for resistance to QSI suggests that resistance mechanisms unrelated to QS, such as increased expression of efflux pumps that may pump out QSIs, already exist in natural and clinic settings (Maeda *et al.*, 2012, Garcia-Contreras *et al.*, 2013). Despite these indications, targeting cooperative behaviors in infections has potential to restrain resistance under the appropriate conditions. If QSI-resistant mutants arose or were present in the population, these public-good-producing cells would be at a very low frequency and may never reach the quorum needed to activate QS-controlled genes. Even if QS-controlled virulence factors were activated in response to slow growth and starvation (as demonstrated in Chapter 4), the population of

cells would likely have too few cooperating cells to reach a critical production level of exoenzymes or toxins for a successful infection.

Our *in vitro* experiment does not address several complex problems associated with real infections. First, we assumed that future QSIs would be broad spectrum inhibitors of acyl-HSL QS and inhibit both *lasR* and *rhlR.* Previous *in vitro* evolution studies have shown that *lasR* mutants can regain protease production, possibly through compensation by the *rhl* system (as observed during phosphorous-limited slow growth in Chapter 4) (van Delden *et al.*, 1998, Sandoz *et al.*, 2007). Second, populations growing *in vivo* may be more structured than a well-mixed batch culture, and this limited dispersal may increase the fitness of QSI-resistant mutants (Diggle *et al.*, 2007b, Kummerli *et al.*, 2009). In addition, the immune system may apply further selective pressure in that QS-proficient cells are protected from polymorphonuclear leukocytes (Bjarnsholt *et al.*, 2005). One future approach to address these issues may be to combine QSIs with traditional antibiotic therapy and potentially reduce the development of resistance to both treatments.

Future studies in antivirulence therapy should focus on appropriately designed experiments with relevant tissue culture and animal models. In addition, studying the interplay between social and non-social production of virulence factors may offer new insights. While QS is a popular target for antivirulence inhibition, different virulence transcriptional regulators, type II secretion, type III secretion, cell adhesion, and toxin function may also be promising targets (Clatworthy *et al.*, 2007). Which of these factors are considered public goods shared in a population of invading pathogens? Type II secretion could be considered an outlet tied to public good export (e.g. exoenzymes such as LasB elastase), but the social impacts of inhibition of other virulence factors remains to be determined. Our work suggests that social conflict is an important factor in the development of drug resistance.

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