AN ABSTRACT OF THE DISSERTATION OF

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Martin Schuster

Cell-cell communication in bacteria is understood to facilitate the coordination of population-wide cooperative behavior in the form of concerted gene expression. The opportunistic pathogen *Pseudomonas aeruginosa* uses such a communication mechanism to regulate a large group of genes important to virulence strategies in this bacterium. This general mechanism of communication is termed quorum sensing (QS) and restricts activation of target genes to high cell density when cooperation is beneficial. QS in *P. aeruginosa*, like many Gram-negative *Proteobacteria*, is mediated through the synthesis of diffusible *N*-acyl-homoserine lactone (AHL) signals by LuxI-type synthases, and recognition by LuxR-type receptors that function as transcriptional regulators. *P. aeruginosa* harbors two complete AHL QS synthase/receptor pairs termed LasI/R and RhlI/R. Here we use *P. aeruginosa* QS as a model system to investigate mechanisms that help maintain cooperative, QS-dependent secretion in the face of non-cooperating cheater mutants, and that define the cell density threshold that triggers the activation of QS target gene expression.

We begin with analysis of an *in vitro* evolution system in which *P. aeruginosa* must express QS-controlled extracellular proteases in order to grow. In this system, QS-deficient cheater mutants evolve over time. They take advantage of protease production by the QS-proficient wild-type. Curiously, QS-deficient cheaters only

reach a frequency of about 25% during the duration of the experiment. They do not enrich to levels that would cause a collapse of the population, generally referred to as a "tragedy of the commons". Genomic sequence analysis revealed a previously unknown mutation in this system in the transcriptional regulator PsdR. Mutations in the gene coding for PsdR derepress growth rate limiting nutrient uptake and metabolism, a non-social adaptation. Combining mutational analysis with phenotypic assays and measurements of relative fitness, we show that rapid fixation of PsdR mutation in evolving populations serves to preserve cooperation and prevent a tragedy of the commons.

Next, we focus on the mechanisms that determine the threshold of QS induction in *P. aeruginosa*. We constructed a set of isogenic mutant strains deficient in one, two, or three anti-activator proteins that serve to delay QS activation: QteE, QscR, and QslA. While these anti-activator proteins are understood to bind LasR and RhlR QS receptors, it is yet unclear why multiple anti-activators are needed, and how they work in concert to achieve the QS threshold. Using phenotypic assays, QS gene activation kinetics, and transcriptomic profiling, we found additive effects in the deletion of multiple anti-activator genes with largely overlapping sets of anti-activator-affected genes. Progressive deletion of anti-activators advances the induction threshold and increases expression levels. Our results suggest some anti-activators may even co-associate with R-proteins in exerting their effect.

Together, these studies contribute new mechanistic understanding of how *P. aeruginosa* uses QS to coordinate cooperative behaviors to specific conditions, and how this cooperative communication system may be safeguarded against social exploitation.

Social Evolution and Regulatory Architecture of *Pseudomonas aeruginosa* Quorum Sensing

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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.

Kyle L. Asfahl, Author

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Chapter 1

SOCIAL INTERACTIONS IN BACTERIAL CELL-CELL SIGNALING

Kyle L. Asfahl and Martin Schuster

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Abstract

Cooperation and conflict in microorganisms is being recognized as an important factor in the organization and function of microbial communities. Many of the cooperative behaviors described in bacteria are governed through a cell-cell signaling process generally termed quorum sensing. Communication and cooperation in diverse microorganisms exhibit predictable trends that behave according to social evolutionary theory, notably that public goods dilemmas produce selective pressures for divergence in social phenotypes including cheating. In this review we relate the general features of quorum sensing and social adaptation in microorganisms to established evolutionary theory. We then describe physiological and molecular mechanisms that have been shown to stabilize cooperation in microbes, thereby preventing a tragedy of the commons. Continued study of the role of communication and cooperation in microbial ecology and evolution is important to clinical treatment of pathogens, as well as to our fundamental understanding of cooperative selection at all levels of life.

1.1 Introduction

Cooperation among individuals is a common strategy affecting natural selection at every level of life, from genes in genomes (Burt & Trivers, 2006) to humans in global societies (Hardin, 1968, Kollock, 1998). Cooperative interactions typify states of stabilization along an evolutionary progression that has ultimately resulted in the complex and interconnected ecology of life we currently observe (Maynard Smith & Szathmary, 1995). With such a fundamental role for cooperation in the underlying ecology of the natural world, understanding the evolutionary origins and maintenance of cooperation has become a primary theme in biological research.

While not required for all cooperative interactions, communication among neighboring individuals is often deployed as a mechanism to coordinate cooperative strategies. At the scale of single cells, cooperation among microorganisms has provided a clear window for viewing complex evolutionary phenomena, enabling insights into mechanisms where similar studies of larger organisms have struggled (West, *et al.*, 2007). We now understand that many bacteria communicate in a process generally referred to as "quorum sensing" (QS). Originally discovered in Gram-negative *Proteobacteria*, the diversity of bacterial taxa harboring QS componentry has grown to include hundreds of species across most known bacterial phyla (Manefield & Turner, 2002, Case, *et al.*, 2008, Pereira, *et al.*, 2013). QS is now understood to mediate cooperative behaviors as diverse as light production during endosymbiosis with cephalopods (Fuqua, *et al.*, 1994), air vesicle formation that allows vertical migration of planktonic bacteria in aquatic habitats (Ramsay, *et al.*, 2011), biofilm formation (Davies, *et al.*, 1998), and virulence factor production (Van Delden & Iglewski, 1998). Many of these QS-regulated phenotypes exhibit the tell-tale signs of a cooperative "public good" and are the result of secreted products that are produced by individuals with benefits that are available to all cells in a population. Examples are exoenzymes for the degradation of biopolymers, exopolysaccharide (EPS) for the formation of biofilms, and antibiotics for microbial warfare (Schuster, *et al.*, 2013, Cook & Federle, 2014).

The particularly well-studied opportunistic pathogen *Pseudomonas aeruginosa* exhibits many such cooperative behaviors. It uses QS-controlled extracellular enzymes and toxins to inactivate host immune agents as well as digest and invade host tissue (Rumbaugh, *et al.*, 2000, Williams & Camara, 2009, Strateva & Mitov, 2011, Jimenez, *et al.*, 2012). Considering the roles of cooperation and communication in the guise of bacterial virulence, their medical relevance is evident.

Transcriptome analysis has found that QS regulation can affect hundreds of genes encoding secreted virulence factors as well as intracellular metabolic enzymes, thereby demonstrating the regulatory scope to be much wider than just easily identifiable cooperative traits (Schuster, *et al.*, 2003, Majerczyk, *et al.*, 2014). Some QS-regulated products appear entirely "private" in nature, such as intracellular nucleoside hydrolase in *P. aeruginosa* (Heurlier, *et al.*, 2005), whose function in the realm of QS is not entirely clear. Other intracellular QS-controlled functions, such as catalase and dehydrogenases in *P. aeruginosa* (Garcia-Contreras, *et al.*, 2015) and enzymes involved in the acetate switch in *Vibrio fischeri* (Studer, *et al.*, 2008) provide an indirect group benefit by reducing environmental oxidative stress and acidification, respectively. Observations of QS-regulated competence in *Streptococcus* (Havarstein, *et al.*, 1995, Mashburn-Warren, *et al.*, 2010) and conjugation in *Enterococcus* (Shokeen, *et al.*, 2010) and *Agrobacterium* (Wang, *et al.*, 2014) highlight examples of QS phenotypes for which the evolutionary implications appear less clear and may diverge from the cooperative scheme. QS circuitry also integrates environmental and nutritional cues, providing cells with additional regulatory input that allows further optimization of metabolic and secretion strategies (Schuster & Greenberg, 2006, Venturi, 2006, Srivastava & Waters, 2012).

The fields of bacterial QS and social evolution have benefitted extensively from theoretical and computational approaches. Mathematical modeling studies have contributed to our understanding of the ecology and social context of QS, just some of which include: the integral role of relatedness in the stability of QS-mediated cooperative behavior (Brown & Johnstone, 2001), definition of the effects of signal stratification on biofilm production and structure in pathogenic bacteria (Nadell, *et al.*, 2008), the roles of nutrient limitation in QS-mediated bacterial swarming (Boyle,

et al., 2015), and even the fundamental premise for the evolution and diversification of signaling (Pacheco, *et al.*, 2015). Recent application of evolutionary game theory has shown the general features of social interactions in bacteria behave according to economic social games, adding a level of predictive power and broader understanding to the field of QS (Damore & Gore, 2012).

Despite the breadth and depth of knowledge we have gained regarding bacterial signaling and social evolution, many significant barriers remain. Relating evolutionary pressures to the social dynamics observed in bacteria presents several conceptual and methodological difficulties. Until recently, much of the social evolutionary literature has focused on higher eukaryotes while failing to appropriate theory for the unique biological constraints of microbes. This review aims to summarize the general themes relating bacterial cell-cell signaling, cooperative behaviors, and applications of evolutionary theory to understand the evolution and maintenance of microbial cooperation in general. We start with a general overview of bacterial QS and cooperative behaviors, followed by theoretical treatments of social evolution. We will then focus on empirical evidence examining the maintenance of cooperative behavior in bacteria and microbes in general. We conclude with applications of social evolutionary research in microbes and highlight some remaining questions and new directions in the field.

1.2 Principles of bacterial signaling and selection

1.2.1 Microbial growth

The general features of microbial growth and selection provide an excellent model system to investigate cooperative and competitive interactions among cells. Bacteria are especially fit for experimentation. Bacteria exhibit the fastest generation times of any independent biological organism, in some cases under 30 minutes with optimal conditions, permitting the observation of evolutionary change essentially in real time. The ability to easily achieve clonality in routine cultivation is an instrumental advantage. These features, when coupled with the ease of selective pressure manipulation, genetic tractability of model microbial organisms, general

ease of handling, and relatively large effective population sizes, extend excellent opportunities for experimental evolution studies (Elena & Lenski, 2003).

1.2.2 Bacterial signaling

Bacterial cell-cell signaling was termed QS in 1994 and has been thoroughly characterized over roughly the past 30 years (Fuqua, *et al.*, 1994). QS is widespread in prokaryotes and much of the literature to date has focused on the two most well-understood mechanisms: acyl-homoserine lactone (AHL) signaling in Gram-negative bacteria, and peptide-QS in Gram-positive bacteria (Waters & Bassler, 2005). With both mechanisms, a small pheromone-like signal is released and received by participating members of a population, allowing surveillance of population density. The molecular architecture and regulatory processes allowing both AHL and peptide-QS signaling have received considerable attention in the literature (Schuster, *et al.*, 2013, Cook & Federle, 2014), so we are restricting our coverage here to include only the concepts that are necessary to understand their role in social evolution. The common QS componentry in both types of signaling includes a signal synthase, the autoinducer signal, and a signal receptor-regulator (Figure 1.1). These components were initially characterized in the QS-archetype *V. fischeri*, yielding a well-studied model of the circuitry (LuxI-type AHL synthase and LuxR-type receptor-regulator) that has served as a guide for defining other systems (Eberhard, *et al.*, 1981, Engebrecht, *et al.*, 1983, Engebrecht & Silverman, 1984). It is important to note that other bacterial QS mechanisms have been described in the literature in addition to AHL- and peptide-based QS. These include hydrophobic signals such as the *Pseudomonas* quinolone signal (PQS) which is packaged into a membrane vesicle for trafficking between members of a population (Mashburn & Whiteley, 2005), and a furanosyl borate diester known as autoinducer-2 (AI-2) that is released by diverse bacteria and is presumed to be a mechanism of interspecies communication (Chen, *et al.*, 2002). Other factors and cues that exhibit signal-like qualities are being discovered (Lindemann, *et al.*, 2011, Kumar, *et al.*, 2013, Brameyer, *et al.*, 2015, Weigel & Demuth, 2015, Zhou, *et al.*, 2015).

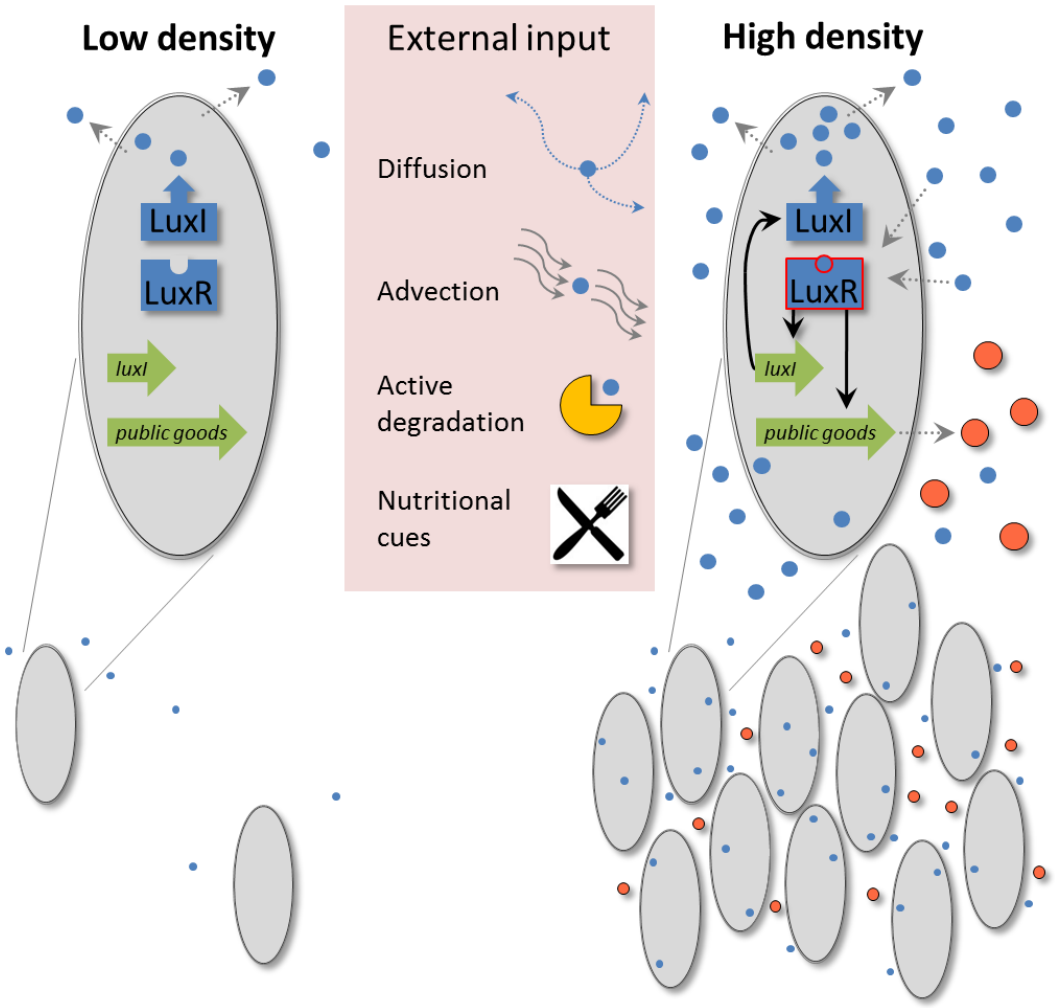

Figure 1.1 Social and environmental context of QS. Representative AHL-QS circuit with a signal synthase of the LuxI family and a signal receptor of the LuxR family (blue). LuxI produces AHL signals (circles) that diffuse out of the cell (gray ellipse) and are bound by LuxR. Several external abiotic and biotic inputs (pink region) influence extracellular signal concentration and consequently, the quorum threshold. As cell density increases, signal levels rise leading to LuxR binding of signal (highlighted in red) and subsequent downstream gene regulation; the positive feedback loop of QS-mediated *lasI* upregulation and QS-regulon genes encoding secreted public goods (orange) are emphasized. Larger cells (top) represent relatively magnified views of cells existing in populations (bottom).

QS is just one of many molecular mechanisms bacteria can use to yield a regulatory strategy that is tuned for economical use of resources in a given set of environmental parameters (Dekel & Alon, 2005). QS regulatory systems can include graded responses, as well as distinct "ON" and "OFF" activation states that impart

bistability on the target regulon. Generally, as a population exists at low density and releases signal, the signal diffuses away before it can bind a receptor-regulator, leaving the QS system OFF (Figure 1.1). As the population grows in density, so does the concentration of signal. At a critical threshold density, enough signal has accumulated to bind the cognate receptor and shift the stoichiometry of signal and receptor in favor of signal-bound, active receptor-regulators, turning the QS system ON (Figure 1.1). Several QS systems employ an autoregulatory positive feedback loop that stimulates the production of additional signal (Choi & Greenberg, 1992, Seed, *et al.*, 1995), thereby amplifying the QS response. When additional network elements are present, namely either receptor dimerization or a second positive feedback-loop, then bistable behavior results that is generally accompanied by hysteresis and associated "memory" of previous states. Such bistability has been observed in a synthetic LuxI/R QS system based on *V. fischeri* components (Williams, *et al.*, 2008). At the single-cell level, QS gene expression in native *V. fischeri* was found to be highly heterogenous, likely due to biochemical noise, leading to a graded QS response at the population level (Perez & Hagen, 2010).

Regardless of the QS mechanism employed, bacterial communication signals and receptors are affected by numerous layers of regulatory control, as well as external biological and physicochemical input. Co-regulation by other cellular pathways is a common theme in this regard; adaptation to stationary-phase stress (Goo, *et al.*, 2012) and the coordination of metabolic processes through prudent regulation of secretion (Xavier, *et al.*, 2011) and starvation responses (Ulitzur, 1989, Mellbye & Schuster, 2014) are clearly integrated into QS circuitry. When released, signals are subject to diffusion, advection, and even active degradation by competing bacteria (Figure 1.1) (Hense & Schuster, 2015). Within the cell there are myriad opportunities for control of a QS circuit with various inputs. Of course, some factors influencing the stability of QS-activation states are specific to a given taxon or QS mechanism. Some oligopeptide signals of peptide-QS require processing for maturation and subsequent secretion (Thoendel & Horswill, 2010), and the employment of a two-component sensor kinase with a subsequent phosphorylation cascade yielding transcription of a major regulatory RNA (for example, RNAIII in

Staphylococcus spp.) can provide additional opportunities for tuning of the QS response (Novick & Geisinger, 2008). The general QS circuitry of signaling and reception of signal must be tightly tuned to ensure the timing and magnitude of gene induction matches the immediate needs of the cell (Hense & Schuster, 2015). One mechanism for this fine-tuning of QS induction is through repression and anti-activation. Negative regulators directly repress AHL synthase transcription in *Pseudomonads* and provide AHL homeostasis (Rampioni, *et al.*, 2007, Venturi, *et al.*, 2011). Anti-activator proteins decrease AHL receptor stability in *P. aeruginosa* (Siehnel, *et al.*, 2010, Fan, *et al.*, 2013) and *Agrobacterium tumefaciens* (Piper & Farrand, 2000, Chen, *et al.*, 2007). Anti-activators control the induction threshold and may ultimately prevent short-circuiting of signaling machinery before induction is advantageous (Goryachev, *et al.*, 2005). In *Vibrio* spp., one endpoint regulatory agent is a set of small regulatory RNAs, providing yet another layer of complexity (Lenz, *et al.*, 2004). All of these factors come together at the intersection of a specific bacterium's biology and the given social and ecological scenarios.

1.2.3 Signaling theory

Careful study of signaling mechanisms is complemented by integration of existing signaling theory. While much of the signaling theory literature to date has focused on signaling and language in higher eukaryotes as models, similar to social evolution theory covered later in this review, the concepts of signaling honesty and information content are applicable to microbes. First, it is important to clearly define what is meant by a "signal" when evaluating social behaviors in bacteria with respect to signaling theory. Not every secreted or otherwise released molecule that affects the behavior of other organisms can be considered a social signal under the same definition. The key features in discerning true signaling lie in the fitness consequences of communication for both the sender and receiver, and whether the system evolved owing to that effect (Table 1) (Maynard Smith & Harper, 2003). In a case where the sender's act did not evolve according to a beneficial effect on sender fitness, but still benefits the receiver to respond, the communication is termed a cue.

Conversely, when the communication did evolve according to the effect on the sender but does not benefit the receiver to respond, the interaction is termed coercion. For the purposes of this review we will assume the standard definition of true signaling to include only communication that both evolved due to the effect on the sender and which benefits the receiver to respond (Diggle, *et al.*, 2007). Of course gray areas exist with consideration of bacterial QS. In the case of the individual AHL-based QS signals of a symbiont (*Sinorhizobium meliloti*) and a facultative pathogen (*P. aeruginosa*) of the model legume *Medicago truncatula*, the plant is able to "eavesdrop" and respond to the different AHLs as cues depending on which bacteria are present (Mathesius, *et al.*, 2003). In this example, a true signal produced by one organism serves as a cue to another. Additionally, divergence in a microbial "species", even over the course of an *in vitro* evolution experiment, could potentially lead to responses formerly evolved as signaling mechanisms being reconfigured as coercion.

Table 1.1 Forms of communication

	Evolved because of effect on sender	Benefits receiver to respond
Signal	+	+
Cue	-	+
Coercion	+	-

Diligence in the proper use of these terms for communication and signaling has allowed more parsimony in microbial social evolution and signaling theory literature, but the nature of a communicative act is not always clear. The information contained in a signal determines what the signal will mean for distinct individuals. AHL synthases are generally thought to produce species-specific signals which yield very specific information, and with the high degree of side-chain modification possible this seems intuitive (Schuster, *et al.*, 2013). However, some LuxR-type receptors show promiscuity that could blur their designation between signals, cues, and coercion, depending on the social context of the interaction. This configuration allows for "cross-talk" or "cross-inhibition" between QS systems. *Burkholderia cepacia* has been shown to respond to the AHLs of *P. aeruginosa* when the two co-

occur in the lungs of cystic fibrosis patients; upregulation of *B. cepacia* virulence factors from this interaction suggest this interaction may be a cue (Eberl & Tümmler, 2004). A similar form of eavesdropping has also been observed in *Vibrio* and separately in *Bacillus*, suggesting the potential for an adaptive role for QS signal diversification where varying signal specificity may benefit the receiver through facultative cheating in the presence of non-kin (Ke, *et al.*, 2015, Even-Tov, *et al.*, 2016). Plasticity of receptor specificity may also foster adaptations in recently diverged species or upon introduction to new ecological niches, where intermediate levels of signal specificity could enable novel response relationships (Ke, *et al.*, 2015). Additionally, the abundance of orphan *luxR* genes in diverse prokaryotic genomes provides opportunity for eavesdropping on the AHL signals produced by other species. Because some LuxR orphans contain a relatively high number of cysteine residues, it has been suggested that they serve an additional role as cellular redox sensors, although more direct, mechanistic experimentation will be necessary to confirm this speculation (Hudaiberdiev, *et al.*, 2015). Similarly, as AI-2 is produced by a wide variety of bacteria, the information content of this signal could be very low aside from providing total community abundance. In many bacteria, it is likely nothing more than a metabolic by-product of the activated methyl cycle, and may be most appropriately classified as a cue (Diggle, *et al.*, 2007). Dedicated AI-2 signaling pathways are currently only known for *Vibrio* and *Salmonella* (Surette, *et al.*, 1999, Taga, *et al.*, 2001). In contrast, autoinducing oligopeptides of the Gram-positive *Staphylococci* can be highly specific to subsets of strains of the same species, yielding very specific information to the communicating population (Novick & Geisinger, 2008).

The centerpiece of signaling theory is perhaps the honesty of a signaling system, or signal reliability. Honest signaling requires a balance in the fitness trade-offs between fitness cost and benefits received by signaler and receiver. For example, overproduction of signal could be utilized to elicit increased cooperative behavior from neighboring cells (exaggeration), while underproduction of signal avoids the metabolic cost of signal production (concealment of information). In order for positive selection of honest signaling, mechanisms preventing the subjugation of

signaling individuals are required. Three principles are thought to maintain signal reliability in theory: (i) an index signal, which is causally related to the quality being signaled and cannot be faked, thereby ensuring reliability, (ii) handicap, which makes signaling inherently costly and therefore expensive to fake, and (iii) common interest, in which relatedness among signaling individuals provides incentive for reliable communication (Maynard Smith & Harper, 2003, Davies, *et al.*, 2012).

The concept of an index signal was originally introduced in the context of mate selection in birds, where plumage quality in a male is directly correlated with genetic quality, providing a reliable signal to females in search of a mate (Davies, *et al.*, 2012). In bacteria, the only current examples of index signals are cases were the signal itself, or a precursor to the signal, serves as the public good normally mechanistically downstream of signaling, obligately linking the signal to the reliability of communication that allows cooperation. An example is lantibiotic production Gram-positive bacteria, where production of competitive agents such as nisin in *Lactococcus lactis* (Kuipers, *et al.*, 1995) or subtilin in *Bacillus subtilis* (Kleerebezem, 2004) is regulated by the respective peptides themselves, obligately linking the signal to the cooperative act and thereby guaranteeing reliable signaling (Dufour, *et al.*, 2007).

Costly signaling was originally thought to provide a "handicap" to honestly signaling individuals (Johnstone, 1998, Zahavi & Zahavi, 1999). In the handicap principle, the cost of signaling adds a degree of reliability to the signaling system; a would-be defector that exaggerates by overproducing signal would incur a greater signaling cost (Számadó, 1999, Zahavi & Zahavi, 1999, Brown & Johnstone, 2001). As many microbial signals must be actively produced and released, the cost of signaling should then be empirically quantifiable, although it is difficult to uncouple the cost of signaling and the cost of cooperation in many systems. Out of two studies with *P. aeruginosa*, one found that QS signal production by a signal-proficient strain significantly reduced growth compared to a signal-blind mutant (Diggle, *et al.*, 2007), whereas the other found no significant difference (Wilder, *et al.*, 2011). Moreover, a theoretical exploration of the fates of variably signaling groups of cooperating

individuals suggested signaling and cooperative behavior may be coupled obligately (Czaran & Hoekstra, 2009).

According to the model of Brown and Johnstone, the level of signaling should initially increase and then decrease as relatedness is reduced, because intermediate relatedness selects for individuals to "coerce" other individuals into cooperation (Brown & Johnstone, 2001). However, this was not observed in a recent empirical test of this hypothesis. Instead, Popat *et al.* found that the level of signaling monotonically decreased with decreasing relatedness. The primary cause appeared to be a loss of responsiveness to the signal, although the tight coupling between signal production level and ability to respond complicated interpretation of results (Popat, *et al.*, 2015). Further disentanglement of the direct fitness costs of signaling with co-occurring behaviors will be necessary to define the role of signaling cost and common interest in the maintenance of honest signaling. Common interest in the form of relatedness between cooperating organisms, the foundation of kin selection theory, is further discussed in the context of social evolution theory later in this review.

1.2.4 Signal network complexity and ecological considerations

Thus far, we have generally discussed QS systems in terms of single synthase-receptor circuits, but many bacteria utilize multiple QS systems simultaneously. For example, *P. aeruginosa* possesses two interconnected QS circuits, consisting of two AHL synthase/receptor pairs LasI/R and RhlI/R, arranged in a hierarchy that places the *las* system largely in control of the QS response (Schuster & Greenberg, 2006). Both QS circuits regulate partially overlapping sets of genes, but induction of the *rhl* system components is generally controlled by LasR (Schuster, *et al.*, 2003, Schuster & Greenberg, 2006). Several ideas have been proposed to explain the purpose of multiple QS systems. In a coupled modeling and experimental approach, Cornforth *et al.* argued that the varying stabilities of the two signal molecules, 3-oxo-C12-HSL and C4-HSL generated by LasI and RhlI, respectively, could enable distinction between social (cell density) versus physical (mass transfer) inputs (Cornforth, *et al.*, 2014). Based on experiments evaluating the relative contributions of the *las* and *rhl*

systems to QS gene induction under specific nutrient limitation, Mellbye and Schuster suggested that multiple QS systems may have evolved to permit distinct levels of signal integration: While the *las* system primarily responds to cell density, the *rhl* system also integrates starvation signals (Mellbye & Schuster, 2014).

In contrast to the hierarchical systems of *P. aeruginosa*, the three QS pathways possessed by *V. harveyi* (LuxM/N, AI-1; LuxS/PQ, AI-2; CqsA/S, CAI-1) function in a parallel fashion (Long, *et al.*, 2009). By analyzing pathways in isolation as well as in combination, Long *et al.* showed AI-1 and AI-2 function in a strictly additive, graded fashion, with near-equal contribution to the total response (Long, *et al.*, 2009). They proposed that such reliable distinction of external autoinducer concentrations and combinations could help synchronize gene expression during distinct developmental steps in a bacterial community (Long, *et al.*, 2009). Another recent study explained the presence of multiple parallel quorum signal-receptor pairs in *V. harveyi* and *B. subtilis* from the perspective of social evolution. Key here is the repression of QS-controlled target genes by the various receptors in the unliganded state. A strain with a novel signal-receptor pair can then invade and exploit the ancestral population as a social cheater. When its frequency increases, it resumes cooperation because the novel signal is produced at sufficiently high levels to de-repress the cognate receptor (Even-Tov, *et al.*, 2016).

The complexity of QS signaling networks also draws into question the ecological role of QS. Most studies of QS involve *in vitro* populations of clonal bacteria growing under conditions that reflect only a limited set of conditions that bacteria may experience in the natural environment. The lack of ecological relevance in the QS literature leaves many questions remaining, particularly the role of natural population diversity in determining signal specificity, inter- and intra-specific cooperation, as well as validation of *in vitro* evolution approaches. Some have argued that the primary principle of cell-density dependence that we have described thus far has actually evolved for a different ecological function, namely sensing the extent of diffusion (or more generally, mass transfer), challenging the notion that QS is a social behavior (Redfield, 2002) (Figure 1.2). Not surprisingly, mass transfer limitation can trigger QS at very low cell densities or even in single cells (Shompole, *et al.*, 2003,

Boedicker, *et al.*, 2009, Lui, *et al.*, 2013). Others have suggested these two explanations are not mutually exclusive and can be unified generally as an "efficiency sensing" principle that integrates information about cell density, mass transfer, and also spatial distribution of cells (Hense, *et al.*, 2007). As indicated above, additional factors such as signal stability and degradation, either enzymatically or abiotically, can further modify the actual extracellular signal concentration that is sensed (Hense & Schuster, 2015). The system response may be disturbed by these factors, or may be tuned to account for them if they are an integral part of the ecology of the organism. Clearly, there is a need to better understand the ecological context of the respective microbe and its QS system in order to evaluate the relative importance of cell density, mass transfer and other parameters, as discussed in more detail by Hense and Schuster (Hense & Schuster, 2015).

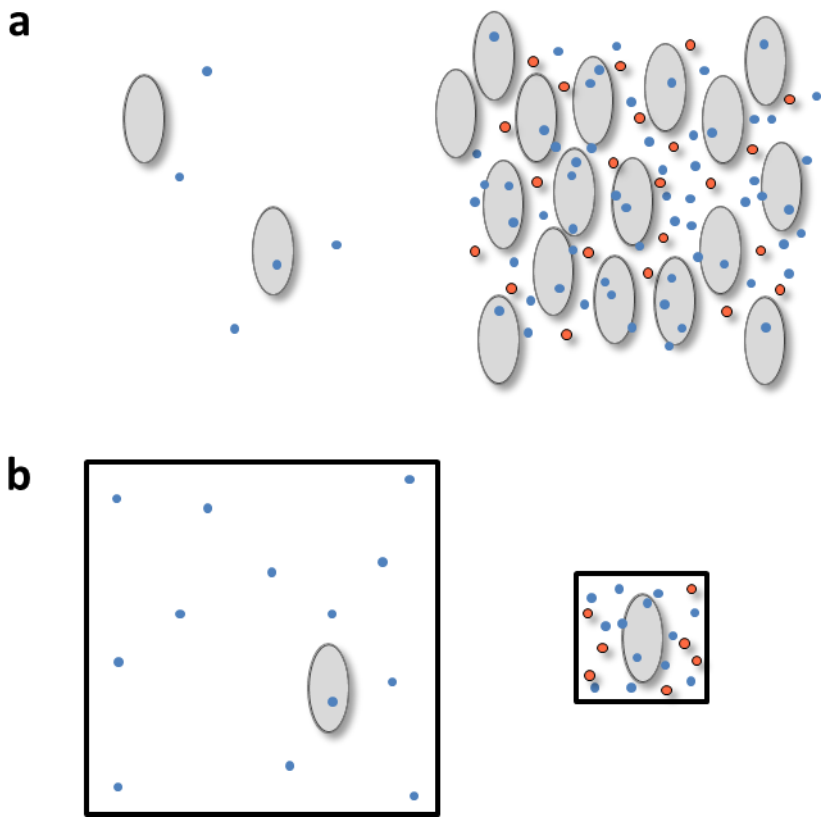

Figure 1.2 Cell-density and diffusion sensing. (**a**) Cell density. Induction (right) of public goods (orange) may occur principally when cells (gray ellipses) are at a high density, such as a densely populated colony or biofilm, where accumulation of signal (blue) has reached a critical threshold. When cell density is low (left), as is the case

with free-living planktonic bacteria, signal does not accumulate leaving the cells uninduced. (**b**) Diffusion. The accumulation of signal and subsequent induction is primarily due to mass transfer limitation by, for example, spatial constraints.

1.2.5 Cooperative growth and selection

In microbial communities, like most living populations, the best, most beneficial strategy for an individual does not necessarily align with the best strategy for the community. Herein lies the source of social conflict; investment by some members of the population produces a common or "public" good that is costly, but can yield fitness benefits for all members of the population, including defectors or "cheaters" that do not suffer the cost of the investment (Keller & Surette, 2006, West, *et al.*, 2006). The microbial world is replete with examples of behaviors that involve these canonical public goods, many of which are potentially vulnerable to cheating. For example, production of secreted proteases (Diggle, *et al.*, 2007, Sandoz, *et al.*, 2007) or iron-scavenging siderophores (Griffin, *et al.*, 2004) by *P. aeruginosa* cooperating under nutrient-limited conditions provides opportunities for non-producers to exploit these public goods for a fitness benefit. Cheating phenotypes are often the result of a loss-of-function mutation in a QS receptor that regulates cooperative traits, such as the central QS regulator LasR in *P. aeruginosa* (Sandoz, *et al.*, 2007) or AgrC in *Staphylococcus aureus* (Pollitt, *et al.*, 2014). Clarity has been gained from *in vitro* studies using nutrient-limited synthetic media. In a minimal medium with a bulky protein as the sole carbon source, extracellular protease regulated through QS is required for growth of *P. aeruginosa* (Diggle, *et al.*, 2007, Sandoz, *et al.*, 2007). Mutants defective in QS are unable to grow to high densities on their own but have a fitness advantage in wild-type co-culture (Diggle, *et al.*, 2007, Sandoz, *et al.*, 2007, Wilder, *et al.*, 2011, Asfahl, *et al.*, 2015). A recent experiment using strains of *P. aeruginosa* engineered to be defective in the secreted protease LasB or in the QS regulator LasR, which is responsible for LasB expression along with other cooperative traits, found that fitness advantages were only realized by the regulatory cheater (Mitri & Foster, 2016). QS regulator mutants were favored over protease mutants because they provided a much larger cost reduction. *In vivo* studies

have also observed potential cheating in bacterial cooperation; *P. aeruginosa* isolates harboring fitness-enhancing mutations in LasR have been observed to increase in frequency in acute burn wound infections in a murine model (Rumbaugh, *et al.*, 2009), as well as acute lung infections of mechanically ventilated patients (Kohler, *et al.*, 2009), and *Staphylococcus aureus* QS cheaters have been observed to behave similarly in model (Pollitt, *et al.*, 2014) and clinical infections (Shopsin, *et al.*, 2008) where they are able to invade and persist in mixed cooperating populations. That a behavior is exploitable by a cheating phenotype has become a hallmark of cooperative behavior (West, *et al.*, 2006, Schuster, *et al.*, 2013). The overarching theme of these examples demonstrates how public goods produced by cooperating populations can yield profound effects on population fitness.

The dilemma of cooperation, namely the conflict between group benefit and personal benefit, has produced a wealth of scientific research and speculation, beginning with Hardin's original treatise, "The Tragedy of the Commons", presented in the context of human economics (Hardin, 1968, Rankin, *et al.*, 2007). In the original example, herdsmen with shared access to a common grazing area make the rational decision to graze their own cattle as much as possible, even though moderate grazing would preserve the cooperative resource for future use. The tragedy arises because natural selection favors overgrazing for each individual herdsman, eventually exhausting the resource and reducing the fitness of all herdsmen (Hardin, 1968). The debate continues through the present with several significant reviews that focus specifically on the "problem of cooperation" in microbial populations (West, *et al.*, 2006, West, *et al.*, 2007, Diggle, 2010). For example, under nutrient limiting conditions, selection can be strong at the level of the individual bacterium to cooperate – without a mechanism to secure further nutrients, the fitness of the individual will approach zero. Alas, executing a cooperative behavior alone or in a population with a high frequency of cheaters does not guarantee a significant return on investment, either. The need for a mechanism like QS to facilitate group behavior is then justified; QS is an optimizing principle, a mechanism that restricts public good production to growth stages and ecological scenarios with the greatest net fitness benefit (Pai, *et al.*, 2012). Facultative cheating may have even contributed to the

diversification and redundancy of QS systems observed today (Eldar, 2011, Pollak, *et al.*, 2016).

Cooperative behaviors generally benefit from a higher density of cooperating members in a population, a feature referred to as density dependence. The density-dependence of cooperation by public goods can be described by two overlapping concepts, "avoidance of dilution" and "strength of effect" (Ng & Bassler, 2009, Darch, *et al.*, 2012, Pai, *et al.*, 2012, Heilmann, *et al.*, 2015, Popat, *et al.*, 2015). In the former, public goods are lost to the environment at low density but benefit neighboring cells at high density; an example is an extracellular degradative enzyme that makes nutrients available to all cells. In the latter, the effect of public goods on the environment depends on their concentration and hence, on cell density; an example is a secreted antibiotic that kills competing microbes in a concentration-dependent fashion, benefitting all contributing cells. That cooperation is density-dependent and has a non-linear effect on population fitness was originally described for *Myxococcus xanthus* proteolytic growth in casein media (Rosenberg, *et al.*, 1977). This effect has been further demonstrated for a variety of different microbial phenotypes and species (Gore, *et al.*, 2009, Ross-Gillespie, *et al.*, 2009), including QS (Darch, *et al.*, 2012, Pai, *et al.*, 2012). The density-dependent benefit of cooperation also affects the relative fitness of invading cheater populations through frequency-dependent selection (Sanchez & Gore, 2013). As has been demonstrated in several microbial systems, the relative fitness benefit of a cheater phenotype is dependent on their frequency in the population (Dugatkin, *et al.*, 2005, MacLean & Gudelj, 2006, Ross-Gillespie, *et al.*, 2007, Wilder, *et al.*, 2011); when cheaters are rarer, their relative fitness is higher.

1.3 Game theory and theoretical approaches to cooperation

Theoretical approaches allow an abstract perspective for viewing central problems in microbial cell-cell signaling, cooperative growth, and the maintenance of these strategies. Here, we review the various contributions of evolutionary theory and game theory to our understanding of social evolution in microbes. A critical review of

theoretical tools useful to the microbiologist has already been published elsewhere (Damore & Gore, 2012), and we direct the reader to that review for origins and derivations of evolutionary theory.

1.3.1 The Prisoner's Dilemma, common themes and game theory

The goal of evolutionary game theory is to reduce the evolutionary outcomes to a simple mathematical model that assigns distinct pay-offs to social interactions (Nowak & Sigmund, 2004). Perhaps the simplest theoretical treatment of cooperative interactions is encapsulated in the Prisoner's Dilemma (PD) game. In the original 2-person PD game, interacting individuals ("players") have a choice to cooperate and pay a cost c to yield a benefit $b > c$ for another individual, or to defect and not incur a cost while benefiting from others' cooperation. Choosing to cooperate yields a modest payoff for each cooperator and the highest mean fitness, but if one player chooses to defect, there is a larger payoff for the defector. If both players defect, the payoff for both players is zero. We have formalized this mathematical approach to the Prisoner's Dilemma with an illustrative model based on the replicator equation, presented in Figure 1.3. The replicator equation is a rate equation for the relative sizes of subpopulations that each play a different strategy. In a simple model, the reproductive success, or "growth rate", is determined by fitness payoffs from pairwise encounters between individuals, and the probability to meet members of different subpopulations is given by their frequencies (Taylor & Jonker, 1978, Schuster & Sigmund, 1983, Nowak & Sigmund, 2004). The underlying dilemma arises because selection typically favors a scenario where both individuals defect (Figure 1.3A). Defection will always yield a higher payoff than cooperation for the individual ($b > b-c$), even in the case of defection with defectors ($0 > -c$) (Figure 1.3A). In this case, defection is an evolutionarily stable strategy (ESS): a strategy that is immune to the invasion of other, initially rare strategies (Smith & Price, 1973, Maynard Smith, 1982, Sigmund, 2011).

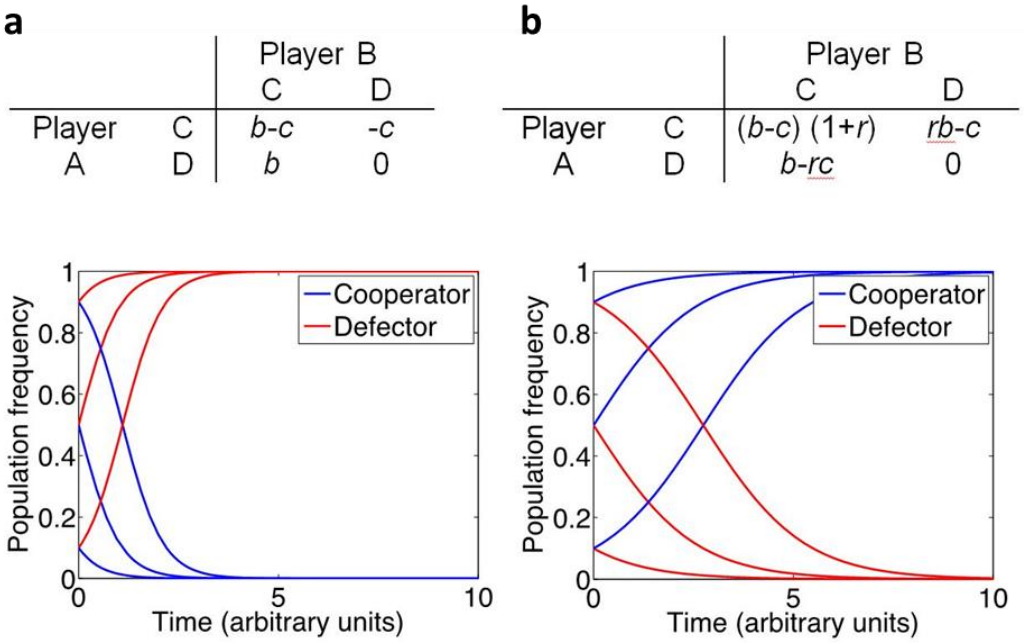

Figure 1.3 Classical Prisoner's Dilemma and kin selection. (a) Classical Prisoner's Dilemma. (**b)** Prisoner's Dilemma with kin selection. Pay-off matrices are shown above each graph with C, cooperator; D, defector. Graphs show population frequencies over time, as modeled by the replicator equation. Initial frequencies of cooperator and defector subpopulations are either 50:50, 90:10, or 10:90. **(a)** parameters $b = 4$, $c = 2$. **(b)** parameters $b = 4$, $c = 2$, $r = 0.7$. Pay-off matrices and the replicator equation were implemented in Matlab (Mathworks, Natick, MA, United States). We employed the deterministic replicator equation to model the relative sizes of subpopulations in a well-mixed population (Taylor & Jonker, 1978; Schuster & Sigmund, 1983). Here, the population frequencies x_i evolve in time according to $dx_i/dt = x_i(f_i(x) - \langle f \rangle)$, where $f_i(x) = \sum_{j=1}^{n} (f_{ij}\, x_j)$ is the average payoff strategy. When an individual of type i meets an individual of type j, it obtains a payoff f_{ij}, and the probability to meet a member of a different subpopulation is given by their frequencies $x_1, x_2, \ldots x_n$, which sum up to 1. $\langle f \rangle = \Sigma_i (x_i\, f_j(x))$ is the time-dependent mean fitness of the entire population, which ensures that the population frequencies remain normalized.

Given that biological interactions are rarely limited to two isolated individuals, the *N*-person PD game, also called the *N*-person Public Goods game, was developed to include interactions of all members of a population simultaneously (Hamburger H., 1973). This approach allows the individual contributions of all cooperating individuals to a central pool of "public goods" to be considered with respect to varying frequencies of defectors. Nevertheless, the ESS in the *N*-person PG

game is also “defect”. The general assumptions of both of these games do not include peculiarities of microbial cooperation, namely non-linear cost-benefit relationships, but they illustrate the central problem of cooperative behavior, and they provide a foundation for understanding the concept of the “Tragedy of the Commons”.

1.3.2 Altruism and kin selection

The leading theoretical explanation for the success of altruistic behavior is kin selection, which suggests altruistic behaviors can be selected if they produce a fitness benefit for relatives. Kin selection was first formalized in Hamilton’s rule, which states a cooperative behavior will be selected, if the cost c of the cooperative behavior is less than the product of the benefit b of the behavior multiplied by the relatedness r between actor and recipient ($rb - c > 0$; Figure 1.3B) (Hamilton, 1964). This creates a scenario where both individuals must share reciprocal altruism in order to suppress competition and maintain cooperation (Dawes, 1980, Axelrod & Hamilton, 1981). Here, kinship between neighboring cooperating individuals ensures that shared genes are favored by natural selection, allowing the cooperative strategy to dominate as long as $r > b/c$ (Figure 1.3b). It is important to note that relatedness refers to the alleles encoding the cooperative behavior that is under selection in a cooperative environment. This approach necessarily assumes some form of assortment to allow interactions between kin, a quality common of many empirically demonstrated cooperative systems which are presented later in this review.

There is empirical support from *in vitro* culturing and from an infection model that kin selection contributes to the maintenance of QS in *P. aeruginosa* (Diggle, *et al.*, 2007, Rumbaugh, *et al.*, 2012). When QS-proficient cells were kept separate from QS-deficient cells, i.e. relatedness was high, then the relative fitness of the QS-proficient strain was high and QS was favored. When QS-proficient cells were mixed with QS-deficient cells, i.e. relatedness was low, then QS-proficient cells could be exploited; their relative fitness was low and QS was not favored.

Recently, a modified form of Hamilton’s rule has accommodated the general features of microbial cooperation that make this framework more useful, including

parameters for non-linearity, strong selection, and non-additivity of fitness effects (Smith, *et al.*, 2010). The utility of this model was tested using measurements of its parameters in a cooperating population of *Myxococcus xanthus* bacteria (Smith, *et al.*, 2010), confirming the role of spatial structure in kin selection for cooperative traits.

1.4 Mechanisms that stabilize cooperation

In addition to kin selection as an evolutionary force, several distinct mechanisms that stabilize cooperation have been defined in microbes. Most of the mechanisms do not directly involve bacterial QS, but we include them here to highlight common principles (Travisano & Velicer, 2004, Foster, *et al.*, 2007, Schuster, *et al.*, 2013, Bruger & Waters, 2015).

1.4.1 Facultative cooperation

As indicated above, the benefit of a cooperative behavior, such as a secreted exoprotease or an antibiotic, generally increases with population density. QS optimizes this cooperative behavior by restricting production of public goods to sufficiently high densities (Pai & You, 2009, Pai, *et al.*, 2012). A recent investigation using a coupled modeling and experimental approach confirmed that *P. aeruginosa* exoenzyme production is restricted, through tightly-regulated QS, to instances where the cooperating bacteria are surrounded by other cooperating bacteria (Allen, *et al.*, 2016). This intrinsic feature of QS-based cell-cell signaling allows the cost incurred by cooperators to be calibrated to the relative abundance of other cooperators in the population (Allen, *et al.*, 2016). In many cases, the linear cost of public good production is coupled with accelerating benefits at the population level, which allows QS to maximize the cost-benefit ratio of a cooperative behavior (Heilmann, *et al.*, 2015). This restriction of cooperation to times when it is most beneficial effectively reduces the strength of selection for non-producing cheats at lower cell densities, allowing QS to bestow a stabilizing effect on the cooperative system (Cornforth, *et al.*, 2012) (Figure 1.4a).

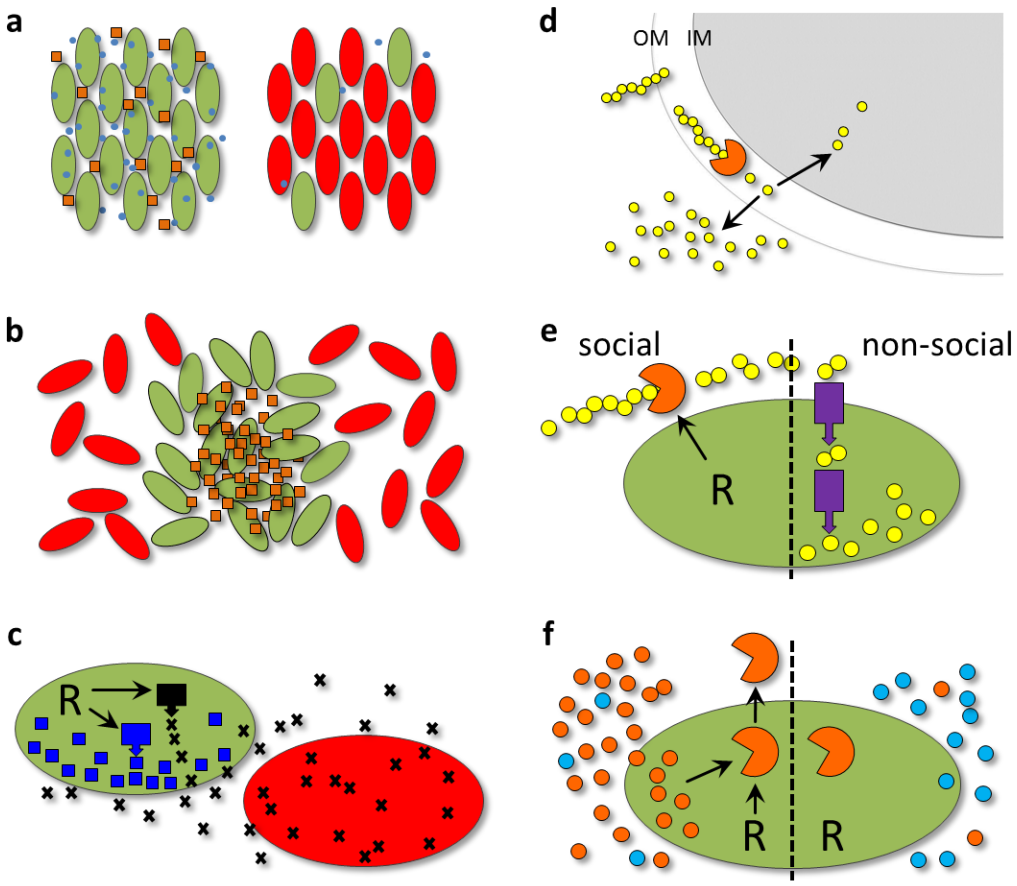

Figure 1.4 Physiological and molecular mechanisms of cooperation stabilization. (**a)** Facultative cooperation through QS. QS signaling (blue dots) in cooperating cells (green) restricts production of secreted public goods (orange squares) to populations where more cooperators are present (left); in contrast, when there is a high relative frequency of cheaters (red) in the population (right), public goods are not secreted. (**b)** Spatial structuring and positive assortment. Growth of structurally isolated groups of cooperators (green) keeps distribution of secreted public goods locally separated from cheaters (red). (**c)** Kin discrimination, policing and pleiotropy. Pleiotropic regulation (R) of secreted and cell-associated products, e.g. a toxin (black X's) and immunity protein or other resistance trait (blue squares), respectively, has different effects on cooperator (green) and cheater (red) fitness. Without the cell-associated resistance trait, cheaters (red) suffer a fitness cost due to the toxin. (**d)** Partial privatization of public goods. Degradative enzymes (orange) associated with the periplasm (between OM, outer membrane, and IM, inner membrane) can hydrolyse complex substrate (yellow chains). Most of the hydrolysis products (individual yellow circles) are lost to the extracellular space, while a portion is retained by the producing cell (gray). (**e)** Non-social adaptation. Growth environments can promote social (left) and non-social (right) selection. In social adaptation, mechanisms like QS (left) provide regulation (R) of secreted public goods (orange) that break down polymeric nutrients such as polypeptides (yellow chains) outside the cell for export.

In non-social adaptation, mutations allow increased uptake and processing (purple) of digested nutrients such as dipeptides and individual amino acids (individual yellow circles), increasing the fitness of the individual. (**f)** Metabolic prudence. QS integrates cues regarding the relative supply of specific nutrients (orange and blue circles); regulating (R) secretion of public goods (orange enzyme) to times when the primary building block of the product is not limiting (left, orange nutrients relatively high), isolating cooperative secretions to periods when metabolic cost is relatively low. Secretion is repressed under specific nutrient limitation (right).

1.4.2 Spatial structuring and positive assortment

That idea that the net benefit of cooperation is maximized at high cell density underlies a second stabilizing mechanism: spatial structure and positive assortment of cooperating individuals. When positioned in a structured habitat cooperative behaviors can be more easily directed at other cooperators, and related cooperators in particular (Figure 1.4b). This mechanism necessarily draws from the theoretical kin selection framework introduced earlier, and several empirical examples of the cooperation-stabilizing effect of spatial structure are available. In the available examples it is not always clear if and how the emergent cooperative behaviors in question are regulated by QS or other mechanisms. A recent examination of cooperative fruiting body production in the social amoeba *Dictyostelium discoideum* showed the importance of the fine-scale spatial positioning of related, cooperating individuals (smith, *et al.*, 2016). The authors found that just millimeters of separation between genetically distinct foraging cells was sufficient to produce clonal fruiting bodies, thereby ensuring the cooperative task of sporulation was shared among relatives (smith, *et al.*, 2016).

Another example of positive assortment is focused on the ecology of biofilm growth. Biofilms are a frequently encountered microbial strategy where cells secrete a stationary EPS matrix that immobilizes the cooperating population, allowing stationary lifestyle in beneficial environments, protection from dessication and predation, and a physical lattice for limiting the diffusion of secreted public goods (Hall-Stoodley, *et al.*, 2004, Nadell, *et al.*, 2009, Drescher, *et al.*, 2014). Models of biofilm growth have predicted this spatial structuring promotes cooperative behavior (Kreft, 2004, Xavier & Foster, 2007, Nadell, *et al.*, 2010). In support of this notion,

Van Gestel *et al.* found that cooperative EPS production in *Bacillus subtilis* provides a competitive advantage over non-producers during *in vitro* biofilm growth when biofilms are initiated at a low cell density, enabling strong positive assortment of related bacteria (van Gestel, *et al.*, 2014). In a recent extension of this approach in *Vibrio cholerae*, Nadell *et al*. showed that invasion of biofilms by non-cooperating cells is prevented through secretion of a binder protein by cooperators that connects related cells within the biofilm, further localizing cooperative populations (Nadell, *et al.*, 2015). With these examples it is clear that spatial structuring and positive assortment of kin provide strong stabilization of cooperative behavior. It must be noted that the sessile lifestyle of biofilms also presents potential conflict in the competition for local resources, even among closely related individuals. While these conflicts are certainly important drivers of selection in biofilms, they reflect more the physical constraints of the biofilm lifestyle (Nadell, *et al.*, 2009). In contrast to the evidence that biofilms promote cooperation, Popat *et al*. demonstrated that QS cheaters lacking the regulator LasR are able to invade QS-proficient populations of a *P. aeruginosa* biofilm and impose a significant burden on overall growth to an extent greater than in planktonic culture (Popat, *et al.*, 2012). It is plausible that exploitation was facilitated in this case by the density of the biofilm, by the initial mixing of the seed population, or by the diffusibility of the public good.

1.4.3 Kin discrimination, policing and pleiotropy

In order to ensure the benefits of secreted public goods are adequately directed to related cooperators, some microbes utilize discrimination of kin and policing of non-producers (Figure 1.4c). Mechanisms of kin discrimination can be generally sorted as either promoting fitness of kin, or punitive treatment of non-kin. For microbes, the term “kind discrimination” has also been proposed to generally define mechanisms that distinguish those who possess a specific trait from those that do not, and contrast them from a more narrow definition in animals that associates kin discrimination with descent due to genealogy (Strassmann, *et al.*, 2011). The social amoeba *Dictyostelium purpureum* preferentially aggregates and migrates with close

relatives when forming a characteristic cooperative fruiting body, thereby promoting fitness of only related individuals (Mehdiabadi, *et al.*, 2006). In an *in vitro* evolution approach, *M. xanthus* bacteria also preferentially aggregate with cooperating relatives when forming cooperative fruiting bodies, even discriminating against cooperating ancestors that evolved in parallel conditions (Rendueles, *et al.*, 2015). In *M. xanthus*, kin discrimination is effectively achieved through recognition of the polymorphic cell-surface protein TraA (Pathak, *et al.*, 2013), isolating the mechanism to a single variable allele. A similar discrimination effect has been observed in cooperative swarming in *B. subtilis* (Stefanic, *et al.*, 2015, Lyons, *et al.*, 2016), and separately in *Proteus mirabilis* (Gibbs, *et al.*, 2008), where a distinct boundary termed a Dienes line forms between conspecific migrating populations of different strains of the same species (Dienes, 1946, Budding, *et al.*, 2009).

Punitive treatment of non-kin through coercion, generally referred to as "policing" owing to a substantial amount of literature examining this behavior in higher eukaryotes (Clutton-Brock & Parker, 1995), takes on several forms in the maintenance of bacterial cooperation. To prevent non-producing cheaters from invading a cooperative biofilm, *Burkholderia* species utilize a toxin-resistance system to punish neighbors lacking relatedness at specific loci (Anderson, *et al.*, 2014). Also described in *Escherichia coli*, these contact-dependent-inhibition (CDI) systems utilize a set of toxic proteins expressed on the outer membrane coupled with expression of immunity proteins that confer resistance to the toxin to indirectly promote kin fitness (Aoki, *et al.*, 2009, Aoki, *et al.*, 2010). In a different form of punishment, policing in *P. aeruginosa* cooperative proteolytic growth is mediated by production of hydrogen cyanide by cooperators (Wang, *et al.*, 2015). QS cheater mutants defective in the QS regulator RhlR, or by extension the master regulator LasR, are punished by sensitivity to cyanide and suffer a fitness cost (Wang, *et al.*, 2015). This mechanism of policing was recently shown to have the added benefit of preventing inter-species cheating, allowing *P. aeruginosa* to also punish neighboring *Burkholderia multivorans* that exploit the same pool of public goods (Smalley, *et al.*, 2015). In this system, like many other toxin-resistance systems widely distributed in

bacteria (Strassmann, *et al.*, 2011), the bulk production of a toxin by cooperators is also associated with resistance to the toxin.

The general effect of co-regulated toxin-resistance systems shares some conceptual similarity with the cooperation-stabilizing mechanism of pleiotropy, a common feature of genes in which a single allele yields multiple traits (Barton, 1990). In the social amoeba *D. discoideum* described earlier in the context of population structuring, pleiotropy also acts to preserve the integrity of cooperative spore formation by constraint: mutants that do not "hear the call" to cooperate are excluded. A single gene confers a phenotype that responds to signals to form a fruiting body stalk, a sacrificial altruistic role, while lack of the gene is also associated with exclusion from the spores (Foster, *et al.*, 2004). QS-dependent cooperative proteolysis in *P. aeruginosa* can be in part safe-guarded from cheater exploitation due to pleiotropic linkage to an allele encoding a private metabolic trait (Dandekar, *et al.*, 2012). Cell-associated nucleoside hydrolase (Nuh) allows growth on adenosine and secreted LasB protease allows growth on casein in a medium with the two compounds supplied as the sole carbon sources (Dandekar, *et al.*, 2012). Pleiotropic linkage of *nuh* and *lasB* through LasR regulation thus confers a metabolic advantage to cooperators under specific growth conditions that prevents enrichment of QS cheaters (Dandekar, *et al.*, 2012). If QS-control of *nuh* evolved for this purpose, however, is not clear. Taken together, in these systems, pleiotropic linkage of contrasting traits serves to link cooperative behavior with the opportunity to reproduce or otherwise increase fitness, effectively stabilizing cooperation in the presence of cheating.

1.4.4 Partial privatization of public goods

In addition to linking private and public good regulation, cooperation can also be stabilized if a small fraction of the public good is retained by the producing cell (Figure 1.4d). For example, the sucrose-hydrolyzing enzyme invertase in yeast is located in the periplasm where roughly 99% of the hydrolysis products are allowed to diffuse away from the individual cell (Gore, *et al.*, 2009). While cheaters are able to

exploit the bulk of the hydrolysis products, the small fraction of products not released are secured by the cooperator and are sufficient to prevent population collapse. Cooperators and cheaters co-exist in a mixed equilibrium resulting from the fact that the rare strategy always invades the common strategy, the defining characteristic of the snow-drift game in evolutionary game theory. This dynamic is echoed in a recent study of a cooperative siderophore, enterochelin, in *Escherichia coli*. Scholz and Greenberg showed that enterochelin is partially cell-associated (private) at low cell densities, but is secreted at high cell densities allowing exploitation by cheaters (Scholz & Greenberg, 2015). Facultative privatization of siderophore production also offers a solution to the problem of cooperation with diffusible public goods at low cell densities.

1.4.5 Non-social adaptation and adaptive race

Exploitable behavior among related cooperators can also be safe-guarded against cheating simply through co-inheritance of other alleles that are subject to separate selective pressures, a feature referred to as genetic hitchhiking (Santos & Szathmary, 2008). While such positive selection through genetic hitchhiking may eventually be lost due to linkage equilibrium, its short-term advantage to a cooperating population can sufficiently prolong cooperation. General, non-social adaptation to a cooperative environment can be under positive selection (Figure 1.4e) (Waite & Shou, 2012, Asfahl, *et al.*, 2015). Observations of *P. aeruginosa* evolving in an environment requiring QS-regulated protease for growth found a non-social adaptation rose to fixation in the population before a cheating subpopulation was even detected (Asfahl, *et al.*, 2015). The underlying mutation allowed increased uptake of proteolysis products in the cooperative environment, elevating the absolute fitness of individuals and allowing the population to saturate faster (Asfahl, *et al.*, 2015). The adapted, cooperating population was still vulnerable to invasion by cheater phenotypes, but the adapted population maintained higher overall yields than defined co-cultures with equivalent cheater load (Asfahl, *et al.*, 2015). In a related example using an engineered mutualistic cooperative system in the yeast *Saccharomyces*

cerevisiae, the adaptive dynamics were reversed (Waite & Shou, 2012). In this contrasting system, the evolutionary dynamics were observed with co-cultures of cooperators and defined obligate cheaters mixed together at the start of each experiment (Waite & Shou, 2012). While obligate cheating phenotypes with a higher fitness than the cooperators typically enriched in co-culture, some replicate populations acquired a mutation that allowed purging of cheaters and concomitant preservation of cooperation (Waite & Shou, 2012). The authors proposed an "adaptive race" model where the fate of the cooperating population depended on acquiring the beneficial allele earlier than the cheats, which positively correlated with the frequency of cooperators (Waite & Shou, 2012). Evidence for the adaptive race mechanism has also been demonstrated in cooperative siderophore production in *Pseudomonas fluorescens*, where numerically dominant cooperators were found more likely to be the subject of strong non-social selection (Morgan, *et al.*, 2012).

1.4.6 Metabolic prudence

A final mechanism that effectively limits cheating of microbial cooperation is the prudent regulation of public goods that minimizes the metabolic cost of their production (Figure 1.4f). In *P. aeruginosa* swarming motility, the production of a QS-dependent secreted biosurfactant was resistant to exploitation by non-producing mutants. Xavier *et al.* found that biosurfactant expression was limited to growth conditions where carbon, the primary nutrient required for biosurfactant synthesis, was not limiting (Xavier, *et al.*, 2011), thus minimizing the metabolic cost of its synthesis. While somewhat similar to examples of facultative cooperation described earlier, metabolically prudent regulation of public good expression in this case is not mediated exclusively by cell-cell signaling, but instead is dependent on nutrient availability (Xavier, *et al.*, 2011). This mechanism received further support when Mellbye and Schuster (Mellbye & Schuster, 2014) showed multiple QS-controlled cooperative secretions are prudently regulated in *P. aeruginosa* according to the availability of the specific building blocks of the public good.

1.5 Applications and future directions

Cooperative strategies mediated by QS are common in bacterial pathogenesis, and understanding the social dynamics of virulence has become an important focus (Rutherford & Bassler, 2012). *P. aeruginosa* uses QS to control expression of many secreted virulence factors (Schuster, *et al.*, 2003). QS mutants defective in the master QS regulator LasR have been isolated from wound infections, the lungs of cystic fibrosis (CF) patients, and other intubated patients (Denervaud, *et al.*, 2004, Schaber, *et al.*, 2004, Hoffman, *et al.*, 2009, Kohler, *et al.*, 2009). However, it is unclear if social selective pressures demonstrated *in vitro* (Sandoz, *et al.*, 2007, Popat, *et al.*, 2012) are also important drivers of population structure *in vivo*. On the one hand, data from a mouse infection model and from intubated patients suggest that social selection plays a role (Kohler, *et al.*, 2009, Rumbaugh, *et al.*, 2009). On the other hand, non-social, physiological factors such as increased antibiotic resistance and growth under oxygen limitation also favor *lasR* mutants (Hoffman, *et al.*, 2010). *In vivo* study of population dynamics can be difficult, and recent development of realistic model systems that correlate with actual infections may help to disentangle the evolutionary trends affecting virulence (Harrison, *et al.*, 2014).

A better understanding of the social and non-social drivers of selection in pathogens could lead to new therapies for treating infections that do not involve the problems of positive selection inherent with traditional antibiotics. Antivirulence strategies using QS inhibitors (QSIs) could effectively reduce QS-mediated virulence by preventing the induction of QS-controlled virulence factors (Hentzer, *et al.*, 2003, Kalia & Purohit, 2011). Even if a QSI-resistant phenotype were to evolve, social conflict in situations where virulence is mediated by QS-dependent public goods should prevent selection of QSI-resistant isolates (Mellbye & Schuster, 2011). The largely QSI-susceptible population would act as social cheaters that attenuate growth and virulence of a QSI-resistant mutant. Recent evidence in an analogous study of *P. aeruginosa* virulence attenuation via siderophore quenching provided support for this notion (Ross-Gillespie, *et al.*, 2014). This particular avenue within the greater field of antivirulence research has seen development of an assortment of strategies for interfering with QS signaling, including targeting of signal molecules directly,

inhibition of signal biogenesis, disruption of signal detection (LaSarre & Federle, 2013), or even introduction of strains with coercive, spiteful, or cheater phenotypes using a "Trojan horse" strategy (Brown, *et al.*, 2009).

The detailed understanding of QS design principles has also received application in synthetic biology. For example, synthetic AHL sender and receiver pairs have been used for the formation of complex spatial patterns (Basu, *et al.*, 2005). In another example, QS circuitry has been employed to precisely control population density via QS-controlled killing (You, *et al.*, 2004). Mechanistic knowledge about QS is being combined with ecological and social evolution concepts in the emerging field of synthetic ecology (Fredrickson, 2015, Hennig, *et al.*, 2015). Synthetic ecology investigates interactions in defined microbial communities for a better understanding of more complex, authentic microbial ecosystems, and it attempts to engineer microbial populations for biotechnological applications. Multiple orthogonal QS signal-response systems that function independently without cross-talk are being developed (Scott & Hasty, 2016). These systems permit the coordination of different metabolic tasks among community members that are not easily accomplished by a single cell. Potential benefits include the yield improvement in typically low-efficiency biosynthesis processes (Zhang, *et al.*, 2015), or optimization and stabilization of communities in wastewater treatment plant systems (Zhang & Li, 2016). While metabolic interactions in complex communities are being increasingly well understood in synthetic ecology (Zelezniak, *et al.*, 2015), the social evolutionary outcomes and competitive interactions between existing and engineered or introduced communities will benefit from further development.

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Conflicts of interest

The authors declare no existing conflicts of interest.

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Chapter 2

RESEARCH OBJECTIVES

The topics of this dissertation lie at the confluence of social evolution, ecology, and gene expression in bacteria. This goal of this dissertation is to uncover poorly understood aspects of the social evolutionary pressures facing communicating bacteria and the molecular architecture of their signaling systems. Cooperation and communication by *P. aeruginosa* has emerged as a popular experimental model system to investigate both aspects. During growth in media requiring QS-controlled proteolysis for growth, strong selection for cooperation puts cooperators at risk of exploitation by non-responding cheats. In Chapter 3 of this dissertation, our focus was to provide systematic evidence for the molecular mechanism allowing *P. aeruginosa* cooperative growth to persist in the presence of naturally evolving cheaters. Using genome sequencing of an evolved isolate from an *in vitro* evolution experiment, we discovered a mutation in a non-social single gene coding for the transcriptional regulator PsdR that allows cooperation to persist. Mutation in PsdR confers a cooperation-stabilizing effect through derepression of growth rate-limiting nutrient uptake and processing, thereby maximizing absolute fitness of cooperators and deferring a tragedy of the commons.

In Chapter 4 of this dissertation, we turned our focus upon the molecular mechanisms defining the threshold of QS activation in *P. aeruginosa.* As discussed in Chapter 1, a considerable amount of research has been dedicated to understanding the circuitry and dynamics of QS. However, it is unclear how multiple anti-activator proteins of QS function together to determine the quorum-activated threshold. Using mutational analysis of all known anti-activators of *P. aeruginosa* coupled with phenotypic measurements, gene induction kinetics, and transcriptional profiling to approach this question. We found an additive effect of multiple anti-activator deletion on QS gene expression, particularly when one mutation was in the anti-activator gene *qslA.* We also found nested, overlapping anti-activator regulons that suggest anti-

activation likely works through LasR or RhlR, and may involve co-binding of these regulators with more than one anti-activator.

Chapter 3

NON-SOCIAL ADAPTATION DEFERS A TRAGEDY OF THE COMMONS IN *Pseudomonas aeruginosa* QUORUM SENSING

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Abstract

In a process termed quorum sensing (QS), the opportunistic bacterial pathogen *Pseudomonas aeruginosa* uses diffusible signaling molecules to regulate the expression of numerous secreted factors or public goods that are shared within the population. But not all cells respond to QS signals. These social cheaters typically harbor a mutation in the QS receptor gene *lasR* and exploit the public goods produced by cooperators. Here we show that non-social adaptation under growth conditions that require QS-dependent public goods increases tolerance to cheating and defers a tragedy of the commons. The underlying mutation is in the transcriptional repressor gene *psdR*. This mutation has no effect on public goods expression but instead increases individual fitness by derepressing growth-limiting intracellular metabolism. Even though *psdR* mutant populations remain susceptible to invasion by isogenic *psdR lasR* cheaters, they bear a lower cheater-load than do wild-type populations, and they are completely resistant to invasion by *lasR* cheaters with functional *psdR*. Mutations in *psdR* also sustain growth near wild-type levels when paired with certain partial loss-of-function *lasR* mutations. Targeted sequencing of multiple evolved isolates revealed that mutations in *psdR* arise before mutations in *lasR*, and rapidly sweep through the population. Our results indicate that a QS-favoring environment can lead to adaptations in non-social, intracellular traits that increase the fitness of cooperating individuals and thereby contribute to population-wide maintenance of QS and associated cooperative behaviors.

3.1 Introduction

Bacterial cell-cell signaling, termed quorum-sensing (QS), often coordinates other cooperative behaviors such as nutrient acquisition, biofilm formation, or virulence in a cell-density-dependent manner (Waters and Bassler 2005, Williams et al 2007). In Gram-negative proteobacteria, QS is generally comprised of a LuxI-type signal synthase that produces a diffusible acyl-homoserine lactone (acyl-HSL) signal, and a cognate LuxR-type receptor that binds the signal and regulates transcription of target genes (Schuster et al 2013, Waters and Bassler 2005, Williams et al 2007). The opportunistic pathogen *Pseudomonas aeruginosa*, a particularly well-understood example, employs two acyl-HSL signaling systems, LasI/R and RhlI/R, arranged in a hierarchical fashion with LasR sitting atop the hierarchy (Jimenez et al 2012, Schuster and Greenberg 2006, Williams and Camara 2009). Together, both systems regulate over 300 genes, many of which encode secreted public goods such as extracellular enzymes or secondary metabolites that have a role in virulence (Hentzer et al 2003, Schuster et al 2003, Wagner et al 2003).

How social behaviors such as QS evolve and are maintained is of intense research and debate, as exploitation of common resources by selfish individuals should be favored and lead to a so-called "tragedy of the commons" (Keller and Surette 2006, West et al 2006). A tragedy of the commons results when the magnitude of selfish exploitation by cheaters exceeds the capacity of a cooperative system, resulting in the collapse of the entire population. Indeed, several studies have demonstrated the emergence of QS-cheaters that reap the benefits of cooperative secretions without metabolic investment both *in vitro* (Dandekar et al 2012, Diggle et al 2007, Sandoz et al 2007, Wilder et al 2011) and *in vivo* (Kohler et al 2009, Rumbaugh et al 2009). These QS-cheaters are defined by a loss-of-function mutation in the gene coding for the primary QS-receptor LasR. We previously showed that *P. aeruginosa lasR* mutant cheaters consistently evolve in a minimal growth medium with casein as the sole carbon source that requires QS-dependent extracellular proteolysis (Sandoz et al 2007). Using defined wild-type and *lasR* mutant co-cultures, we further showed that these cheaters do better when they are rare (i.e. display negative frequency-dependent fitness), and that they impose a burden on population

growth (Sandoz et al 2007, Wilder et al 2011). Intriguingly, however, this negative effect on group fitness was generally not observed during *in vitro* evolution experiments initiated solely with the wild-type strain, suggesting evolution of a mechanism that stabilized QS (Dandekar et al 2012, Sandoz et al 2007, Wilder et al 2011).

To identify and characterize the underlying mechanism, we used a combination of whole-genome sequencing, genetic analysis, and growth experiments. We found a single mutation in a transcriptional repressor, PsdR, that rapidly dominates the population, enhances intracellular dipeptide metabolism, increases both individual and group fitness, provides immunity against cheaters that do not themselves carry a *psdR* mutation, and lessens the detrimental effect of certain *lasR* mutations on group fitness. Our results show that QS-favoring conditions can select for non-social adaptations that improve group fitness and defer a tragedy of the commons.

3.2 Materials and methods

3.2.1 Strains and culture conditions

Pseudomonas aeruginosa PAO1 was used as the wild-type isogenic parent at the start of all original *in vitro* evolution experiments (Sandoz et al 2007, Wilder et al 2011). All mutants were created via allelic exchange using a suicide vector containing either evolved alleles or in-frame deletions constructed by splicing-overlap-extension PCR (SOE-PCR) (Hoang et al 1998, Horton 1995) (see Table 3.1 for a comprehensive list of strains). For routine culturing, we grew strains at 37°C on Lennox LB agar or with shaking in Lennox LB broth buffered with 50 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS), pH 7.0. Plates were supplemented with 100 µg/mL tetracycline when necessary for the selection of marked strains. For fitness, competition, substrate utilization, and expression assays, M9 minimal medium supplemented with either 1% caseinate, 0.5% casamino acids (CAA), or 10 mM GlyGlu dipeptide was used (Kiely et al 2008, Sandoz et al 2007). In the case of caseinate fitness experiments with supplemented exoprotease, porcine elastase

(Sigma) was added at the beginning of growth, in principle as described previously (Diggle et al 2007). As determined with a FITC-casein assay (see below), the caseinolytic activity of the elastase concentration used (0.21 U/ml) was 20% of that found in the supernatant of wild-type cultures grown in M9-caseinate medium for 24 h. All experiments were performed using a minimum of three biological replicates with independent inocula.

Table 3.1 Bacterial strains and plasmids.

Strain or plasmid	Relevant properties	Reference or origin
Pseudomonas aeruginosa		
PAO1	Wild-type (obtained from M Vasil and U Ochsner)	(Holloway 1955)
PAO-HC	PAO1 derivative; evolved hybrid cooperator containing *lasR5*, *psdR1*, and *abcB1* mutations	(Sandoz et al 2007)
PAO1 *lasR5*	PAO1 derivative; *lasR5*, unmarked mutant in which wild-type *lasR* was replaced with *lasR5*	This study
PAO1 *psdR1*	PAO1 derivative; *psdR1*, unmarked mutant in which wild-type *psdR* was replaced with *psdR1*	This study
PAO1 Δ*lasR*	PAO1 derivative; Δ*lasR*, unmarked in-frame deletion from amino acid 102 to 216	(Wilder et al 2011)
PAO1 Δ*psdR*	PAO1 derivative; Δ*psdR*, unmarked in-frame deletion from amino acid 11 to 124	This study
PAO1 *psdR1 lasR5*	PAO1 *psdR1* derivative; *psdR1 lasR5*, unmarked mutant in which wild-type *psdR* and *lasR* were replaced with *psdR1* and *lasR5*, respectively	This study
PAO1 *psdR1* Δ*lasR*	PAO1 Δ*lasR* derivative; *psdR1* Δ*lasR*, unmarked mutant in which wild-type *psdR* and *lasR* were replaced with *psdR1* and Δ*lasR*, respectively	This study
Escherichia coli		
DH5α	F- Φ80*lacZYA-argF* U169 *recA1 hsdR17* (rk-, mk+) *phoA supE44* λ- *thi-1 gyrA96 relA1*	Invitrogen
SM10	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km λpir	(Simon et al 1983)
Plasmids		
pEX18Gm	Conjugative suicide plasmid; Gm^R	(Hoang et al 1998)
pEX18Gm.*psdR1*	pEX18Gm with the evolved *psdR1* allele	This study
pEX18Gm.Δ*psdR*	pEX18Gm with Δ*psdR* containing an in-frame deletion from amino acid 11 to 124	This study
pEX18Gm.*lasR5*	pEX18Gm with the evolved *lasR5* allele	This study
pUC18R6KT-mini-Tn7T-Tet	Broad host range mini-Tn7 vector with Tc resistance gene cassette	Courtesy of Herbert P Schweizer

3.2.2 Whole genome sequencing and targeted DNA sequencing

For genome sequencing, we selected an evolved "hybrid cooperator" (HC) isolate from our previous long-term growth experiment (Sandoz et al 2007) and its wild-type PAO1 parent strain. The evolved isolate was dubbed HC because of its partially positive QS phenotypes (see the Results section for details). Both strains were grown individually overnight (18 h) in MOPS-buffered LB medium as described above. Genomic DNA was isolated using the Qiagen Puregene Yeast/Bacteria Kit B (Qiagen Sciences, Germantown, MD, USA) and assessed for quality on a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). 454 pyrosequencing was carried out using unpaired reads on a Genome Sequencer FLX instrument with GS FLX Titanium series reagents (454 Life Sciences, Branford, CT, USA) by the Dhingra Genomics Lab at Washington State University in Pullman, Washington, USA. Sequencing of the HC isolate produced 507094 reads covering approximately 187 Mb, while the ancestral PAO1 produced 501270 reads covering approximately 200 Mb. Raw 454 reads were assembled using the Roche 454 Newbler assembler with the PAO1 genome as a reference (Margulies et al 2005, Stover et al 2000, Winsor et al 2011). The HC assembly utilized an average map length of 370 bp and average sequence depth of 29.5, while the ancestral PAO1 assembly utilized an average map length of 401 bp and average sequence depth of 31.7. Differences between the HC and ancestral PAO1 assemblies were discovered using SNP/INDEL calling in SAMtools (Li et al 2009). To confirm the identified mutations, and to sequence specific loci of interest, standard dideoxy sequencing of PCR-amplified and purified chromosomal DNA was employed at the Center for Genome Research and Biocomputing at Oregon State University in Corvallis, Oregon, USA. Primers are listed in Table S3.4.

3.2.3 Fitness assays and cheater-load

Wild-type, evolved HC, and defined mutants (tagged with an antibiotic-resistance marker where applicable) were grown in caseinate minimal medium. Overnight cultures of individual strains in MOPS-buffered LB were used as inocula

for experiments and diluted to starting OD_{600} values of 0.02 (1 cm pathlength, approximately 2×10^7 CFU/mL). In the case of co-culture experiments, the combined total starting OD_{600} was 0.02. All fitness experiments were allowed to proceed for 24 h with shaking at 37°C. For rich media (LB+MOPS, M9-CAA) and dipeptide media co-cultures, conditions were kept identical to caseinate experiments with the exception that co-cultures in dipeptide media were grown for 7 d to allow the cultures to reach saturation. Colony forming units (CFU) per mL were determined by dilution-plating at t = 0 and 24 h, with an additional plating at t = 12 h during absolute fitness experiments. For enumeration in co-culture experiments, differential plating on tetracycline-supplemented LB agar was used. Fitness was calculated according to the Malthusian growth model (Lenski et al 1991, Wilder et al 2011). Absolute fitness is expressed as the average rate of increase or Malthusian parameter (m), with $m = \ln(N_1/N_0)/t$, where N_1 and N_0 are the final and initial strain densities, respectively, and t is the culturing time in days. Relative fitness is expressed as the ratio of the Malthusian parameters (w) of two competing strains. Cheater-load experiments were performed as previously described (Sandoz et al 2007), with the exception that for this set of experiments total starting OD_{600} values of 0.02 were identical for all treatments.

3.2.4 Extracellular proteolysis

Extracellular caseinolytic activity was determined using an established FITC-casein assay (Twining 1984, Wilder et al 2011). Briefly, starter cultures of each strain were grown overnight in MOPS-buffered LB at 37°C and diluted to an OD_{600} of 0.02 in fresh CAA medium. Supernatants were harvested after 12 h of growth, sterile filtered and incubated with the FITC-conjugated casein substrate (Sigma). Digestion was allowed to proceed for 3 h at 37°C. Fluorescence was measured at λ_{ex} = 490 nm and λ_{em} = 525 nm in a 96-well format on a Tecan Infinite M200 plate reader (Tecan Group Ltd., Männedorf, Switzerland).

To predict the cleavage pattern of our caseinate substrate (a mixture of α-s1-casein, α-s2-casein, β-casein, and κ-casein) by LasB elastase, we employed ExPASy

PeptideCutter (http://web.expasy.org/peptide_cutter/) for *in silico* digestion with thermolysin, the closest LasB elastase family member available in the database (Gasteiger et al 2005).

3.2.5 Expression analysis

Strains were initially grown overnight in MOPS-buffered LB liquid culture, and then diluted to an OD_{600} of 0.02 in fresh CAA medium. Expression cultures were harvested at OD_{600} values of 0.5 and 1.5, corresponding to exponential and early stationary phases in this medium, respectively. Total RNA was isolated and cDNA synthesized as previously described (Schuster and Greenberg 2007, Schuster 2011). Quantitative reverse-transcriptase PCR (qRT-PCR) was carried out according to established protocols (Schuster and Greenberg 2007, Schuster 2011) using an Applied Biosystems 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). Identical amounts of cDNA were used as template. Transcript levels were quantified using the relative standard curve method.

3.3 Results

3.3.1 Genome sequencing of *in vitro*-evolved *P. aeruginosa*

In our previous *in vitro* evolution studies, we cultured the PAO1 wild-type strain in caseinate medium for 20 days, subculturing into fresh medium each day (Sandoz et al 2007, Wilder et al 2011). We used two phenotypic screens as a proxy for QS-proficiency, namely 1) protease production on skim milk agar plates, and 2) growth on minimal agar plates with adenosine as the sole carbon source. Negative results in the phenotypic screens correspond to mutations in the gene coding for the primary QS regulator, *lasR*, thereby conferring a cheater phenotype.

To confirm that QS-controlled extracellular proteolysis is solely responsible for the growth deficiency of the pleiotropic *lasR* mutant in caseinate medium, we cultured the Δ*lasR* single mutant in the presence of purified elastase. Addition of elastase restored Δ*lasR* mutant fitness, expressed as Malthusian growth parameter, *m*

(Lenski et al 1991), to a level indistinguishable from wild-type and significantly above that of the Δ*lasR* mutant without elastase (Figure 3.1).

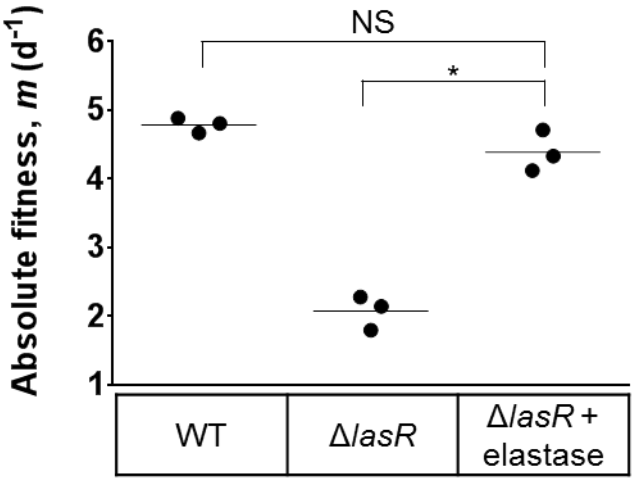

Figure 3.1 Effect of elastase addition on the absolute fitness of a *lasR* mutant. Absolute fitness of the *P. aeruginosa* Δ*lasR* mutant and its wild-type parent was calculated as Malthusian growth parameter (*m*) after 24 h of growth in caseinate medium. In the case of Δ*lasR* + elastase, 0.21 U/ml porcine elastase was added. Bars represent means ($n = 3$). * Denotes significant difference as determined by unpaired t-test ($p = 0.00010$). NS, difference not significant (unpaired t-test, $p = 0.096$).

While cheaters can exploit the public goods produced by the cooperating population in a way that eventually leads to a population crash, we only observed this outcome in one of our five replicate evolution experiments (Figure 3.2a-f). Instead, we found that *lasR* cheater frequencies as high as 60% are tolerated and do not significantly affect the growth yield of the population (Figure 3.2a-f). This was surprising, considering our previous co-culture experiments with specific initial frequencies of defined *lasR*-mutant cheaters and wild-type cooperators, where we demonstrated the burden of cheaters on the productivity of a population manifests at cheater levels as low as 25% (Sandoz et al 2007). We also observed a subpopulation of isolates deficient in growth on adenosine, but not in skim-milk proteolysis. Based on their phenotypes, we dubbed these isolates "hybrid cooperators" (HCs). The HC subpopulation rose in frequency similar to the cheaters, representing up to 20% of the total population (Figure 3.2a), and also harbored mutations in *lasR* (Sandoz et al 2007). We reasoned that this HC phenotype had an important role in the maintenance

of cooperative population growth. We hypothesized that the HC phenotype was caused by an independent second-site mutation that occurred either before or after mutation of *lasR*, and that this mutation partially restored QS proficiency.

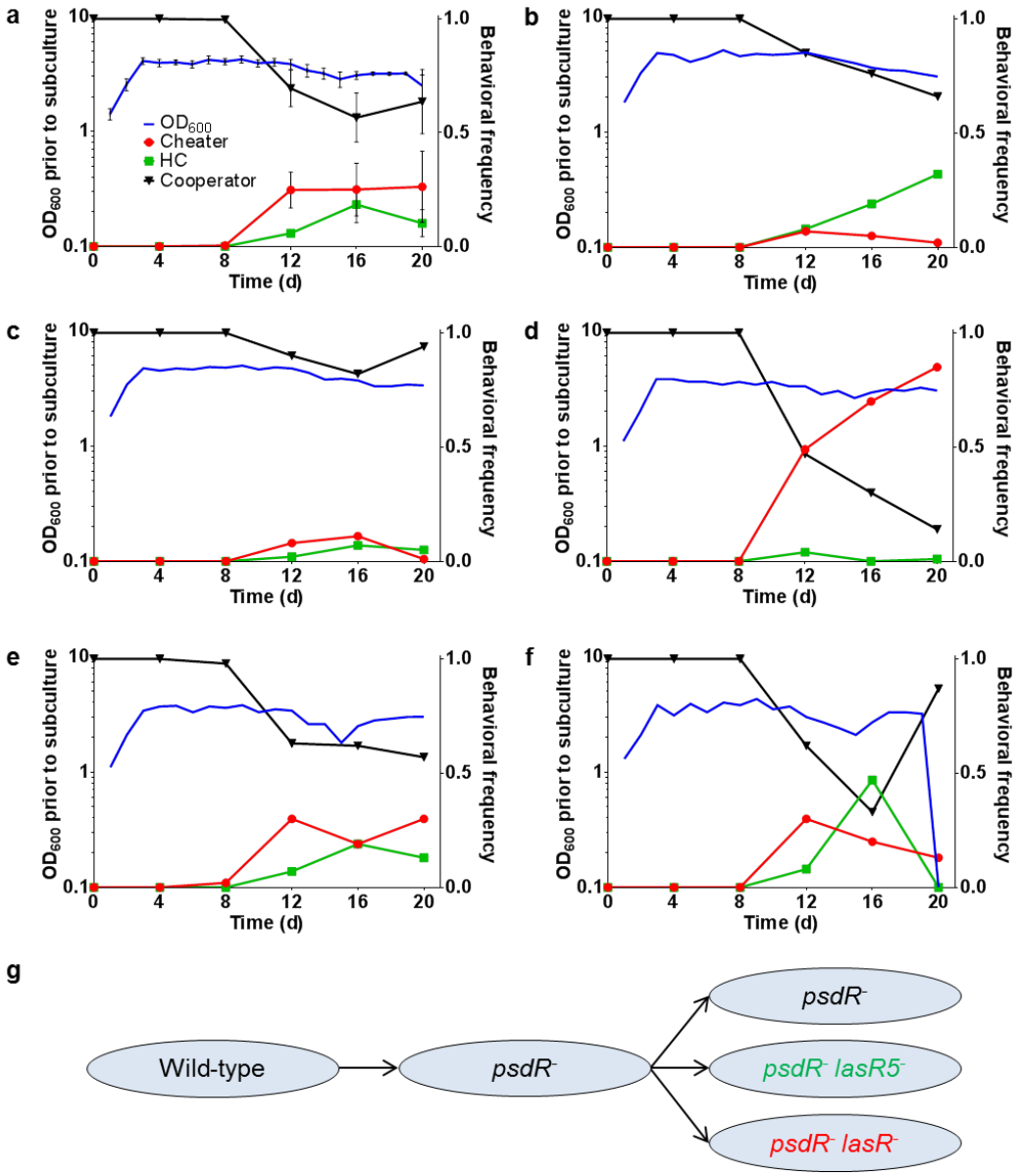

Figure 3.2 *In vitro* evolution of *P. aeruginosa* populations under conditions that require QS. (a-f) Population growth yield and phenotypic frequencies. OD_{600} values measured daily prior to subculture are plotted on the left vertical axis (blue line). Frequencies of cooperator (black triangles), cheater (red circles), and hybrid cooperator (green squares) phenotypes are plotted on the right vertical axis. **(a)** Means and SEM of all replicate experiments (n = 5). In some cases error bars are too small to be seen. **(b-f)** Individual, independent biological replicates. Based on raw

data from Wilder *et al.*, 2011 (panels b, c) and Sandoz *et al.*, 2007 (panels d-f). **(g)** Schematic of evolutionary trajectories of individual mutations.

To address our hypothesis, we sequenced the genome of a representative HC isolate from day 12 of one replicate experiment. We also sequenced the genome of the ancestral PAO1 wild-type strain for comparison. Genomes were assembled using the published PAO1 genome sequence as a reference (Stover et al 2000, Winsor et al 2011). In all, our analysis showed the HC harbored only three mutations when compared to the wild-type ancestor, including *lasR*. The mutations were single nucleotide polymorphisms (SNPs) in *lasR* (PA1430) and in PA2408, as well as an 18 base-pair truncation in *psdR* (PA4499). PA2408 encodes a probable ATP-binding component of an ABC-transporter, and *psdR* encodes a transcriptional regulator (Kiely et al 2008, Winsor et al 2011) (Table 3.2). Targeted Sanger sequencing of all three loci in two additional HC and cheater isolates from day 12 of two independent *in vitro* evolution experiments revealed that mutations in *lasR* and *psdR* are ubiquitous in isolates displaying both phenotypes, but mutations in PA2408 are not (Table S3.5). We therefore concluded that the PA2408 mutation is not likely to be relevant to the HC phenotype. Additional evidence for this conclusion is presented below.

Table 3.2 Mutations in a sequenced *Pseudomonas aeruginosa* HC genome.

Gene (name)[a]	Function[a]	Mutation[b]	Allele ID
PA1430 (*lasR)*	*luxR*-type transcriptional regulator	C→T (683)	*lasR5*
PA4499 (*psdR)*	Putative transcriptional regulator	Δ18 bp (514)	*psdR1*
PA2408	Probable ATP-binding component of ABC transporter	T→C (337)	*abcB1*

[a]Gene names and functions as annotated in the *Pseudomonas* Genome Database.

[b]Numbers in parentheses indicate position or beginning of a given mutation relative to the translational start site.

The presence of *psdR* mutations in cheater isolates in addition to the HC isolates indicated that a mutation in *psdR* is not a distinguishing feature of the HC phenotype, and that mutation of *psdR* may have arisen prior to mutation of *lasR*. To elucidate the evolutionary trajectories of the *psdR* mutation, and to assess whether *psdR* mutations are also present in cooperator phenotypes, we sequenced the *psdR*

locus of evolved isolates positive for both skim-milk proteolysis and adenosine utilization from the day 4, 8, and 12 archives of two replicates (5 isolates per day, 30 total). Surprisingly, we found 100% of sequenced isolates harbored a non-synonymous mutation in *psdR* (Table 3.3). Even as early as the first phenotypic screen at day 4, the entire sampled population that originally appeared to be "wild-type" with respect to QS actually had acquired point mutations, insertions or deletions in *psdR*. Isolates from the same replicate culture often harbored different *psdR* mutations, and some *psdR* mutations in early cooperator isolates were identical to those in later HC and cheater isolates. These results therefore suggest that the HC phenotype is primarily defined by the nature of the *lasR* mutation itself. In general, our sequencing data indicate a strong selection against a functional *psdR* during cooperative growth in caseinate medium, and show that the evolutionary trajectories of cheater, HC and cooperator phenotypes all start with a mutation in *psdR* (Figure 3.2g). This result is consistent with the presumed function of PsdR. PsdR has been characterized as a transcriptional repressor of genes involved in the uptake and intracellular degradation of dipeptides in *P. aeruginosa* (Kiely et al 2008). Thus, derepression of dipeptide metabolism through mutation and inactivation of PsdR could potentially increase the fitness of *P. aeruginosa* during proteolytic growth in caseinate medium. Such a mutant would take up and process the dipeptides generated by the cocktail of secreted proteases (including LasB elastase, alkaline protease, and protease IV) more rapidly. Consistent with this idea, *in silico* digestion of bovine casein by thermolysin, a homolog of *P. aeruginosa* LasB elastase with similar cleavage properties (Jiang and Bond 1992, Morihara and Tsuzuki 1971), indeed produces up to 6 dipeptides per casein molecule.

Table 3.3 *psdR* mutations in evolved *Pseudomonas aeruginosa* isolates.

	Number of mutations (Replicate)[b]			
Mutations[a]	Day 4	Day 8	Day 12	Change[c]
Cheater (4 sequences total)				
Δ505-end			2 (2)	Deletion
T166C			2 (1)	S56P
HC (4 sequences total)				
Δ145-148			2 (2)	Deletion

Table 3.3 (continued)

Δ261-422			1 (1)	Deletion
Δ261-end			1 (1)	Deletion
Cooperator (30 sequences total)				
Δ261-422	1 (1)	1 (2)		Deletion
Δ505-end		1 (2)		Deletion
Δ147-159		1 (1)	1 (1)	Deletion
C74T	1 (2)	1 (2)		A25V
T100C	1 (2)			F34L
C109A	1 (1)		1 (1)	Q37K
T166C	1 (1)			S56P
G397A	2 (1)	2 (1)		G133R
C411A			3 (1)	STOP at 137
A431C	1 (2)	1 (1)	2 (2)	Y144S
Insert A at 3		1 (1)		Frameshift
Insert A at 378	1 (2)	1 (2)	1 (2)	Frameshift
No amplicon	1 (2)	1 (2)	2 (2)	Unknown

[a]Mutations are sorted by cheater, hybrid cooperator, and cooperator phenotypes. Numbers indicate nucleotide position or beginning of a given mutation relative to the translational start site.
[b]The individual replicate of the *in vitro* evolution experiment is indicated in parentheses. Same-day isolates with identical mutations were always from the same replicate. Replicates 1 and 2 correspond to Fig. 2 panels b and c, respectively.
[c]Numbers indicate amino acid position relative to the translational start site.

3.3.2 Absolute fitness of evolved and defined strains

Next, we investigated the fitness contributions of each mutant allele to the cooperator, cheater, and HC phenotypes. We constructed defined single and double mutants by transferring the evolved *lasR5* and *psdR1* alleles into the parental PAO1 strain background, and compared their phenotypes to those of in-frame deletion mutants. This approach also allowed us to assess whether the nature of the mutation in *lasR* distinguishes a HC from a cheater. As characterized in our previous study, all evolved cheaters deficient in skim-milk proteolysis and adenosine utilization were also fully deficient in other QS-dependent phenotypes, identical to a *lasR* in-frame deletion mutant (Sandoz et al 2007).

We first assessed growth of individual strains by measuring their population densities during clonal growth in caseinate medium that requires QS-dependent proteolysis (Figure 3.3a). The corresponding absolute fitness values and statistical

data are shown in Figure S3.8. The wild-type is capable of two logs of growth within 24 h, from approximately10^7 to 10^9 CFU/mL, whereas a *lasR* deletion mutant shows little growth. Interestingly, the defined *lasR5* mutant displayed an intermediate level of growth and fitness significantly above that of the Δ*lasR* mutant at 24 h, indicating that *lasR5* retains partial function. The defined *psdR1* mutant and the Δ*psdR* mutant displayed similar growth and fitness levels significantly above that of the wild-type at 12 h, indicating that the *psdR1* mutation completely inactivates gene function. The defined *psdR1 lasR5* double mutant and the evolved HC showed identical growth characteristics, similar to that of the wild-type at 24 h, supporting our sequencing data suggesting the PA2408 mutation does not play a part in the HC phenotype. In contrast, the *psdR1* mutation, when paired with the Δ*lasR* mutation, did not support growth to levels beyond the Δ*lasR* single mutant, strengthening the role of the *lasR5* allele in the HC phenotype. Taken together, these results show that inactivation of *psdR* increases absolute fitness, and that this effect can compensate for the reduced level of cooperation from partial loss-of-function in *lasR5*.

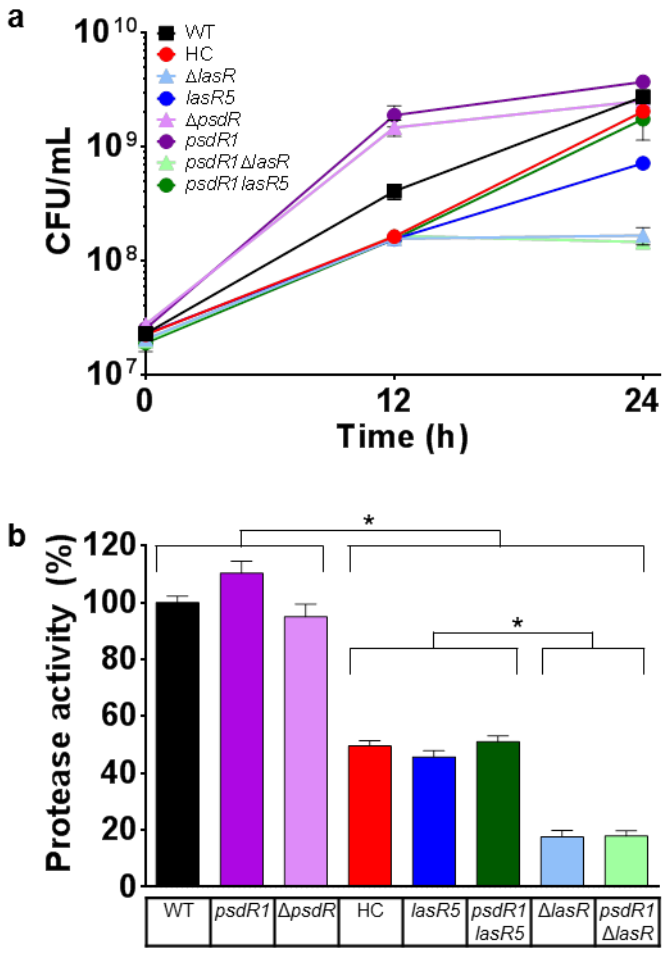

Figure 3.3 Growth and proteolysis in pure culture. (a) Growth in caseinate medium measured at 12 and 24 h, expressed as CFU/mL. Means and SEM are shown (n = 3) and in some cases error bars are too small to be seen. Starting CFU/mL are statistically the same (one-way ANOVA, Tukey's multiple comparisons test, α = 0.05). **(b)** Caseinolytic activity of cultures grown in CAA medium for 12 h, as measured by FITC-casein assay. Caseinolytic activity is shown per OD_{600} to correct for slight variations in the final culture densities in CAA medium. Means and SEM are shown (n = 3). * Denotes significant differences as determined by one-way ANOVA, Tukey's multiple comparisons test, α = 0.05. Results of similar magnitude are grouped for clarity.

3.3.3 Exoprotease activity

To correlate the absolute fitness of each strain with its exoprotease activity, we quantified caseinolysis of culture supernatants using a fluorescein isothiocyanate (FITC)-casein assay (Twining 1984, Wilder et al 2011). This method is more precise than the qualitative skim milk plate assay used previously (Sandoz et al 2007). In

order to uncouple exoprotease activity from its effect on growth, we replaced caseinate in our growth medium with casamino acids (CAA), a C-source that does not require QS-dependent proteolysis. All strains harboring the *lasR5* allele showed intermediate levels of extracellular caseinolysis at half the levels of the wild-type *lasR* alleles and roughly three times higher than the Δ*lasR* alleles (Figure 3.3b). Strains containing *psdR* mutations did not show elevated caseinolysis compared with the wild-type. These results confirm that *lasR5* is a partial-loss-of-function mutation, and further show that *psdR* has no effect on QS-dependent exoprotease production, consistent with its role in regulating intracellular dipeptide metabolism.

3.3.4 Transcriptional regulation of dipeptide transport and processing

We have provided evidence that *psdR* mutations are explicitly linked to significant increases in the fitness of *P. aeruginosa* in a cooperative environment. As indicated above, PsdR is a transcriptional repressor of several neighboring genes involved in the transport and processing of dipeptides in *P. aeruginosa* (Kiely et al 2008). Specifically, PsdR represses transcription of *mdpA*, which codes for the cytoplasmic dipeptidase MdpA, as well as *dppA3*, the first gene in a dipeptide transport gene cluster annotated *dpp* for a homologous region in the *Escherichia coli* K12 genome (Kiely et al 2008). Associated with this gene cluster is a gene coding for the porin OpdP, which is implicated in the uptake of single amino acids as well as dipeptides in *P. aeruginosa* (Tamber and Hancock 2006).

Interestingly, our previous transcriptome analysis of *P. aeruginosa* grown in rich medium indicated that *mdpA* expression was affected by *rhl*-QS. Addition of 3OC12-HSL to a signal synthesis mutant only induced expression 1.6-fold, but addition of both acyl-HSL signals induced expression 9-fold (Schuster et al 2003). Thus, it was conceivable that *lasR* affected dipeptide transport and processing mainly indirectly, through its effect on the *rhl* system, although this regulation is nutritionally conditional (Dekimpe and Deziel 2009, Medina et al 2003, Mellbye and Schuster 2014). To investigate a possible link between QS and dipeptide metabolism in our experimental system, we quantified the transcript levels of *dppA3* and *mdpA* during

growth in CAA medium for our set of eight *P. aeruginosa* strains used in the previous sections. Using qRT-PCR, we assessed transcription in exponential and early stationary phases, corresponding to OD_{600} values of 0.5 and 1.5, respectively. We found that for either gene at any growth phase tested, relative expression could be sorted by *psdR* allele, with at least an order of magnitude separating functional *psdR* alleles from those harboring *psdR1* or Δ*psdR* (Figure 3.4). Importantly, none of the *lasR* alleles substantially influenced expression of *dppA3* or *mdpA* in our experiments. This result indicated that, under the growth conditions employed here, the regulation of dipeptide transport and processing is dependent on *psdR* but independent of *lasR*. Hence, control of the relevant 'private' goods – the cellular dipeptide uptake and processing machinery – occurs independently of QS-mediated 'public' goods in our system.

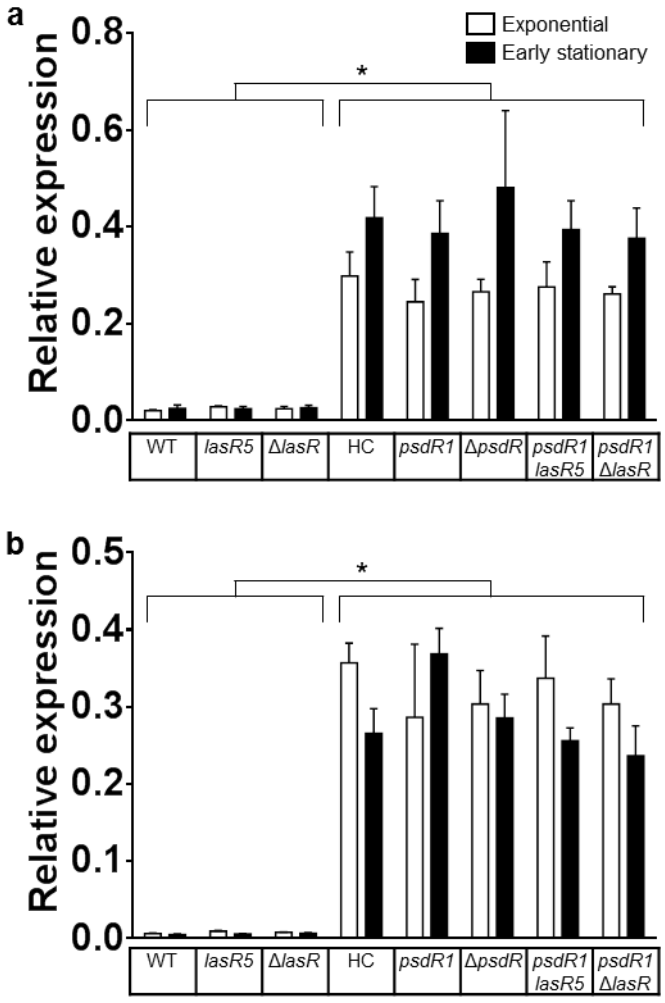

Figure 3.4 Expression of *dppA3* and *mdpA*. Relative transcript levels of *dppA3* **(a)** and *mdpA* **(b)**, as determined by qRT-PCR. Relative expression during exponential (OD_{600} = 0.5, empty bars) and early stationary (OD_{600} = 1.5, filled bars) growth phases in CAA medium are shown. Means and SEM are shown (n = 3). * Denotes significant differences as determined by one-way ANOVA, Tukey's multiple comparisons test, $\alpha = 0.05$. Results of similar magnitude are grouped for clarity.

3.3.5 Relative fitness of evolved and defined strains in co-culture

Next, we measured the relative fitness of our set of strains through pairwise comparisons in co-culture, again employing caseinate medium that requires QS-dependent cooperation. We introduced an antibiotic resistance marker into one of the two strains at a neutral chromosomal site to allow differentiation in co-culture. The marker itself has no effect on growth (Wilder et al 2011). With the marked strain at an initial frequency of 0.01 (1%) in starting populations of approximately 2×10^7 CFU/mL total, we allowed competitions to proceed for 24 hours, and calculated the relative fitness (w) as the ratio of the average growth rates (Malthusian growth parameters) (Lenski et al 1991, Wilder et al 2011).

First, we sought to ensure that selection for *psdR* mutants in the original *in vitro* evolution experiments was not just a general feature of prolonged growth but was tied to the specific growth medium. We therefore initiated defined co-cultures of the Δ*psdR* mutant and the wild-type in different growth media, at a mutant frequency of 0.01. We used a complex medium (MOPS-buffered LB) and M9 minimal medium with essentially fully hydrolyzed casein (CAA) as the sole C-source. The Δ*psdR* mutant did not enrich in either LB+MOPS or CAA media, confirming that adaptive mutation of *psdR* is linked to the cooperative media we employed (Figure 3.5a). To confirm that the increased absolute fitness of a *psdR* mutant can be attributed in part to increased uptake and metabolism of dipeptides, we also employed M9-minimal medium with the dipeptide GlyGlu as the sole carbon source (Kiely et al 2008). The Δ*psdR* mutant exhibited a high degree of relative fitness in this medium very similar to the *psdR1* mutant in caseinate medium (Figure 3.5a and first column of Figure 3.5b), indicating that dipeptide uptake and metabolism is indeed a target of selection in our cooperative growth environment.

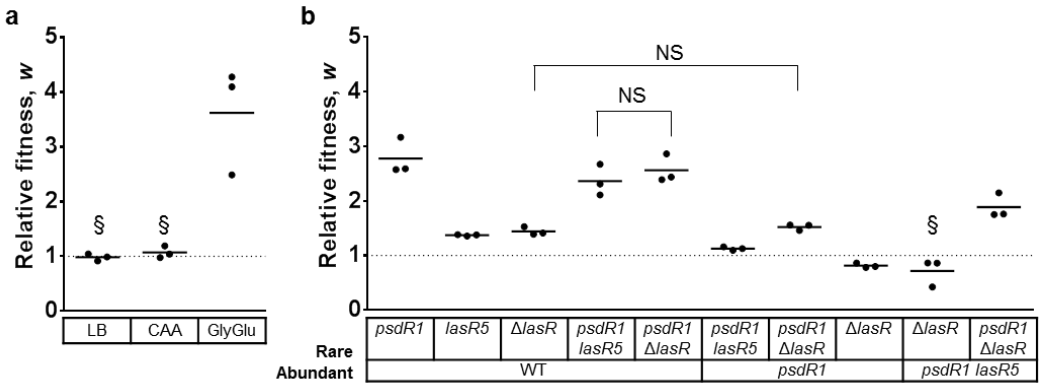

Figure 3.5 Relative fitness. (a) Relative fitness of a Δ*psdR* mutant in co-culture with its wild-type parent in rich and defined media, initiated at a mutant frequency of 0.01. LB, LB+MOPS; CAA, M9-CAA; GlyGlu, M9-GlyGlu. **(b)** Relative fitness of defined mutants in caseinate co-culture. Pairs of the respective rare and abundant strain were initiated at a ratio of 1:99. Relative fitness values were calculated as the ratio of Malthusian growth parameters (*w*) after 24 h, with the exception that experiments in M9-GlyGlu were allowed to proceed for 7 d to allow the co-cultures to reach saturation. Values of *w* signify whether the rare strains grow faster ($w > 1$) or grow slower ($w < 1$) than the respective abundant strains. Bars represent means ($n = 3$), and means are significantly different from $w = 1$ (one sample t-test, $p < 0.05$), unless designated by §. NS, difference between two conditions not significant (unpaired t-test, $p > 0.05$). Difference in mean relative fitness of Δ*psdR* in M9-GlyGlu co-culture with WT (a) and *psdR1* in caseinate co-culture with WT (b) is not significant (unpaired t-test, $p = 0.23$).

Second, we sought to compare the relative fitness of mutant alleles (initial frequency of 0.01) against the wild-type ancestor to better understand the population dynamics at the beginning of our previous *in vitro* evolution experiment. The *lasR5* mutant modestly enriched in wild-type co-culture, as did the Δ*lasR* mutant in accordance with previous studies (Figure 3.5b) (Sandoz et al 2007, Wilder et al 2011). This result was expected as an individual that decreases investment in a secreted "public good" while still taking advantage of its production by cooperators should exhibit higher relative fitness (West et al 2006). A *psdR1* mutant had a tremendous relative fitness advantage with respect to the wild-type, consistent with its high absolute fitness, and mirroring the early dominance of *psdR* mutants during *in vitro* evolution (Figure 3.5b). When we combined either of the *lasR* mutant alleles with *psdR1*, the average relative fitness again was well above that of the *lasR* mutants

alone, demonstrating the independence of *psdR* fitness from LasR regulation (Figure 3.5b).

We then aimed to understand the relative fitness dynamics after the emergence and dominance of *psdR* mutations in the evolved populations. This time we initiated competitions with the *psdR1* defined mutant in majority (initial frequency of 0.99). Both *psdR1 lasR5* and *psdR1* Δ*lasR* double mutants displayed relative fitness above 1.0, as would be required for their enrichment in the original in *in vitro* evolution experiments (Figure 3.5b). The difference in the relative fitness between the two strains is reflected in their relative abundances during *in vitro* evolution (Figure 3.2a). Interestingly, a defined mutant with the Δ*lasR* allele alone was not able to enrich against the defined *psdR1* mutant, further demonstrating the effect that a large increase in absolute fitness can have on relative fitness against an obligate cheater.

Fourth, to investigate resistance of the HC to obligate cheating, we initiated competitions with the HC genotype (*psdR1 lasR5*) in majority (initial frequency of 0.99). We observed resistance to invasion by the Δ*lasR* cheater, but when the obligate cheater allele is paired with the evolved *psdR* allele, the *psdR1* Δ*lasR* relative fitness again rose above 1.0 (Figure 3.5b).

Taken together, we confirmed our original predictions of the evolutionary trajectories (Figure 3.2g) of an evolving *P. aeruginosa* population. However, these relative fitness measurements do not fully explain the sustained cooperative growth of the evolved population. While even the *psdR* mutant was susceptible to subsequent invasion by *psdR1* Δ*lasR* mutants, it is plausible that it would tolerate a higher proportion of cheaters, thereby maintaining high population growth in cooperative growth environments.

3.3.6 Cheater-load

To finally determine the effect of increasing fractions of cheaters on the mean group fitness of the entire population, or cheater-load, we again used defined co-culture experiments in caseinate medium. We varied the initial frequencies of an

obligate cheater, Δ*lasR* or *psdR1* Δ*lasR*, with respect to the cooperating parent strain, wild-type or *psdR1*, respectively, and quantified total population growth after 24 h. As expected, we found the burden of cheaters was significantly lower for the *psdR1* mutant compared with the wild-type. Significant decreases in population productivity in the *psdR1* background did not occur until the cheater was at a frequency of 0.75 or greater, compared to 0.25 for the wild-type (Figure 3.6). This result demonstrated that the *psdR1* mutation helps stabilize cooperative, proteolytic growth as long as obligate cheaters are not the majority.

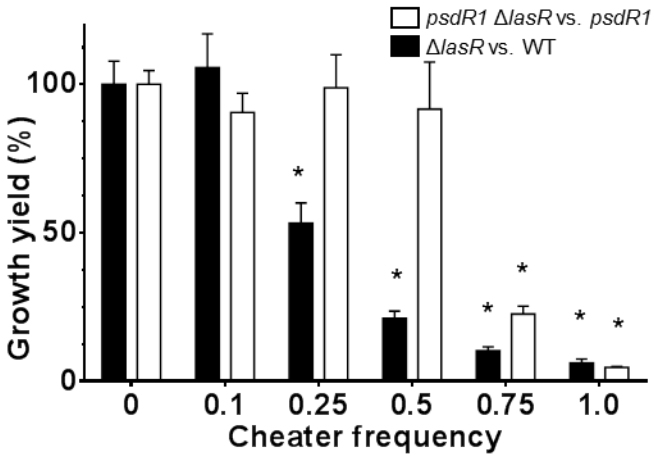

Figure 3.6 Cheater-load. Cheater-load expressed as relative growth yield of the entire population. Co-cultures of a Δ*lasR* mutant cheater and its wild-type parent (filled bars), and of a *psdR1* Δ*lasR* double mutant cheater and its *psdR1* single mutant parent (empty bars) were grown for 24 h in caseinate medium. Starting cheater frequencies are indicated on the horizontal axis. Growth yield of each parent strain culture without cheater is set to 100%. Means and SEM are shown (n = 3). * Denotes significant difference from respective parent strain without cheater as determined by unpaired t-test ($p < 0.05$).

3.4 Discussion

In this paper, we identified and characterized a mechanism that helps transiently stabilize cooperative behavior in the QS pathway of the opportunistic pathogen *P. aeruginosa*. Growth in the QS-dependent cooperative environment described here strongly selects for non-social mutations that increase absolute fitness, thereby leading to increased tolerance to cheaters. Such adaptive mutation has been

described in other microbial systems. Morgan *et al.* theoretically and experimentally showed that siderophore-producing populations of *Pseudomonas fluorescens* grown under iron-limiting conditions cannot be invaded by non-producing mutants (Morgan et al 2012). The authors proposed that this occurred because the numerically dominant cooperators had a greater chance of obtaining a beneficial mutation that could sweep through the population. However, the underlying mutation was not identified. In a related study, Waite and Shou (Waite and Shou 2012) engineered a system with obligatory mutualistic cooperation between two non-mating yeast strains. The addition of an obligate cheater strain that exploits a common good shared between the two mutualistic cooperators lead to an adaptive race to either preserve cooperation or fail through population collapse. In the cases where cooperation was preserved, the cooperating subpopulation acquired a beneficial mutation that helped purge the cheater phenotype from the population. Here, the genetically engineered nature of the cooperative system raised questions about its relevance.

Our *in vitro* evolution experiments were initiated with pure cultures of wild-type bacteria. Under these conditions, there was essentially no adaptive race between cooperators and cheaters initially, because the non-social adaptation emerged first and quickly dominated the population. Of course, these mutants with non-social adaptations were then subject to invasion by cheaters that also carry the adaptation. It is possible that an adaptive race between these two evolved genotypes would eventually result in a second non-social mutation that further sustained cooperative growth, consistent with previous work on long-term microbial adaptation (Wiser et al 2013). This stochastic scenario may explain why we observed a collapsing population in only one out of five *in vitro* evolution experiments (Figure 3.2b-f). Genome sequencing of late isolates beyond day 12 would be required to confirm this notion. As in the study by Waite and Shou, we found that in defined co-cultures, non-social adaptation conferred resistance to cheaters with an otherwise wild-type background (Waite and Shou 2012). However, in contrast to Waite and Shou, evolved cheaters were still able to invade their cooperating parent strains. Our result is plausible in that cooperators, no matter how evolved, inevitably divert a portion of their resources into

the secretion of public goods, resulting in an inherent growth disadvantage compared with non-producing strains.

An increase in the absolute fitness of *P. aeruginosa* during proteolytic growth was realized through a loss-of-function mutation in the transcriptional repressor PsdR, which in turn increases intracellular dipeptide transport and processing. This adaptation suggests that QS-dependent extracellular proteolysis is not growth-rate limiting during *in vitro* evolution, at least not exclusively. Presumably, proteolysis is only limiting during an initial lag-period at the beginning of each growth cycle in caseinate medium. Abundant protease secretion during this period may lead to an excess in proteolytic break-down products that await uptake and processing later in growth. Here, *psdR* mutants would benefit. This effective separation in cooperative and non-cooperative selective targets during QS-dependent *in vitro* evolution of *P. aeruginosa* is illustrated in Figure 3.7. The *psdR* mutation proportionally increased the growth rates of cooperators and cheaters in co-culture, because the *psdR lasR* mutant showed the same relative fitness in *psdR* mutant co-culture as did the *lasR* mutant in wild-type co-culture (Fig. 5b). This general impact on growth is nevertheless sufficient to explain its cooperation-stabilizing effect during *in vitro* evolution: Mixed cooperator/cheater populations deficient in PsdR reach saturation faster than those with functional PsdR and are consequently more robust to cycles of dilution and regrowth.

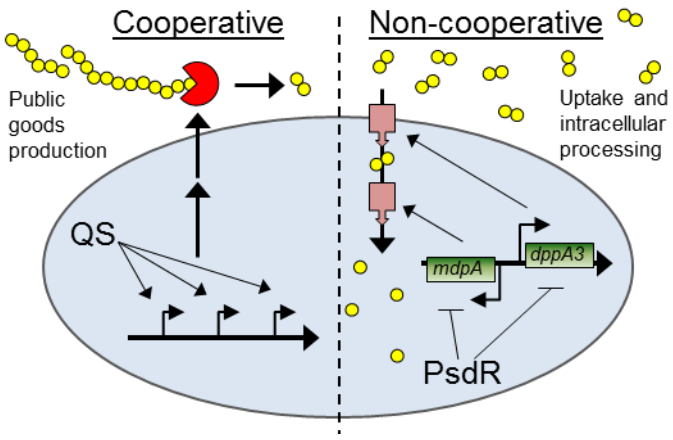

Figure 3.7 Targets of selection during *P. aeruginosa* QS-dependent *in vitro* evolution. Cooperative (left) and non-cooperative (right) targets of selection are

illustrated in this schematic model. QS-controlled public goods, specifically extracellular proteases (red) that degrade polypeptides outside the cell (chains of yellow circles, each of which represent individual amino acids), constitute the cooperative target. PsdR-mediated repression of genes (green) coding for proteins (pink) that facilitate the uptake (DppA3) and intracellular processing (MdpA) of dipeptides constitutes the non-cooperative target. Temporal separation of these selective targets likely accounts for the evolutionary dynamics observed in this study.

The high number of independent *psdR* mutations during *in vitro* evolution was a surprise, raising the possibility that this locus is a mutational hot spot. However, this notion is not supported by the analysis of published *P. aeruginosa* genomes. Out of 18 genomes in the NCBI database, 17 contain a *psdR* homologue with 99% or greater identity, nine of which show 100% identity (Altschul et al 1990) (http://blast.ncbi.nlm.nih.gov/). This ubiquity and sequence conservation implies that a functional PsdR is likely necessary for the evolutionary success of *P. aeruginosa* in its natural environment, although the situation may be different for other *Pseudomonas* species with *dpp* operons. *P. protegens* Pf-5 (formerly *P. fluorescens*) contains a truncated, presumably inactive *psdR* allele, while several other *Pseudomonas* spp. do not carry *psdR* at all (Kiely et al 2008). The maintenance of a functional PsdR in natural *P. aeruginosa* isolates suggests that proteolysis may limit growth more often than subsequent peptide processing, or that PsdR activity may be modulated through a natural ligand and derepression would sufficiently increase dipeptide metabolism. PsdR is a Mer-type regulator with a helix-turn-helix DNA-binding domain and a cupin sensor domain that has the potential to respond to a variety of environmental stimuli (Brown et al 2003, Kiely et al 2008).

A second, beneficial effect of the *psdR* mutation was that it was able to promote cooperative growth near wild-type levels when paired with a partial loss-of-function *lasR* allele, *lasR5*. This mutation in *lasR*, by itself, conferred intermediate levels of proteolysis and proteolytic growth in culture. Thus, the random emergence of certain *lasR* mutations, particularly in an adapted parent, is not detrimental to cooperative growth. Although *lasR5* affects LasR-dependent phenotypes other than caseinolysis (Sandoz et al 2007), the precise impact on the entire regulon is not clear. The *lasR5* mutation substitutes a valine for a nonconserved alanine at position 228 in

the DNA-binding domain of LasR, presumably weakening, but not completely eliminating, interaction with target promoters.

Given the properties of the *lasR5* mutation, one might expect a HC to exhibit lower relative fitness than a fully *lasR*-deficient cheater when paired with a cooperator. We observed this difference with strains harboring the *psdR1* mutation, but not with those harboring functional PsdR. A possible explanation for this discrepancy could be the large size of the *lasR*-controlled QS-regulon and the difference in the relative burdens it imposes on cooperators with and without the *psdR* mutation. Potential fitness differences between *lasR5* and Δ*lasR* alleles stemming from the variable costs of cooperative extracellular proteolysis (and other LasR-dependent behaviors) could be effectively masked by PsdR-mediated repression of dipeptide uptake and processing, and only manifest after this rate-limiting step has been removed through mutation of *psdR*. Thus, genetic context may be important when considering the relative fitness contributions from a cooperative allele. This idea is also supported when interpreting our results in the framework of kin selection theory.

Kin selection theory, encapsulated in Hamilton's rule, states that cooperation evolves if $rb - c > 0$, where b is the benefit of cooperation, c is the cost of cooperation, and r is the genetic relatedness between actors and recipients (Hamilton 1964a, Hamilton 1964b). It has been shown that the cost c of bacterial cooperation may decrease with increased resource supply (Brockhurst et al 2008). Analogously, *psdR* mutation appears to alleviate c by increased use of the products of protease digestion. This reduction in c does not require a direct mechanism, i.e. a direct effect of *psdR* on the cooperative trait itself, but merely reflects the context in which the behavior is performed. Given the non-linearity of fitness effects in our system (including a synergistic effect from QS induction and a saturating effect from protease secretion), further analysis of frequency-dependent relative fitness in the context of a generalized form of Hamilton's rule would be required to precisely quantify b and c (Smith et al 2010).

More broadly, cycles of non-social, genetic adaptation and cheating are unlikely to maintain cooperative behavior in the long-term as environmental adaptation is expected to eventually reach an optimum. Non-social adaptation through mutation likely works in concert with other mechanisms that stabilize cooperative behavior, and may be particularly beneficial early in the evolution of cooperative behavior. The generally high phenotypic plasticity of present-day microbes with unpredictable life histories would appear sufficient for coping with most changes in their natural environment. In microbes, other stabilizing mechanisms include positive assortment of cooperating individuals through, for example, colonial growth (Fletcher and Doebeli 2009), the linkage of cooperative behaviors with other essential traits through pleiotropy (Foster et al 2004), and metabolically prudent regulation of public goods such that their production is only initiated if the limiting nutrient is not also a building block of the good (Mellbye and Schuster 2014, Xavier et al 2011). It seems that pleiotropic control of extracellular proteolysis and subsequent intracellular metabolism via QS would be a reasonable strategy to curtail cheating, and a recent investigation using a similar *in vitro* evolution system has provided support for this notion (Dandekar et al 2012). QS cheaters that do not contribute to proteolysis would be punished with reduced nutrient uptake and processing. We found that *lasR* indeed controls *mdpA* transcription in rich medium (Schuster et al 2003), but not in the minimal medium used in this study. Of course, the relative fitness advantage of *lasR* mutant cheaters is consistent with the latter. Even if pleiotropic control played a role here, our results suggest that QS regulation of *mdpA* or related genes would be subject to strong counterselection whenever dipeptide uptake and processing was growth-rate limiting.

In summary, we have shown that non-social, genetic adaptation to a new growth environment that requires QS can help maintain cooperative behavior in *P. aeruginosa* populations. The adapted population is still vulnerable to invasion by cheaters that also carry the adaptation. However, a higher intrinsic growth rate affords higher tolerance to these cheaters, and some *lasR* mutations even contribute to cooperative growth.

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Conflicts of interest

The authors declare no existing conflicts of interest.

Author contributions

The author(s) have made the following declarations about their contributions. Conceived and designed the experiments: KLA, JW, MS. Performed the experiments: KLA, JW. Analyzed the data: KLA. Contributed reagents/materials/analysis tools: KLA, JW, KG, MS. Wrote the paper: KLA, MS.

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3.6 Supplementary Material

Table S3.4 Primers used in this study.

Primer Name	Sequence	Region amplified[a]	Source
Mutant Construction			
del_*lasR*_1	5'-N_6GAGCTCACAGACGTCTGCGCCTCGG-3'	-396 *(lasR)*	(Wilder et al 2011)
del_*lasR*_4	5'-N_6AAGCTTCGCCTCCAGCGTACAGTCG-3'	+1456 *(lasR)*	(Wilder et al 2011)
PA4499_KO_1	5'-N_6GAGCTCACGCTCGACGTGGRGGTGCTC-3'	-557 (*psdR)*	This study
PA4499_KO_2	5'-CAGGCGTTCGCCGATGCGGTCTAC-3'	-386 (*psdR)*	This study
PA4499_KO_3	5'-CGTAGACCGCATCGGCGAACGCCTGGCCGAGTTCTCCTACGTCCTGTCCGGG-3'	+6 (*psdR)*	This study
PA4499_KO_4	5'-N_6TCTAGATCTGGTAGCGGCTCAGGATGAAAGGC-3'	+1478 (*psdR)*	This study
Sequencing Primers			
PA1486_forward	5'-GGTGGTGATGGAGACCTT-3'	+371 - +528 (PA1486)	This study
PA1486_reverse	5'-CTTGAACTCGTGACAGATCAT-3'		
PA2875_forward	5'-CGGTATCCGTCGGTTCAGC-3'	+970 - +859 (PA2875)	This study
PA2875_reverse	5'-CGACCAGGCGGACCCCAC-3'		
PA2976_forward	5'-GCGAGGAACGCAGCGAACG-3'	+1552 - +1761 (PA2976)	This study
PA2976_reverse	5'-TCGTCCTGCTCGTCCTGCTC-3'		
PA2727_forward	5'-CAGCGACCCGTCCCAGGAG-3'	+2850 - +3034 (PA2727)	This study

Table S3.4 (continued)

PA2727_reverse	5’-GCTTGTGTACCACTTCCAGG-3’		
PA3317_forward	5’-GAGAGCCTGGTGATCGAGG-3’	+475 - +673 (PA3317)	This study
PA3317_reverse	5’-GAAATGCCTGCGGTCCGTC-3’		
PA3749_forward	5’-TACGACAGCATCGGCTACTGG-3’	+343 - +580 (PA3749)	This study
PA3749_reverse	5’-ACTCACGGAACTGCTCCTCG-3’		
PA4606_forward	5’-GCGGTCTGGGTGAGTTGCTC-3’	+1111 - +1289 (PA4606)	This study
PA4606_reverse	5’-ATGCTGATGGAGTCCTTCGTGG-3’		
PA5425_forward	5’-CGACGGCGACCACCTGAGC-3’	+823 - +949 (PA5425)	This study
PA5425_reverse	5’-AGCCAGTTCGAGAACCACTTGC-3’		
PA1765_forward	5’-CATGGGCAGGAGCTTCTACG-3’	+792 - +989 (PA1765)	This study
PA1765_reverse	5’-ATGAAAGCGTAGCGATACCAGG-3’		
PA2278_forward	5’-GACCTTCGTCGTCGGTGGC-3’	+666 - +869 (PA2278)	This study
PA2278_reverse	5’-TACATGCCCAGCGAGAAGACC-3’		
PA5024_forward	5’-TGCTGATGGGCCTGTACATCCTGA-3’	+302 - +739 (PA5024)	This study
PA5024_reverse	5’-TTGTGTTCGCCGCTTATGCCTGT-3’		
PA3760_forward	5’-TGCCGGTGGAAGAAAACCCAGCA-3’	+1865 - +2174 (PA3760)	This study
PA3760_reverse	5’-TCGTTGGTGCCGATGGAGAGGAA-3’		
PA5100_forward	5’-ATTCAGCAGGGCATTCAGCAGCG-3’	+1003 - +1434 (PA5100)	This study
PA5100_reverse	5’-GGGGTGGCCAATGCCTTCGATTT-3’		
PA4499_forward	5’-CGACCAAGACCCATTGCCTG-3’	+467 - +605 (psdR)	This study
PA4499_reverse	5’-ACGTTTGCCTGACAGGATGG-3’		

Table S3.4 (continued)

PA2408_forward	5’-GCCTGCCGCTCACCGTCG-3’	+281 - +399 (PA2408)	This study
PA2408_reverse	5’-CATGCCGACCCGTTCCAGG-3’		
PA1430_full_F	5’-GTGCCGGATATCGGGTGCCG-3’	-68 - +764 (lasR)	This study
PA1430_full_R	5’-AGGGCAAATTACCGATCGCCAGC-3’		
PA4499_full_F	5’-AACACCCACGGTCATTTGT-3’	-182 - +685 (psdR)	This study
PA4499_full_R	5’-GATTCGCTGATGCCGAAATTAAG-3’		
PA2408_full_F	5’-CCGGCAAGTACGAGGAAGAA-3’	-124 - +775 (PA2408)	This study
PA2408_full_R	5’-AGTTGTTCGTAGGCGTCGT-3’		
Intergenic1_F	5’-GCGAAGCGCTCCGTAAGGTTTCA-3’	1467321 - 1467775	This study
Intergenic1_R	5’-ATCCCGGCCGACTGGAAAGACAA-3’		

[a]Region amplified is given in relation to the gene’s start site. In the case of mutant construction primers, only the primer annealing position is given. The gene name or number is indicated in parentheses. In the case of intergenic regions, genome position is given.

Table S3.5 Targeted sequencing results from selected evolved *Pseudomonas aeruginosa* isolates.

		Gene (name)[c]					
		PA1430 (*lasR*)		PA4499 (*psdR*)		PA2408 (*abcB*)	
Isolate (Replicate)[a]	Cooperative phenotype[b]	Mutation	Change	Mutation	Change	Mutation	Change
5	HC	C683T	A228V	Δ514-531	Deletion	T337C	F113L
13 (1)	Cheater	Δ107-108	Deletion	T166C	S56P	None	None
14 (1)	Cheater	Δ107-108	Deletion	T166C	S56P	None	None
15 (1)	HC	A634G	M212V	Δ261-422	Deletion	None	None
16 (1)	HC	A634G	M212V	Δ261-end	Deletion	None	None
17 (2)	Cheater	Δ331-343	Deletion	Δ505-end	Deletion	None	None
18 (2)	Cheater	Δ331-343	Deletion	Δ505-end	Deletion	None	None
19 (2)	HC	A605T	N202I	Δ145-148	Deletion	None	None
20 (2)	HC	A605T	N202I	Δ145-148	Deletion	None	None

[a]The individual replicate of the *in vitro* evolution experiment is indicated in parentheses. Isolates 13-16 (replicate 1) and isolates 17-20 (replicate 2) are from experiments depicted in Fig. 2 panels b and c (Wilder et al 2011), respectively. Isolate 5, the evolved "hybrid cooperator" (PAO-HC) used for genome sequencing, was isolated from replicate 1 of the Sandoz et al (2007) experiments, Fig. 2 panel d.

[b]Phenotypes as originally determined using two qualitative screens for QS proficiency: 1) protease production on skim milk agar and 2) growth on minimal agar with adenosine as the sole carbon source. HC, hybrid cooperator.

[c]Gene names and functions as annotated in the *Pseudomonas* Genome Database. Numbers indicate nucleotide or amino acid position relative to the translational start site.

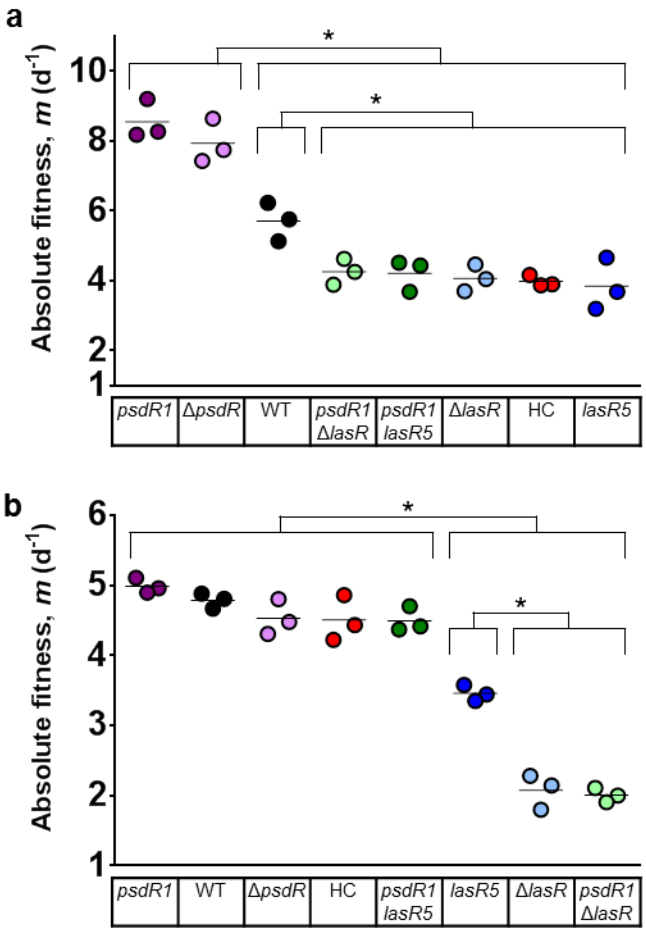

Figure S3.8 Absolute fitness in pure culture. Absolute fitness of individual strains after **(a)** 12 h and **(b)** 24 h of growth in caseinate medium. Bars represent means (n = 3). * Denotes significant differences (one-way ANOVA, Tukey's multiple comparisons test, $\alpha = 0.05$). Results of similar magnitude are grouped for clarity.

Chapter 4

ADDITIVE EFFECTS OF ANTI-ACTIVATOR PROTEINS IN CONTROL OF THE *Pseudomonas aeruginosa* QUORUM SENSING INDUCTION THRESHOLD

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Abstract

In the opportunistic pathogen *Pseudomonas aeruginosa,* quorum sensing (QS) via acyl-homoserine lactone (AHL) signals coordinates virulence gene expression. AHL signals must reach a critical threshold before enough is bound by cognate regulators LasR and RhlR to drive transcription of target genes. In addition, three anti-activator proteins, QteE, QscR, and QslA, sequester QS regulators to increase the threshold for induction and delay expression of QS target genes. It remains unclear how multiple anti-activators work together to achieve the quorum threshold. Here, we employed a combination of mutational, kinetic, phenotypic, and transcriptomic analysis to examine regulatory effects and interactions of the three distinct anti-activators. We observed additive, combinatorial effects on QS gene expression. As measured by reporter gene fusion, individual deletion of each anti-activator gene increased *lasB* expression and QS-controlled virulence factor production. Deletion of *qslA* in combination with the deletion of any other anti-activator gene resulted in the greatest increase and earliest activation of *lasB* gene expression. RNA-seq of the previously uncharacterized QslA and QteE regulons revealed overlapping, yet distinct groups of differentially expressed genes. Simultaneous inactivation of *qteE* and *qslA* had the largest effect on gene expression with 999 genes induced in the double mutant and 798 genes repressed in the double mutant vs. wild-type. We found that LasR and RhlR-activated QS genes form a subset of the genes induced in the *qteE*, *qslA*, and double mutant. The activation of almost all of these QS genes was advanced from stationary phase to logarithmic phase in the *qteE qslA* double mutant. Taken together, our results identify additive effects of anti-activation on QS gene expression, likely via LasR and RhlR, but also suggest QS-independent effects.

4.1 Introduction

Bacterial cell-cell signaling is a widespread mechanism of communication, allowing coordination of behavior among cells in a population (Asfahl and Schuster, 2017). This intercellular signaling is generally termed quorum sensing (QS), but the signaling mechanisms and behaviors regulated by QS in different bacteria are diverse (Schuster et al., 2013; Cook and Federle, 2014; Mashburn and Whiteley, 2005). The environmental bacterium and opportunistic human pathogen *Pseudomonas aeruginosa* has been established as a premier model system for studying QS regulation via diffusible acyl-homoserine lactone (AHL) signals. Hundreds of target genes, including many virulence genes, are controlled through a hierarchy of two complete, interconnected LuxI/R-type AHL circuits (Hentzer et al., 2003; Schuster et al., 2003; Wagner et al., 2003). The LasI synthase produces the *N*-3-oxo-dodecanoyl-homoserine lactone (3OC12-HSL) signal received by the LasR receptor-regulator (an 'R-protein'), and the RhlI synthase produces the *N*-butanoyl-homoserine lactone (C4-HSL) signal received by the RhlR receptor-regulator (Schuster and Greenberg, 2006). *P. aeruginosa* also harbors an orphan LuxR-type regulator QscR that binds and responds to 3OC12-HSL, as well as the PQS signaling system, both of which contribute to virulence gene regulation (Chugani et al., 2001; Diggle et al., 2006; Lee et al., 2006b; Lequette et al., 2006; Lintz et al., 2011; Chugani and Greenberg, 2014).

LasR and RhlR function as transcriptional activators by binding to conserved sequence elements upstream of target promoters. Most QS target genes are activated at the beginning of stationary phase in batch culture (Schuster et al., 2003). QS signal accumulation is necessary but not sufficient for QS gene induction. Additional regulatory inputs, such as the general stress response, are required for expression (Schuster et al., 2004). Serving as an opposing force to QS activation in the presence of signal, anti-activation of QS components can effectively delay the QS response (Hense and Schuster, 2015). Anti-activation was originally discovered in *Agrobacterium tumefaciens*, where a TraM anti-activator protein binds the LuxR-type receptor TraR, suppressing AHL-QS activation and transcription of TraR target genes (Fuqua et al., 1995; Hwang et al., 1995). Anti-activator proteins bind and destabilize LuxR-type regulators (Piper and Farrand, 2000; Siehnel et al., 2010; Fan et al., 2013),

imposing constraints on the activation threshold and allowing the timing and magnitude of QS response to be tuned. Deletion of *A. tumefaciens* TraM activates QS at a much lower cell density (Hwang et al., 1995), possibly representing constitutive activation. It has therefore been proposed that anti-activation could prevent intracellular self-activation of receptors, also termed "short-circuiting" (Goryachev et al., 2005). In this model, the stoichiometry of LuxR-type receptors with anti-activators determines the induction threshold. More generally, anti-activation tunes the induction threshold to optimize the benefits attained from costly secretions (Pai et al., 2012; Gupta and Schuster, 2013).

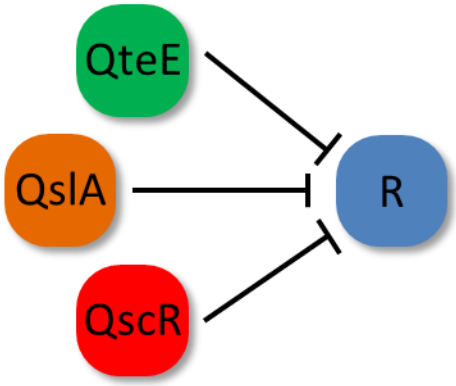


Figure 4.1 Anti-activators responsible for suppressing R-protein (R) activation in *P. aeruginosa*.

Three anti-activator proteins that work to suppress QS-activation have been identified in *P. aeruginosa* thus far: QteE, QscR, and QslA (Figure 4.1). The orphan LuxR homolog QscR (PA1898, *qscR*) has been observed in the formation of heteromultimeric complexes with both LasR and RhlR (Ledgham et al., 2003; Lintz et al., 2011). QscR suppresses key gene clusters of the quorum regulon such as *hcn* (hydrogen cyanide biosynthesis) and *phz* (phenazine biosynthesis)(Chugani et al., 2001). Microarray analysis shows that *qscR* represses 329 genes, although it also activates a small, separate set of target genes (Lequette et al., 2006). The structurally unrelated anti-activator protein QteE (PA2593, *qteE*) may also form a heterodimer with LasR that prevents signal binding and destabilizes LasR (Siehnel et al., 2010). The authors of that study also found that in addition to LasR, QteE can reduce RhlR

QS-transcriptional activity independently, as well as destabilize the RhlR protein (Siehnel et al., 2010). A third protein QslA (PA1244, *qslA*) acts as a potent anti-activator of LasR through heterotrimer formation that can dissociate previously formed LasR-DNA complexes (Seet and Zhang, 2011). This effect is achieved through direct binding of QslA to the ligand-binding-domain (LBD) of LasR in a 2:1 ratio, obscuring the dimerization interface and thereby preventing activation (Fan et al., 2013). Despite the contributions of these studies, the roles of individual anti-activators and the scope of their collective influence on the QS induction threshold are not fully understood. It is unclear in an evolutionary context why *P. aeruginosa* would maintain multiple similarly functioning anti-activator proteins. Gupta and Schuster found that mutations in either *qteE* or *qscR* can produce virtually identical phenotypes under certain conditions (Gupta and Schuster, 2013). However, considering the inherent metabolic constraints on superfluous protein production in bacteria, individual anti-activators are unlikely to have equivalent effects on the QS regulon. At 191, 238, and 114 amino acids long, respectively, QteE, QscR, and QslA are not structurally related and may bind R-proteins differently (Siehnel et al., 2010; Lintz et al., 2011; Fan et al., 2013). In consideration of this evidence together, several important open questions remain. Why does *P. aeruginosa* maintain multiple anti-activators? Does deletion of multiple anti-activators produce a stronger affect than loss of a single gene, and how do different anti-activators affect the QS regulon? Deletion of any single anti-activator produces a general increase in QS activation (Chugani et al., 2001; Siehnel et al., 2010; Seet and Zhang, 2011; Gupta and Schuster, 2013), indicating their functions are not completely redundant. Interactions between anti-activators are possible, a scenario that could produce additive or synergistic effects. Given current mechanistic information, it is plausible that most, if not all of the genes affected by anti-activator deletions are those activated by LasR and RhlR. Here we use mutational analysis of anti-activator genes in combination with phenotypic measurements, gene induction kinetics, and transcriptome profiling to address these questions.

4.2 Materials and methods

4.2.1 Strains and culture conditions

Pseudomonas aeruginosa PAO1 was used as the wild-type isogenic parent in mutant construction and as the control strain in all experiments. PAO1 and the isogenic, markerless Δ*qteE* knockout were obtained from R. Siehnel (Univ. Washington, USA) (Siehnel et al., 2010). See Table 4.1 for a comprehensive list of strains and plasmids. The Δ*qslA* and Δ*qslA* Δ*qteE* mutants were created using a pEX18-based suicide vector (Hoang et al., 1998). We subcloned an in-frame deletion constructed by splicing-overlap-extension PCR (SOE-PCR) into pEX18Gm for use in allelic exchange (Horton, 1995; Hoang et al., 1998). The PAO-R3 (*qscR*-Gm^R) strain was obtained from S. Chugani (Univ. Washington, USA)(Chugani et al., 2001). PAO-R3 genomic DNA was used to introduce the *qscR*-Gm^R allele into PAO Δ*qteE qscR*-Gm^R and PAO Δ*qteE* Δ*qslA qscR*-Gm^R strains via whole-genome transformation and subsequent homologous recombination of the *qscR*-Gm^R fragment (Choi et al., 2006). All routine and experimental cultures were carried out at 37°C. We grew strains on Lennox LB agar solid media or with shaking at 250 rpm in Lennox LB broth buffered with 50 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS), pH 7.0, for routine propagation. When necessary, plates were supplemented with 100 µg/ml tetracycline or 100 µg/ml gentamicin for the selection of marked strains. Reporter plasmids were maintained using 200 µg/ml carbenicillin in routine cultures, but not in experimental cultures. When necessary, cells were washed, resuspended, and diluted in M9 minimal medium with no carbon added (M9-salts)(Gupta and Schuster, 2013). For inoculation of all experimental cultures, we modified a previously described (Siehnel et al., 2010; Gupta and Schuster, 2013) recursive growth-dilution preculture scheme to effectively dilute carryover GFP-fluorescence from previously activated LasR and its associated P_{lasB}-*gfp* reporter activity. First, fresh colonies from plates were suspended in M9-salts, optical density was measured at 600 nm (OD_{600}) and then diluted to allow initial inoculation of 4 ml LB-MOPS at OD_{600}=0.0001 in glass culture tubes. After incubation at 37°C with shaking, cells were harvested in log phase (OD_{600} < 0.2), washed in M9-salts, and re-diluted into 4 ml fresh LB-MOPS at OD_{600}=0.0000001. After another incubation at 37°C with shaking, cells were again

harvested in log phase (OD_{600} < 0.2), washed in M9-salts, and then diluted to compose experimental inocula. For transcriptional reporter assays, endpoint phenotypic assays, and transcriptomic analysis, M9 minimal medium was supplemented with 0.5% (w/v) casamino acids (CAA) as the sole carbon source (Gupta and Schuster, 2013). CAA medium serves as a semi-defined rich medium in which all required amino acids are present. Growth experiments conducted in the plate reader were terminated at 800 min due to evaporation in this configuration and corresponding increased variability beyond this time point. All experiments were performed using a minimum of three biological replicates with independently prepared inocula.

Table 4.1 Bacterial strains and plasmids.

Strain or plasmid	Relevant properties	Reference or origin
Pseudomonas aeruginosa		
PAO1	Wild-type, PAO1 UW library strain	(Jacobs *et al.*, 2003)
PAO Δ*qteE*	Markerless *qteE* deletion mutant, in PAO1 UW background; '*qteE*'	(Siehnel *et al.*, 2010)
PAOR3	PAO1 derivative; *qscR*-Gm^R, null mutant marked with Gm cassette inactivating *qscR*; '*qscR*'	(Chugani *et al.*, 2001)
PAO Δ*qslA*	PAO1 derivative; Δ*qslA*, unmarked in-frame deletion from amino acid 6 to 111 ; '*qslA*'	This study
PAO Δ*qteE* Δ*qslA*	PAO1 Δ*qteE* derivative; unmarked double-null deletion mutant in which both *qteE* and *qslA* harbor in-frame deletions; '*qteE qslA*'	This study
PAO *qscR*-Gm^R Δ*qslA*	PAOR3 derivative; marked double-null mutant which harbors both Δ*qslA* and *qscR*-Gm^R alleles; '*qscR qslA*'	This study
PAO Δ*qteE qscR*-Gm^R	PAO1 Δ*qteE* derivative; marked double-null mutant harbors both Δ*qteE* and *qscR*-Gm^R alleles; '*qteE qscR*'	This study
PAO Δ*qteE* Δ*qslA qscR*-Gm^R	PAO1 Δ*qteE* Δ*qslA* derivative; marked triple-null mutant harbors Δ*qteE*, Δ*qslA*, and *qscR*-Gm^R alleles; '*qteE qslA qscR*'	This study
DA6	PAO1 derivative; Δ*lasR* Δ*rhlR*, unmarked double-null deletion mutant in which both *lasR* and *rhlR* harbor in-frame deletions; '*lasR rhlR*'	(Siehnel *et al.*, 2010)
Escherichia coli		

Table 4.1 (continued)

DH5α	F$^-$ Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *deoR recA1 endA1 hsdR17*(r_K^-, m_K^+) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Invitrogen
SM10	*thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^R λpir	(Simon et al 1983)
Plasmids		
pEX18Gm	Conjugative suicide plasmid; Gm^R	(Hoang et al 1998)
pEX18Gm.Δ*qslA*	pEX18Gm with Δ*qslA* containing an in-frame deletion from amino acid	This study
pProbeAT	Broad-host-range vector with a promoterless *gfp*, Cb^R	(Miller *et al.*, 2000)
pRG13	240bp *lasB* promoter cloned into pProbeAT	(Gupta *et al.*, 2013)

4.2.2 GFP-transcriptional reporter assays

A plasmid-borne fusion of the QS-controlled *lasB* (PA3724) promoter sequence (240 bp) and GFP was used to assess promoter activity in our collection of mutants. We used fluorescence spectroscopy for detection as previously described (Gupta and Schuster, 2013). Briefly, pRG13 (P_{lasB}-*gfp*) and pProbeAT (promoterless *gfp* negative control) were individually introduced into each strain background. Following our recursive growth-dilution scheme, precultured cells were inoculated at a starting OD_{600}=0.01 in 200 µL of CAA medium in black-walled (fluorescence) 96-well plates (Greiner bio-one, Cat. No. 655090). Cell density (absorbance at 600 nm) and fluorescence (GFP, $\lambda_{excitation}$=480 nm, $\lambda_{emission}$=535 nm, gain setting=60) were measured in 15 min intervals as cultures were incubated with shaking at 37°C in a Tecan Infinite (M200) multifunction plate reader. P_{lasB}-*gfp* promoter activity (reported as *lasB* expression) for individual strains was corrected for background fluorescence by subtracting the OD-normalized fluorescence of a strain harboring pProbeAT from the OD-normalized fluorescence of the corresponding strains with the active reporter for each time point. P_{lasB}-*gfp* expression rates were calculated as the time derivative of GFP fluorescence over OD_{600} ($dGFP/dt/OD_{600}$) over a 30 minute period as described previously (Zaslaver et al., 2006; Gupta and Schuster, 2013). Data were smoothed by reporting the mean of 3 consecutive measurements.

4.2.3 Pyocyanin production assay

Pyocyanin production of individual strains was assessed essentially as described previously (Essar et al., 1990; Mellbye and Schuster, 2014). Starting with our recursive dilution scheme, we inoculated each precultured strain into 5 ml CAA medium at a starting OD_{600}=0.01 and allowed cultures to grow with shaking at 37°C for 18 h (stationary phase). Pyocyanin was extracted from 5 ml supernatant using 3 ml chloroform, followed by extraction of pyocyanin from chloroform using 1 ml 0.2 M HCl. After separation of the acidified pyocyanin from the top of the mixture, ABS_{520} of 200 µl aliquots was measured in a Tecan plate reader and reported as fold-change vs. wild-type production.

4.2.4 Elastase activity assay

Elastolytic activity of stationary phase supernatants was determined using the elastin congo red (ECR) assay as previously described (Diggle et al., 2002), but modified to allow high throughput. Starting with our preculture scheme, we inoculated each strain into 800 µl CAA medium at a starting OD_{600}=0.01 and allowed cultures to grow at 37°C with shaking in 96-well deep-well blocks (VWR North America, Cat. No. 82006-448) covered with Breath Easy® sealing membranes (Diversified Biotech, Cat. No. BEM-1). After 18 h, OD_{600} was measured in a Tecan plate reader, and separately cells were pelleted at 4000 rpm for 10 min, followed by sterile filtration of 250 µl supernatant in AcroPrep™ 96-well filter plates (Pall Life Sciences, Cat. No. 5045). Forty µl cell-free supernatant was combined with 360 µl ECR buffer (100 mM Tris, 1 mM $CaCl_2$, pH 7.5) containing 20 mg/ml ECR (Sigma-Aldrich Co., Cat. No. E0502) in sealed 96-well deep-well blocks and incubated at 37°C with shaking for 3 h. After pelleting insoluble ECR at 4000 rpm for 10 min, 200 µl supernatant was transferred to a 96-well plate for measurement of absorbance at 495 nm in a Tecan plate reader. Elastolytic activity of supernatants is reported as fold-change vs. wild-type activity.

4.2.5 RNA sequencing transcriptome generation

RNA sequencing (RNA-seq) was carried out on a subset of 5 of our strains; WT, *lasR rhlR*, *qteE*, *qslA*, and *qteE qslA* were each examined at 2 time-points with 3 biological replicates made from separate preparations on separate days, producing a total of 30 samples. Starting with our recursive dilution scheme, we inoculated each strain into 4 ml CAA medium at a starting OD_{600}=0.01 and incubated cultures with shaking at 37°C, periodically measuring OD_{600} to monitor growth. Approximately 2×10^9 cells were harvested at OD_{600} values of 0.2 (log phase) and 1.6 (early stationary phase), immediately preserved using RNAprotect Bacteria Reagent (Qiagen, Cat. No. 76506), pelleted by centrifugation, and frozen at -80°C until RNA extraction. Total RNA was isolated as previously described (Schuster et al., 2003) using sonication and column-based purification (RNeasy Mini Kit, Qiagen, Cat. No. 74106), followed by treatment with DNase I (RNAse-free, New England Biolabs, Cat. No. M0303S), and RNeasy-based purification. Total RNA was subjected to rRNA-depletion using the Ribo-Zero™ protocol (Illumina Inc.), followed by cDNA synthesis and indexed, stranded library preparation using the WaferGen protocol on the robotic Apollo instrument (WaferGen Bio-systems Inc.). All 30 sample libraries were then pooled and evenly multiplexed into a single lane of paired-end 2×100 bp sequencing on the HiSeq3000 instrument (Illumina Inc.). cDNA libraries were prepared and sequenced at the Center for Genome Research and Biocomputing at Oregon State University (Corvallis, Oregon, USA). Sequences were separated according to index and filtered of contaminating adapter content bioinformatically. Raw .FASTQ files (containing sequence "reads") were inspected for general quality (per base sequence quality > Q28) and sequence contamination using FastQC (http://www.bioinformatics.babraham.ac.uk), confirming no further pre-processing was necessary.

4.2.6 Transcriptome data analysis

We used the Burrows-Wheeler aligner (BWA-MEM; see ref. (Li and Durbin, 2010)) to map processed reads to the *P. aeruginosa* PAO1 reference genome ORFs

(PAO1_107; available at http://www.pseudomonas.com), followed by optical-duplicate removal and count matrix generation in samtools with default parameters (Li et al., 2009). rRNA, tRNA, and tmRNA ORFs (http://www.pseudomonas.com) were manually removed yielding a count matrix of 5622 genes × 30 samples that was then loaded into the RStudio statistical environment (https://www.rstudio.com). Differential expression analysis was carried out using the DESeq2 package under standard settings using each strain-growth phase combination as a factor level (Love et al., 2014). Hypothesis testing was carried out in DESeq2 using the Benjamini Hochberg adjustment for multiple comparisons and a false-discovery rate (FDR) α=0.05 with no high or low $\log_2$fold-change limits. Functional annotations were assigned using the most recent list of 22 predicted classes produced using publicly available PAO1 COG mappings (http://www.pseudomonas.com). Absolute expression comparisons were made using the regularized log transformation (rlog) in DESeq2 (Love et al., 2014). Data were visualized using the Heatmapper webtool (Babicki et al., 2016), ClustVis webtool (Metsalu and Vilo, 2015) and ggplot package in RStudio (Wickham, 2009).

4.3 Results

4.3.1 *lasB* promoter activity among anti-activator mutants

The effects of individual *qteE*, *qscR*, or *qslA* gene deletions on the induction of QS target genes have been examined by different research groups (Chugani et al., 2001; Siehnel et al., 2010; Fan et al., 2013; Gupta and Schuster, 2013), but a direct comparison of their individual effects and the effects of multiple deletions on timing and magnitude of QS expression has not been made. We assembled a set of anti-activator-null strains of PAO1 representing each possible combination of anti-activator-null alleles (7 mutants total; see Table 4.1 for a comprehensive list of strains and plasmids used in this study) to allow comparisons of anti-activator effects. *P. aeruginosa* LasB-elastase is a well-described Las- and Rhl-responsive proteolytic virulence factor, making *lasB* promoter activity an appropriate proxy for QS gene induction in this context (Pearson et al., 1997). We recorded *lasB* promoter activity

through utilization of an established plasmid-borne P_{lasB}-*gfp* (*lasB*) transcriptional reporter (Gupta and Schuster, 2013). We evaluated accumulation of P_{lasB}-*gfp*-derived fluorescence during growth (Figure 4.2). All strains showed similar growth in CAA medium (Figure 4.2a). From these data, we also calculated specific expression rates (Figure 4.2c). The *qteE* and *qscR* single mutants showed 30- and 15-fold increases in maximum expression levels and rates (Figure 4.2a and c) compared to the wild-type. The *qslA* mutant only showed increases of roughly 7-fold in expression levels and rates. The *qteE qscR* double mutant registered values nearly identical to mutants harboring just a single one of these mutations, indicating a lack of additivity with these two anti-activators. However, with any other combination of deleted anti-activator alleles *(qteE qslA* or *qteE qscR),* P_{lasB}-*gfp* induction is increased further in both total expression levels and rates, with the triple anti-activator mutant showing a slightly lower increase (Figure 4.2b and c). The timing of induction only changed for our three strains showing the highest expression levels. *qscR qslA, qteE qslA,* and *qteE qslA qscR* mutants all showed P_{lasB}-*gfp*-activation and rapid increases in expression rates starting at approximately 200 min, with all other mutants and the wild type showing activation occurring roughly 60-120 min later (Figure 4.2b, inset). In summary, all measurements of overall mutant P_{lasB}-*gfp* expression levels and rates were higher in the mutants than the wild-type, with three groups emerging with similar profiles: the *qslA* mutant with the smallest increase in expression, the *qteE, qscR*, and *qteE qscR* mutants with moderate increases, and the *qscR qslA*, *qteE qslA*, and *qteE qslA qscR* mutants showing the highest expression.

A closer look at the expression rates of the wild-type reveals a biphasic pattern with two distinct peaks (Figure 4.2c, inset) at approximately 310 and 500 min, likely corresponding to the sequential induction of the Las and Rhl QS systems, respectively (Gupta and Schuster, 2013). In all mutants except *qslA*, we observed a general shift in the relative expression rates to favor much higher expression rates during the initial, presumably Las-dependent, rate peak. This induction pattern suggests that anti-activator proteins primarily target LasR rather than RhlR.

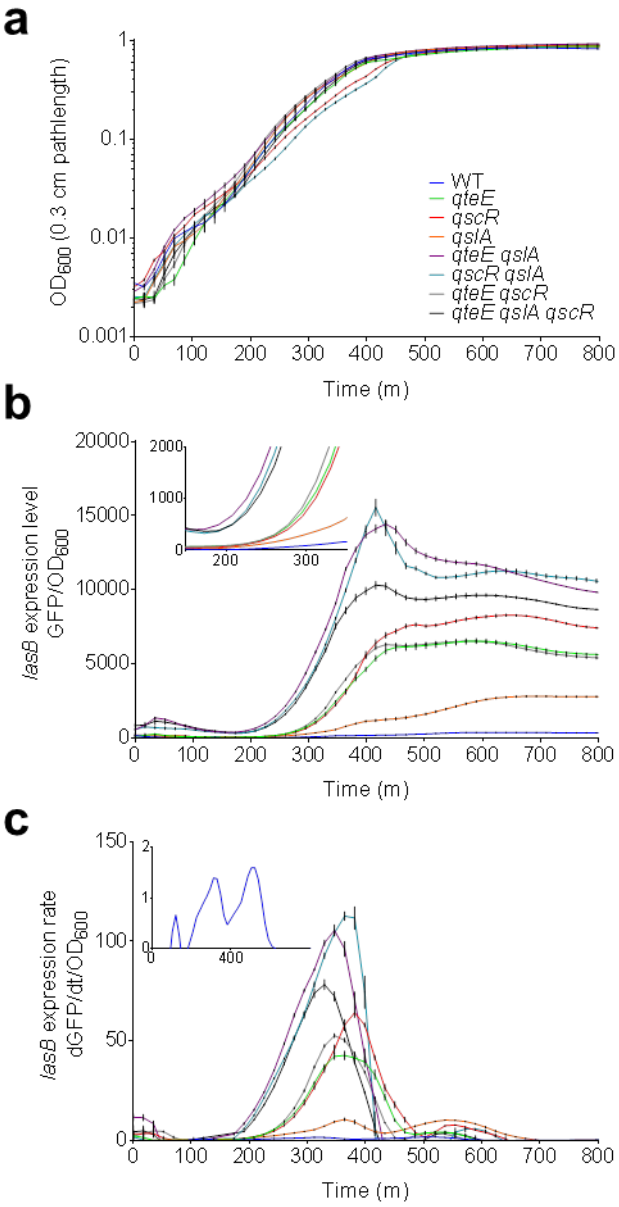


Figure 4.2 Effects of anti-activator gene deletion on P_{lasB}-*gfp* expression kinetics. **(a)** Growth of strains in CAA medium. **(b)** P_{lasB}-*gfp* expression levels. Expression levels are normalized to OD_{600}. Inset has reduced *y*- and *x*-axes to emphasize expression timing. **(c)** P_{lasB}-*gfp* expression rates. Time derivatives of expression levels are normalized to OD_{600}. Inset has a reduced *y*-axis to emphasize expression rate peaks in the wild-type. In all panels, values represent means of three biological replicates. Error bars indicate s.e.m. (*n*=3).

4.3.2 Elastase and pyocyanin production in anti-activator mutants

To support our observations of differing effects of some anti-activator combinations on P_{lasB}-*gfp* expression, we examined two characteristic QS phenotypes in *P. aeruginosa*, pyocyanin production and elastase activity in CAA stationary-phase cultures. Levels of pyocyanin produced by the wild-type rose only 2-fold over the *lasR rhlR* QS mutant and elastase activity was roughly equivalent between the two strains (Figure 4.3a and b; not significantly different, α=0.05). Elastase and pyocyanin production levels in the different anti-activator mutant combinations generally mirrored that observed with P_{lasB}-*gfp* fusions. Single mutants produced intermediate levels, and the double mutants harboring a *qslA* deletion as well as the triple mutant produced the most. The *qteE, qscR* double mutant grouped together with most single mutants. Notably, the *qslA* mutant produced as much elastase as the other single mutants, but significantly less pyocyanin.

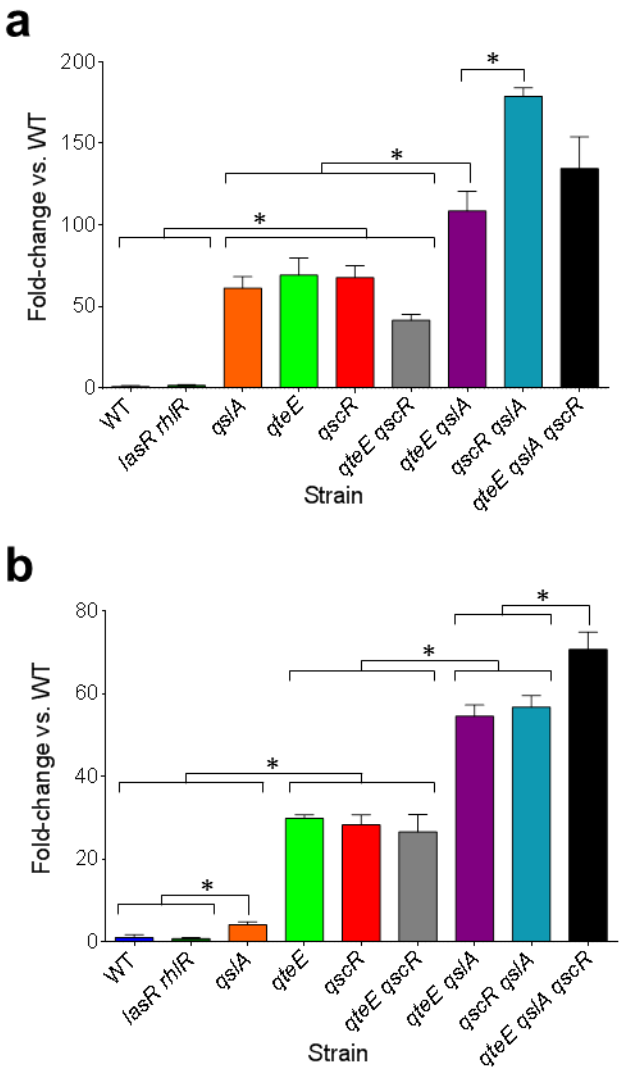


Figure 4.3 Effects of anti-activator gene deletion on QS phenotypes. (a) Elastase activity. **(b)** Pyocyanin production. Each assay was performed separately after 18 h growth in CAA medium. In both panels, values represent means of three biological replicates. Error bars indicate s.e.m. (*n*=3). Bars are grouped for clarity. Significant differences (*) in selected individual pairwise comparisons were determined using a two-tailed T-test (α=0.05).

4.3.3 Identification of QteE and QslA regulons

Having demonstrated anti-activator effects on QS phenotypes in addition to promoter activity dynamics of a QS gene, we sought to uncover the global scope of QS anti-activators with transcriptome profiling. We focused on *qteE* and *qslA* mutants, alone and in combination, which produced the most consistent additive

effects on gene expression based on our analysis above, and which had not been previously profiled. Using an RNA-seq-based transcriptomics approach, we identified all genes that were differentially expressed (DE, α=0.05) when mutants (*qteE*, *qslA*, *qteE qslA*) were compared to wild-type in both logarithmic and early stationary phase. Both single anti-activator mutants showed differential expression of hundreds of genes, with the *qteE* mutant showing 415 differentially expressed genes, and the *qslA* mutant showing roughly double that quantity at 770 genes (Table 4.2). We observed a synergistic effect of deletion of both anti-activators with the *qteE qslA* mutant differentially expressing a total of 1797 genes, corresponding to roughly 31 percent of all *P. aeruginosa* genes. Consistent with a common functional role, the three different gene sets showed substantial overlap in both log and early stationary phase (Figure 4.4), and most genes affected by anti-activator gene deletion showed activation (Table 4.2). Sets of anti-activator-affected genes were effectively nested; regardless of growth phase, more than 75 percent of *qteE*-affected genes were also *qslA*-affected, and more than 85 percent of *qslA*-affected genes were also affected in the double mutant (Figure 4.4). This finding is consistent with anti-activators strictly functioning by sequestering QS activators to different degrees, with the *qteE qslA*-affected gene set encompassing both single-mutant gene sets. In addition, numerous genes were repressed by anti-activator gene deletion. These genes are either indirectly regulated by QS, through LasR or RhlR-dependent activation of a transcriptional repressor, or they are regulated completely independently of the presumed R-protein sequestration mechanism. (Figure 4.4).

Table 4.2 Differentially expressed genes

	Log			Early stationary			All unique		
Strain	**Induced**	**Repressed**	**Total**	**Induced**	**Repressed**	**Total**	**Induced**	**Repressed**	**Total**
wild-type	1	-	1	79	59	138	79	59	138
qteE	52	2	54	298	101	399	312	103	415
qslA	82	8	90	477	265	742	500	270	770
qteE qslA	214	52	266	934	757	1691	999	798	1797

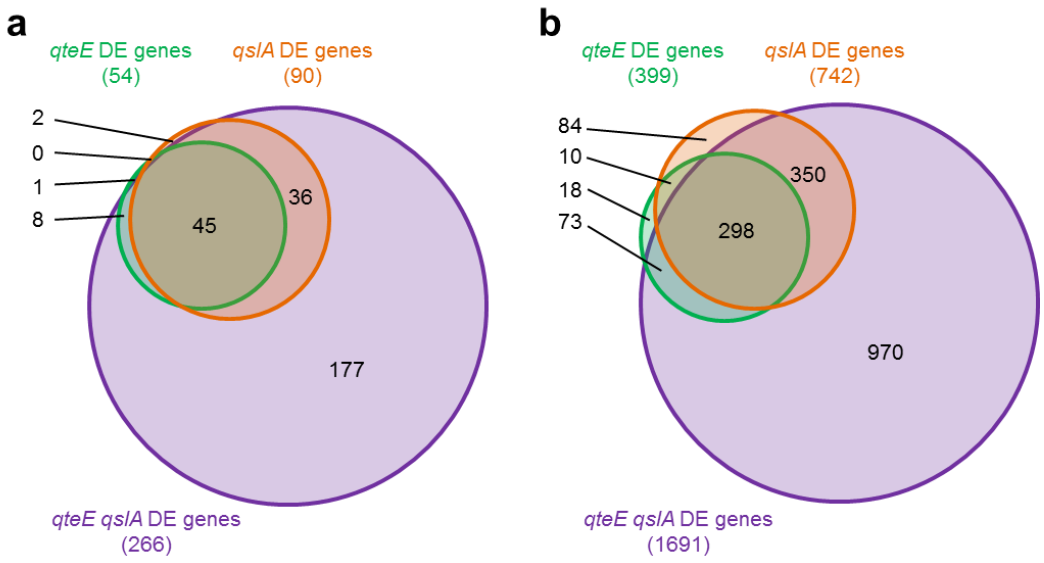
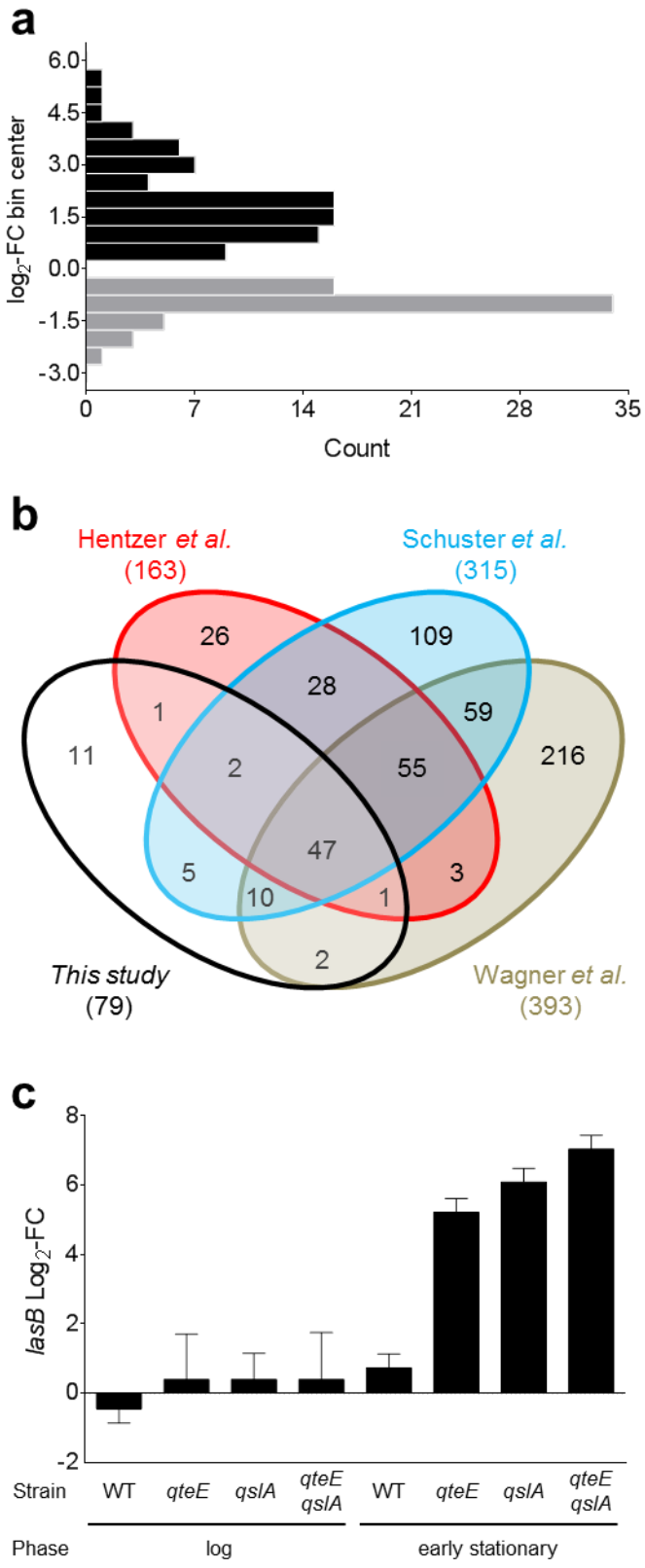


Figure 4.4 Comparison of differentially expressed genes among anti-activators. **(a)** Log phase. **(b)** Early stationary phase. Differentially expressed (DE) genes were determined in DESeq2 using three biological replicates (false discovery rate α=0.05, *n*=3). Venn diagram variables are roughly scaled to reflect quantities to visualize nesting.

4.3.4 Identification of a QS regulon

Next, to evaluate the relationship between our anti-activator-affected genes and QS, we determined a QS regulon for the wild-type strain under our culture conditions. We identified all differentially expressed (DE, α=0.05) genes between our wild-type strain and an isogenic *lasR rhlR* mutant in both log and early stationary phase. Based on previous studies, we expected few DE genes in the log phase comparison as this represents a quorum "OFF" state, while induction of QS in early stationary phase represents a QS "ON" state and should produce differential expression in many genes (Schuster et al., 2003; Wagner et al., 2003). We found 138 differentially expressed genes in early stationary phase between the wildtype and *lasR rhlR* mutant, including 79 quorum-activated and 59 quorum-repressed genes (Figure 4.5a, Tables 4.3 & 4.4). The only DE gene detected in our log phase comparison was *lasR* itself, supporting the design of our log phase (QS "OFF") vs early stationary phase (QS "ON") comparison. As genes activated in the quorum regulon are consistent with the established function of LasR and RhlR as transcriptional

activators (Whiteley et al., 1999; Kiratisin et al., 2002; Hentzer et al., 2003; Schuster et al., 2003; Schuster and Greenberg, 2007) and the established function of anti-activators as factors for R-protein destabilization and degradation (Piper and Farrand, 2000; Siehnel et al., 2010; Fan et al., 2013), we focused our subsequent analysis on only activated genes.


a
6.0
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3.0
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log2-FC bin center
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7
14
21
28
35
Count
b
Hentzer et al.
(163)
Schuster et al.
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26
109
28
1
59
11
2
55
216
47
5
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This study
(79)
Wagner et al.
(393)
c
8
6
4
2
0
-2
lasB Log2-FC
Strain
WT
qteE
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qteE qslA
WT
qteE
qslA
qteE qslA
Phase
log
early stationary

Figure 4.5 A QS-controlled regulon. (a) Histogram of genes differentially expressed in a wild-type vs *lasR rhlR* mutant comparison from early stationary phase cultures grown in CAA medium. Differentially expressed (DE) genes activated (black bars) and repressed (gray bars) in the comparison were determined in DESeq2 using three biological replicates (false discovery rate α=0.05, n=3). **(b)** Comparison of QS regulons among previous microarray results and the present study. Venn diagram variables are not quantitatively scaled. **(c)** Comparison of *lasB* (PA3724) fold-change in the RNA-seq experiment. Wild-type (WT) fold-change represents results from the wild-type vs. *lasR rhlR* comparison; all others represent results of individual comparisons with each strain vs. the wild-type.

Table 4.3 Quorum-activated genes

			Early stationary phase fold change[2]			
Locus tag[1]	**Name[1]**	**Annotation[1]**	**WT**	***qteE***	***qslA***	***qteE qslA***
PA0026	*plcB*	phospholipase C, PlcB	2.5	NC	2.3	2.1
PA0027		hypothetical protein	2.2	1.8	**3.1**	**2.4**
PA0052		hypothetical protein	2.3	4.6	9.6	14.9
PA0143	*nuh*	purine nucleosidase Nuh	1.9	**1.9**	**3.8**	**3.8**
PA0178		probable two-component sensor	2.4	NC	NC	1.7
PA0524	*norB*	nitric-oxide reductase subunit B	7.8	NC	NC	-10.7
PA0572		hypothetical protein	3.6	2.9	**4.5**	**2.8**
PA1130	*rhlC*	rhamnosyltransferase 2	4.3	5.4	5.6	**6.8**
PA1131		probable major facilitator superfamily (MFS) transporter	11.0	6.1	5.3	**7.0**
PA1246	*aprD*	alkaline protease secretion protein AprD	3.3	NC	NC	**2.5**
PA1248	*aprF*	Alkaline protease secretion outer membrane protein AprF precursor	2.6	NC	NC	**3.0**
PA1249	*aprA*	alkaline metalloproteinase precursor	3.6	3.6	4.5	7.0
PA1250	*aprI*	alkaline proteinase inhibitor AprI	3.7	**3.1**	**3.6**	**3.5**
PA1251		probable chemotaxis transducer	3.3	**2.5**	**3.3**	**3.7**
PA1430	*lasR*	transcriptional regulator LasR	**40.7***	1.7	**2.5**	**3.6**
PA1431	*rsaL*	regulatory protein RsaL	6.1	**1.9**	**3.1**	4.1
PA1432	*lasI*	autoinducer synthesis protein LasI	28.0	**NC**	**-2.5**	-1.6
PA1433		conserved hypothetical protein	1.6	**NC**	**NC**	NC
PA1656	*hsiA2*	HsiA2	3.5	**12.0**	**8.0**	**5.8**
PA1663	*sfa2*	Sfa2	2.3	9.2	4.5	**2.8**
PA1668	*dotU2*	DotU2	1.8	8.6	**3.6**	**2.4**
PA1784		hypothetical protein	2.3	3.5	7.2	13.4
PA1869		probable acyl carrier protein	3.5	**25.1**	**20.5**	**23.6**
PA1871	*lasA*	LasA protease precursor	3.4	22.9	22.9	36.6
PA1893		hypothetical protein	2.5	**1.6**	NC	**2.3**
PA1894		hypothetical protein	3.9	**2.4**	**NC**	**3.2**
PA1895		hypothetical protein	2.3	**2.1**	NC	**2.9**
PA1896		hypothetical protein	2.4	**1.7**	NC	**2.9**
PA1897		hypothetical protein	2.5	**2.3**	**NC**	**3.2**

Table 4.3 (continued)

PA2076		probable transcriptional regulator	1.8	**1.6**	**2.4**	**2.2**
PA2080	*kynU*	kynureninase KynU	1.7	**1.7**	**2.8**	**2.1**
PA2081	*kynB*	kynurenine formamidase, KynB	2.5	**1.9**	**2.7**	**2.5**
PA2193	*hcnA*	hydrogen cyanide synthase HcnA	4.9	12.1	19.3	**7.4**
PA2194	*hcnB*	hydrogen cyanide synthase HcnB	5.4	12.7	**19.0**	**7.3**
PA2195	*hcnC*	hydrogen cyanide synthase HcnC	3.8	13.8	20.8	**8.9**
PA2301		hypothetical protein	4.0	2.7	3.0	3.5
PA2302	*ambE*	AmbE	18.9	**3.0**	**3.9**	**4.2**
PA2303	*ambD*	AmbD	25.6	**3.0**	**3.7**	**4.3**
PA2304	*ambC*	AmbC	13.3	**2.9**	**3.3**	**4.3**
PA2305	*ambB*	AmbB	12.2	**3.3**	**4.8**	**4.8**
PA2423		hypothetical protein	3.1	**NC**	**3.1**	**3.9**
PA2587	*pqsH*	probable FAD-dependent monooxygenase	8.1	**3.9**	**4.3**	**5.6**
PA2588		probable transcriptional regulator	1.9	**7..9**	**7.0**	**24.4**
PA2591	*vqsR*	VqsR	7.1	**2.4**	**3.0**	**2.3**
PA2592		probable periplasmic spermidine/putrescine-binding protein (*potF5*)	3.7	**5.2**	**4.1**	**4.7**
PA2607		conserved hypothetical protein	1.6	NC	NC	NC
PA2608		conserved hypothetical protein (*yccK*)	1.5	NC	NC	NC
PA2939		probable aminopeptidase (*pepB*)	2.7	4.1	9.1	11.5
PA2949		probable lipase	1.4	NC	NC	NC
PA3326	*clpP2*	ClpP2	2.5	7.2	7.3	**7.0**
PA3327		probable non-ribosomal peptide synthetase	3.3	16.1	8.1	**3.5**
PA3328		probable FAD-dependent monooxygenase	4.5	21.9	12.4	**5.4**
PA3329		hypothetical protein	3.6	25.1	13.9	**5.9**
PA3330		probable short chain dehydrogenase	4.1	18.3	11.1	**4.4**
PA3331		cytochrome P450	3.5	20.3	11.6	**4.8**
PA3332		conserved hypothetical protein	3.3	23.4	13.4	**4.8**
PA3333	*fabH2*	3-oxoacyl-[acyl-carrier-protein] synthase III	4.4	22.9	11.9	**4.0**
PA3336		probable major facilitator superfamily (MFS) transporter	2.6	18.5	10.0	**41.0**
PA3346		two-component response regulator	1.7	NC	2.0	**2.8**
PA3391	*nosR*	regulatory protein NosR	8.6	NC	NC	-21.7
PA3392	*nosZ*	nitrous-oxide reductase precursor	10.7	NC	NC	-13.5
PA3476	*rhlI*	autoinducer synthesis protein RhlI	10.5	**4.1**	**2.9**	**4.3**
PA3477	*rhlR*	transcriptional regulator RhlR	7.2	**2.3**	**3.0**	**4.0**
PA3479	*rhlA*	rhamnosyltransferase chain A	2.2	36.7	24.3	**74.0**
PA3535		probable serine protease (*eprS*)	2.8	**2.2**	**5.2**	**6.0**
PA3615		hypothetical protein	1.6	NC	NC	-1.5
PA3904		hypothetical protein	15.0	**2.6**	**3.3**	**2.5**
PA3905		hypothetical protein	10.5	**2.4**	**3.0**	**1.6**
PA3906		hypothetical protein	17.4	**NC**	**3.1**	**NC**

Table 4.3 (continued)

PA3907		hypothetical protein	8.4	**2.7**	**4.1**	**NC**
PA3908		hypothetical protein	5.8	**2.9**	**4.3**	**2.4**
PA4117	*bphP*	bacterial phytochrome, BphP	1.8	**1.7**	**2.9**	**4.3**
PA4190	*pqsL*	probable FAD-dependent monooxygenase	2.5	**NC**	**NC**	**NC**
PA4594		probable ATP-binding component of ABC transporter	1.9	NC	2.1	2.6
PA4677		hypothetical protein	1.8	3.7	**3.1**	**3.6**
PA4778	*cueR*	CueR (*ybbI*)	1.8	**2.4**	**3.5**	**5.0**
PA4869		hypothetical protein	1.7	**NC**	**2.4**	**2.7**
PA4955		hypothetical protein	1.6	NC	NC	NC
PA5255	*algQ*	Alginate regulatory protein AlgQ (*algR2*)	1.5	NC	NC	NC

[1]Locus tags, gene names, and gene annotations from the Pseudomonas Genome Database (https://www.pseudomonas.com).

[2]Wild-type (WT) represent the WT vs. *lasR rhlR* contrast, while all anti-activator mutant contrasts are vs. the wild-type. **BOLD** denotes genes of the quorum-activated regulon also differentially expressed in log phase. Negative values indicate repression, positive values indicate activation. NC, no change.

*This fold change estimate does not represent a fold change increase *per se*, but rather the native expression of LasR in the wild-type vs. *lasR rhlR* contrast.

Table 4.4 Quorum-repressed genes

			Early stationary phase fold change[2]			
Locus tag[1]	**Name[1]**	**Annotation[1]**	**WT**	***qteE***	***qslA***	***qteE qslA***
PA0045		hypothetical protein	-2.2	NC	-2.9	-3.3
PA0047		hypothetical protein	-2.3	NC	-1.9	-2.3
PA0592	*ksgA*	rRNA (adenine-N6,N6)-dimethyltransferase	-1.6	NC	NC	-1.4
PA0944	*purN*	phosphoribosylaminoimidazole synthetase	-1.8	NC	NC	NC
PA1302		probable heme utilization protein precursor (*hxuC*)	-2.1	NC	NC	NC
PA1303		signal peptidase	-2.4	NC	NC	NC
PA1542		hypothetical protein	-1.8	NC	1.7	1.9
PA1580	*gltA*	citrate synthase (*cisY*)	-1.6	NC	NC	NC
PA1595		hypothetical protein	-1.9	NC	NC	NC
PA1757	*thrH*	homoserine kinase	-2.0	NC	NC	NC
PA1791		hypothetical protein	-1.9	NC	**-2.1**	-3.1
PA2583		probable sensor/response regulator hybrid	-1.7	NC	NC	NC
PA2665	*fhpR*	Transcriptional activator of P. aeruginosa flavohemoglobin, FhpR (*ygaA*)	-1.7	NC	NC	NC
PA2770		hypothetical protein	-1.7	NC	NC	2.3
PA2780	*bswR*	bacterial swarming regulator BswR	-1.5	**NC**	**NC**	**NC**
PA2930		probable transcriptional regulator	-2.4	NC	NC	NC

Table 4.4 (continued)

PA2950	*pfm*	proton motive force protein, PMF	-1.6	NC	NC	NC
PA2964	*pabC*	4-amino-4-deoxychorismate lyase	-1.5	NC	NC	NC
PA2970	*rpmF*	50S ribosomal protein L32	-2.1	NC	NC	NC
PA2998	*nqrB*	Na+-translocating NADH:ubiquinone oxidoreductase subunit Nrq2	-1.8	NC	NC	NC
PA3079		hypothetical protein	-1.9	NC	NC	NC
PA3111	*folC*	folylpolyglutamate synthetase	-1.6	NC	NC	NC
PA3174		probable transcriptional regulator	-1.9	-2.3	-2.7	**-3.2**
PA3268		probable TonB-dependent receptor	-3.4	NC	NC	**NC**
PA3284		hypothetical protein	-3.3	-5.5	-7.4	-8.8
PA3362		hypothetical protein (*amiS*)	-2.3	8.0	12.4	7.3
PA3473		hypothetical protein	-1.7	NC	NC	NC
PA3609	*potC*	polyamine transport protein PotC	-2.0	NC	NC	NC
PA3820	*secF*	secretion protein SecF	-2.5	NC	NC	NC
PA3823	*tgt*	queuine tRNA-ribosyltransferase	-1.8	NC	NC	-1.8
PA3827	*lptG*	Lipopolysaccharide export system permease protein LptG (*yjgQ*)	-1.5	NC	NC	NC
PA3979		hypothetical protein	-1.6	NC	NC	NC
PA4045		conserved hypothetical protein (*btuF*; *yadT*)	-1.7	NC	NC	NC
PA4046		hypothetical protein	-1.5	NC	NC	NC
PA4375	*mexW*	Resistance-Nodulation-Cell Division (RND) multidrug efflux transporter MexW	-1.7	NC	NC	NC
PA4479	*mreD*	rod shape-determining protein MreD	-2.8	NC	NC	NC
PA4519	*speC*	ornithine decarboxylase	-1.8	1.9	2	3.2
PA4562		conserved hypothetical protein (*mviN*)	-1.7	NC	NC	NC
PA4569	*ispB*	octaprenyl-diphosphate synthase (*cel*)	-1.8	NC	NC	-1.9
PA4628	*lysP*	lysine-specific permease	-1.7	NC	NC	NC
PA4630		hypothetical protein	-2.0	-1.6	-2.6	-2.5
PA4672		peptidyl-tRNA hydrolase (*pth*)	-2.1	NC	NC	NC
PA4757		conserved hypothetical protein (*yeaS*)	-1.5	NC	NC	-1.4
PA4840		conserved hypothetical protein (*yciH*)	-1.6	NC	NC	NC
PA5072		probable chemotaxis transducer	-1.5	NC	NC	NC
PA5081		hypothetical protein	-2.0	-1.8	NC	-1.5
PA5117	*typA*	regulatory protein TypA (*bipA*)	-1.7	NC	NC	NC
PA5139		hypothetical protein	-2.2	NC	NC	-3.3
PA5156		hypothetical protein	-1.8	NC	NC	NC
PA5167	*dctP*	DctP	-3.9	NC	NC	NC

Table 4.4 (continued)

PA5168	*dctQ*	DctQ	-4.3	NC	NC	NC
PA5169	*dctM*	DctM	-4.9	NC	NC	NC
PA5194		hypothetical protein	-1.7	NC	NC	NC
PA5250		conserved hypothetical protein	-1.7	NC	NC	NC
PA5251		hypothetical protein	-1.7	NC	NC	NC
PA5320	*coaC*	Phosphopantothenoylcysteine synthase/(R)-4'-phospho-N-pantothenoylcysteine decarboxylase (*coaB*; *coaBCI*; *dfp*)	-1.4	NC	NC	-1.3
PA5361	*phoR*	two-component sensor PhoR	-1.6	NC	NC	NC
PA5492		conserved hypothetical protein (*ysxC*; *yihA*)	-1.9	NC	NC	NC
PA5560	*atpB*	ATP synthase A chain (*papD*, *uncB*)	-1.8	NC	NC	-2.4

[1]Locus tags, gene names, and gene annotations from the Pseudomonas Genome Database (https://www.pseudomonas.com).

[2]Wild-type (WT) represents the wild-type vs. *lasR rhlR* contrast, while all anti-activator mutant contrasts are vs. the wild-type. **Bold** denotes genes of the quorum-repressed regulon also differentially expressed in log phase. Negative values indicate repression, positive values indicate activation. NC, no change.

We then compared our quorum-activated genes with those published previously using microarrays (Hentzer et al., 2003; Schuster et al., 2003; Wagner et al., 2003). While media choice, growth phases tested, and strain backgrounds vary among these studies, previous comparisons suggest a core QS regulon in *P. aeruginosa* that may be activated in most strains (Schuster and Greenberg, 2006). In our 4-way comparison we found 68 of our 79 genes were shared with at least one previous study, and a core regulon of 47 quorum-activated genes is shared among all 4 studies (Figure 4.5b). The large overlap of the quorum-activated regulon described here with those in previous microarray experiments validated our approach, as well as the general observation of a core QS-regulon among different *P. aeruginosa* strains and growth conditions (Chugani et al., 2012). The core QS-regulon determined here includes many well-studied targets of QS activation: *rhlA* (PA3479), encoding rhamnosyl transferase; the *apr* cluster (PA1246-50), encoding alkaline protease; *rsaL* (PA1430), a transcriptional repressor of LasR; *rhlI* and *rhlR* (PA3476-7), encoding the Rhl QS machinery and *pepB* (PA2939), encoding the aminopeptidase PepB (Table 4.3). We did not identify the *lasB* gene (PA3724) as differentially expressed in our QS regulon, which contrasts with the expected pattern of this quorum-activated gene based on P_{lasB}-*gfp* expression analysis (Figure 4.2). Our transcriptome sampling

time in early stationary phase was guided in part by these *lasB* expression data, although we recognize that accumulation of stable GFP expressed from a multi-copy plasmid likely exaggerates gene expression changes obtained by transcriptomics. In addition, our sampling scheme was guided by a previous microarray study in LB medium (Schuster and Greenberg, 2007), where the vast majority of QS genes showed high induction in early stationary phase. In comparison with those results, it appears that induction levels in CAA medium are lower than those in LB medium. This is consistent with the fact that the QS regulon of cells grown in CAA medium is smaller than that in LB, not considering differences in statistical analysis. We also observed relatively low levels of elastase activity in the wild-type strain used here (Figure 4.2b). Additionally, examination of *lasB* expression values alone in our transcriptome dataset is in agreement with these phenotypic results. In the transcriptome results, the wild-type shows a modest increase in expression (2-fold, not significant, α=0.05), while the presence of any anti-activator mutation drives *lasB* expression beyond 37-fold (*qteE*) and up to roughly 130-fold (*qslA*)(Figure 4.5c). Thus, sampling times and growth conditions likely explain the absence of *lasB* in our experimentally determined quorum-activated regulon.

With the above expression patterns under consideration, we asked whether variable proportions of QS componentry may be responsible for the observed differences among anti-activator deletion mutants. We sought to answer this question by evaluating absolute expression of the typical QS machinery (*lasR, lasI, rhlR, rhlI*) and anti-activator (*qteE, qscR, qslA*) genes in log and early stationary phase in the wild-type RNA-seq data. All genes encoding the typical QS machinery, including the gene coding for the orphan R-protein/anti-activator *qscR*, showed significant increases in absolute expression in early stationary phase (α=0.05, Figure 4.6), consistent with established mechanisms of QS autoregulation of synthase genes and stationary phase upregulation of R-proteins (Schuster et al., 2003; Schuster and Greenberg, 2006). *qslA* and *qteE* were both unchanged between log and early stationary phase.

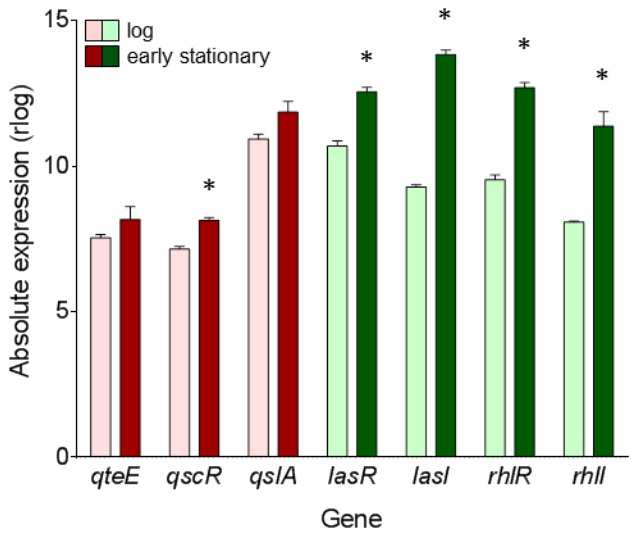

Figure 4.6 Absolute expression of genes coding for QS machinery and anti-activators. Absolute expression is presented as regularized log (rlog) values generated in DESeq2 using three biological replicates (*n*=3). Absolute gene expression in log phase (light bars) and early stationary phase (dark bars) are grouped by gene as QS machinery (red bars) or anti-activators (green bars). Bars represent means + s.e.m. (*n*=3). * indicates significantly higher expression in early stationary phase than log phase, two-tailed T-test (α=0.05).

4.3.5 Deletion of *qteE* and *qslA* advance timing and increase magnitude of QS gene expression

The quantity of differentially expressed genes was drastically higher in anti-activator mutants than the wild-type in log phase (Table 4.2), so we reasoned many of those genes were genes from our quorum-activated regulon that exhibited advanced timing. To test this, we compared expression of genes induced in the *qteE*, *qslA*, and *qteE qslA* mutants in log phase with our quorum-activated regulon in early stationary phase. Genes listed in the quorum-activated regulon that are differentially expressed in anti-activator mutants in log phase can then be said to be the result of advancement of timing in the quorum threshold due to absence of QS anti-activation. The large majority of quorum-induced genes (61 of 79, 77 %) in early stationary phase were advanced to log phase through deletion of *qteE*, *qslA*, or both (Figure 4.7a). In addition, the nested character of the anti-activator regulons, as mentioned above, was

again apparent here. Both features reinforce the notion that QteE and QslA function by R-protein sequestration.

We continued analysis of anti-activator effects through comparison of the 79 quorum-activated genes with anti-activator mutant gene expression in early stationary phase. In all, 74 of the 79 quorum-activated genes, or 93 %, were differentially expressed by a mutant deficient in at least one anti-activator protein (Figure 4.7b). Fifty-one of those 74 were differentially expressed in all mutants tested. We then questioned whether absolute expression of the quorum-activated regulon as a whole differs among our mutants. Deletion of *qteE* or *qslA* appears to produce a similar pattern of increased absolute expression among quorum-activated genes in both log and early stationary phase (Table 4.2, Figure 4.8). Loss of anti-activation shows a step-wise increase in absolute expression of several QS genes during log phase moving from *qteE* to *qslA* to the *qteE qslA* double mutant. These genes include: *nuh* (PA0143), encoding the purine nucleosidase Nuh; *rsaL* (PA1431); *kynU* (PA2080) encoding the kynureninase KynU; *cueR* (PA4778), encoding the copper toxicity transcriptional regulator CueR; and a cluster of relatively evenly expressed genes (PA3904-8) encoding hypothetical proteins. A select group of nitrate respiration genes (*norB*, PA0524; *nosR*, PA3391) exhibited a nearly opposite pattern, showing maximal absolute expression in the *qteE* mutant, lower expression in the *qslA* mutant, and lowest absolute expression in the *qteE qslA* mutant.

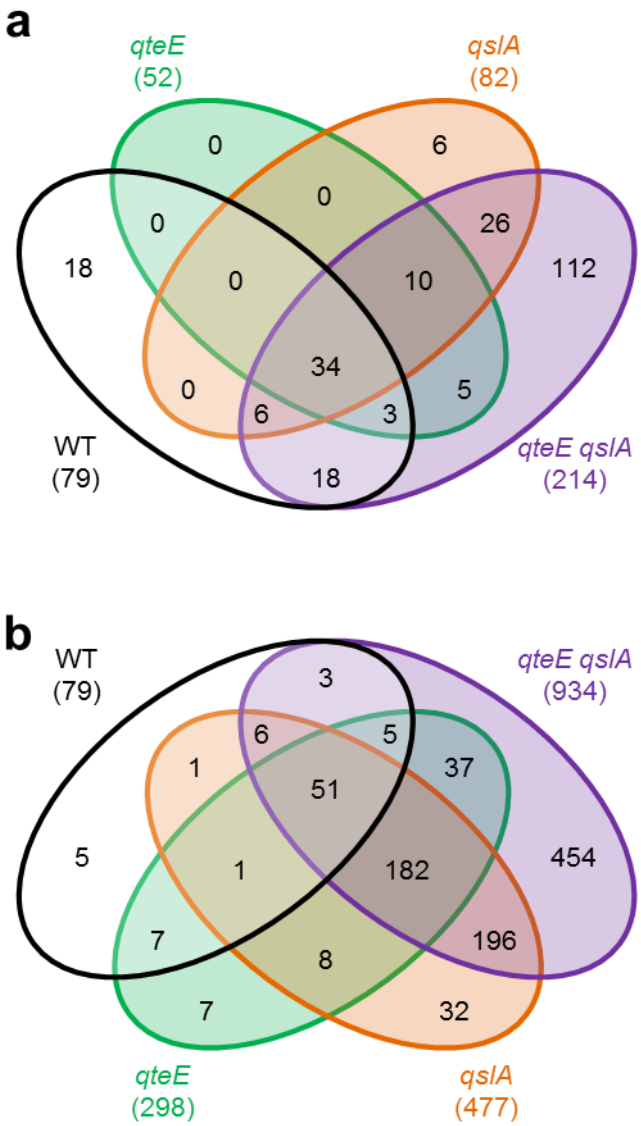

Figure 4.7 Overlap of induced genes in QS and anti-activator regulons. (a) Log phase anti-activator regulons and early stationary phase QS regulon. **(b)** Early stationary phase anti-anti-activator regulons and early stationary phase QS regulon. For both panels: Differentially expressed (DE) genes were determined in DESeq2 (see Materials and Methods) using three biological replicates (false discovery rate α=0.05, n=3), values represent only induced genes, and Venn diagram variables are not quantitatively scaled.

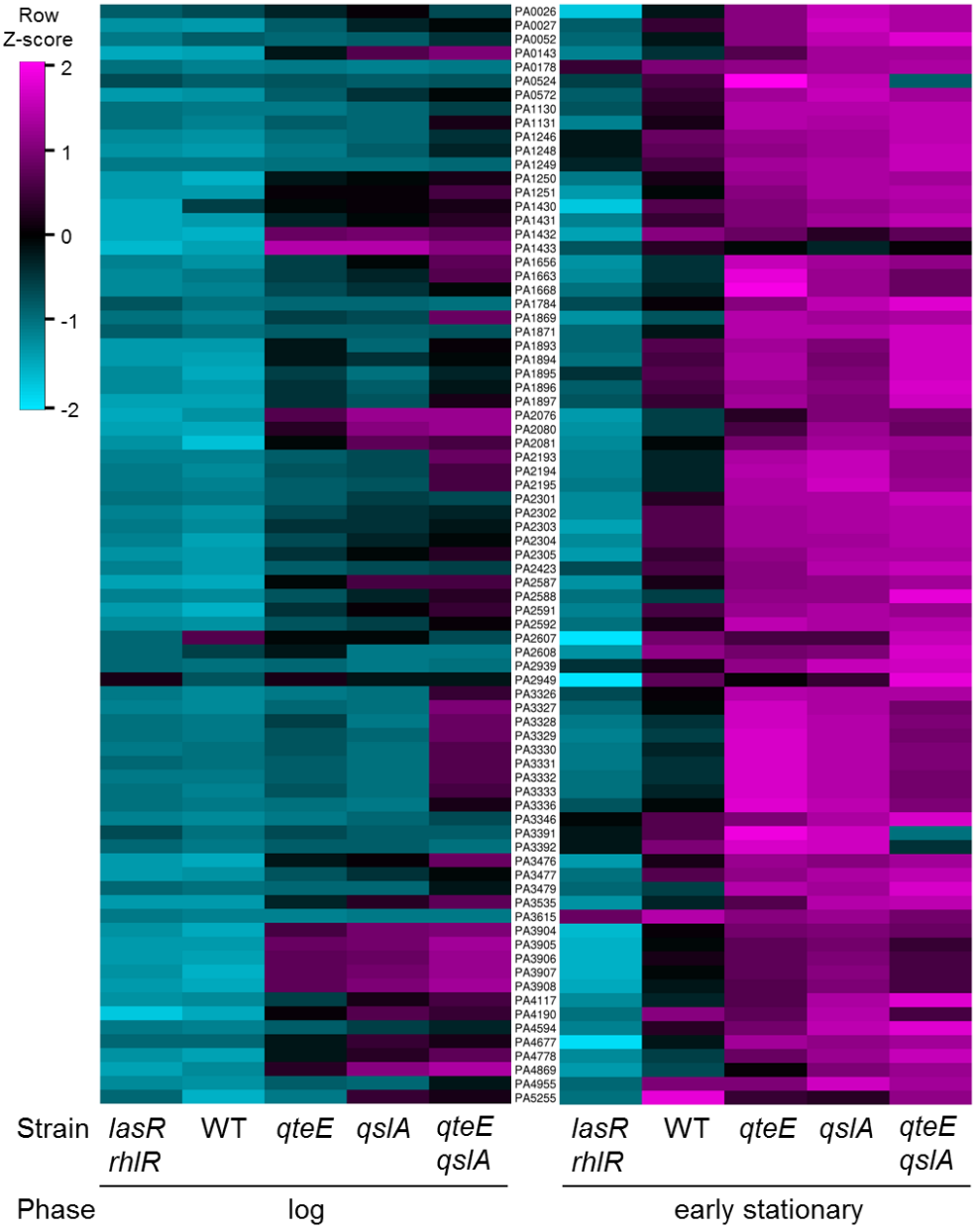

Figure 4.8 Absolute expression of the QS regulon. Absolute expression was calculated as the Z-score for individual samples among rows of both log and early stationary phase regularized log (rlog) values generated in DESeq2 (see Materials and Methods) using three biological replicates (*n*=3). Rows selected represent only induced genes in the QS regulon and are ordered by locus tag (middle column) for reference. WT, wild-type.

4.3.6 Global relationships between regulons

Deletion of anti-activators generally increased expression of the quorum-activated regulon, so we questioned if the subset of all induced genes identified in our study in early stationary phase also showed a similar trend. We defined a list of all induced genes from the wild-type vs. *lasR rhlR* comparison in early stationary phase, and from the comparison of each anti-activator vs. the wild-type in early stationary phase, yielding a subset list of 1002 unique genes. We compared the absolute expression of this list among each strain for both growth phases tested. Clustering of similarly expressed genes in our differential expression analysis allowed discovery of three distinct expression pattern groups (Figure 4.9a). Group I genes showed a general stationary phase-dependent pattern of low log phase expression combined with high expression in early stationary phase. This is in contrast to Group II which showed a general pattern of both anti-activator- and growth phase-dependent expression. With most genes remaining minimally expressed except in the anti-activator mutants in early stationary phase, Group II highlights a set of genes not normally induced in the wild-type in early stationary phase (as in Group I). Group III was the smallest and exhibited a pattern roughly the opposite of Group I, with most genes activated in log phase and only a few relatively activated by the *qteE qslA* mutant. Group I and II genes showed a pattern of successive induction that is particularly evident in stationary phase, with lowest levels in the *lasR rhlR* mutant and highest levels in the *qteE qslA* mutant. We used principle component analysis (PCA) to generalize our observations of differences among all of our strain-growth phase expression profiles. All log phase profiles clustered tightly, with early stationary phase-strain combinations driving nearly 80 percent of the variation in our data set (Figure 4.9b). Stationary phase profiles clearly segregated into two groups representing those strains with anti-activators and those without. This clustering is consistent with their general expression patterns in the heatmap (Figure 4.9a).

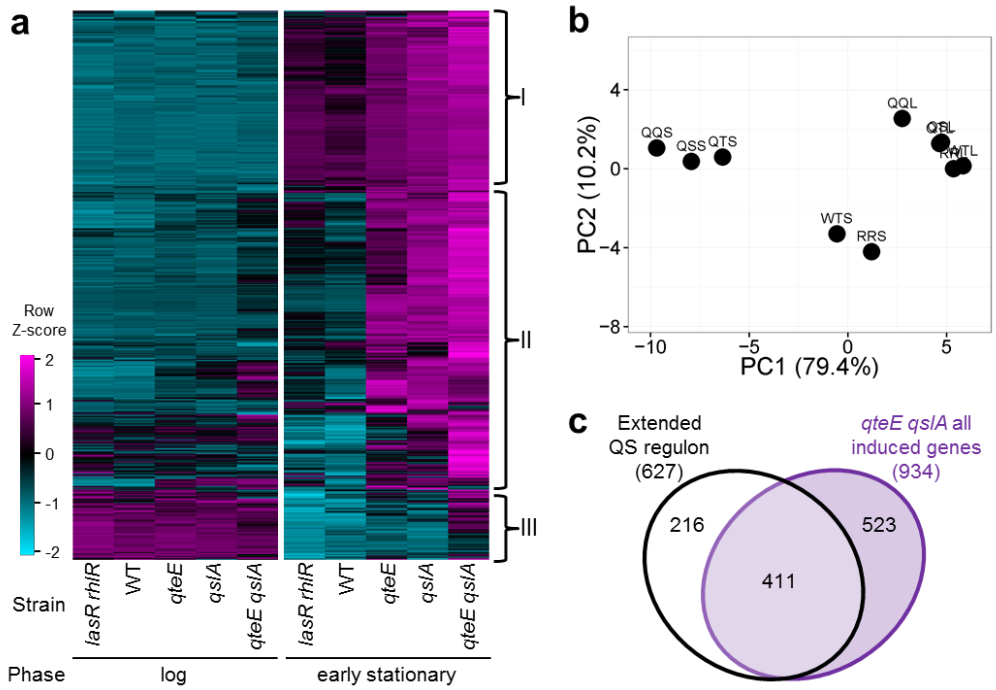

Figure 4.9 Absolute expression of all induced genes. (a) Absolute expression was calculated as the Z-score for individual samples among rows of both log and early stationary phase regularized log (rlog) values generated in DESeq2 using three biological replicates (*n*=3). Rows selected represent only genes induced in our stationary phase analysis (1002 unique loci total) and are clustered by average linkage. I, Group I genes; II, Group II genes; III, Group III genes; WT, wild-type. **(b)** Principal component analysis (PCA) of absolute expression results depicted in (A). WTL and WTS, wild-type in log and early stationary phase; QTL and QTS, *qteE* in log and early stationary phase; QSL and QSS, *qslA* in log and early stationary phase; QQL and QQS, *qteE qslA* in log and early stationary phase; RRL and RRS, *lasR rhlR* in log and early stationary phase, respectively. Superimposition of tightly clustered samples obscures some labels. **(c)** Comparison of all induced genes in QS and *qteE qslA* anti-activator regulons in early stationary phase. Venn diagram variables are not quantitatively scaled.

Most of the quorum-activated regulon determined in this study showed overlap with genes affected by anti-activation, so we questioned whether other genes induced in the absence of both anti-activators also correspond to other larger, previously identified QS regulons (Hentzer and Givskov, 2003; Schuster et al., 2003; Wagner et al., 2003). We assembled a list of all genes identified as quorum-activated in our analysis along with those of the other three studies identified in Section 4.3.4. This yielded a list of 627 unique genes in an ‘extended’ QS regulon. Comparison with

the 934 genes induced in the *qteE qslA* mutant showed that 411 genes are shared with the extended QS regulon (Figure 4.9c). This large overlap represents fully two-thirds of all genes of the extended QS regulon and nearly half of those induced in the *qteE qslA* mutant. Despite considerable reorganization and updates since previous functional profiles of QS were published, we found good agreement in the functional distribution of genes between each anti-activator regulon (Figure 10). Generally, removal of anti-activators simply increased the number of genes in groups already represented in the WT QS regulon. This is consistent with the idea that most genes in the anti-activator regulons are QS-dependent. It is plausible that anti-activator deletion allows an increase in the levels of active R-protein to an extent that is not normally achieved under physiological conditions. The set of genes activated under these conditions could then still be considered "quorum-sensing dependent".

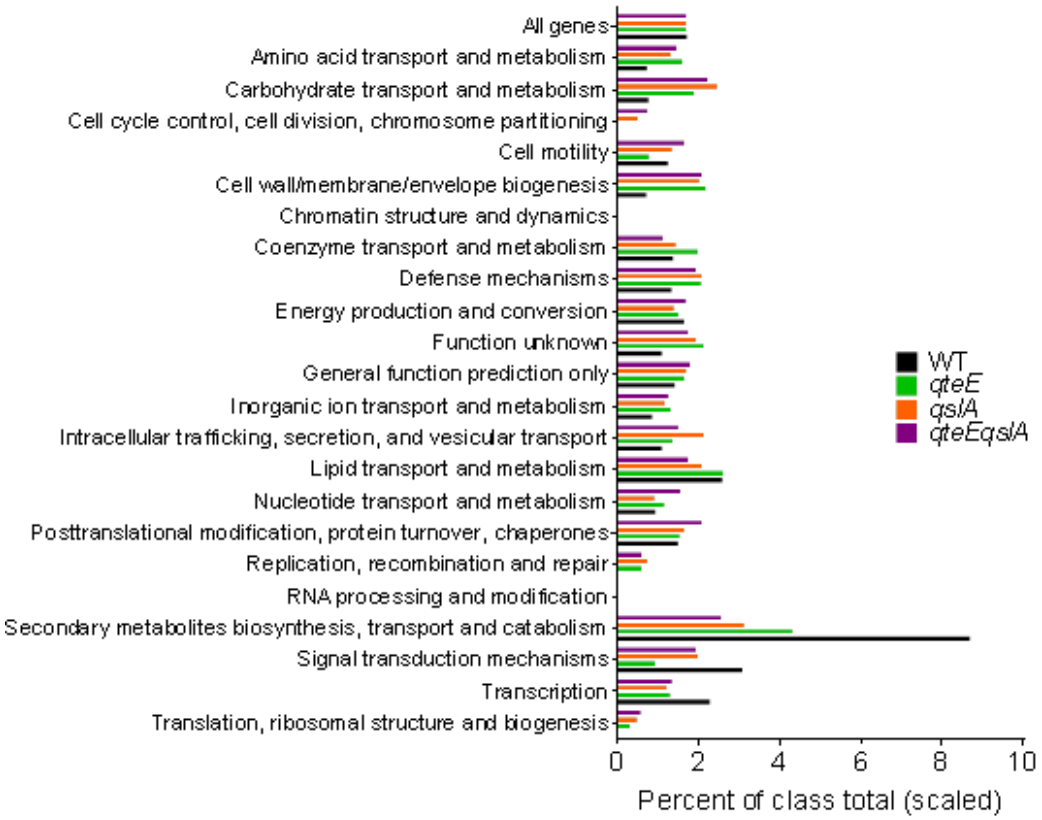

Figure 4.10 Functional classification of induced genes. Functional classes and annotations were retrieved from the Pseudomonas Genome Database. Bars represent percent of each functional class represented in induced gene lists, scaled to the wild-type percentage of all genes. Induced gene lists for each sample were assembled from

differentially expressed (DE) genes in early stationary phase as determined in DESeq2 using three biological replicates (false discovery rate α=0.05, n=3).

4.4 Discussion

Anti-activation through binding of R-proteins is a potent mechanism in modulation of the quorum-activation threshold in *P. aeruginosa* QS. The currently known collection of anti-activator proteins, QteE, QscR, and QslA, was previously shown to have somewhat parallel effects in their roles of preventing premature activation of QS (Siehnel et al., 2010; Seet and Zhang, 2011; Gupta and Schuster, 2013; Chugani and Greenberg, 2014). Here, we demonstrate additive, overlapping effects for each anti-activator in the modulation of the quorum-activation threshold. Our results draw on evidence of QS promoter activity, QS phenotypes, and anti-activator transcriptional profiles to show a group of anti-activator proteins confer a distinct, but combinatorial effect in their regulation of QS. These results paint a considerably more complex picture of the factors influencing the *P. aeruginosa* QS activation threshold than previously presented.

Removal of anti-activators increased the magnitude of P_{lasB}-*gfp* expression in every strain tested (Figure 4.2). This result confirms a critical role for the mechanism of anti-activation in the general control of the quorum-activation threshold in *P. aeruginosa*. Owing to the social nature of QS-mediated intercellular communication and gene regulation, several QS-controlled products including LasB elastase are metabolically costly to produce, providing vulnerability to overinvestment of resources or exploitation by neighboring cells (Frank, 2010; Schuster et al., 2013). Indeed, deletion of QteE or QscR individually may yield increased LasB secretion that provides increased population fitness in media requiring QS-regulated protease for growth, but those mutants also suffer a fitness cost (Gupta and Schuster, 2013). The presence of multiple anti-activators implies a redundant 'failsafe' mechanism for ensuring this fitness cost is kept low, but our observations of P_{lasB}-*gfp* expression indicate each may have a distinct role in this mechanism. Our results are consistent with the notion of anti-activators effectively preventing short-circuiting. However, removal of all three anti-activators did not drive P_{lasB}-*gfp* expression levels higher

than that of the *qteE qslA* and *qslA qscR* mutants in a 'short-circuit' effect. It is possible that yet another regulator is preventing constitutive activation in the absence of our selected anti-activators in these conditions. In the case of nutrient starvation for example, higher levels of RelA further increase the alternative stationary phase sigma-factor RpoS, leading to premature *lasB* induction (van Delden et al., 2001). The stringent response is not active in CAA medium, so the maximum lasR level in our conditions may not be sufficient for demonstrating short-circuiting. Our results are consistent with the notion of anti-activators effectively preventing short-circuiting. Removal of QslA only produced increases in expression half that seen in strains lacking QteE or QscR alone (Figure 4.2c). However, removal of either QteE or QscR from the strain lacking QslA drove levels to nearly three times that of any single anti-activator mutant. These data suggest a synergistic effect between anti-activators may be possible. This synergism could be due to cooperative protein-protein interactions among anti-activators in their binding of R-proteins, a mechanism in contrast to the current model of independent anti-activator binding and repression of R-proteins (Figure 4.1). QscR binds LasR forming a heterodimer presumably similar to LasR dimer formation (Lintz et al., 2011), but QscR is not particularly closely related to LasR (Chugani and Greenberg, 2014), so their exact binding may in fact be distinct. QslA dimers obscure the LasR dimerization interface in the N-terminal ligand binding domain (Fan et al., 2013). The direct binding orientation of QteE to R-proteins has not been determined. With these potentially disparate avenues of R-protein and anti-activator binding, a cooperative mechanism of interaction is plausible. Further evidence for this possibility is presented later in our discussion. In contrast, removal of QscR and QteE together produces an expression profile nearly superimposable with the corresponding single mutants. Together, these results are consistent with our initial hypothesis that individual anti-activators may have distinct roles in the modulation of the QS threshold. For example, the list of regulatory inputs that effect QS regulation is extensive (Schuster and Greenberg, 2006), and individual anti-activators could be regulated dynamically and independent of each other, thereby permitting multiple pathways of QS threshold modulation. Our analysis of absolute expression of QS components and anti-activators in our wild-type RNA-seq data

found generally constitutive expression of anti-activators, while R-proteins are up-regulated in stationary phase (Section 4.3.4, Figure 6). However, the limited resolution of our absolute expression data to two time points in a single growth medium leaves room for further definition in the regulation of anti-activators. In a separate study, qRT-PCR analysis of *qslA* transcription revealed a constitutive expression pattern in LB medium (Seet and Zhang, 2011), but the regulatory dynamics of *qteE* and *qscR*, in addition to those of *qslA* under varying growth conditions, are not entirely clear.

Anti-activation is part of a larger group of QS-dampening mechanisms that include transcriptional repression (RsaL) and dilution or environmental degradation of signal to prevent advancement of the QS threshold (de Kievit et al., 1999; Hense and Schuster, 2015). Here, we show that anti-activation alone can prevent premature activation of QS. Deletion of anti-activator genes can reduce the time and hence, the cell density, until high-level activation of QS genes is achieved (Figures 4.2, 4.4 and 4.7). Key here is expression magnitude at a given time point, but also expression rate – accelerating expression rates and high-level expression early in growth effectively represent advancement. This allows full wild-type levels of expression to be reached much earlier in mutants than when anti-activators are present. All anti-activator mutants showed increases in expression rates, but the most dramatic rate increases that effectively demonstrate threshold advancement were characteristic of multi-deletion mutants lacking QslA (Figure 4.2). These observations suggest a key role for QslA in determining not only the timing and magnitude of QS activation, but also the expression rate. Further evidence for the QS advancement effect was mirrored in absolute expression of the quorum-activated regulon of 79 genes, were maximal expression of many genes is reached in log phase in the absence of anti-activators (Figure 4.8, left panel). Our observations of P_{lasB}-*gfp* induction kinetics are similar to those published previously for *qteE* and *qscR* single mutants in that deletion of either anti-activator alone produces stronger effects on magnitude than timing of expression (Gupta and Schuster, 2013)(Figure 4.2c). However, our results show that deletion of some combinations of anti-activators can advance timing of QS activation, and QslA in particular is implicated in this role.

Sequential peaks in P_{lasB}-*gfp* expression rates in our kinetic experiments are of similar magnitude in wild-type, but expression skews towards the earlier, presumably Las-controlled peak in all anti-activator mutants (including multiple deletions) with the exception of the *qslA* single mutant. Similar kinetic experiments conducted with both the wild-type and an isogenic *lasR* mutant showed the first expression peak disappears in the absence of LasR (Gupta and Schuster, 2013), providing further support for the notion of sequential wild-type expression peaks corresponding to Las and Rhl system induction. In light of this, our observations indicate that anti-activators may primarily target LasR rather than RhlR. Such a relationship is intuitive considering that typical Las induction comes earlier than Rhl, and that induction of the Rhl system is generally subordinate to Las (Schuster and Greenberg, 2006). However, more direct evidence is needed to support this interaction model, as the independent effects of anti-activators on LasR and RhlR activity are not entirely clear. QteE is known to interact and destabilize both LasR and RhlR, but interaction with the latter was shown in the absence of LasR where competition between the two R-proteins for QteE binding was absent (Siehnel et al., 2010). QscR was also reported to associate with both LasR and RhlR *in vitro* in the absence of signal using fluorescence anisotropy (Ledgham et al., 2003). However, direct evidence of the QscR-RhlR interaction *in vivo*, as well as the biological relevance of this association, is still needed. On the other hand, QslA was not shown to significantly abrogate RhlR-mediated transcription of *rhlI* in the *E. coli* heterologous host (Seet and Zhang, 2011), further supporting the Las-dominant interaction model described above.

QscR is different than other anti-activators in that it can also respond to signals and effectively act as a transcriptional activator on its own (Lequette et al., 2006). QscR exhibits promiscuity in its response to AHL signals; in addition to 3oxoC12-HSL generated by LasI, QscR responds to 3oxoC10 similarly, and an even stronger response was observed for C10 and C12HSL ligands, adding an additional layer of complexity to QscR activity (Lee et al., 2006a). Transcription of *qscR* may also be under tighter control than other anti-activators. QscR transcription is regulated by both the global regulator VqsR (Liang et al., 2012) and LasR itself, but a *qscR* mutant also affects Las system induction through repression of *lasI* transcription

(Chugani et al., 2001), creating an interconnected negative regulatory feedback loop reminiscent of RsaL. How other anti-activators are regulated in response to growth phase, signal concentration, nutritional status, and environmental conditions remain open questions. Future experimentation in determining anti-activator induction kinetics in variable conditions and the effects of over-expression will aide in this pursuit.

Our transcriptome analysis produced a list of 79 quorum-activated genes, or roughly 1.4% of all *P. aeruginosa* genes, notably smaller than previous microarray studies that suggest "hundreds" of QS-activated genes (6 – 10% of genome) (Hentzer et al., 2003; Wagner et al., 2003; Schuster and Greenberg, 2006). Considering our choice of a semi-defined medium (CAA) that limits the final densities of bacteria to almost half that of previous studies (using LB broth), this difference is perhaps unsurprising. However, almost 90% of the genes we identified were also identified in at least one of the microarray studies (Figure 4.5b), supporting the notion of a core QS regulon conserved in *P. aeruginosa* suggested elsewhere (Schuster and Greenberg, 2006; Chugani et al., 2012). The large number of additional genes induced in anti-activator mutants could draw into question if these genes are all QS-dependent. It is possible that some of the genes identified as induced in anti-activator mutants are not regulated directly through canonical QS. Considering roughly half of all genes induced in the strain lacking QteE and QslA were not shared with previously identified QS-activated gene sets (Figure 4.9c), a subset of these genes could conceivably be induced through a yet undetermined QS-independent mechanism. It is also possible that the large number of genes affected by simultaneous *qteE* and *qslA* inactivation, but not present in the extended QS regulon, are activated through canonical QS but are not induced under standard culture conditions as R-protein levels are not high enough. All possible environmental conditions for QS gene expression have not yet been explored, and it is plausible that high levels of R-proteins are achieved under some relevant physiological conditions. The differences between each of our anti-activator regulons and the QS regulon could simply stem from the fact that each deletion results in a different level of free, active LasR: the higher the level of free LasR, the more promoters are bound and activated

due to decreased competition for active LasR. This mechanism is most plausible with the nested anti-activator differentially expressed genes identified in the logarithmic phase of growth (Figures 4.4a and 4.6a), where almost all *qteE* genes are a subset of *qslA* genes, and almost all *qslA* genes are a subset of *qteE qslA* genes. Differential interaction of anti-activators with RhlR could also play a role. Epistasis analysis could be used to address these possibilities, an approach that showed epistatic interactions in the functioning of parallel QS circuits in *V. harveyi* (Henke and Bassler, 2004). In our case, *lasR* and/or *rhlR* mutations would need to be introduced into strains harboring mutations in *qteE* and/or *qslA*. Such analyses could enable a better understanding of the regulatory interactions and dependencies of anti-activation in a QS-independent context. Transcriptome profiling experiments utilizing mutants lacking both anti-activators and LasR or RhlR or both will allow exploration of this possibility.

Finally, evaluation of functional annotations of induced genes in our strains showed few substantive differences in their overall functional class distribution, and all were largely similar to the quorum-activated regulon distribution (Section 3.7, Figure 10). Our results were generally consistent with previous analyses of the content of QS regulons (Schuster et al., 2003; Schuster and Greenberg, 2006). QS is responsible for global gene regulation in *P. aeruginosa* (Schuster and Greenberg, 2006), including genes involved in growth and central metabolism, biosynthesis and transport of secondary metabolites, and signal transduction mechanisms, so our findings are also in support of the proposed mechanisms of anti-activators as suppressors of QS regulon expression, specifically.

We conclude that anti-activation mechanisms conferred by QteE, QscR, and QslA differentially suppress the magnitude of QS-gene activation. Loss of anti-activation advances the effective timing of QS-gene activation, but the magnitude of this effect is dependent on the specific combination of anti-activators deleted, with loss of QslA in combination with another anti-activator conferring the greatest effect. Anti-activators affect an overlapping but distinct set of genes largely governed by QS, and do so in a combinatorial fashion. This study further supports the concept of a core QS regulon in *P. aeruginosa*, and provides the ground work for multiple directions of

fundamental investigation of anti-activation and gene regulation in bacteria. Our transcriptome results will likely aide studies of seeking to determine the roles of anti-activators in *P. aeruginosa* pathogenesis, clinical avenues for inhibiting QS, and regulation of virulence gene expression. More broadly, our results will also contribute to a more detailed understanding of the factors influencing the QS threshold in bacteria.

Conflicts of interest

The authors declare no existing or potential conflicts of interest.

Author contributions

The author(s) have made the following declarations about their contributions. Conceived and designed the experiments: KLA, MS. Performed the experiments: KLA. Analyzed the data: KLA. Contributed reagents/materials/analysis tools: KLA, MS. Wrote the paper: KLA, MS.

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Chapter 5

CONCLUSIONS

Interest in the ways populations of bacteria use communication and cooperation in interspecific competition in the natural word has increased in recent years. Cooperation among bacteria through the cell-cell communication mechanism of acyl-homoserine-lactone (AHL) quorum sensing (QS) is now understood to include many behaviors, and the QS control of *P. aeruginosa* virulence factors is of particular clinical importance. In Chapters 3 and 4 of this doctoral dissertation, we identified and addressed key open questions related to QS mechanism and social implications. Our goals were namely, 1) to determine the molecular genetic basis for the preservation of QS-mediated cooperation in the presence of QS cheaters, and 2) to investigate the interactions between QS anti-activator proteins in determining the quorum sensing threshold.

In our first inquiry, we discovered that non-social adaptation in a cooperative growth environment can stabilize QS cooperation. Mutations in the transcriptional repressor PsdR maximize absolute fitness of individuals as they take up proteolysis products. This allows evolved cells to saturate quicker in a mixed population, and quickly leads to the fixation of *psdR*-null mutations in the population. In combination with cooperative alleles, isolates with these mutations are still vulnerable to social exploitation by non-producing cheats, but they are also able to tolerate a higher cheater load. This result highlights a scenario where cycles of social and non-social adaptation may allow a temporary stabilization of cooperation, but under strong selection for cooperative growth cheaters may persist. In this way, non-social adaptation to a growth environment may defer a tragedy of the commons, but not eliminate its threat. Other studies have also found this form of adaptive race to provide a stabilizing effect on cooperation between synthetic yeast (Waite and Shou, 2012) and in *Pseudomonas fluorescens* siderophore production (Morgan et al., 2012). Non-social adaptation likely works in concert with environmental variables and other

mechanisms to preserve cooperation, including kin selection (smith et al., 2016), pleiotropic constraint (Foster et al., 2004), metabolic prudence (Xavier et al., 2011).

Our second inquiry focused on the role of anti-activation in determining the QS activation threshold in *P. aeruginosa.* We found deletion of any anti-activator significantly increased QS gene expression, but strains with combinations of deletions displayed a QS threshold that can be effectively advanced in time in addition to expression magnitude. This effect was largely visible in double - or triple-anti-activator mutants with deleted QslA. Rates of QS gene expression where much higher in anti-activator mutants compared with the wild-type. Based on timing of induction and maximum expression rate in the wild-type, the greatest increases in expression rate appear to correspond to Las system induction. Anti-activator mutants harboring QteE and/or QslA deletions all showed differentially expressed genes that overlap well with established QS-controlled gene sets, including our own experimentally determined wild-type QS regulon. Moreover, QteE and/or QslA deletion showed an additive, nested effect where QteE-affected genes are also affected by QslA, which in turn were largely also affected in the double mutant. These results paint a picture where anti-activation works in an additive, combinatorial fashion to suppress QS in a way that may be synergistic in the conditions tested. Future work would be wise to examine the potential for epistatic effects through introduction of *lasR* and/or *rhlR* deletions into anti-activator backgrounds. This type of epistasis analysis could provide further evidence for the dependence of anti-activator differential expression on LasR. Our work also lays a mechanistic framework for future biochemical studies to evaluate heteromultimer formation between LasR and anti-activators.

Our results provide a candid view into the molecular genetics and social evolutionary context of *P. aeruginosa* QS. Future studies will benefit from our detailed genetic and evolutionary analysis in Chapter 3, and our mechanistic regulatory analysis in Chapter 4. Together, our results contribute novel insight into a clinically important and fundamentally relevant process in microbiology.

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