AN ABSTRACT OF THE DISSERTATION OF

Cara N. Wilder for the degree of Doctor of Philosophy in Microbiology presented on April 28, 2011.
Title: Cooperation and Conflict in *Pseudomonas aeruginosa* Quorum Sensing

Abstract approved:

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Martin Schuster

Pseudomonas aeruginosa is an environmental bacterium as well as an opportunistic pathogen that primarily infects immunocompromised individuals, including those suffering from cystic fibrosis. The density-dependent regulation of gene expression via cell-to-cell communication, also termed quorum sensing (QS), is an important virulence determinant in this organism. Generally, *P. aeruginosa* uses three hierarchically-arranged QS systems, *las*, *rhl*, and *pqs*, to coordinate the expression of hundreds of genes required for both virulence and nutrient acquisition. Each QS system consists of genes involved in autoinducer synthesis, *lasI*, *rhlI*, and *pqsABCDH*, and cognate regulatory genes *lasR*, *rhlR*, and *pqsR*. Although QS is required for infection, QS-deficient isolates, often carrying mutations in the central regulator *lasR*, have been isolated from clinical populations. Recent *in vitro* studies provide evidence indicating that *lasR* mutants can arise by social exploitation. As social cheaters, *lasR* mutants cease the production of public goods, such as extracellular proteases, and take advantage of their production by the QS-proficient majority. In this study, we sought to compare the social behavior of the *lasR* mutant to that of the *rhlR* and *pqsR* mutants

in vitro. We also sought to understand whether social conflict plays a role in shaping the instantaneous QS diversity of both clinical and environmental populations. Under *in vitro* growth conditions requiring QS, both the *lasR* and *pqsR* mutants, but not the *rhlR* and signal-negative mutants, invaded wild-type populations. In contrast to the *lasR* and *rhlR* mutants, the *pqsR* mutant also grew well on its own. While the *lasR* mutant exhibited typical cheating behavior as previously reported, the *pqsR* and *rhlR* mutants demonstrated more complex behaviors that can be attributed to positive and negative pleiotropic effects through the differential regulation of *pqs* gene expression. Therefore, if the selective forces associated with each of these social phenotypes are similar *in vivo*, then *lasR* and *pqsR*, but not *rhlR*, mutants may thrive in both clinical and natural populations. Upon the analysis of clinical *P. aeruginosa* populations obtained from cystic fibrosis lung infections, we found that these predictions were partially substantiated. Generally, these populations demonstrated high QS diversity both within and between patients, suggesting diverse selection pressures within the lung. In contrast, environmental populations isolated from natural composts exhibited minimal QS diversity, indicating that social conflict may not play a predominant role in some natural populations. Overall, our study highlights the effects of social interactions on QS diversity within various *P. aeruginosa* populations.

Cooperation and Conflict in *Pseudomonas aeruginosa* Quorum Sensing

by
Cara N. Wilder

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degree of

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APPROVED:

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Major Professor, representing Microbiology

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Chair of the Department of Microbiology

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Dean of the Graduate School

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

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Cara N. Wilder, Author

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CONTRIBUTION OF AUTHORS

Dr. Martin Schuster and Cara N. Wilder were involved with the design, data interpretation, and writing of Chapters 2-4. Dr. Stephen P. Diggle was involved with writing and interpretation of the data for Chapter 2. Dr. Gopal Allada provided us with samples as well as assisted us in the writing for Chapter 3.

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DEDICATION

I would like to dedicate this dissertation to my family, who taught me the value of hard work, perseverance, and a good education. I would also like to dedicate this dissertation to the love of my life, Keith D. Moodhe, for his never-ending support and encouragement.

Cooperation and Conflict in *Pseudomonas aeruginosa* Quorum Sensing

General introduction and literature review

Overview

Many bacteria are social organisms capable of coordinating their cooperative behaviors through the emission and detection of self-produced low molecular weight chemical signals known as autoinducers. This phenomenon, termed quorum sensing (QS), permits bacteria to monitor cell density in order to synchronize the regulation of gene expression (Fuqua *et al.* 1994). This process is used by numerous bacterial species, and can vary between organisms in signal processing, the structure of autoinducer signaling molecules, and the regulation of a diverse array of physiological activities such as competence, virulence and sporulation (Atkinson and Williams 2009). Currently, some of the best-studied QS autoinducers are acyl-homoserine lactones (acyl-HSL) found in Gram-negative proteobacteria (Atkinson and Williams 2009). Conversely, in Gram-positive QS systems, bacteria often employ secreted peptides as autoinducers. Within both Gram-negative and -positive bacteria QS, species-specific autoinducers are generated that can vary in length and modifications (Fuqua and Greenberg 2002, Miller and Bassler 2001, Ni *et al.* 2009).

It is assumed that QS evolved as a means to coordinate cooperative behaviors to provide a benefit at the population level (Diggle *et al.* 2007a). However, evolutionary theory predicts that the participation in social behavior is metabolically expensive, leaving the population vulnerable to social cheating (West *et al.* 2006). Cheaters are individuals that benefit from a social trait, such as the production of a

public good, while contributing less than average to production costs (Greig and Travisano 2004, Velicer *et al.* 2000). In addition to the social complexities associated with QS, various exogenous conditions can skew autoinducer levels, leading to an incorrect estimation of cell density. To account for these problems, QS has been extended by the concept of efficiency sensing (ES), a unifying theory for autoinducer sensing encompassing both individual and group benefits. In ES, cells use autoinducers as a proxy to estimate the efficiency of producing more expensive public goods by not only taking into account cell density, but also diffusion and spatial cell distribution (Hense *et al.* 2007). In the following, we will use QS terminology with "quorum" indicating the minimum amount of autoinducer required to elicit gene regulation, regardless of how it was achieved.

Generally, acyl-HSL QS has been studied within several model organisms including *Pseudomonas aeruginosa*. This opportunistic pathogen requires QS to regulate over three hundred genes, many of which encode extracellular virulence factors (Schuster *et al.* 2003). Recent studies have shown that *P. aeruginosa* QS populations can be affected by social cheating, resulting in reduced virulence as well as a metabolic burden on the bacterial community (Diggle *et al.* 2007b, Rumbaugh *et al.* 1999, Rumbaugh *et al.* 2009, Sandoz *et al.* 2007). We further analyze this phenomenon within *in vitro*, clinical, and environmental *P. aeruginosa* populations in the following chapters. This introductory chapter focuses on the diversity and evolutionary implications of QS systems, as well as QS circuitry and regulation in *P. aeruginosa*.

Prokaryotic QS systems

The evolution of QS

Altruistic behaviors, such as cooperation and communication, are described as actions that increase a recipient's fitness at a cost to the actor. Though altruistic populations are subject to invasion by selfish and uncooperative individuals (i.e. social cheaters) (Hamilton 1963, Hamilton 1964a, Hamilton 1964b), cooperative behaviors have been demonstrated across the bacterial domain and appear to be relatively stable (Crespi 2001). A primary example of bacterial cooperation and communication can be found in the diverse array of QS systems found in various Gram-positive and -negative bacteria (Diggle *et al.* 2007a). Specifically, QS is density-dependent bacterial cell-to-cell communication used to coordinate cooperative behaviors (Fuqua *et al.* 1994).

It is believed that QS may have evolved as a means to provide a benefit to the population as a whole; however, like other altruistic behaviors, QS is vulnerable to invasion by selfish individuals (Diggle *et al.* 2007a). Despite this susceptibility, QS is still evolutionarily stable. One explanation for this is provided by the Kin Selection theory (Hamilton 1964a). This theory of altruism between relatives describes a mechanism by which an individual can pass its genes to the next generation, though indirectly (Hamilton 1963). Generally, an individual performs altruistic, cooperative behaviors as a means to promote the growth and replication of closely related relatives (Hamilton 1963). An alternate explanation of the stability of QS can be extended to the multiple functions of signaling molecules, which would alter the relative cost and

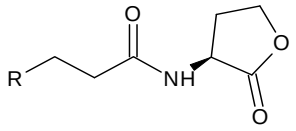
benefit of their production (Brown and Johnstone 2001). For examples, autoinducers can also function as iron-scavenging molecules (Kaufmann *et al.* 2005) or antibiotics (Stein 2005). Below, we describe the various types of bacterial QS systems currently known.

Acyl-HSL-mediated QS systems

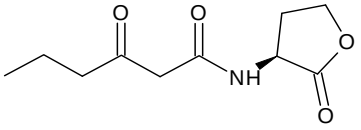
Acyl-HSL QS was first discovered forty years ago in the Gram-negative bacterium *Vibrio fischeri* (Eberhard 1972, Nealson *et al.* 1970). This organism is found both globally in marine environments as well as symbiotically in the light organs of various squid and teleost fish. Within the light organ, *V. fischeri* is supplied with nutrients as well as a protected environment. In return, the bacteria supply their host with light which can be used for either attracting prey, assisting in escaping or diverting predation, or communication (Nealson and Hastings 1979). The production of light by *V. fischeri* is concomitant with high bacterial cell density, which can be achieved within the confines of the light organ (10^{10} CFU/ml) but not in open sea water ($<10^{2}$ CFU/ml) (Engebrecht *et al.* 1983, Kaplan and Greenberg 1985, Nealson and Hastings 1979).

Light production by *V. fischeri* requires the translation of luciferase, an enzyme which catalytically generates light through the oxidation of the reduced form of flavin mononucleotide, $FMNH_2$, and a long chain aldehyde by diatomic oxygen (Engebrecht and Silverman 1984). In *V. fischeri*, genes involved in the regulation and production of luciferase are encoded within a 9 kB section of DNA consisting of *luxR* and *luxI*, as

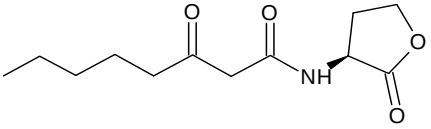
Generic acyl-HSL structure



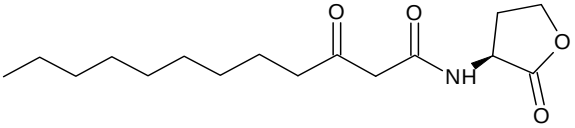
Vibrio fischeri
3-oxo-C6-HSL



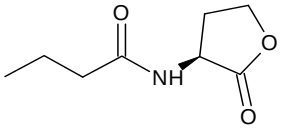
Agrobacterium tumefaciens
3-oxo-C8-HSL



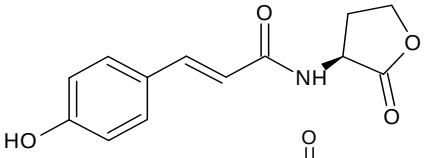
Pseudomonas aeruginosa
3-oxo-C12-HSL



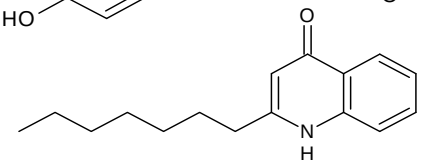
Pseudomonas aeruginosa
C4-HSL



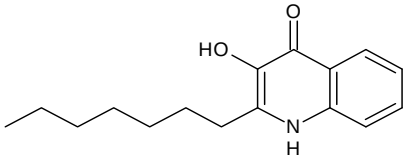
Rhodopseudomonas palustris
p-coumaroyl-HSL



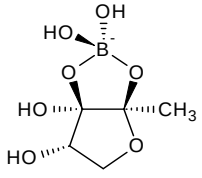
Pseudomonas aeruginosa
HHQ



Pseudomonas aeruginosa
PQS



Autoinducer-2 (AI-2)



Staphylococcus aureus
AIP-1

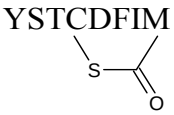


Figure 1.1: Select Gram-negative and -positive QS autoinducer structures.

well as *luxCDABE*, respectively (Engebrecht and Silverman 1984). The *luxI* gene encodes an autoinducer synthase that generates *N*-(3-oxo-hexanoyl)-homoserine lactone (3OC6-HSL; Figure 1.1.) through the fusion of a six-carbon length fatty acid chain to the amino nitrogen of *S*-adenosylmethionine (SAM) (Fuqua and Greenberg 2002, Kaplan and Greenberg 1985, Nealson and Hastings 1979). Once synthesized, the autoinducer molecule freely diffuses across the cell membrane into the surrounding environment. Once a critical autoinducer threshold is met, 3OC6-HSL binds to its cognate receptor LuxR, encoded by *luxR*, to activate the transcription of the *lux* operon (Figure 1.2) (Kaplan and Greenberg 1985, Nealson and Hastings 1979). Specifically, the LuxR:3OC6-HSL complex binds to a <20 bp *cis*-acting palindromic sequence, often referred to as a *lux*-box, localized within the promoter region of the *lux* operon (Fuqua and Greenberg 2002). Once bound to the *lux*-box, the receptor-autoinducer complex functions as a transcription factor and interacts with RNA polymerase (RNAP) to stimulate transcription of the operon (Devine *et al.* 1989, Fuqua and Greenberg 2002).

Presently, over 70 genera of proteobacteria are known have LuxI and LuxR homologs (Boyer and Wisniewski-Dye 2009). For many of these species studied, only one QS system has been found; however, some bacteria, such as *P. aeruginosa*, contain two or more LuxI/R homologs (Lazdunski *et al.* 2004). Generally, LuxI homologs are able to produce unique acyl-HSLs through the linkage of SAM to variable acyl chains derived from fatty acid biosynthesis (general acyl-HSL structure depicted in Figure 1.1). The length of the acyl chain can vary in size from four to18

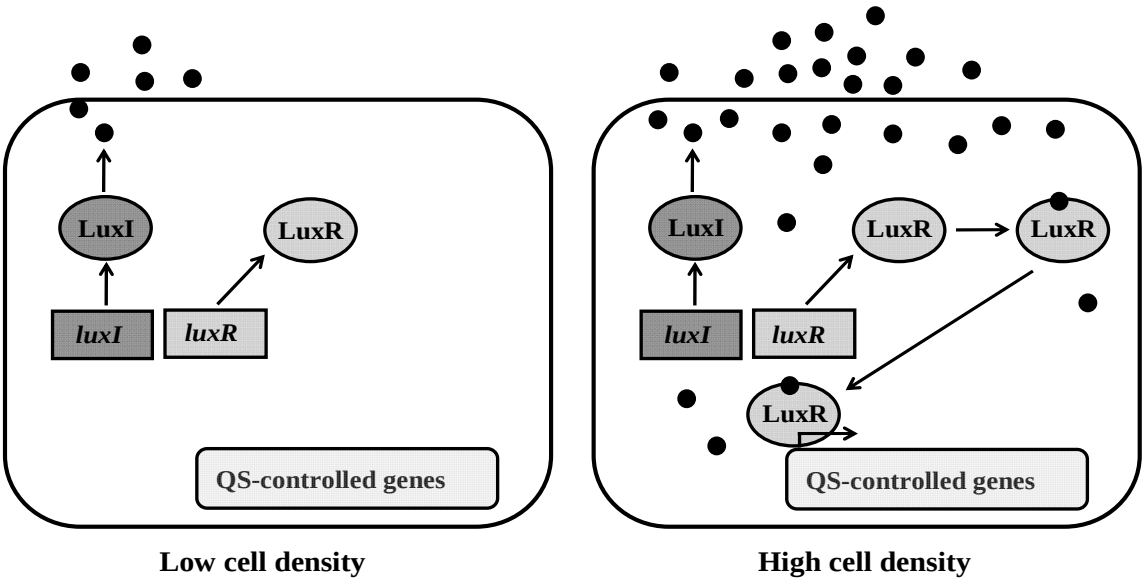


Figure 1.2: Acyl-HSL QS circuitry. The gene *luxI* encodes an autoinducer synthase, LuxI, which generates an autoinducer signaling molecule (black circle). At high cell density, the autoinducer binds to its cognate regulator, LuxR, encoded by *luxR*. This complex induces the transcription of QS-controlled genes. In *V. fischeri*, these genes include the up-regulation of *luxR* and *luxICDABE*. The increase in LuxI production allows for the up-regulation of QS gene expression in a positive feedback loop.

carbons, generally in 2-carbon increments, with larger autoinducers excreted by efflux pumps (Fuqua and Greenberg 2002, Pearson *et al.* 1999). In addition, the third carbon of the acyl chain may be either a fully oxidized carbonyl, carry a hydroxyl group, or be fully reduced (Fuqua and Greenberg 2002).

LuxR homologs, though divergent in sequence, have conserved tertiary structures including an N-terminal acyl-HSL binding domain and a C-terminal helix-turn-helix DNA-binding domain (Fuqua and Greenberg 2002, Stevens and Greenberg 1997, Whitehead *et al.* 2001). These LuxR homologs bind with high affinity to autoinducers synthesized by a cognate LuxI homolog (Schaefer *et al.* 1996), although

some non-cognate autoinducers can elicit partial responses (Lazdunski *et al.* 2004). Additionally, the binding of autoinducers to their cognate LuxR homolog can be either reversible or irreversible. For example, in *V. fischeri*, the binding of 3OC6-HSL to LuxR is reversible, while in *A. tumefaciens*, the binding of *N*-(3-oxo-octanoyl)-homoserine lactone (3OC8-HSL; Figure 1.1.) to its LuxR homolog, TraR, is irreversible. In *A. tumefaciens*, this binding is irreversible because the autoinducer binding site is buried within the protein, requiring 3OC8-HSL to associate with TraR during protein synthesis (Zhang *et al.* 2002). Lastly, these acyl-HSL QS systems differ between species in the mediation of a variety of responses such as plasmid transfer, production of exoenzymes, or biofilm formation (Lazdunski *et al.* 2004).

Aryl-HSL-mediated QS systems

Recently, a new class of autoinducers, aryl-HSLs, has been discovered in the phototrophic soil bacterium, *Rhodopseudomonas palustris.* This bacterium uses a LuxI homolog, RpaI, to synthesize *p*-coumaroyl-homoserine lactones (Figure 1.1) using an acyl group derived from the aromatic monomer of plant lignin, *p*-coumarate (Schaefer *et al.* 2008). This contrasts with the previous concept that only acyl moieties from fatty acid biosynthesis can serve as autoinducer synthase substrates in acyl-HSL QS. The discovery of a non-fatty acyl-HSL (aryl-HSL) autoinducer expands the range of possible acyl moieties to any environmental organic acid. Additionally, this unique system allows the bacteria to use signal production to integrate sensing population density as well as the availability of a particular

exogenous substrate to tightly regulate QS-controlled genes (Schaefer *et al.* 2008). Overall, this ability may be beneficial in inter-kingdom communication with a host plant or in bacteria that require QS-controlled functions under specific environmental conditions.

Oligopeptide-mediated QS systems

In response to increasing population size, Gram-positive bacteria regulate a variety of processes such as competence, sporulation, and virulence. However, in contrast to acyl-HSL mediated QS found in Gram-negative bacteria, Gram-positive organisms use secreted oligopeptides as autoinducers (Thoendel and Horswill 2010). In general, these oligopeptide autoinducers can vary in length from 5-17 amino acids and are often post-translationally modified through the incorporation of an isoprenyl, lactone, thiolactone, or lanthionine group. These differences allow for species-specific signal binding and processing (Ni *et al.* 2009). In *Staphylococcus aureus*, for example, the regulation of virulence gene transcription requires a thiolactone-modified oligopeptide autoinducer (AIP-1; Figure 1.1) (Antunes *et al.* 2010, Thoendel and Horswill 2010).

Generally, bacteria secrete processed oligopeptide autoinducers into the surrounding environment using an ATP-binding cassette (ABC) transporter (Figure 1.3). When signal concentration reaches a critical threshold, the oligopeptide autoinducers initiate signal processing via binding to the histidine kinase portion of a two-component signal transduction system (Bassler 1999, Lazazzera and Grossman 1998, Miller and Bassler 2001, Ni *et al.* 2009). The activation of the histidine kinase

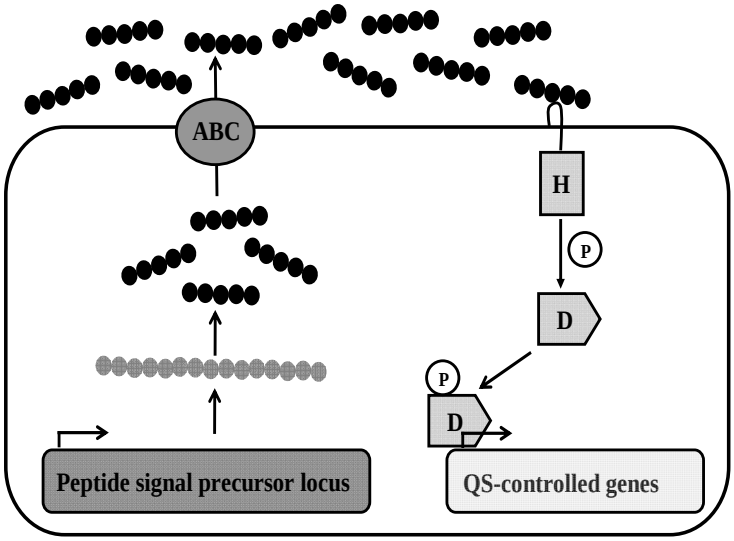


Figure 1.3: Oligopeptide-mediated QS circuitry. An oligopeptide signal precursor (grey circles) is translated and cleaved to produce the oligopeptide autoinducer (black circles). The autoinducer is then secreted into the surrounding environment by an ABC transporter (ABC). At high cell density, the oligopeptide is detected by the histidine kinase portion of a two-component regulatory system. Once activated, the sensor kinase autophosphorylates on a conserved histidine residue (H). Subsequently, the phosphoryl group (P) is transferred to a conserved aspartate residue (D) on a cognate response regulator. The phosphorylated response regulator then activates transcription of QS-controlled genes.

sensor initiates a phosphorylation cascade, eventually leading to the phosphorylation and activation of a cognate response regulator protein. Once activated, the response regulator initiates the transcription of target genes (Miller and Bassler 2001, Thoendel and Horswill 2010).

Autoinducer-2 mediated QS systems

Gram-positive and -negative bacteria use oligopeptide and acyl-HSL mediated QS, respectively, to regulate gene expression in response to environmental cues

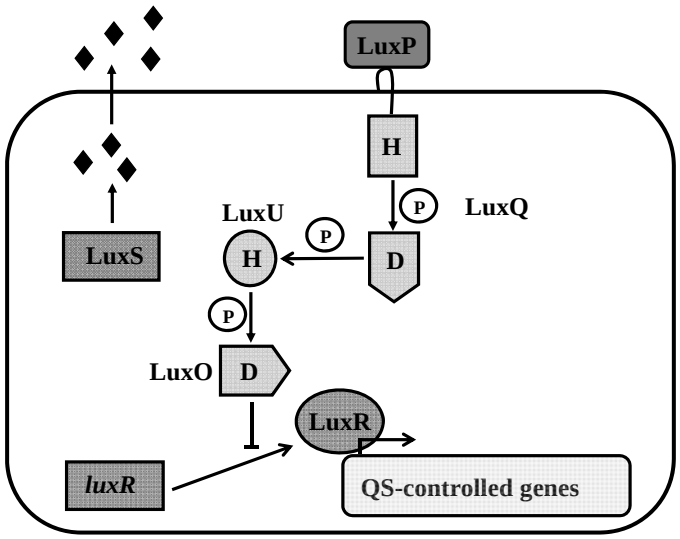


Figure 1.4: AI-2 mediated QS in *V. harveyi*. The AI-2 signaling molecule (black diamond) is produced as a by-product by LuxS during the activated methyl cycle (AMC). At high cell density, AI-2 interacts with the periplasmic binding protein LuxP, which subsequently interacts with the histidine protein kinase, LuxQ. In turn, LuxQ functions as a phosphatase, which reverses the flow of phosphoryl groups (P), resulting in LuxR-initiated transcription of QS-controlled genes that regulate bioluminescense. In the absence of AI-2, LuxQ autophosphorylates and transfers a phosphoryl group to LuxU, which subsequently passes it to the response regulator, LuxO. The phosphorylated LuxO protein activates the expression of small regulatory RNA, which, along with the chaperone Hfq, destabilizes LuxR mRNA (not shown). For simplicity, the LuxLM autoinducer synthase and LuxN regulator of the acyl-HSL mediated QS system of *V. harveyi* is not shown.

(Atkinson and Williams 2009). These QS systems are often specific for intraspecial communication; however, the discovery of autoinducer-2 (AI-2) mediated QS has provided a mechanism by which Gram-positive and -negative bacteria can participate in interspecial communication (Vendeville *et al.* 2005). This novel QS system was originally discovered in the marine organism *Vibrio harveyi* (Figure 1.4). Since then, the production of the AI-2 signal has been identified in over 50 bacterial species (Atkinson and Williams 2009, Vendeville *et al.* 2005).

From an evolutionary standpoint, AI-2 mediated interspecies cross-talk is difficult to explain as kin selection is not important across species (Diggle *et al.* 2007a). It is plausible that cooperation can be favored between species under various conditions such as when there are byproduct benefits, i.e. cooperation as an incidental consequence of selfish action (Sachs *et al.* 2004), or to avoid punishment (West *et al.* 2002). However, AI-2 may not be a true autoinducer signal as there is very little evidence linking it to the direct activation of specific genes. Currently, only the regulation of an ABC transporter in *Salmonella typhimurium* (Taga *et al.* 2001) and bioluminescence in *V. harveyi* (Surette *et al.* 1999) have been shown to be directly regulated by AI-2.

The production of AI-2 requires LuxS, an enzyme used in the activated methyl cycle (AMC). This metabolic cycle is used in the generation and recycling of the methyl donor, SAM (Vendeville *et al.* 2005). Through multiple steps in the AMC, SAM is converted to the LuxS substrate, *S*-ribosyl-homocysteine (SRH). When SRH is cleaved by LuxS to generate homocysteine, 4, 5-dihydroxy-2, 3-pentanedione (DPD) is formed as a byproduct. Spontaneously, DPD will cyclize to yield a number of furanones that are collectively known as AI-2 (Atkinson and Williams 2009, Vendeville *et al.* 2005). In *V. harveyi*, AI-2 is a furanosyl-borate diester (Figure 1.1) that activates a receptor-mediated sensor kinase/phosphorelay system as a means to regulate bioluminescense (Figure 1.4) (Lenz *et al.* 2004, Neiditch *et al.* 2005). This pathway, however, can differ between bacteria in which AI-2 furanone is used as well as how this signal is processed. *S. typhimurium*, for example, uses the AI-2 furanone,

(*2R*, *4S*)-2-methyl-2,3,3,4-tetra-hydroxytetrahydrofuran (*R*-THMF), which lacks boron. Additionally, instead of using a phosphorelay system, *S. typhimurium* transports *R*-THMF (AI-2) into the cytoplasm using an ABC transporter (Vendeville *et al.* 2005). Overall, these differences in AI-2 structure and processing may be reflective of environmental diversity, types of QS-controlled functions, and the use of LuxS in AMC-regulated metabolism (Atkinson and Williams 2009, Vendeville *et al.* 2005, Winzer *et al.* 2002).

QS in *P. aeruginosa*

QS circuitry

P. aeruginosa is a model organism used to study acyl-HSL QS. These ubiquitous Gram-negative bacteria thrive in a variety of environmental niches including both terrestrial and aquatic habitats. *P. aeruginosa* also has the ability to transition from the environment to become an opportunistic pathogen, causing severe nosocomial and community-acquired infections (Schuster and Greenberg 2006, Venturi 2006). Immunocompromised individuals, such as those with cystic fibrosis (CF), are particularly susceptible to both chronic and acute *P. aeruginosa* infections (Doring 2010, Moreau-Marquis *et al.* 2008, Wagner and Iglewski 2008). QS circuitry, among other regulatory elements, regulates this metabolic versatility demonstrated by *P. aeruginosa* (Goodman and Lory 2004, Stover *et al.* 2000).

In the development of an acute infection, *P. aeruginosa* uses QS to regulate the expression of virulence factors; including extracellular proteases, toxins, and

secondary metabolites (Hentzer and Givskov 2003, Rumbaugh *et al.* 2000, Smith and Iglewski 2003, Whiteley *et al.* 1999). In *P. aeruginosa*, these QS-controlled functions are regulated by three distinct QS systems, the *las*, *rhl*, and *pqs* systems (Figure 1.5). These QS pathways are arranged hierarchically with the *las* system positively regulating both the *rhl* (Latifi *et al.* 1996, Pesci and Iglewski 1997) and *pqs* (Wade *et al.* 2005) systems. Additionally, the *rhl* system negatively regulates the *pqs* system (McGrath *et al.* 2004, Wade *et al.* 2005). Variants with disruptive mutations in either the *las*, *rhl*, or *pqs* systems display reduced or no pathogenicity in plants, insects and mice (Cao *et al.* 2001, Jander *et al.* 2000, Lesprit *et al.* 2003, Rahme *et al.* 1997).

The *las* and *rhl* systems are acyl-HSL mediated signaling pathways. These systems both consist of the LuxI/LuxR homologs LasI/LasR and RhlI/RhlR, respectively. The LasI autoinducer synthase produces *N*-(3-oxo-dodecanoyl)-homoserine lactone (3OC12-HSL; Figure1.1) (Gambello and Iglewski 1991, Passador *et al.* 1993, Pearson *et al.* 1994), which binds irreversibly to the cognate receptor, LasR. The LasR:3OC12-HSL transcriptional complex regulates specific genes including *rhlR* and *rhlI,* thus activating the *rhl* system (Figure 1.5) (Gilbert *et al.* 2009). The RhlI autoinducer synthase directs the production of *N*-butanoyl-homoserine lactone (C4-HSL; Figure 1.1), which interacts with the cognate regulator, RhlR, to activate transcription of certain genes (Figure 1.5) (Venturi 2006). In addition to global gene regulation, both systems auto-regulate gene transcription through the up-regulation of their respective autoinducer synthase, thus creating a positive feedback loop (Figure 1.5) (Seed *et al.* 1995).

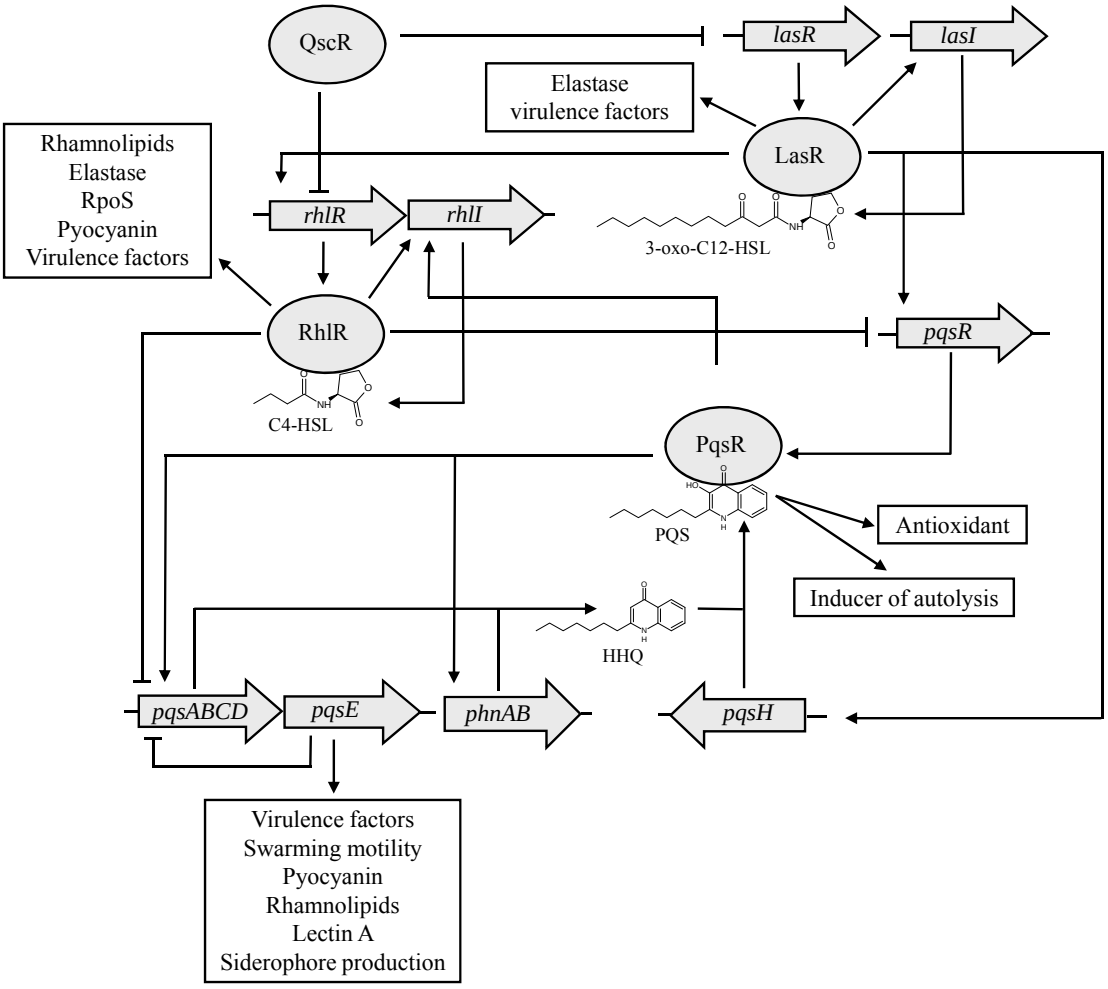


Figure 1.5: QS signal integration in *P. aeruginosa*. Arrows and T bars indicate positive and negative regulation, respectively. The gene *lasI* encodes an autoinducer synthase used to generate 3-oxo-C12, which binds to LasR. This complex up-regulates the *rhl* and *pqs* QS systems. The gene *rhlI* encodes an autoinducer synthase used to generate C4-HSL, which binds to RhlR. This complex negatively regulates the *pqs* QS system. QscR, an orphan QS regulator, down-regulates both the *las* and *rhl* systems. Genes *pqsABCDH* are involved in the generation of PQS, which binds to PqsR. This complex up-regulates the *rhl* QS system. Additionally, PQS plays a dual role in bacterial survival as an antioxidant and inducer of autolysis. Each of these systems regulates genes required for virulence.

Together, the *las* and *rhl* systems regulate 315 genes, with the *rhl* system regulating a subset of approximately 112 genes (≥ 2-fold activation by both signals

versus 3OC12-HSL alone) (Hentzer *et al.* 2003, Schuster *et al.* 2003, Wagner *et al.* 2003). Though many of these QS-controlled genes are specifically regulated by either the *las* or *rhl* system, some genes are co-regulated by both systems (Latifi *et al.* 1996, Pesci *et al.* 1997). To activate gene transcription, the LasR and RhlR transcriptional complexes bind *lux*-box-like sequences, referred to as *las*-boxes. These regulatory elements can be bound by either LasR, RhlR, or both (Schuster *et al.* 2004b, Whiteley *et al.* 1999). Interestingly, LasR can bind *las*-boxes as a multimer only in the presence of 3OC12-HSL, whereas RhlR can bind as a dimer in the presence or absence of C4-HSL. In the absence of C4-HSL, RhlR functions as a repressor while binding to the same promoter region (Medina *et al.* 2003b, Ventre *et al.* 2003). Thus far, 35 *las*-boxes that are directly bound by the LasR:3OC12-HSL complex have been identified; this represents a fraction of all *las*-controlled genes (Gilbert *et al.* 2009). Lastly, though the *las* and *rhl* systems together form a hierarchical regulatory cascade, expression of *rhl*-controlled genes may be *las*-independent during late stationary phase and under specific environmental conditions (Dekimpe and Deziel 2009, Diggle *et al.* 2003, Medina *et al.* 2003a, Van Delden *et al.* 1998).

In addition to LasR and RhlR, there is a third, orphan LuxR homolog, QscR (Figure 1.5) (Chugani *et al.* 2001). This regulator has no cognate acyl-HSL synthase gene; however, LasI-generated 3OC12-HSL can serve as a signaling molecule (Lequette *et al.* 2006). Previous studies have shown that mutations in *qscR* lead to the premature production of several QS-controlled virulence factors, thus leading to a hypervirulent phenotype (Chugani *et al.* 2001). During early phases of growth when

autoinducer levels are low, QscR may function as a QS repressor through the formation of heterodimers between itself and either LasR or RhlR (Figure 1.5) (Ledgham *et al.* 2003). Overall, QscR both directly and indirectly affects a distinct regulon as well as genes that are co-regulated by the *las* and *rhl* systems (Lequette *et al.* 2006).

Another QS pathway in *P. aeruginosa*, the *pqs* system, is mediated by 2-alkyl-4-quinolones (AQ) instead of acyl-HSL (Figure 1.5). This system consists of the autoinducers 2-heptyl-4 quinolone (HHQ; Figure 1.1) and 2-heptyl-3-hydroxy-4 quinolone (PQS; Figure 1.1) as well as a cognate regulator, PqsR (MvfR) (Dubern and Diggle 2008). PqsR, a LysR-type regulator, controls the expression of operons *pqsABCDE* and *phnAB*; encoding proteins required for AQ and anthranilate synthesis, respectively (Deziel *et al.* 2004, Gallagher *et al.* 2002). Biosynthesis of HHQ occurs via the condensation of anthranilate and the products of *pqsABCD* (Bredenbruch *et al.* 2005, Rampioni *et al.* 2010). HHQ can be subsequently oxidized to PQS by the putative monooxygenase PqsH, encoded by *pqsH* (Figure 1.5) (Deziel *et al.* 2004, Gallagher *et al.* 2002). Both PQS and HHQ can bind PqsR, though with high and low affinity, respectively (Wade *et al.* 2005, Xiao *et al.* 2006a). This binding leads to activation of the *pqsA* promoter, creating a positive feedback loop that increases expression of genes required for signal production (McGrath *et al.* 2004, Wade *et al.* 2005, Xiao *et al.* 2006b). In addition to functioning as an autoinducer, PQS is also an anti-oxidant and pro-oxidant (Haussler and Becker 2008), an inducer of autolysis (D'Argenio *et al.* 2002), an iron chelator (Bredenbruch *et al.* 2006, Diggle *et al.*

2007a), and is required for biofilm maturation (D'Argenio *et al.* 2002) and outer membrane vesicle formation (Mashburn and Whiteley 2005).

The *pqs* system regulates 141 genes, many of which are dependent on PqsE, a metallo-β-hydrolase encoded by *pqsE* (Rampioni *et al.* 2010). PqsE also negatively controls its own expression through the repression of the *pqsABCDE* operon (Rampioni *et al.* 2010). Recent transcriptome studies revealed that approximately 55% the PqsR regulon is regulated by QS, and that many of these genes are also co-regulated by the *rhl* system (Deziel *et al.* 2005). In general, regulation by the *pqs* system is tightly controlled; in addition to auto-regulation, the expression of the *pqs* system is modified by both the *las* and *rhl* systems (Figure 1.5). These acyl-HSL QS systems exert control over the *pqs* system through up-regulation of *pqsR* and *pqsH* (Wade *et al.* 2005), and down-regulation of *pqsA* and *pqsR* (McGrath *et al.* 2004), respectively. Additionally, PqsR positively regulates the *rhl* system (McKnight *et al.* 2000), thus providing an additional layer of control. This complex regulation of the *pqs* system provides an additional link between the *las* and *rhl* systems as well as allows for the precise timing of virulence gene expression (Deziel *et al.* 2005, Wade *et al.* 2005).

Quorum-independent gene regulation

Expression of *P. aeruginosa* QS genes can be modulated independently of cell density through transcriptional and posttranscriptional regulation in response to abiotic and biotic environmental factors (Boyer and Wisniewski-Dye 2009). Previous QS studies

have demonstrated that there is a delay in the induction of certain quorum-controlled genes that is unrelated to signal concentration (Diggle *et al.* 2002, Schuster *et al.* 2003, Whiteley *et al.* 1999). These observations suggest that additional factors are required for gene regulation other than quorum size. One such level of regulation is to control the timing of gene activation in response to growth phase (Diggle *et al.* 2002, Schuster *et al.* 2003). Presently, multiple regulators have been identified in the involvement in gene induction and repression throughout bacterial growth (Diggle *et al.* 2002, Schuster and Greenberg 2006). One such regulator is RpoS, the stationary phase sigma factor, which regulates genes under a variety of stresses including starvation associated with the onset of stationary phase (Fujita *et al.* 1994). RpoS directs the expression of 40% of all quorum-controlled genes through the recruitment of RNAP to the respective promoter region (Schuster *et al.* 2004a, Schuster and Greenberg 2006).

QS gene expression can also be controlled by various exogenous environmental conditions such as prolonged starvation, limitations in specific nutrients, or low oxygen and anaerobic conditions. During prolonged amino acid or carbon starvation, bacterial genes are regulated through the stringent response. The stringent response is controlled by the alarmone guanosine tetraphosphate, ppGpp, which regulates gene expression through binding to RNAP (Artsimovitch *et al.* 2004). In contrast, under nutrient limited conditions, such as phosphate or calcium limitation, QS gene regulation is enhanced through the up-regulation of *rhlR* (Jensen *et al.* 2006), and *lasR* (Albus *et al.* 1997, Kanack *et al.* 2006), respectively. Lastly, under anaerobic or low oxygen conditions, the transcriptional regulator ANR (anaerobic

regulator) functions synergistically with LasR and RhlR to regulate genes such as the hydrogen cyanide biosynthesis operon, *hcnABC* (Pessi and Haas 2000). Overall, these various environmental conditions exert both transcriptional and posttranscriptional control over QS genes via one- and two- component signal transduction systems (Boyer and Wisniewski-Dye 2009, Gooderham and Hancock 2009). The two-component signal transduction systems consist of an inner membrane-spanning sensor histidine kinase which detects various environmental cues and a cytoplamic response regulator that regulates gene expression in response to these cues (Stock *et al.* 2000). In contrast, the one-component signal transduction system transfers signaling information using a single protein that contains both an input and output domain that lack histidine kinase and receiver domains (Ulrich *et al.* 2005). In *P. aeruginosa*, there are approximately 64 sensor kinases and 72 response regulators (Gooderham and Hancock 2009); an example of which includes the PhoB-PhoR system, which mediates gene expression during phosphate limiting conditions as described above (Jensen *et al.* 2006).

Environmental factors that affect the stability, diffusion, and perception of acyl-HSLs can also modulate QS gene expression in *P. aeruginosa* (Boyer and Wisniewski-Dye 2009). Abiotic factors, such as temperature and pH, affect the stability of acyl-HSLs through the opening of the lactone ring, thus inactivating the autoinducer. This occurs at high temperatures and at alkaline pH (Decho *et al.* 2009, Yates *et al.* 2002). Acyl-HSL stability can also be affected by biotic factors within the bacterial community via interspecies competition and eukaryotic defense. Interspecies

inactivation of acyl-HSL, referred to as quorum quenching, is accomplished through the use of bacterial enzymes such as acyl-HSL-lactonases and acyl-HSL-acylases/amidohydrolases (Dong *et al.* 2000, Leadbetter and Greenberg 2000). The hydrolysis of acyl-HSLs provides bacterial competitors with a nutrient source as well as prevents QS signaling (Leadbetter and Greenberg 2000). In contrast, eukaryotes inactivate acyl-HSLs, possibly as a defense mechanism, to impede the activation of QS-controlled virulence factors (Stoltz *et al.* 2007, Teiber *et al.* 2008). This can occur in the serum of animals expressing paraoxonase; an enzyme that hydrolyzes the lactone ring (Ozer *et al.* 2005, Stoltz *et al.* 2007).

Another factor affecting *P. aeruginosa* QS gene regulation is mass transfer, or the diffusion of the signal. Bacteria rely on autoinducer concentration as a way to detect quorum size; however, diffusion of the signal can be affected by the nature of the signal and the hydrophobicity of the surrounding medium. In highly hydrophobic media, such as the exopolysaccharide-rich biofilm matrix, acyl-HSLs are less likely to diffuse (Charlton *et al.* 2000). To account for problems associated with diffusion of the signal, QS has been recently extended by the concept of efficiency sensing (ES), which uses signal production to assess the efficiency of producing public goods. This unifying theory not only accounts for cell density, but also signal diffusion and spatial cell distribution (Hense *et al.* 2007).

Lastly, *P. aeruginosa* QS gene expression can be modified through altered perception of autoinducer levels. Increased or decreased signal levels can be perceived through interspecies communication, where different co-existing bacterial

species produce identical or structurally related autoinducer molecules (Eberl *et al.* 1996, Winson *et al.* 1995). For example, within the cystic fibrosis lung both *P. aeruginosa* and *Burkholderia cepacia* often occur together, resulting in high mortality (Govan and Deretic 1996). *B. cepacia* has been shown to up-regulate virulence factor production in response to acyl-HSL production by *P. aeruginosa*; however, this cross-talk is not reciprocated, thus resulting in decreased perception of acyl-HSLs (Govan and Deretic 1996). Overall, the regulation of the QS network incorporates a multitude of factors leading to the efficient adaptation of *P. aeruginosa* in response to a variety of environmental cues.

The evolutionary implications of QS in *P. aeruginosa*

It has been hypothesized that QS evolved to coordinate cooperative behaviors as a means to acquire group fitness benefits. In *P. aeruginosa* populations, considerable evidence points to QS as being invaluable for pathogenesis due to quorum-controlled production of numerous virulence factors (Juhas *et al.* 2005, Winzer and Williams 2001). In various animal infection models, such as burn wound infections (Rumbaugh *et al.* 1999) and chronic lung infections (Wu *et al.* 2001), QS-deficient isolates have displayed reduced virulence as compared to the QS-proficient parent strain. These studies suggest that QS gives *P. aeruginosa* isolates a competitive advantage during pathogenesis. However, though QS is required for virulence, many *P. aeruginosa* QS-deficient isolates have been obtained from clinical and environmental populations (Table 1.1). These isolates, which display a variety of QS phenotypes, appear to be

Table 1.1: QS-deficient *P. aeruginosa* from infections and the environment

QS mutation	Number of isolates	Origin of isolates	References
lasR	68	Wound, trachaea, cystic fibrosis lung, pneumonia, environment, bacteremia	(Cabrol *et al.* 2003, Denervaud *et al.* 2004, Hamood *et al.* 1996, Heurlier *et al.* 2005, Hoffman *et al.* 2009, Salunkhe *et al.* 2005, Wilder *et al.* 2009)
lasR lasI	2	Cornea	(Zhu *et al.* 2002, Zhu *et al.* 2004)
lasR rhlR	4	Wound, respiratory tract	(Schaber *et al.* 2004)
rhlR	1	Urinary tract	(Sokurenko *et al.* 2001)
rhlI	1	Cystic fibrosis lung	(Wilder *et al.* 2009)
lasI rhlI	3	Cystic fibrosis lung	(Wilder *et al.* 2009)
pqsR	0	N/A[a]	N/A

[a]N/A indicates that the information is not applicable; the information is not known

predominantly subject to mutations in QS regulator genes (signal-blind), rather than in genes required for autoinducer synthesis (signal-negative). The signal-negative phenotype may be less prevalent because the loss of autoinducer production can be compensated by coexisting autoinducer-proficient cells whereas the signal-blind phenotype directly inactivates QS machinery regardless of the presence of QS-proficient cells (Heurlier *et al.* 2006).

The emergence and maintenance of *lasR* mutants may be due to a selective, intrinsic advantage over the QS-proficient wild type strain. For example, when grown aerobically in unbuffered, rich media, *lasR* mutants have a selective advantage over the wild type in that they are more resistant to cell lysis and death during stationary phase at alkaline conditions (Heurlier *et al.* 2005). Additionally, in nutrient-rich

environments, such as within the CF lung, *lasR* mutants are predicted to emerge due to the confluence of selective forces found in this environment. For example, the abundance of nitrate and nitrite (Palmer *et al.* 2007) and low oxygen concentrations (Worlitzsch *et al.* 2002) provide optimal conditions for the selection of *lasR* mutants. This was further observed *in vitro* where *lasR* mutants, but not the wild type, confer a growth advantage on certain carbon and nitrogen sources, including amino acids (D'Argenio *et al.* 2007). Lastly, *lasR* mutants may arise within the CF lung due to increased resistance to tobramycin and ciprofloxacin, two of the antibiotics used most frequently in CF treatment (Hoffman *et al.* 2010). This antibiotic resistance can be attributed to a metabolic shift associated with the *lasR* mutant, resulting in increased nitrate utilization and decreased use of oxygen (Hoffman *et al.* 2010). Both classes of antibiotics, aminoglycosides and fluoroquinolones, respectively, rely on the production of reactive oxygen species (ROS) to promote bacterial killing. However, decreased utilization of oxygen as well as increased resistance to ROS demonstrated by *lasR* mutants leaves them less susceptible to these antibiotics (Hoffman *et al.* 2010).

Another process by which *lasR* mutants can arise is through exploitation of cooperative behaviors. In microorganisms, cooperative behaviors often include the production of public goods, such as the synthesis of extracellular proteases, siderophores, and exopolysaccharides (Keller and Surette 2006). Evolutionary theory suggests that these social behaviors, including communication (i.e. QS), are subject to exploitation by social cheaters, who gain the benefit of cooperation without sharing in

the cost (West *et al.* 2006). The phenomenon of social cheating has been shown to arise in several microbial systems (Ennis *et al.* 2000, Greig and Travisano 2004, Velicer *et al.* 2000). The bacterium *Myxococcus xanthus*, for example, uses cooperative behavior to aggregate cells into spore-producing fruiting bodies during starvation. This social behavior was found to be exploited by evolved cell lines that were deficient in fruiting body development, yet out-competed the developmentally proficient wild type in the production of spores (Velicer *et al.* 2000). In *P. aeruginosa*, social cheating has been demonstrated in reference to the production of siderophores, an extracellular iron scavenging molecule (Griffin *et al.* 2004). In mixed populations, strains that did not produce siderophores out-competed proficient wild type strains under iron starvation conditions. In addition, it was found that the extent of cheating varied with the quantity of siderophore production, where isolates that produced little to no siderophores were better cheaters than those that produced many (Jiricny *et al.* 2010).

In QS, communication is required to coordinate other cooperative behaviors; thus it is subject to social exploitation (Diggle *et al.* 2007b). In general, QS populations are at risk for invasion by social cheaters that either do not produce autoinducers or are deficient in the production of QS regulators. As described above, it is predicted that signal-blind isolates, such as *P. aeruginosa lasR* mutants, are more likely to arise due to higher metabolic costs associated with gene regulation (Heurlier *et al.* 2006). Recent experimental evidence by us and others has confirmed these predictions, demonstrating that the *lasR* mutant is a social cheater (Diggle *et al.*

2007b, Rumbaugh *et al.* 2009, Sandoz *et al.* 2007). During *in vitro* conditions that require QS for growth, *lasR* mutants emerge over the course of evolution (Sandoz *et al.* 2007). Additionally, these *lasR* mutants exhibit negative frequency-dependent relative fitness within wild-type co-culture *in vitro* and *in vivo*, indicating that they have higher fitness when rare (Diggle *et al.* 2007b, Rumbaugh *et al.* 2009).

For the current study, we sought to further understand the social behavior associated with the *lasR* mutant, and compare this behavior to that of the *rhlR* and *pqsR* mutants *in vitro*. Additionally, we sought to understand whether social conflict plays a role in shaping the QS diversity of *P. aeruginosa* populations. Generally, the following chapters investigate the complex social behaviors of *P. aeruginosa* signal-blind isolates as well as the contribution of these behaviors to QS diversity in both environmental and clinical populations.

Cooperation and cheating in *Pseudomonas aeruginosa*: The roles of the *las, rhl,* and *pqs* quorum-sensing systems

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Abstract

Pseudomonas aeruginosa coordinates the transcription of hundreds of genes, including many virulence genes, through three hierarchically-arranged quorum-sensing (QS) systems, *las*, *rhl*, and *pqs*. Each system consists of genes involved in autoinducer synthesis, *lasI*, *rhlI*, and *pqsABCDH*, as well as cognate regulatory genes *lasR*, *rhlR*, and *pqsR*. In this study, we analyzed the social behavior of signal-blind (Δ*lasR*, Δ*rhlR*, Δ*pqsR*) and signal-negative (Δ*lasI*, Δ*rhlI*, Δ*pqsA*) mutants from each QS system. Because each system controls extracellular common goods but differs in the extent of regulatory control, we hypothesized that all signal-blind mutants can behave as cheaters that vary in their ability to invade a QS-proficient population. We found that *lasR* and *pqsR* but not *rhlR* mutants evolve from a wild-type ancestor *in vitro* under conditions that favor QS. Accordingly, defined *lasR* and *pqsR* mutants enriched in wild-type co-culture, whereas *rhlR* and all signal-negative mutants did not. Both *lasR* and *pqsR* mutants enriched with negative frequency-dependence, suggesting social interactions with the wild-type, although the *pqsR* mutant also grew well on its own. Taken together, the *lasR* mutant behaved as a typical cheater, as previously reported. The *pqsR* and *rhlR* mutants, however, exhibited more complex behaviors that can be sufficiently explained by positive and negative pleiotropic effects through differential regulation of *pqs* gene expression in the interconnected QS network. The evolutionary approach taken here may account for the prevalence of naturally occurring QS mutants.

Introduction

Cooperation can be defined as a behavior that benefits another individual and which is maintained because of its beneficial effect on the recipient (West *et al.* 2006, West *et al.* 2007). In microbes, cooperation generally involves the production of public goods in the form of extracellular products (e.g. toxins, proteases, surfactants and siderophores) that are expensive to produce for the individual but benefit all members of the local group (Keller and Surette 2006, West *et al.* 2006). Behaviors that involve such public goods include foraging, virulence, and biofilm formation. In bacteria, cooperative behaviors are often regulated by quorum sensing (QS), a cell-density-dependent communication system mediated by small signaling molecules (Ng and Bassler 2009, Williams *et al.* 2007).

Pseudomonas aeruginosa is a model organism used to study QS (Juhas *et al.* 2005, Schuster and Greenberg 2006). It is known for its ability to cause disease in immunocompromised individuals, including those suffering from cystic fibrosis. *P. aeruginosa* uses QS to regulate the transcription of hundreds of genes, many of which encode extracellular virulence factors (Hentzer *et al.* 2003, Schuster *et al.* 2003, Wagner *et al.* 2003). There are three known QS systems in *P. aeruginosa*, *las*, *rhl*, and *pqs*. These systems are arranged hierarchically with the *las* system positively regulating both the *rhl* (Latifi *et al.* 1996, Pesci *et al.* 1997) and *pqs* (Wade *et al.* 2005) systems. Under certain conditions, however, the *rhl* and *pqs* systems can also be activated in the absence of *las* QS (Dekimpe and Deziel 2009, Diggle *et al.* 2003, Medina *et al.* 2003). In addition, the *rhl* system negatively regulates the *pqs* system

(McGrath *et al.* 2004, Wade *et al.* 2005). These three QS systems are each comprised of a diffusible autoinducer signaling molecule and a regulator protein. When population density is high, the autoinducer binds to its cognate receptor to form a complex that regulates gene transcription. In the *las* and *rhl* system, autoinducer synthases LasI and RhlI produce the acyl-homoserine lactone (acyl-HSL) signals *N*-(3-oxododecanoyl)-HSL (3OC12-HSL) and *N*-butyryl-HSL (C4-HSL), respectively. These signals interact with their cognate receptors LasR and RhlR, respectively (Juhas *et al.* 2005, Schuster and Greenberg 2006). Transcriptome analysis revealed that these two systems together regulate 315 genes, with the *rhl* system regulating a subset of approximately 112 genes (≥ 2-fold activation by both signals versus 3OC12-HSL alone) (Schuster *et al.* 2003).

In the *pqs* system, the products of *pqsABCD* and *pqsH* are involved in the synthesis of the autoinducer 2-heptyl-3-hydroxy-4-quinolone (PQS) and other classes of alkyl quinolones (AQ) (Deziel *et al.* 2005); PQS binds to its cognate LysR-type receptor PqsR with high affinity (Wade *et al.* 2005, Xiao *et al.* 2006). The *pqs* system controls 141 genes, most of which are co-regulated by acyl-HSL QS, primarily the *rhl* system (Deziel *et al.* 2005).

Evolutionary theory predicts that the cost of performing a cooperative behavior leaves a population vulnerable to social cheating (Lehmann and Keller 2006, West *et al.* 2006). Cheaters are individuals that cease (or reduce) the production of public goods and benefit from the cooperative actions of others. QS populations are therefore at risk of invasion by either signal-negative cheaters that do not produce

signals or by signal-blind cheaters that avoid production of QS-controlled extracellular factors. In *P. aeruginosa*, the latter is predicted to be more favorable (Diggle *et al.* 2007b, Sandoz *et al.* 2007) because QS controls the expression of hundreds of genes. Signal-negative strains would not avoid expression of QS target genes as they would be triggered by the autoinducers produced by surrounding wild-type cells. Consistent with this prediction, *P. aeruginosa* strains with mutations in *lasR* (signal-blind) have been predominately isolated from infections (D'Argenio *et al.* 2007, Fothergill *et al.* 2007, Heurlier *et al.* 2006, Hoffman *et al.* 2009, Kohler *et al.* 2009, Smith *et al.* 2006, Tingpej *et al.* 2007), and emerge during *in vitro* evolution (Sandoz *et al.* 2007). Other experimental studies have shown that both *lasR* and *lasI* (signal-negative) mutants enrich *in vitro* (Diggle *et al.* 2007b) and in a mouse infection model (Rumbaugh *et al.* 2009). These mutants have a growth advantage over wild-type cells; however, they exhibit negative frequency-dependent relative fitness because as their proportion increases in a population, there are less wild-type cells to exploit. Taken together, these results are indicative of *las* mutants arising as social cheaters.

Initial social cheating studies focused on the *las* system because of its dominant position within the QS circuitry. In the present study, we investigated the social behavior of signal-blind (*rhlR*, *pqsR*) and signal-negative (*rhlI*, *pqsA*) isolates from the *rhl* and *pqs* systems and compared them to *lasR* and *lasI* mutant strains. These experiments were performed under defined conditions, in growth medium requiring and not requiring QS-regulated extracellular proteases, resembling “social” and “non-social” conditions, respectively. While the common goods relevant to

cheating are exoproteases supplied by the wild-type cooperator (Diggle *et al*. 2007a), variation in the relative fitness among mutant strains is expected to result from differences in the number and expression levels of all the genes controlled by each system. We tested the hypothesis that both the *pqsR* and *rhlR* signal-blind mutants, in addition to the *lasR* mutant, would behave as cheaters under social conditions. In the simplest case, one might expect that *rhlR* and *pqsR* mutants invade wild-type populations less than *lasR* mutants because *rhlR* and *pqsR* only control subsets of the QS regulon, although other outcomes are conceivable given the complexity of the QS network, including the conditionality of the regulatory hierarchy. Lastly, we tested the hypothesis that the *pqsA* and *rhlI* mutants would cheat less or not at all compared with the respective *pqsR* and *rhlR* mutants.

Materials and methods

Bacterial strains, plasmids and culturing conditions

Bacterial strains and plasmids are shown in Table 2.1. For general liquid culture, we used Lennox LB broth buffered with 50 mM 3-(N-morpholino)-propanesulfonic acid (MOPS), pH = 7.0. For QS assays we used M9 minimal medium containing either 1% caseinate or 0.5% casamino acids (CAA) as the sole carbon source (Sandoz *et al*. 2007), designated M9-caseinate ("QS medium") or M9-CAA, respectively. All experiments were performed with true biological replicates on different days with different inocula. Further details are described in Supplemental materials and methods.

Table 2.1: Bacterial strains and plasmids

Lab strain or plasmid	Relevant property	Reference or origin
Strain		
PAO1	*P. aeruginosa* wild-type (Vasil/Ochsner)	(Holloway *et al.* 1979)
PAO1 *lasR*	PAO1 derivative; Δ*lasR*, unmarked in-frame deletion from amino acid 102 to 216	This study
PAO1 *rhlR*	PAO1 derivative; Δ*rhlR*, unmarked in-frame deletion from amino acid 102 to 225	This study
PAO1 *pqsR*	PAO1 derivative; Δ*pqsR*, unmarked in-frame deletion from amino acid 19 to 252.	This study
PAO1 *lasI*	PAO1 derivative; Δ*lasI*, unmarked in-frame deletion from amino acid 12 to 176.	This study
PAO1 *rhlI*	PAO1 derivative; Δ*rhlI*, unmarked in-frame deletion from amino acid 13 to 185.	This study
PAO1 *pqsA*	PAO1 derivative; Δ*pqsA*, unmarked in-frame deletion from amino acid 32 to 419.	This study
PAO1 Δ*pqsA* CTX-*lux::pqsA*	AQ biosensor strain; chromosomal deletion of the *pqsA* gene in PAO1 containing mini CTX-*lux*::*pqsA*	(Diggle *et al.* 2007b)
Plasmids		
pJN105	*araC*-pBAD cloned in pBBR1 MCS-5; Gm^R	(Newman and Fuqua 1999)
pJN105.*pqsR*-H	*pqsR* coding region in pJN105	This study
pJN105.*pqsR*-N	*pqsR* coding region and upstream regulatory region in pJN105 devoid of *araC*-pBAD	This study
pJN105.*lasR*-H	*lasR* coding region in pJN105	(Lee *et al.* 2006)
pJN105.*lasR*-N	*lasR* coding region and upstream regulatory region in pJN105 devoid of *araC*-pBAD.	This study
pJN105.*rhlR*-H	*rhlR* coding region in pJN105	(Schuster and Greenberg 2007)

Continued on next page

Table 2.1-*Continued*

Lab strain or plasmid	Relevant property	Reference or origin
pJN105.*rhlR*-N	*rhlR* coding region and upstream regulatory region in pJN105 devoid of *araC*-pBAD	This study
pEX18Tc	Tc^R; *oriT*, *sacB*, gene replacement vector with MCS from pUC18	(Hoang *et al.* 1998)
pEX18Tc.Δ*pqsR*	pEX18Tc with the *pqsR* gene containing an in frame deletion from residue 19 to 252.	This study
pEX18Tc.Δ*lasR*	pEX18Tc with the *lasR* gene containing an in frame deletion from residue 102 to 216	This study
pEX18Tc.Δ*rhlR*	pEX18Tc with the *rhlR* gene containing an in frame deletion from residue 102 to 225	This study
pEX18Tc.Δ*pqsA*	pEX18Tc with the *pqsA* gene containing an in frame deletion from residue 32 to 419.	This study
pEX18Tc.Δ*lasI*	pEX18Tc with the *lasI* gene containing an in frame deletion from residue 12 to 176.	This study
pEX18Tc.Δ*rhlI*	pEX18Tc with the *rhlI* gene containing an in frame deletion from residue 13 to 185.	This study
pUC18T-mini-Tn7T-Tp	Broad host range mini-Tn7 vector with Tp resistance gene cassette.	(Choi and Schweizer 2006)
pUC18R6KT-mini-Tn7T-Tet	Broad host range mini-Tn7 vector with Tc resistance gene cassette.	Courtesy of Herbert P. Schweizer

In vitro evolution and analysis of QS mutant phenotypes

In vitro evolution of the PAO1 wild-type in liquid batch cultures containing M9-caseinate medium was performed as previously described (Sandoz *et al*. 2007). Culture aliquots were removed after days 4, 8, 12, 16 and 20 of culturing. To determine the colony forming units (CFU)/ml as well as the types of mutants arising, aliquots were appropriately diluted in 1xM9 salts and subsequently plated onto LB agar plates. For 92 colonies from this plating, along with a PAO1 positive control and

appropriate isogenic negative controls (Table 2.1), we determined growth on adenosine as the sole C-source (Heurlier *et al*. 2005, Sandoz *et al*. 2007), proteolysis on skim-milk plates (Sandoz *et al*. 2007), rhamnolipid production on methylene blue plates (Kohler *et al*. 2000), and AQ production by bioassay (Fletcher *et al*. 2007), using a 96-well microplate format. The AQ bioassay detects both PQS and its precursor 2-heptyl-4-quinolone (HHQ). AQ levels were normalized to the OD_{600} of the respective cultures at the time of harvest.

Results were scored as follows: For AQ production < 20% of the wild-type was considered negative. For adenosine plates, growth was scored as positive, while the absence of growth was scored as negative. For skim-milk proteolysis and rhamnolipid production, the formation of a halo similar to the PAO1 control was

Table 2.2: Analysis of *P. aeruginosa* QS mutant phenotypes

Strain	Skim milk[a]	Rhamnolipid[a]	Adenosine[b]	AQ[c]
Wild-type	+	+	+	100 ± 12
lasR	-	+	-	43 ± 21
lasI	-	+	-	24 ± 11
rhlR	+	-	+	230 ± 21
rhlI	+	+	+	310 ± 60
pqsR	+	+	+	6.7 ± 2.0
pqsA	+	+	+	0.99 ± 1.8

[a] "+" indicates presence and "-" indicates absence of a halo on indicator plates.
[b] "+" indicates growth and "-" indicates no growth.
[c] Percent of the wild-type.

scored as positive and the absence of a halo was scored as negative. These assays were performed in the same manner with all the control strains shown in Table 2.2.

Single culture and co-culture growth assays

The PAO1 wild-type and the respective antibiotic-tagged mutant strains were grown in M9-CAA or M9-caseinate media, in single or co-culture. Both media were inoculated at a starting OD_{600} of 0.015 with a single strain or appropriate mixtures from individual 18 h LB-MOPS overnight cultures. For single-culture growth assays, the CFU/ml of each culture was determined at 0, 2, 5, 8, 12, 18, 24, and 30 h after inoculation. For the co-culturing experiments, the M9-CAA and M9-caseinate cultures were harvested after 12 h and 24 h, respectively. The number of mutant cells tagged with antibiotic resistance gene cassettes and the total number of cells in co-culture were distinguished by plating on media with and without the corresponding antibiotic, respectively. Further details are described in Supplemental materials and methods.

The relative fitness (v) of each mutant in co-culture was calculated first by comparing its initial and final frequency over the duration of the experiment (Diggle *et al*. 2007b, Ross-Gillespie *et al*. 2007a). Specifically, $v = [x_1(1-x_0)]/[x_0(1-x_1)]$, where x_0 and x_1 are the initial and final mutant frequencies, respectively. The values of v therefore signifies whether mutants increase in frequency ($v > 1$), decrease in frequency ($v < 1$) or remain at the same frequency ($v = 1$) over the duration of the experiment. We also calculated an alternative measure of relative fitness (w), namely

the ratio of Malthusian growth parameters, which is essentially the ratio of the number of doublings by the mutant and wild-type populations (Lenski 1991). Here, $w = \ln(X_1/X_0)/\ln(Y_1/Y_0)$, where X_0 and X_1 are the initial and final mutant CFU/ml, and Y_0 and Y_1 are the initial and final wild-type CFU/ml, respectively.

Complementation analysis, DNA sequencing, and FITC casein assay

These procedures are described in Supplemental materials and methods.

Results

Both lasR and pqsR mutants arise during in vitro evolution, but rhlR mutants do not

Current evidence suggests that *lasR* mutants are social cheaters that cease production of QS factors and take advantage of their production by the surrounding cooperative wild-type population (Diggle *et al*. 2007b, Rumbaugh *et al*. 2009, Sandoz *et al*. 2007). The main objective of the present study was to further analyze the social behavior of signal-blind and signal-negative mutants from the two other major QS systems in *P. aeruginosa*, *rhl* and *pqs*. We previously showed that *lasR* mutants emerge during *in vitro* evolution in defined medium that favors QS (Sandoz *et al*. 2007), but we did not investigate the emergence of other QS mutants. For a more comprehensive assessment, we repeated this long-term growth experiment and screened for mutants in all three QS systems. We grew the PAO1 wild-type in M9-caseinate medium for twenty 24h-cycles, sub-culturing after each cycle into fresh medium. Growth in this medium requires the production of QS-dependent proteases such as LasB elastase, and

thus cooperative behavior (Sandoz *et al*. 2007, Van Delden *et al*. 1998). Under standard growth conditions (LB liquid culture), *las*, *rhl* and *pqs* mutants all show reduced expression of LasB elastase (Brint and Ohman 1995, Deziel *et al*. 2005, Diggle *et al*. 2003, Pearson *et al*. 1997, Schuster *et al*. 2003).

We employed a screening scheme for distinct QS phenotypes that allowed us to distinguish between the different *las, rhl*, and *pqs*-deficient variants (Table 2.2). After eight days of *in vitro* evolution we observed the emergence of AQ-deficient isolates. By day 12, isolates with other QS deficiencies arose, some with deficiencies for more than one phenotype (Figure 2.1, Table S2.2). Our previous work had shown

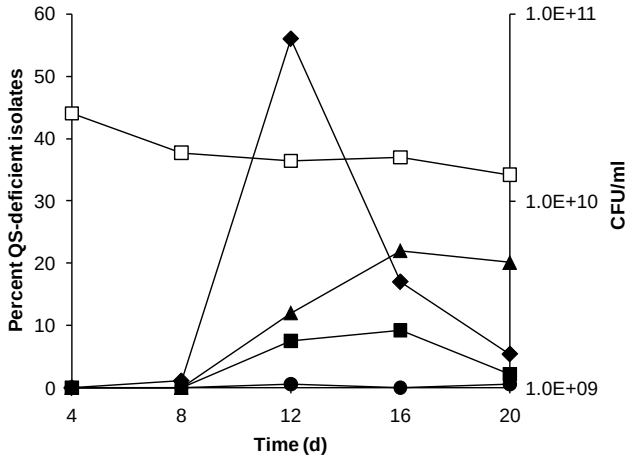

Figure 2.1: Emergence of QS-deficient isolates during *in vitro* evolution of the *P. aeruginosa* wild-type. Deficiencies in skim milk proteolysis (solid squares), growth on adenosine (solid triangles), rhamnolipid production (solid circles), and AQ production (solid diamonds) are shown. CFU/ml (open squares) are indicated on the right y-axis. Skim milk proteolysis and growth on adenosine are *las*-dependent, rhamnolipid production is *rhl*-dependent, and AQ production is *pqs*-dependent. The discrepancy in the number of adenosine and protease-negative isolates is due to the fact that some *lasR* mutants regain the ability to degrade skim milk (Sandoz *et al*. 2007). Data are averages of two independent *in vitro* evolution experiments.

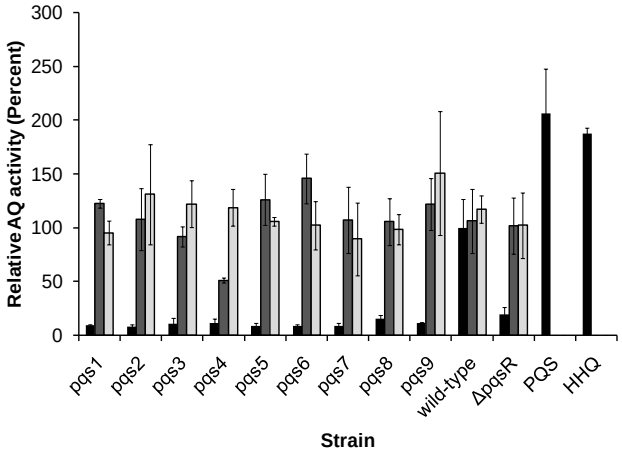

Figure 2.2: Complementation analysis of AQ deficient *in vitro* evolution isolates. AQ production of *P. aeruginosa* AQ-deficient variants containing pJN105 (black), pJN105.*pqsR*-H controlling *pqsR* from a heterologous promoter (dark grey), or pJN105.*pqsR*-N controlling *pqsR* from the native *P. aeruginosa* promoter (light grey). "PQS" and "HHQ" are synthetic signal controls. Data is given as percent of PAO1/pJN105. Error bars indicate standard deviation of the mean of three replicates.

that all isolates that did not grow on adenosine were *lasR* mutants (Sandoz *et al*. 2007). We therefore did not further analyze candidate *lasR* mutant isolates from this *in vitro* evolution study.

Of the AQ-deficient isolates that emerged, nine were selected for complementation and designated *pqs1* to *pqs9*. We transformed each isolate with plasmids containing *pqsR* expressed either from its native promoter (pJN105.*pqsR*-N) or from the heterologous pBAD-*araC* promoter (pJN105.*pqsR*-H). Each isolate showed complementation with both the heterologous and the native promoter, indicating that the mutation resides in the *pqsR* coding or upstream regulatory region (Figure 2.2). Indeed, sequencing analysis revealed that seven isolates harbor an insertion, deletion, or single nucleotide change within the *pqsR* gene (Table 2.3).

Table 2.3: *pqsR* sequence analysis of selected AQ-deficient *in vitro* evolution isolates

Isolate	Non-synonymous mutation[a]	Protein sequence change
pqs1	A→C (+236)	Q79P
pqs2	Δ (+170 to +183)	Non-sense
pqs3	G→A (+672)	M224I
pqs4	None	None
pqs5	TCGGCGGTCAGC (+108)	SAVS in-frame insertion
pqs6	Δ (+703 to +704)	Non-sense
pqs7	Δ (+108 to +119)	SAVS in-frame deletion
pqs8	None	None
pqs9	G→A (+784)	G262S

[a]Nucleotide substitution, deletion (Δ) or insertion at the indicated position relative to the translational start site of the *P. aeruginosa* PAO1 *pqsR* gene.

The *in vitro* evolution experiment revealed only two rhamnolipid-deficient isolates (Figure 2.1), designated *rhl1* and *rhl2*. Both isolates were transformed with plasmids containing *rhlR* expressed from the pBAD-*araC* promoter (pJN105.*rhlR*-H) or the native promoter (pJN105.*rhlR*-N) and were tested for complementation on rhamnolipid detection plates. Both isolates could not be complemented, nor were pyocyanin levels in *rhl2* similar to a defined *rhlR* mutant (Table 2.4). Sequencing analysis of the *rhlR* gene from both rhamnolipid deficient isolates further confirmed that these isolates are not *rhlR* mutants (Table 2.4). We did not test these isolates for

mutations in *rhlI* because this type of mutation does not cause a deficiency in rhamnolipid production (Table 2.2).

The pqsR mutant, but not the lasR and rhlR mutants, grows well in QS media due to protease production

The emergence of *lasR* and *pqsR* mutants during *in vitro* evolution indicates that these mutant strains have a growth advantage compared with the PAO1 wild-type. The lack of *rhlR* mutants, on the other hand, suggests that they do not have a growth advantage. To begin to test these predictions, we first grew defined signal-blind deletion mutants individually in growth media requiring (M9-caseinate) and not requiring QS (M9-CAA). In M9-caseinate, both the *lasR* and *rhlR* mutants grew to a maximum density about 30-40 fold less than that of the PAO1 wild-type after 24 h, indicating a severe

Table 2.4: Analysis of rhamnolipid-deficient *in vitro* evolution isolates of *P. aeruginosa*

Isolate	Rhamnolipid complementation[a]			Pyocyanin production[b]	*rhlR* non-synonymous mutation
	pJN105	pJN105.*rhlR*-H	pJN105.*rhlR*-N		
Wild-type	+	+	+	100 ± 12	N/A[c]
rhlR	-	+	+	3.6 ± 3.8	N/A
rhl1	-	-	-	2.9 ± 2.6	None
rhl2	-	-	-	160 ± 35	None

[a] "+" indicates presence and "-" indicates absence of a halo on indicator plates.
[b] Percent of the wild-type.
[c] Not applicable, lab strains were not sequenced.

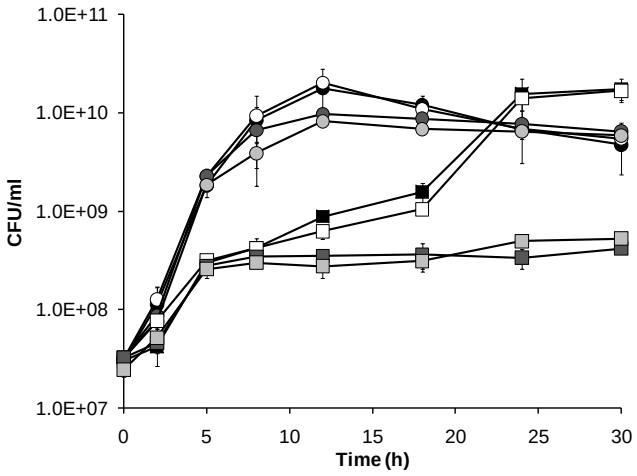

Figure 2.3: Single-culture-growth of signal-blind *P. aeruginosa* strains. Growth of the wild type (black), *lasR* (dark grey), *rhlR* (light grey), and *pqsR* (open) strains in M9-CAA and in M9-caseinate media (circle and square symbols, respectively). Error bars indicate standard deviation of the mean of three replicates, and are too small to be visible in some cases.

growth deficiency (Figure 2.3). This is consistent with the fact that both *lasR* and *rhlR* mutants are impaired in the production of proteases (Brint and Ohman 1995, Pearson *et al*. 1997, Schuster *et al*. 2003). Both mutants did exhibit some growth during the first 5 h of incubation, which might be attributed to the presence of impurities (amino acids, oligopeptides) in the caseinate stock used to prepare the M9 medium. Interestingly, the *pqsR* mutant strain grew at a similar rate as the wild-type over the course of 24 h, indicating that the *pqsR* mutant has high intrinsic fitness in QS medium (Figure 2.3). All strains grew similarly in M9-CAA.

To further examine the unexpected growth of the *pqsR* mutant, we used a FITC-casein assay to quantify protease production of each signal-blind strain and the

PAO1 wild-type (Figure 2.4). This assay contains the same protein source as our growth medium. After 12 h of growth in M9-CAA, all signal-blind mutants produced significantly less proteases as compared to the wild-type, consistent with previous findings (Brint and Ohman 1995, Deziel *et al*. 2005, Diggle *et al*. 2003, Pearson *et al*. 1997, Schuster *et al*. 2003). After 24 h of growth in M9-caseinate, the *pqsR* mutant showed caseinolytic activity that was indistinguishable from the wild-type, whereas *lasR* and *rhlR* mutants showed much lower activity. Thus, only the *las* and *rhl* systems but not the *pqs* system is required for the production of proteases to digest caseinate in QS medium.

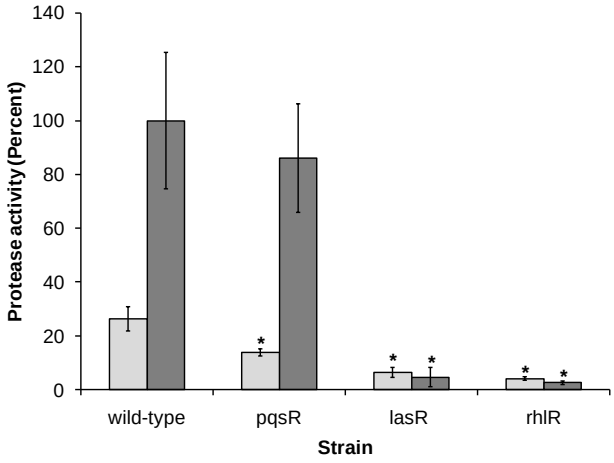

Figure 2.4: Caseinolytic activity of signal-blind *P. aeruginosa* strains. Degradation of FITC-casein by the indicated strains grown in either M9-CAA (light grey bars) or M9-caseinate (dark grey bars). Data are given as percent of the wild-type grown in M9-caseinate. Error bars indicate standard deviation of the mean of three replicates. Statistical significance of the data was determined using a two-tailed unpaired *t* test with "*" indicating *P* values of <0.05. Protease production of each mutant was compared to that of the wild type in the respective media.

In addition, it is worth noting differences in protease production when bacteria are grown in solid versus liquid media. This is most apparent with the *rhlR* mutant, which showed high caseinolytic activity on plates (Table 2.2) but low activity in liquid culture (Figure 2.4). This difference may be due to up-regulation of *lasR* or *lasI* on solid media, permitting enhanced exoprotease expression that would otherwise require both *las* and *rhl* QS.

In QS co-culture, both lasR and pqsR mutants have a growth advantage over the wild-type cooperator, but rhlR mutants do not

To determine each mutant's relative fitness when rare in a population, we co-cultured each defined mutant with the wild-type parent in M9-CAA and M9-caseinate at an initial mutant frequency of approximately 1%. Each mutant contained an antibiotic resistance cassette at a neutral chromosomal site to distinguish it from the wild-type by plating on selective media. The growth rates of these tagged strains were indistinguishable from those of the unmarked parent strains (data not shown). After 24 h of growth in M9-caseinate, the *lasR* and *pqsR* mutants enriched (Figures 2.5A and 2.5B), but the *rhlR* mutant did not (Figure 2.5C). To confirm that the *rhlR* mutant does not exhibit a subtle growth advantage that cannot be captured after a single 24 h incubation period, we co-cultured an unmarked *rhlR* mutant with the wild type for three consecutive 24 h cycles. This *rhlR* mutant also did not enrich (data not shown). The three signal-negative mutants, *lasI*, *pqsA*, and *rhlI* showed little to no enrichment in M9-caseinate (Figures 2.5A-C). The same held true for all signal-negative and

Figure 2.5: Enrichment of defined QS mutant strains in co-culture. Cultures were grown in M9-CAA (light grey bars) and M9-caseinate (dark grey bars) media with initial mutant frequencies of approximately 1%. Values above each bar indicate fold-enrichment (the ratio of final frequency versus initial frequency). (A) *lasR* or *lasI* mutants in wild-type co-culture. (B) *pqsR* or *pqsA* mutants in wild-type co-culture. (C) *rhlR* or *rhlI* mutants in wild-type co-culture. (D) *lasR*, *pqsR*, and *rhlR* mutants combined (each at an initial frequency of 1%) in wild-type co-culture. (E) *lasR* or *rhlR* mutants (each at an initial frequency of 1%) in *pqsR* mutant co-culture. Error bars indicate standard deviation of the mean of three replicates. Statistical significance of the data was determined using a two-tailed unpaired *t* test, with "**" indicating *P* values of <0.05 and "*" indicating *P* values of <0.1. For each individual condition, the initial frequency was compared to the final frequency. For between-condition comparisons, fold-change was compared. For panels A-C, brackets indicate *p*-values from comparisons between growth in M9-caseinate versus M9-CAA as well as growth of the signal-blind versus signal-negative strains.

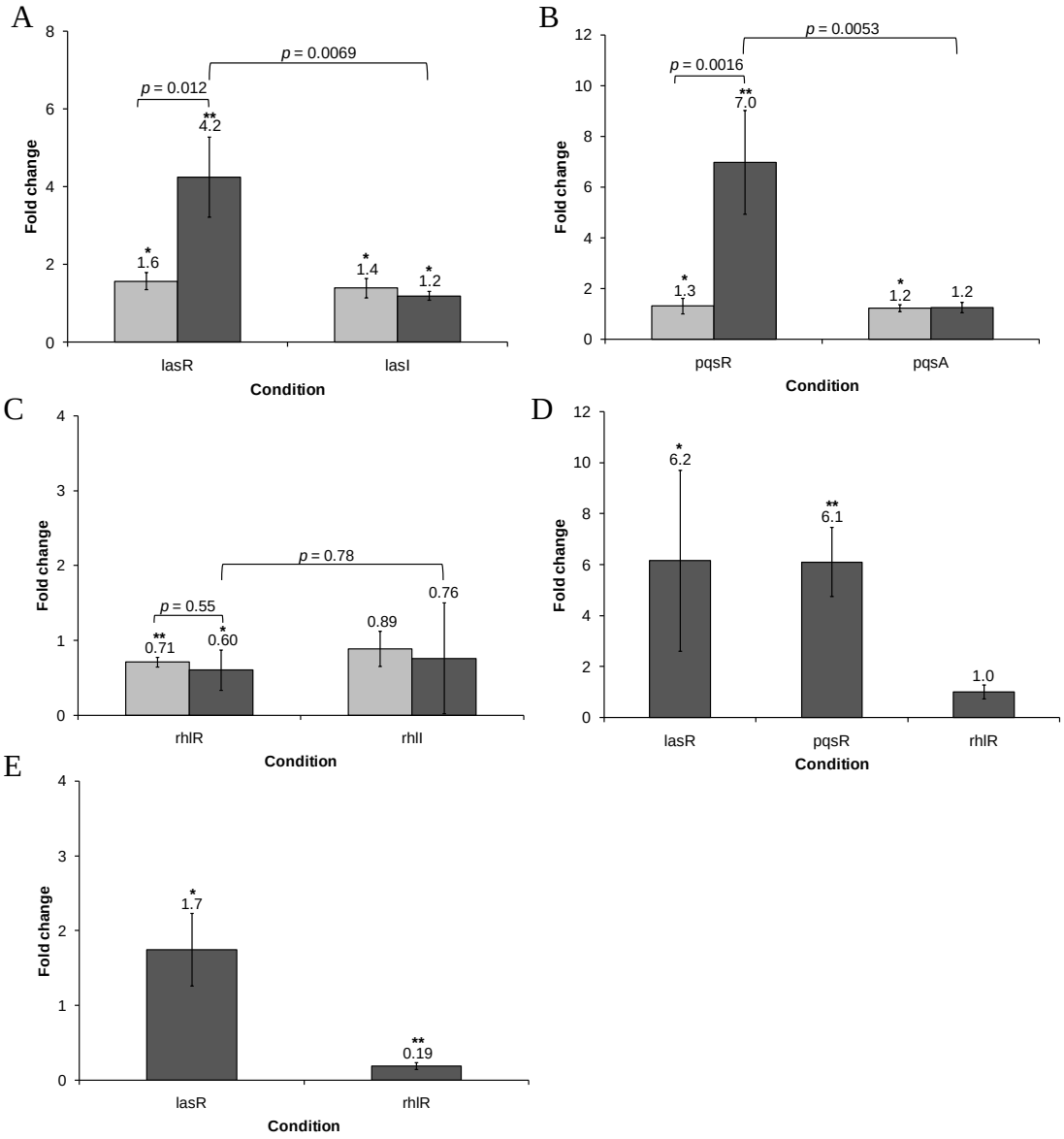

Figure 2.5. Enrichment of defined QS mutant strains in co-culture.

signal-blind mutants in M9-CAA. In addition, we analyzed the enrichment of all signal-blind strains together with the PAO1 parent in one single M9-caseinate co-culture, with initial mutant frequencies of 1% (Figure 2.5D). Interestingly, both the *lasR* and *pqsR* mutants enriched while the *rhlR* mutant did not. Overall, these results indicate that both *lasR* and *pqsR* have a growth advantage and can exploit the PAO1 wild-type under conditions that require QS. This fitness advantage is due to loss of signal reception, not signal production. In the following, we therefore focus on investigating the signal-blind variants in more detail.

In the analysis of our long-term evolution study, we not only observed a large increase in *pqsR* mutants on day 12 but also a large decrease by day 16, coinciding with an increase in *lasR* mutants. To assess whether this pattern could be due to exploitation of the *pqsR* mutant by the *lasR* mutant, we initiated a co-culture of both strains with a *lasR* mutant frequency of 1%. After 24 h of growth in M9-caseinate, the *lasR* mutant enriched almost 2-fold (Figure 2.5E). For comparison, the *rhlR* mutant showed no enrichment (Figure 2.5E). These results indicated that the *lasR* mutant has a modest growth advantage over the *pqsR* mutant and can invade both the *pqsR* mutant and wild-type populations.

The relative fitness of the signal-blind strains shows negative and positive frequency-dependence

To determine the frequency-dependent relative fitness of each signal-blind mutant, we

Figure 2.6: Frequency-dependent relative fitness of signal-blind strains. Relative fitness of the *lasR* mutant (A), the *pqsR* mutant (B), and the *rhlR* mutant (C and D) calculated as comparison of initial and final mutant frequency (*v*, left panels) or as the ratio of mutant and wild-type Malthusian growth parameters (*w*, right panels). Data in left panels A-C are on a double logarithmic scale and are fitted with either a power regression line (A, C) or an exponential regression line (B). Data in right panels A-C are on a semi-logarithmic scale and are fitted with either a logarithmic regression line (A, C) or an exponential regression line (B). Panels D include initial *rhlR* mutant frequencies of 90% and 99% (in addition to 1%, 10% and 50%) plotted on either a semi-logarithmic scale (left) or a linear scale (right), resulting in a unimodal regression. Goodness of fit is indicated by R^2. Fitness trends were considered significant by one-way ANOVA (using log-transformed values for *v*); *p*-values are indicated. Assays were done in quadruplicate.

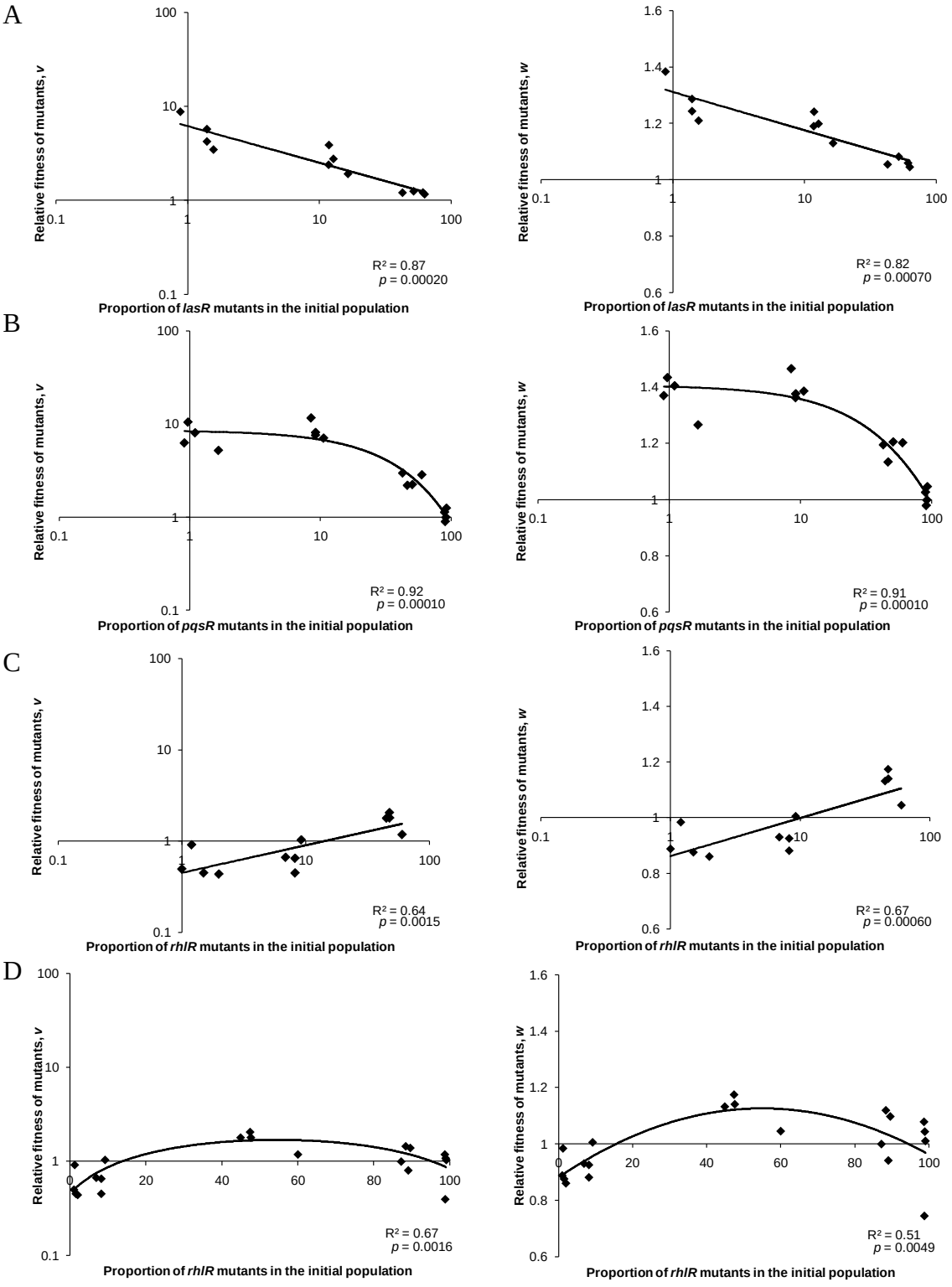

Figure 2.6: Frequency-dependent relative fitness of signal-blind strains.

co-cultured each mutant and its PAO1 parent in M9-caseinate for 24 h at starting mutant frequencies of approximately 10% and 50%, in addition to 1%. Social evolution theory predicts that the relative fitness of cheaters will decrease with increasing frequency in a population as there are less cooperators to exploit (MacLean and Gudelj 2006, Ross-Gillespie *et al*. 2007b). As previously observed, the *lasR* mutant exhibits negative frequency-dependence (Diggle *et al*. 2007b) (Figure 2.6A). Two different established fitness calculations based either on mutant frequency (Ross-Gillespie *et al*. 2007a) or relative growth rates (Lenski 1991) elicited similar trends. The *pqsR* mutant showed negative frequency-dependent fitness that it is much more pronounced at higher than at lower mutant frequencies, which we substantiated by an additional fitness measurement at 90% mutant frequency (Figure 2.6B). This was unexpected as the *pqsR* mutant displays high intrinsic fitness (Figure 2.3). In contrast, the *rhlR* mutant appeared to exhibit positive frequency-dependence (Figure 2.6C), indicating that its relative fitness increases with increasing abundance in a population (Molofsky *et al*. 2001). This result was equally surprising in light of the observation that the *rhlR* mutant displays low intrinsic fitness similar to the *lasR* mutant (Figure 2.3). We therefore further analyzed the fitness of the *rhlR* mutant in wild-type co-culture at even higher mutant frequencies of 90% and 99% (Figure 2.6D) and observed a decline to levels similar to those at low frequencies. Taken together, relative fitness of the *rhlR* mutant centered around 1 with modest enrichment only at intermediate frequency.

Discussion

To understand the forces that select for QS deficiency, we chose a reductionist approach that compares the relative fitness of individual QS mutants *in vitro* under growth conditions that do and do not require QS. In our previous long-term evolution study, we focused on the identification and characterization of *lasR* mutants (Sandoz *et al*. 2007). In this study, we extended our analysis to include the *rhl* and *pqs* systems. A screening scheme for distinct QS phenotypes (Table 2.2) allowed us to detect variants with mutations in all three QS systems. Interestingly, we identified *lasR* and *pqsR* but not *rhlR* mutants during *in vitro* evolution of *P. aeruginosa* under growth conditions that require QS (Figures 2.1-2, Tables 2.2-4). Of the nine complemented AQ-deficient variants, seven showed a mutation in *pqsR* (Table 2.3). Curiously, isolates *pqs4* and *pqs8* were not *pqsR* mutants even though complementation analysis indicated a mutation within the upstream regulatory region or coding region of *pqsR* (Figure 2.2). Of note, only *pqs4* is predicted to harbor an additional mutation in the *lasR* gene (data not shown); however, this mutation would not fully diminish AQ production (Table 2.2) and thus does not explain the AQ-deficient phenotype. The reason for this phenotype is not ultimately clear. It is possible that these isolates are unable to produce AQ due to a constitutively active *rhl* system, which would repress *pqsR* (Wade *et al*. 2005). Consequently, the introduction of multiple plasmid-borne copies of *pqsR* might be sufficient to overcome the repressive effect by *rhlR*.

We found a good correlation between our long-term evolution study and co-culturing results in QS medium. In both experimental approaches the *lasR* and *pqsR* but not *rhlR* mutant had a growth advantage over the wild-type. This was particularly apparent when we co-cultured the wild-type strain with all signal-blind isolates at once (Figure 2.5D). Indicative of social cheating and consistent with previous studies, our defined *lasR* mutant demonstrated substantial enrichment in wild-type co-culture (Figure 2.5A), had negative frequency-dependence (Figure 2.6A), and displayed a growth deficiency when grown individually (Figure 2.3) (Diggle *et al*. 2007b, Rumbaugh *et al*. 2009, Sandoz *et al*. 2007). The substantial fitness advantage of the *lasR* mutant in co-culture is probably a consequence of the energy saved from not expressing extracellular proteases resulting in cheating and from not expressing other dispensable *lasR*-controlled factors; if there is also a cost (e.g. a metabolic imbalance) associated with the lack of certain, probably intracellular, *lasR*-controlled factors, it is relatively minor. In contrast to growth in M9-caseinate, the *lasR* mutant showed no enrichment in M9-CAA because in this medium QS-controlled genes are only induced at low-levels upon cessation of growth (Sandoz *et al*. 2007), and therefore do not impose a high metabolic burden on the wild-type.

On the other hand, we found that our defined *lasI* mutant as well as other signal-negative strains showed virtually no enrichment in co-culture (Figures 2.5A-C). We had predicted that these signal-negative strains, in contrast to signal-blind strains, would not behave as social cheaters because the mutant phenotype can be compensated by surrounding autoinducer-producing cells, and because the cost of

producing acyl-HSL or AQ is negligible compared to the cost of synthesizing hundreds of proteins. However, in previous studies by Rumbaugh *et al*. and Diggle *et al*., *lasI* mutants displayed significantly higher relative fitness than the wild type, similar to that observed for *lasR* mutants (Diggle *et al.* 2007b, Rumbaugh *et al.* 2009). *In vivo* competition experiments by Rumbaugh *et al*. were performed using an animal model mimicking either a chronic or an acute burn wound (Rumbaugh *et al.* 2009). Diggle *et al*. used minimal media supplemented with 1% BSA as the sole carbon source and a starting inoculum that was approximately 20-fold higher than ours (Diggle *et al.* 2007b). It is plausible that in both studies, *lasI* mutant enrichment resulted from a combination of social cheating and other mechanisms. The latter may include an intrinsic growth advantage on some carbon and nitrogen sources (D'Argenio *et al.* 2007), for example, increased levels of free amino acids associated with severe thermal injury (Fong *et al.* 1991), as well as enhanced survival in alkaline conditions during prolonged stationary phase (Heurlier *et al.* 2005). These benefits, however, would only manifest in the absence of complementing acyl-HSL produced by wild type cells. It is conceivable that reduced mixing during infection limits interactions between acyl-HSL deficient and proficient cells, and that acyl-HSL levels are low in stationary phase cultures due to lactonolysis (Yates *et al.* 2002).

We initially predicted that all signal-blind isolates would be social cheaters that invade a wild-type population to varying degrees. While our work with the *lasR* mutant was consistent with previous studies and our hypothesis (Diggle *et al.* 2007b, Rumbaugh *et al.* 2009, Sandoz *et al.* 2007), the social behaviors of the *pqsR* and *rhlR*

mutants were more complex. The *pqsR* mutant enriched substantially in co-culture (Figure 2.5B) and showed overall negative frequency-dependence (Figure 2.6B), a pattern characteristic of social cheating. Individually, however, the *pqsR* mutant grew as well as the wild-type parent (Figure 2.3). The production of wild-type levels of protease accounts for its high intrinsic fitness (Figure 2.4), but does not explain its frequency-dependent dominance over the wild-type. We further determined that the enrichment of the *pqsR* mutant is due to the lack of AQ reception rather than the lack of AQ production (Figure 2.5B). We therefore propose that the fitness pattern of the *pqsR* mutant is a combination of the following: this strain saves energy as it does not express *pqsR*-dependent factors, including those that are under control of *pqsE* (Deziel *et al.* 2005, Rampioni *et al.* 2010). Most of these factors would be dispensable for growth. On the other hand, the loss of PQS production itself incurs a fitness cost through the loss of the involvement in the oxidative stress response (Bredenbruch *et al.* 2006, Diggle *et al.* 2007c, Haussler and Becker 2008). As an anti-oxidant, PQS decreases the oxidative stress induced by oxidation reactions through activity comparable to that of ascorbate (Haussler and Becker 2008). Therefore, when the *pqsR* mutant exceeds a certain frequency in co-culture, the quantity of PQS produced by the wild-type would be insufficient to provide adequate oxidative stress protection, resulting in decreased fitness. If the selective forces are similar *in vivo*, then *pqsR* mutants might abound in infections and other natural environments, although they would eventually be superseded by *lasR* mutants (Figures 2.1 and 2.5E). To our

knowledge, a systematic analysis on the prevalence of natural *pqs* mutants has not been published.

Similar to the *pqsR* mutant, the *rhlR* mutant did not conform to our prediction of social cheating. Although it displayed a similar individual growth pattern as the *lasR* mutant in QS medium, the *rhlR* mutant was unable to invade a wild-type population (Figures 2.3 and 2.5C). The non-invasive nature of the *rhlR* mutant could be due to a large cost associated with the up-regulation of *pqs*-dependent factors (those not also co-regulated by the *rhl* system) that out-weighs the benefits from not producing *rhl*-dependent factors (Table 2.2, Figure 2.4) (McGrath *et al.* 2004). Specifically, synthesis of PQS itself and transcription of *pqsA* have been shown to be up-regulated 6- and 7-fold, respectively, in *rhl* mutant strains (McGrath *et al.* 2004). Additionally, certain *rhl*-controlled factors might also be beneficial for the individual, and their lack might be contributing to an overall decrease in fitness.

Furthermore, the *rhlR* mutant displayed both positive and negative frequency-dependence with maximal fitness at intermediate frequency (Figure 2.6D). Such a pattern could result from a frequency-dependent effect of the wild type on the up-regulation of costly *pqs*-dependent products in the *rhlR* mutant, or, perhaps more intriguingly, from spiteful behavior of the *rhlR* mutant towards the wild-type. Spiteful behaviors, such as the production of bacteriocins, are costly to both the producer and the susceptible recipient. Consequently, they are predicted to evolve to maximal levels when the spiteful phenotype is at an intermediate frequency in the population (Inglis *et al.* 2009, West and Buckling 2003). If the behavior of the *rhlR* mutant was

indeed spiteful, it could be in relation to the up-regulation of PQS (McGrath *et al.* 2004). In addition to its beneficial effects at low concentration, PQS can be deleterious and promote autolysis when over-produced (D'Argenio *et al.* 2002, Haussler and Becker 2008). If the *rhlR* mutant were to gain an advantage through this process, the wild-type would have to be more sensitive to PQS-mediated killing than the *rhlR* mutant. Regardless of the mechanism, however, the positive frequency-dependence of the *rhlR* mutant when rare, together with its low relative fitness overall, may explain why it is not often found in nature.

Taken together, our data represent a comprehensive analysis of QS from an evolutionary point of view. They show that the inactivation of global regulators, as integral parts of an intricate regulatory network, can lead to complex social phenotypes. Pleiotropic effects likely play a role in modulating the behaviors of the *pqsR* and *rhlR* mutant strains.

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Supplemental materials and methods

Bacterial growth conditions

Antibiotics were used at the following concentrations: trimethoprim (Tp), 100 µg/ml; tetracycline (Tc), 100 µg/ml; gentamicin (Gm) 50 µg/ml. Synthetic signals were used at the following concentrations: 3OC12-HSL, 2 µM; C4-HSL, 10 µM; HHQ, 25 µM; PQS, 25 µM. To induce the pBAD-*araC* promoter on pJN105, we supplemented solid media with 50 mM arabinose and liquid media with 0.5 mM arabinose.

Strain construction

The *lasR*, *lasI*, *rhlR*, *rhlI*, *pqsR*, and *pqsA* in-frame deletion mutants were constructed in the *P. aeruginosa* PAO1 wild-type background using spicing-by-overlap-extension polymerase chain reaction (SOE-PCR) (Horton 1995) and gene replacement (Hoang *et al.* 1998, Schweizer and Hoang 1995) with the pEX18Tc plasmid (Table 2.1). Primers used to construct each gene deletion are shown in Supplemental Table S2.1. The proper construction of each mutant strain was confirmed by PCR amplification and sequencing of chromosomal DNA. In addition, each signal-blind mutant (*lasR*, *rhlR*, and *pqsR*) was complemented with plasmid pJN105 carrying the respective regulatory gene (Table 2.1), and each signal-negative mutant (*lasI*, *rhlI*, and *pqsA*) was complemented with synthetic 3OC12-HSL, C4-HSL, or PQS. Each pJN105 construct carrying *lasR*, *rhlR*, or *pqsR* under control of their own promoter, respectively, was generated without the pBAD-*araC* promoter by restriction and ligation. All markerless in-frame deletion mutants were tagged by inserting antibiotic resistance

cassettes at a neutral site using plasmids pUC18T-mini-Tn7T-Tp or pUC18R6KT-mini-Tn7T-Tet (Table 2.1) as previously described (Choi *et al.* 2005, Choi and Schweizer 2006). The *lasI*, *lasR*, *pqsA* and *pqsR* mutants were each tagged with a Tp cassette and the *rhlI* and *rhlR* mutants were each tagged with a Tc cassette.

Single culture and co-culture growth assays

Harvest times of 12 h in M9-CAA and 24 h in M9-caseinate were chosen based on the growth of the wild-type in individual culture, which reached a similar density and growth phase in the respective media. It was our intent to measure the relative fitness of actively growing bacterial strains; we therefore considered it important to harvest cells before they entered stationary phase. Late-stationary-phase effects such as the increased resistance of *lasR* mutants to alkaline lysis (Heurlier *et al.* 2005) could potentially confound results.

In the co-culturing assay involving wild-type and tagged *pqsR*, *lasR*, and *rhlR* mutants, trimethoprim (Tp)-tagged *pqsR* and *lasR* mutants were distinguished by patching 100 Tp-resistant colonies on skim-milk plates. The *pqsR* mutant is skim-milk positive under these conditions, whereas the *lasR* mutant is negative. We calculated the percentage of these mutants by scoring for the presence or absence of skim milk proteolysis after selection on LB agar supplemented with Tp.

Complementation analysis

Rhamnolipid-deficient and AQ-deficient isolates were transformed with pJN105 or its derivatives (Table 2.1, Supplemental Table S2.1) using chemical transformation (Chuanchuen *et al.* 2002). To assess rhamnolipid production, transformants were patched onto rhamnolipid detection agar (Kohler *et al.* 2000) supplemented with Gm and arabinose. Pyocyanin production was quantified after 17 h of growth in LB-MOPS (Essar *et al.* 1990). AQ production was measured as described in the main text. Strains were grown with arabinose, but without Gm due to deleterious effects on the biosensor strain. We determined that there is no significant plasmid loss in the absence of Gm (data not shown).

DNA sequencing

PCR fragments amplified from the DNA of isolates *rhl1*, *rhl2*, and *pqs1-9* were directly sequenced at the Center for Genome Research and Biocomputing at Oregon State University. The 1.35-kb region of *rhlR* was amplified using forward and reverse primers as previously described (Wilder *et al.* 2009). The 1.7-kb region of *pqsR* was amplified using the primers *pqsR* forward and *pqsR* reverse (Supplemental Table S2.1). Appropriate sequencing primers were included with each sequencing reaction.

FITC-casein assay

This assay (Twining 1984) was used to quantify proteolytic activity of *P. aerugionsa lasR, rhlR,* and *pqsR* mutants and the wild-type parent grown in M9-caseinate and M9-

CAA for 24 h and 12 h, respectively. Filter-sterilized culture supernatants (at various dilutions) were incubated with elastase buffer (20 mM borate, pH = 8.8, 150 mM NaCl) and FITC-casein for 10 h at 37^{o}C. FITC fluorescence was measured at λ_{ex} = 490 nm and λ_{em} = 525 nm with a Tecan M200 plate reader (Tecan, Switzerland). Fluorescence intensity was normalized to dilution factor and cell density (CFU/ml). At the respective supernatant dilutions used for quantification, the levels of residual caseinate transferred from the growth medium were too small to affect measurements.

Supplemental Tables

Table S2.1: Primers used in this study

Construct	Primer name	Primer sequence (5'-3')	Amplicon size (kB)
Plasmid construction			
pJN105.*pqsR*-H	pJN105.*pqsR*-H forward	N_6GAATTCGCCACCCAATAAAAGGAATAAG	1.0
	pJN105.*pqsR*-H reverse	N_6TCTAGAGGAGAACGCTCTACTCTGGTG	
pJN105.*pqsR*-N	pJN105.*pqsR*-N forward	N_6CTCGAGTGACGATTGCAGGTTTCGGGCACG	1.7
	pJN105.*pqsR*-N reverse	N_6GAATTCCGTCTGCTGGAGAACGCTCTACTCTGG	
pJN105.*rhlR*-N	pJN105.*rhlR*-N forward	N_6GGTACCGCCACTGGGAGCCTTGCTGC	1.3
	pJN105.*rhlR*-N reverse	N_6CTGCAGGGAGGATGAACGGCAGGCAAC	
pJN105.*lasR*-N	pJN105.*lasR*-N forward	N_6CTCGAGCCGAACTGGAAAAGTGGCTATG	1.2
	pJN105.*lasR*-N reverse	N_6GAATTCGGCAAGATCAGAGAGTAATAAGAC	
Construction of deletion mutants by SOE PCR			
Δ*lasR*	*lasR* del 1	N_6GAGCTCCACAGACGTCTGCGCCTCGG	1.5
	lasR del 2	GAACTCGTGCTGCTTTCGCGT	

Continued on next page

Table S2.1-*Continued*

Construct	Primer name	Primer sequence (5'-3')	Amplicon size (kB)
	lasR del 3	ACGCGAAAGCAGCACGAGT TCCGGAAGTTCGGTGTGACC TCC	
	lasR del 4	N_6AAGCTTTCGCCTCCAGCG TACAGTCG	
Δ*lasI*	*lasI* del 1	N_6TCTAGAGCGTACTGCCGA TTTTCTGGGAAC	1.7
	lasI del 2	GAACTCTTCGCGCCGACCAA TTTG	
	lasI del 3	CAAATTGGTCGGCGCGAAG AGTTCCGCATCGAACTCAAT GCCAAGACC	
	lasI del 4	N_6AAGCTTGTCCTCCACCAC AAGGTCCTCG	
Δ*rhlR*	*rhlR* del 1	N_6TCTAGATGGTGTTCACCC AGGGCTCGAC	1.8
	rhlR del 2	GAACAGGCTGTCGCTCCAG AC	
	rhlR del 3	GTCTGGAGCGACAGCCTGTT CCCGAACAAGACGCTGGCT GCC	
	rhlR del 4	N_6AAGCTTGGCTGACGACCT CACACCG	
Δ*rhlI*	*rhlI* del 1	N_6TCTAGACCGATCCACGAC AGCCAGGG	1.75
	rhlI del 2	AAGCCCTTCCAGCGATTCAG AGAG	
	rhlI del 3	CTCTCTGAATCGCTGGAAGG GCTTCGCTACCACCCGGAAT GGCTG	
	rhlI del 4	N_6AAGCTTGCGAAGTCGAAG GGTTGCTGC	

Continued on next page

Table S2.1-*Continued*

Construct	Primer name	Primer sequence (5'-3')	Amplicon size (kB)
Δ*pqsR*	*pqsR* del 1	N_6TCTAGACGCTCTCCTGCTC GCTCAACACAC	1.85
	pqsR del 2	GGAGGCGATGACCTGGAGG AACAT	
	pqsR del 3	ATGTTCCTCCAGGTCATCGC CTCCGTCCTCAGCGAACTCT ACGAACCG	
	pqsR del 4	N_6CTGCAGCCAGCATCGTCA TCCGCTCCGC	
Δ*pqsA*	*pqsA* del 1	N_6TCTAGACCGAATGGAATG GTGAGCAGGG	1.8
	pqsA del 2	GCTGAGAGTCTGGCCCCGAT AGTG	
	pqsA del 3	CACTATCGGGGCCAGACTCT CAGCCGTCATCTGCCGGAAG TGAGCGAG	
	pqsA del 4	N_6AAGCTTGTTGAGGTGTCC CTTGACGTCGC	
Gene amplification for sequencing			
Δ*pqsR*	*pqsR* forward	ATTGCAGGTTTCGGGCACGG CAC	1.7
	pqsR reverse	TCTCACCACCCACGGCCAGC GTC	
Δ*rhlR*	*rhlR* forward	GGAGCCTTGCTGCCATCGTG CG	1.4
	rhlR reverse	CCAAGTCCCCGTGTCGTGCC G	

Table S2.2: In-depth look at *in vitro* evolution phenotypes

QS phenotype[a]				Percent negative isolates at the respective day				
Growth on adenosine	Skim milk proteolysis	AQ production	Rhamnolipid production	4	8	12	16	20
+	-	+	+	0	0	0.54	0.54	0.54
+	+	+	-	0	0	0	0	0.54
+	-	+	-	0	0	0.54	0	0
+	+	-	+	0	1.1	53	16	4.9
-	-	+	+	0	0	3.8	8.2	1.1
-	-	-	+	0	0	2.7	0.54	0.54
-	+	+	+	0	0	4.8	14	18
-	+	-	+	0	0	0.54	0	0

[a] "+" indicates presence and "-" indicates absence of the indicated condition.

Instantaneous within-patient diversity of *Pseudomonas aeruginosa* quorum-sensing populations from cystic fibrosis lung infections

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Abstract

In the opportunistic pathogen *Pseudomonas aeruginosa*, acyl-homoserine lactone quorum sensing (acyl-HSL QS) regulates biofilm formation and the expression of many extracellular virulence factors. Curiously, QS-deficient variants, often carrying mutations in the central QS regulator *lasR*, are frequently isolated from infections, in particular cystic fibrosis (CF) lung infections. Very little is known about the proportion and diversity of these QS variants in individual infections. Such information is desirable to better understand the selective forces that drive the evolution of QS phenotypes, including social cheating and innate (non-social) benefits. To obtain insight into the instantaneous within-patient diversity of QS, we assayed a panel of 135 concurrent *P. aeruginosa* isolates from eight different adult CF patients (9-20 isolates per patient) for various QS-controlled phenotypes. Most patients contained complex mixtures of QS-proficient and deficient isolates. Between all patients, deficiency in individual phenotypes ranged from 0 to about 90%. Acyl-HSL, sequencing, and complementation analysis of variants with global loss-of-function phenotypes revealed dependency upon central QS circuitry genes *lasR*, *lasI*, and *rhlI*. Deficient and proficient isolates were clonally related, implying evolution from a common ancestor *in vivo*. Our results show that the diversity of QS types is high within and between patients, suggesting diverse selection pressures in the CF lung. A single selective mechanism, be it of social or non-social nature, is unlikely to account for such heterogeneity. The observed diversity also shows that conclusions

about the properties of *P. aeruginosa* QS populations in individual CF infections cannot be drawn from the characterization of one or few selected isolates.

Introduction

Pseudomonas aeruginosa is a ubiquitous opportunistic pathogen that causes a range of acute and chronic infections in immunocompromised individuals, including those suffering from the genetic disorder cystic fibrosis (CF). In these patients, *P. aeruginosa* persistently colonizes the lungs, forming antibiotic-resistant biofilms (Moreau-Marquis *et al*. 2008, Singh *et al.* 2000, Wagner and Iglewski 2008). Many of the factors implicated in *P. aeruginosa* virulence are controlled by quorum sensing (QS) (Bjarnsholt and Givskov 2007a, Girard and Bloemberg 2008, Winstanley and Fothergill 2009). This includes biofilm formation, although the impact of QS is environmentally conditional (de Kievit 2009, Kirisits and Parsek 2006, Winstanley and Fothergill 2009). Thus, QS has been considered as a promising alternative drug target (Bjarnsholt and Givskov 2007b, Clatworthy *et al*. 2007). In QS, bacteria are able to coordinate diverse group behaviors through the production of chemical signals in a density-dependent manner (Waters and Bassler 2005). In *P. aeruginosa* and other Gram-negative bacteria, the majority of these signals are acylated homoserine lactones (acyl-HSL) (Fuqua and Greenberg 2002, Whitehead *et al*. 2001).

The *P. aeruginosa* acyl-HSL QS circuitry is particularly well understood. It is comprised of two acyl-HSL synthases, LasI and RhlI, that generate *N*-3-oxododecanoyl-HSL (3OC12-HSL) and *N*-butyryl-HSL (C4-HSL), respectively. As

the bacterial population increases, the signals accumulate intra- and extracellularly until they bind to their cognate transcriptional regulators, LasR and RhlR, respectively, to activate the expression of target genes (Juhas *et al*. 2005, Schuster and Greenberg 2006). These two QS systems are arranged hierarchically with the LasR-LasI circuit in control of the RhlR-RhlI circuit (Latifi *et al.* 1996, Pesci *et al.* 1997), although under certain conditions expression of RhlR-RhlI can occur independently (Dekimpe and Deziel 2009, Medina *et al*. 2003). Another orphan regulator, QscR, exists that also responds to 3OC12-HSL (Lee *et al*. 2006).

Transcriptome analysis of *P. aeruginosa* quorum-controlled genes revealed that both systems together control the expression of hundreds of genes (Hentzer *et al*. 2003, Schuster *et al*. 2003, Wagner *et al*. 2003), many of which encode virulence factors. In animal models of acute and chronic *P. aeruginosa* infection, mutations in central QS genes result in attenuated virulence as compared to the wild type strain (Lesprit *et al*. 2003, Pearson *et al*. 2000, Rumbaugh *et al*. 1999, Wu *et al*. 2001). However, although QS is required for full virulence in many model systems, clinical and environmental *P. aeruginosa* isolates are often QS deficient (D'Argenio *et al.* 2007, Fothergill *et al*. 2007, Heurlier *et al*. 2006, Hoffman *et al*. 2009, Smith *et al*. 2006, Tingpej *et al*. 2007). Loss-of-function mutations in the central regulator *lasR* gene are most frequent. This apparent paradox may be explained by a physiological advantage of the *lasR* mutant under certain growth conditions (D'Argenio *et al*. 2007, Heurlier *et al*. 2005). Alternatively, *lasR* mutants may emerge by social exploitation, as initially shown *in vitro*. Under conditions that require QS, *lasR* mutants evolve

from a wild type ancestor during long-term culturing, and have a selective advantage when co-cultured with the wild type parent (Diggle *et al*. 2007, Sandoz *et al*. 2007). These mutants also increase in frequency during acute *P. aeruginosa* infection of mechanically ventilated patients (Kohler *et al* 2009), and during experimental co-infection in a mouse burn wound model (Rumbaugh *et al*. 2009). This is because *lasR* mutants benefit from the extracellular products made by the QS-proficient wild type without paying the metabolic cost. Thus, QS-deficient variants can arise because, rather than despite, the importance of QS. Social conflict should be of particular significance in a localized, long-term infection such as CF, which according to evolutionary theory, would result in increased competition and selection for cheaters (Diggle *et al*. 2007, West *et al*. 2006). If QS-deficient variants in CF lung infection indeed arise by social exploitation, then populations of *P. aeruginosa* cells isolated from individual patients should consist of mixtures of wild type and mutant cells. Moreover, if there is a single benefit to QS mutants, be it social or non-social, then we would expect to find one predominant mutant phenotype and little diversity.

Although several studies have analyzed QS in CF isolates, they have generally focused on either between-patient diversity or longitudinal within-patient diversity, rarely involving more than one isolate per patient at any one time (Fothergill *et al*. 2007, Salunkhe *et al*. 2005, Schaber *et al*. 2004, Smith *et al*. 2006, Tingpej *et al*. 2007). Very little attention has been given to the issue of instantaneous within-patient heterogeneity, that is, diversity among isolates collected from a single patient at any one time. One recent study screened an average of three *P. aeruginosa* isolates from

58 young CF patients, many concurrently isolated, for the presence of *lasR* mutations (Hoffman *et al*. 2009). Overall, 31% of the isolates collected were mutated in *lasR*. In several cases, individual patients harbored both wild type and *lasR* mutant isolates at the same time. The distinguishing feature of the present study is that a large number of concurrent isolates were comprehensively characterized, phenotypically and genotypically, from individual adult CF patients, allowing a more complete description of instantaneous diversity and types of QS-related features.

Materials and Methods

CF patients, bacterial strains and plasmids

Laboratory strains and plasmids used in this study are shown in Table 3.1. Clinical *P. aeruginosa* isolates were obtained concurrently from the sputa of eight adult CF patients at Oregon Health and Science University (OHSU). Sputum samples were collected from CF patients by expectoration during a routine hospital visit. Patients were between 20 and 48 years old. Infection with *P. aeruginosa* had been documented since their treatment at OHSU (between 1 and 10 years), although actual infection is likely to be longer. Patients' lung functions ranged from mildly to severely obstructed. Lung function was measured by spirometry as forced expiratory volume during the first second (FEV1) (Miller *et al*. 2005a). Greater than 90% FEV1 is considered normal, 70-89% is mild obstruction, 49-69% is moderate obstruction, and less than 40% is considered severe obstruction (see Table 3.2) (Miller *et al*. 2005a).

Table 3.1: Bacterial strains and plasmids

Lab strain or plasmid	Relevant property	Reference or origin
Strains		
PAO1	*Pseudomonas aeruginosa* wild type	(Holloway *et al*. 1979)
PDO300	*mucA22* derivative of PAO1	(Mathee *et al.* 1999)
PAO *lasR*	PAO1 derivative; *lasR*::Tc^R	(Rahim *et al*. 2001)
PAO *rhlR*	PAO1 derivative; *rhlR*::Gm^R	(Rahim *et al*. 2001)
PAO *lasR rhlR*	PAO1 derivative; *lasR*::Tc^R *rhlR*::Gm^R	(Rahim *et al*. 2001)
PAO *lasI rhlI*	PAO1 derivative; *lasI*::Tc^R *rhlI*::Tn501	(Whiteley *et al*. 1999)
Plasmids		
pJN105	*araC*-pBAD cloned in pBBR1 MCS-5; Gm^R	(Newman and Fuqua 1999)
pJN105.*lasR*	*lasR* in pJN105	(Lee *et al*. 2006)
pJN105.*lasR*D65G	*lasR*D65G from patient B isolate in pJN105	This study

Bacterial isolation and species identification

Sputum samples were instantly stored on ice, express-shipped from OHSU to Oregon State University, and processed immediately. Sputa were liquefied by the addition of an equal volume of sputolysin (Calbiochem), diluted and plated onto *Pseudomonas* Isolation Agar (BD Biosciences), which permits growth of *P. aeruginosa* and other *Pseudomonas* species. Twenty isolates were randomly picked from a single sputum sample per patient and saved as frozen stock cultures. PCR amplification with *P. aeruginosa*-specific 16S ribosomal DNA primers was used to confirm species identity (Spilker *et al*. 2004). Only isolates identified as *P. aeruginosa* were used for this

study. Mucoidy was evaluated based on distinct colony morphology (Mathee *et al*. 1999). Relative abundance of *P. aeruginosa* compared with other microbial flora was estimated based on routine four-quadrant plating of CF sputum samples by the Kaiser Permanente Clinical Microbiology Laboratory (Portland, OR).

Production of extracellular proteases, rhamnolipids, and nucleoside hydrolase

These assays were performed in high-throughput format. Skim-milk proteolysis (Sandoz *et al*. 2007), rhamnolipid production (Kohler *et al*. 2000), and growth on adenosine, indicative of nucleoside hydrolase production (Heurlier *et al*. 2005, Sandoz *et al*. 2007), were determined semi-quantitatively through the use of agar plate assays. Isolates were initially grown on Lennox LB agar plates at 37^{o}C for 24-48 hours. Colonies were subsequently replica-plated onto the respective agar test plates and evaluated after 24h of growth. The scoring scheme was as follows: For adenosine plates, growth was scored as "+", while the absence of growth was scored as "-". For skim-milk proteolysis and rhamnolipid production, the formation of a halo similar to the PAO1 wild-type was scored as "+", the formation of a halo significantly smaller than that of the PAO1 wild-type was scored as "○", and the absence of a halo was scored as "-". Severe growth deficiencies were indicated as "no growth".

LasA and LasB protease activities were measured from Lennox LB liquid cultures grown in deep-well titer blocks for 24h. All cultures had reached stationary phase by the time of measurement, as determined by OD_{600}. Assays were adapted for high-throughput analysis in 96-well plate format using a microplate reader (Infiniti

M200, Tecan, USA). LasA protease activity was determined by measuring the rate at which filter-sterilized *P. aeruginosa* culture supernatants could lyse boiled *Staphylococcus aureus* cells (Diggle *et al*. 2002, Kessler *et al.* 1993). Twenty µl of bacterial supernatant were added to each well of a 96-well plate followed by the addition of 180 µl of boiled *S. aureus* cells to each well. The optical density (OD_{600}) was measured every 5 minutes for 75 min. LasB (elastolytic) activity of bacterial supernatants was determined using an elastin congo red assay as previously described (Diggle *et al*. 2002). Twenty-two µl of bacterial supernatant were added to 200 µl of ECR buffer in 96-well plates. Absorbance was read at 495 nm. For both assays, ≥80% activity compared with the PAO1 wild-type control was scored as "+", 10-79% activity was scored as a "○", and <10% activity was scored as a "-" (see Supplemental Table S3.1 and Supplemental Figure S3.1). Additional controls included in all assays were defined *lasR*, *rhlR*, and *lasR rhlR* mutants (Table 3.1).

For our initial screen, we chose the aforementioned approach of assaying QS-controlled phenotypes of all clinical isolates at one particular time, although these isolates exhibited a range of different growth rates. Few grew as fast as the wild type, most grew significantly slower. However, when dealing with 135 samples in our initial screen, it would not have been feasible to specifically adjust the time of harvest for every single isolate. Instead, we chose a time (24 h), at which even the slowest isolate had well reached stationary phase. Because the quorum-controlled products assayed are typically induced at the transition from logarithmic to stationary phase, every isolate that did not produce QS factors was thereby correctly classified as QS-

negative. Individual isolates that produced QS factors essentially served as internal "controls" for other isolates from the same patient that were scored as QS-negative but exhibited very similar growth characteristics (Supplemental Table S3.1 and Supplemental Figure S3.1). For the next step - the in-depth characterization of isolates from two selected patients - we chose an approach that takes growth-rate differences into account (see below). These results nevertheless were in good agreement with the initial screening results.

Acyl-HSL detection

Production of 3OC12-HSL and C4-HSL was quantified from ethyl acetate extracts of culture supernatants using *E. coli* bioassays (Passador *et al*. 1996, Pearson *et al*. 1995, Pearson *et al*. 1997). MOPS-buffered Lennox LB cultures were inoculated to a starting OD_{600} of 0.01 and grown until stationary phase. To correct for differences in growth, growth curves were determined in a preliminary experiment for all clinical isolates from patients A and B. Doubling times for most patient A and B isolates were 60 min compared with 30 min for the PAO1 control strain. Thus, for acyl-HSL analysis most clinical isolates were grown for up to 25 h compared with 15 h for PAO1 and mutant derivatives. At the indicated times, all strains spent the same amount of time in stationary phase and reached similar culture densities. Diluted acyl-HSL extracts were added to *E. coli* biosensor strain MG4/pKDT17 to detect 3-oxo-C12-HSL and to strain DH5α/pECP61.5 to detect C4-HSL. β-galactosidase production by these *E. coli* strains was measured with a microplate reader using a

luminescence assay (Galacto Light Plus, Tropix) (Sandoz *et al*. 2007, Whiteley *et al*. 1999).

Random Amplified Polymorphic DNA analysis (RAPD)

RAPD analysis was performed as previously described using Primer 208 and 40 ng of genomic DNA per PCR reaction (Mahenthiralingam *et al*. 1996, Mereghetti *et al*. 1998). Chromosomal DNA was isolated from *P. aeruginosa* liquid cultures using the PURGENE^{TM} DNA Purification Kit (Gentra Systems). The PCR-amplified DNA products were resolved by agarose gel electrophoresis and stained with ethidium bromide. Gel images were taken using the BioDoc-It^{TM} Imaging System (UVP). Banding patterns were analyzed using GelCompar II software (Applied Math). This program aligns sample lanes to a reference ladder using pattern recognition. It then creates a dendrogram based on the similarity of banding patterns between samples. This entire process was performed twice with independently amplified DNA. In both cases, very similar results were obtained.

DNA sequencing

PCR fragments amplified from isolated *P. aeruginosa* DNA were directly sequenced at the Center for Genome Research and Biocomputing at Oregon State University. Each allele was sequenced twice using independently amplified DNA. The 1.28-kb region of *vfr* was amplified using forward primer 5'-AAG GCT TCG CAG CTC TCC ACC G-3' and reverse primer 5'-CTG GTT GGC GAG GCG TTC ATG CGG-3'.

The 1.24-kb region of *lasR* was amplified using primers las1 and las2 (Heurlier *et al*. 2005). The 1.1-kb region of *lasI* was amplified using forward primer 5'-GGA AGT TCG GTG TGA CCT CCC GC-3' and reverse primer 5'-CGG ATT CGG CAT CGA CGC CAG G-3'. The 1.35-kb region of *rhlR* was amplified using forward primer 5'-GGA GCC TTG CTG CCA TCG TGC G-3' and reverse primer 5'-CCA AGT CCC CGT GTC GTG CCG-3'. The 1.11-kb region of *rhlI* was amplified using forward primer 5'-TGC TGA CCC AGA AGC TGA CCG ACC-3' and reverse primer 5'-GCA GGG GAA CGC CCG ATG ACG C-3'. Appropriate sequencing primers were included with each sequencing reaction.

Complementation analysis

Experimental cultures were grown as described for acyl-HSL analysis. Chemical complementation of patient A isolates was performed by addition of either 3OC12-HSL, C4-HSL, both signals, or neither signal to cultures upon inoculation. The concentrations of 3OC12-HSL and C4-HSL were 2 and 10 μM, respectively, as previously described (Schuster *et al*. 2003). 3OC12-HSL was custom-synthesized by RTI International (Research Triangle Park, NC) and C4-HSL was custom-synthesized by Vertex Pharmaceuticals (Coralville, IA). For *lasR* complementation of patient B isolates, we used the low-copy number plasmids pJN105 and pJN105.*lasR*. In the latter, expression of *lasR* is under control of the arabinose-inducible *araBAD* promoter (Lee *et al*. 2006, Newman and Fuqua 1999). Plasmids were introduced into clinical strains by transformation of chemically competent cells. For strains containing

plasmids, 50 μg/ml gentamicin and 5 mM L-arabinose were added to the medium. The concentration of arabinose chosen results in LasR expression levels similar to those of the native chromosomal gene in the PAO1 wild type (data not shown). LasB elastase activity in culture supernatants was measured using the elastin congo red assay as described above.

Cloning and characterization of lasRD65G

The *lasR* gene from an isolate from patient B harboring the D65G mutation was amplified with flanking primers (Lee *et al*. 2006) containing *Eco*RI and *Xba*I restriction sites and cloned into appropriately digested pJN105 (Lee *et al*. 2006). Proper construction was confirmed by restriction analysis and DNA sequencing. The resulting plasmid pJN105.*lasR*D65G was transformed into the defined *lasR* mutant, PA0 *lasR::*Tc^R (Rahim *et al*. 2001). This transformant and appropriate controls were analyzed for proteolysis on 4% skim milk plates supplemented with arabinose and gentamicin as described (Sandoz *et al*. 2007).

Results

Assembly of a library of P. aeruginosa isolates from CF patients

The main objective of this study was to determine the proportion of QS-deficient and QS-proficient clinical isolates within an individual CF lung infection. Our goal was to characterize numerous (up to 20) concurrent isolates per patient to obtain sufficient information about instantaneous QS diversity. To our knowledge, such a broad

collection of concurrent isolates from single patients does not exist, necessitating new isolation of *P. aeruginosa* from CF patients. Sputum was collected from adult patients. The propensity for the emergence of QS-deficient variants is expected to be highest in these patients with long-term lung infection. In the sputum samples, *P. aeruginosa* was present at typical bacterial load (10^5-10^8 CFU/ml) (Hogardt *et al*. 2000), and was in most cases the predominant, although not exclusive, organism. *P. aeruginosa* was isolated by plating on *Pseudomonas* isolation agar. Twenty candidate isolates were randomly picked, irrespective of morphotype, for further analysis. Species identity was verified by 16s rRNA analysis (Spilker *et al*. 2004). In most cases, less than 20 of the original sputum isolates were confirmed as *P. aeruginosa* (Tables 3.2 and S3.1). Some *P. aeruginosa* populations were exclusively mucoid or non-mucoid, while others contained mixtures of both (Tables 3.2 and S3.1).

Cystic fibrosis patients are commonly infected with a large subpopulation of QS-deficient variants

Our collection of *Pseudomonas aeruginosa* CF isolates was screened for various QS-dependent phenotypes. All isolates were tested for growth on adenosine, skim milk proteolysis, rhamnolipid production, elastolytic (LasB) activity, and staphylolytic (LasA) activity. In the PAO1 reference strain, utilization of adenosine as sole carbon source and skim-milk proteolysis are *las*-dependent phenotypes (Sandoz *et al*. 2007); LasB and LasA activities are dependent on both *las* and *rhl* systems, whereas rhamnolipid production is primarily *rhl*-dependent (Schuster *et al*. 2003). We identified mixed populations of isolates with either partially or fully attenuated QS

phenotypes in seven out of eight patients (Table 3.2, Supplemental Table S3.1, and Supplemental Figure S3.1). Many isolates were fully deficient in some but not all of the QS phenotypes tested. All *P. aeruginosa* isolates from patient D displayed QS phenotypes similar to the PAO1 control strain. However, there was no apparent correlation between QS proficiency and total bacterial load. The QS-proficient *P. aeruginosa* population from patient D was not more abundant than other, largely QS deficient populations.

Some isolates did not grow on adenosine medium and also did not grow on rhamnolipid detection medium (Supplemental Table S3.1 and Supplemental Figure S3.1). As these assays utilize minimal media, it is possible that some mutants did not grow because they are amino acid auxotrophs. Thus, lack of growth on adenosine does not necessarily indicate a QS defect. For the purpose of this study, we felt that it was unnecessary to distinguish between the two possibilities as our phenotypic analysis included several other, independent assays that allowed us to evaluate QS proficiency.

P. aeruginosa populations from two selected patients are largely clonal, deficient in acyl-HSL production and harbor mutations in lasR, lasI, and rhlI.

Most *P. aeruginosa* isolates from two patients (patients A and B) were negative for all of the QS phenotypes tested (Supplemental Table S3.1 and Supplemental Figure S3.1), indicating mutations in central QS-regulatory genes. These isolates were therefore selected for further in-depth analysis. Clonal relatedness among isolates was

Table 3.2: Characteristics of *P. aeruginosa* populations from CF patients

Patient	Severity of lung obstruction	CFU/ml in sputum	Number of isolates	Percent mucoidy	Percentage of negative isolates in the QS assay indicated				
					Adenosine	Skim milk	Rhamnolipid	Staphylolysis	Elastin Congo Red
A	Mild	10^8	18	0.0	89	83	83[a]	83	83
B	Severe	10^7	18	100	78	78	78[a]	83	78
C	Mild	10^5	19	79	95	58	95[a]	5.3	5.3
D	Moderate	10^7	19	0.0	0.0	0.0	0.0	0.0	0.0
E	Moderate	10^6	20	100	15	70	70	30	20
F	Severe	10^8	9	0.0	0.0	78	44	44	44
G	Moderate	10^6	14	79	71	14	64[a]	64	71
H	Moderate	10^5	18	83	83	83	89	61	67

[a] Includes "no growth" isolates (see Supplemental Table S3.1 and Supplemental Figure S3.1).

assessed by random amplification of polymorphic DNA (RAPD) (Figure 3.1). All isolates from patient A were highly related (>80%). Most isolates from patient B were also highly related, whereas two isolates, B6 and B18, showed lower relatedness to other patient B isolates (40% and 70%, respectively). There were no strain variants common to both patients. Importantly, there was no apparent correlation between RAPD profiles and QS phenotypes. There were QS-deficient and (partially) proficient isolates with the same RAPD type, suggesting that both types evolved from a common ancestor during infection.

Next, we quantified production of 3OC12-HSL and C4-HSL using an *E. coli* bioassay (Figure 3.2). Patient A isolates fell into two groups. Some produced very

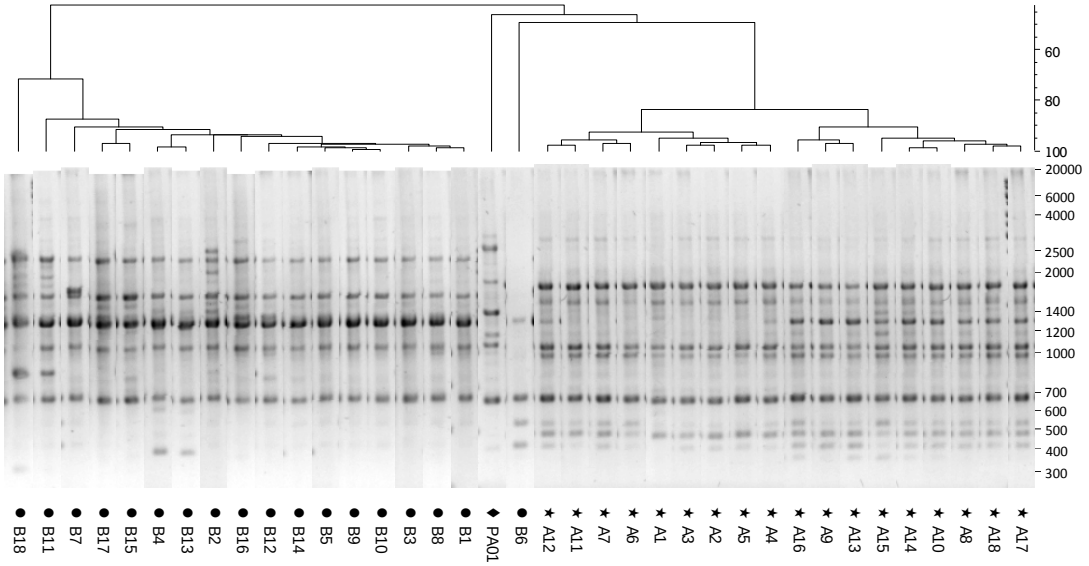

Figure 3.1: RAPD analysis of *P. aeruginosa* isolates from patients A and B. Isolates A1-A18 and B1-B18 are isolates 1-18 from patient A and patient B, respectively. The upper scale next to the dendrogram indicates relatedness of isolates in percent, whereas the lower scale indicates size of DNA fragments in base pairs.

little to no 3OC12-HSL and C4-HSL, while others produced some 3OC12-HSL but very little to no C4-HSL (Figure 3.2A). Complete lack of acyl-HSL production is consistent with mutations in either *lasI* and *rhlI,* or *lasR* and *rhlR*. Most isolates from patient B showed substantially reduced 3OC12-HSL but only slightly reduced C4-HSL levels (Figure 3.2B). This acyl-HSL profile is similar to that of a defined *lasR* mutant. Curiously, isolates scored as partially QS-proficient (Supplemental Table S3.1 and Supplemental Figure S3.1) also produced little to no acyl-HSL (patient A isolates 3, 17, 18, and patient B isolates 16, 17, 18).

To obtain further insights into the mutations responsible for the observed phenotypes, we sequenced *lasR*, *rhlR*, *lasI*, and *rhlI* from selected patient A isolates, and we sequenced *lasR* from selected patient B isolates (Table 3.3). These isolates

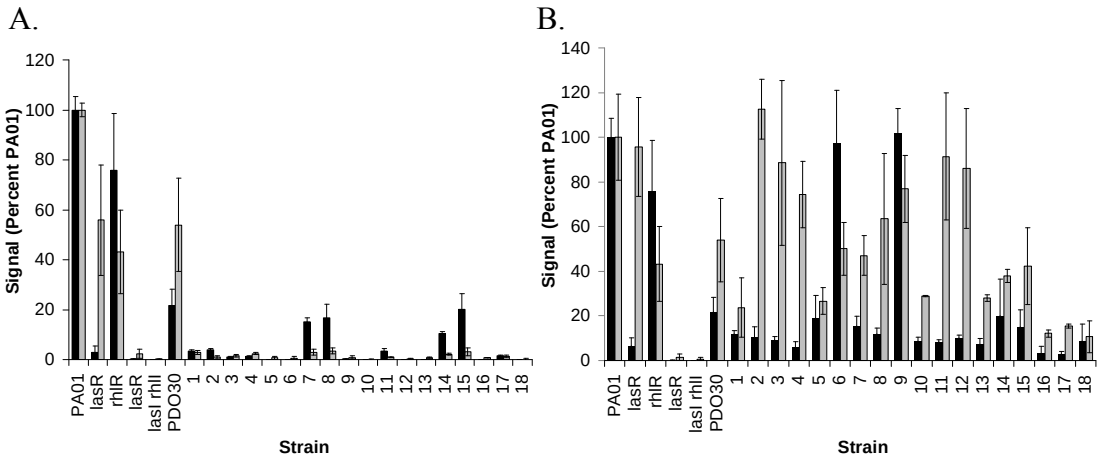

Figure 3.2: Acyl-HSL assay of *P. aeruginosa* isolates. (A) Patient A; (B) patient B. Black and grey bars designate 3OC12-HSL and C4-HSL, respectively. Acyl-HSL levels were normalized to the optical density of the respective culture at the time of measurement. The genotypes of the respective *P. aeruginosa* mutant control strains are indicated. Values represent the averages of three independent replicates. Error bars indicate standard deviations of the mean.

were selected based on their QS phenotypes and acyl-HSL profiles (Supplemental Table S3.1, Supplemental Figure S3.1, Figure 3.2). All sequenced isolates from patient A have missense mutations in *rhlI*, resulting in a Leu substitution of conserved Phe 28, and a Glu substitution of non-conserved Asp 83. Substitution F28L in *rhlI* was predicted to be not tolerated by the SIFT algorithm (Ng and Henikoff 2003). QS-negative isolates 5 and 13 also harbor a frameshift mutation in *lasI*, resulting in a truncated protein. QS-negative isolate 8, which produced some 3OC12-HSL, and partially QS-proficient isolate 18, which produced no acyl-HSL, showed no mutation in *lasI*. Of note, whereas defined *rhlR* mutants are completely deficient in the standard rhamnolipid plate assay employed, *rhlI* mutants are only partially deficient (Kohler *et al*. 2001) (data not shown). This could explain why isolate 18 was scored as partially rhamnolipid proficient (Supplemental Table S3.1 and Supplemental Figure S3.1) despite carrying a mutation in *rhlI*.

Table 3.3: Sequence analysis of selected *P. aeruginosa* CF isolates

Patient/Isolate[a]	Gene	Non-synonymous mutation[b]	Change[c]
Patient A			
5	*lasR*	None	None
5	*lasI*	A insertion (+43)	Truncation
5	*rhlR*	None	None
5	*rhlI*	T→C (+82), C→A (+249)	F28L*[†], D83E
8	*lasR*	None	None
8	*lasI*	None	None
8	*rhlR*	None	None
8	*rhlI*	T→C (+82), C→A (+249)	F28L*[†], D83E

Continued on next page

Table 3.3-*Continued*

Patient/Isolate[a]	Gene	Non-synonymous mutation[b]	Change[c]
13	*lasR*	None	None
13	*lasI*	A insertion (+43)	Truncation
13	*rhlR*	None	None
13	*rhlI*	T→C (+82), C→A (+249)	F28L*†, D83E
18	*lasR*	None	None
18	*lasI*	None	None
18	*rhlR*	None	None
18	*rhlI*	T→C (+82), C→A (+249)	F28L*†, D83E
Patient B			
4	*lasR*	A→G (+194)	D65G†
4	*vfR*	None	None
6	*lasR*	A→G (+194)	D65G†
6	*vfR*	None	None
7	*lasR*	A→G (+194)	D65G†
7	*vfR*	None	None
17	*lasR*	A→G (+194)	D65G†

[a]Individual clinical isolates
[b]Nucleotide substitution or insertion at the indicated position relative to translational start site
[c]Amino acid changes relative to the PAO1 protein sequence. *, indicates that the respective amino acid residue is conserved in LuxR and LuxI-type proteins from a range of different bacterial species (Whitehead *et al*. 2001). †, indicates that the respective amino acid residue is conserved in *P. aeruginosa* PAO1, PA7, PA14, and LESB58 according to www.pseudomonas.com.

All sequenced isolates from patient B carry a missense mutation in *lasR* resulting in an Asp to Gly substitution at position 65, which is adjacent to highly conserved Tyr 64. The fact that the same mutation was also present in partially QS-proficient isolate 17 suggests that it may not be fully responsible for the observed loss-

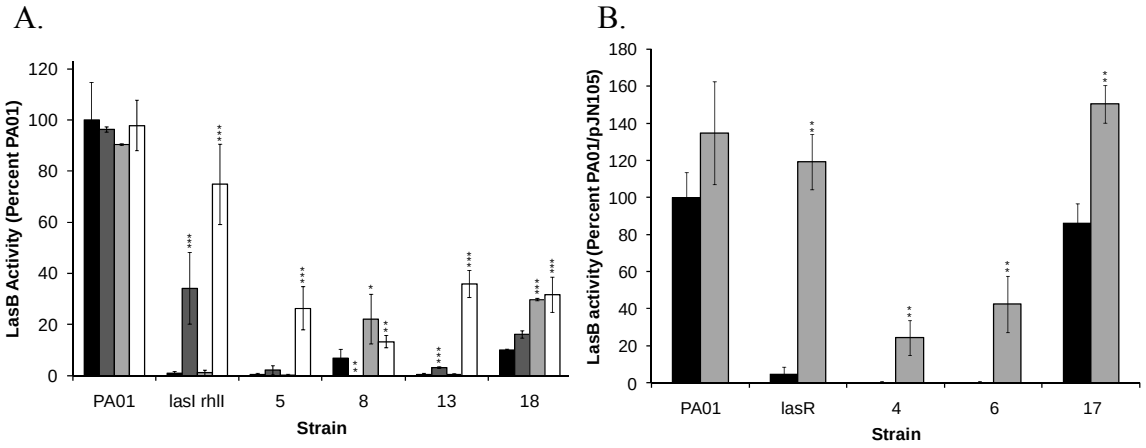

Figure 3.3: Complementation analysis of selected *P. aeruginosa* isolates. Elastolytic (LasB) activity of culture supernatants was measured using the elastin congo red assay and was normalized to culture density (OD_{600}). (A) Complementation of patient A isolates with exogenous acyl-HSL. Black bars indicate the absence of acyl-HSL, dark grey bars indicate the presence of 3OC12-HSL, light grey bars indicate the presence of C4-HSL, and white bars indicate the presence of both signals. (B) Complementation of patient B isolates with *lasR*. Black bars indicate the presence of pJN105, and grey bars indicate the presence of pJN105.*lasR*. Values represent the averages of three independent replicates. Error bars indicate standard deviations of the mean. Statistical significance of the data was determined using a two-tailed unpaired T-test with "***" indicating $p < 0.017$ (treatment-level Bonferroni correction to adjust for multiple comparisons) "**" indicating $0.017 \leq p < 0.05$ and "*" indicating $0.05 \leq p < 0.1$. Within each isolate, expression in the presence of either one or both acyl-HSL was compared to no acyl-HSL (panel A), and expression with pJN105.*lasR* was compared to the vector control (panel B).

of-function phenotypes in QS-deficient isolates, although this mutation was predicted to be not tolerated by the SIFT algorithm (Ng and Henikoff 2003).

Complementation analysis confirms significance of lasR and acyl-HSL-dependent phenotypes, but not of the lasR mutation itself

Complementation analysis was performed in order to determine whether the identified mutations are responsible for the observed phenotypes. LasB elastase production was

used as a representative QS assay because it is controlled by both the *las* and *rhl* QS systems. Selected patient A isolates mutated in *lasI* or *rhlI* (Table 3.3) were chemically complemented using synthetic acyl-HSL signals (Figure 3.3A). Isolates 5 and 13, which carry mutations in both *lasI* and *rhlI,* were complemented by the addition of both 3OC12-HSL and C4-HSL. Isolates 8 and 18, which carry a mutation in *rhlI* only, were complemented by the addition of C4-HSL alone. Curiously, the low-level elastase production by isolate 8 was completely inhibited by the addition of 3OC12-HSL.

Isolates from patient B were complemented with a plasmid (pJN105) carrying *lasR* under control of an arabinose-inducible promoter (Lee *et al*. 2006). Although the relevance of the *lasR*D65G mutation itself was questionable, we nevertheless reasoned that the observed loss-of-function phenotypes were *lasR*-dependent because acyl-HSL profiles in many isolates were similar to that of a defined *lasR* mutant. Indeed, in QS-deficient isolates 4 and 6 (Tables 3.2, S3.1 and 3.3), elastase production increased significantly by introducing *lasR in trans* (Figure 3.3B). Moreover, the *lasR*D65G allele, when cloned in pJN105, was able to fully complement skim milk proteolysis of a defined *lasR* mutant (Figure 3.4). This result indicates that the D65G mutation itself is not responsible for the observed QS phenotype. Instead, our results indicate that

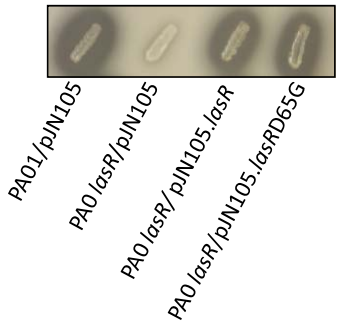

Figure 3.4: Complementation of a defined *lasR* mutant with the *lasR*D65G allele. Proteolysis was measured on skim-milk plates.

lasR expression is abolished due to an upstream regulatory defect, because [1] *lasR* expression from a heterologous promoter was able to restore QS, and [2] there were no mutations in the *lasR* promoter region. We found no mutations in *vfr*, which encodes a major regulator of *lasR* transcription that is often mutated in CF isolates (Albus *et al*. 1997, Smith *et al*. 2006), suggesting a defect in another regulatory gene (Table 3.3).

Discussion

In the present study, we characterized *P. aeruginosa* populations concurrently isolated from chronically colonized adult CF patients to obtain insight into the within-patient diversity of QS. In seven out of eight patients, we found mixtures of isolates with distinct QS-proficient and QS-deficient phenotypes. Deficiency in individual QS-dependent phenotypes was not the result of attenuated growth of some of the clinical isolates. In our initial phenotypic screens, isolates were grown long enough to reach the stationary phase of growth; the PAO1 control strain shows high-level expression of QS products as soon as cells transition from logarithmic to stationary phase. In subsequent acyl-HSL and complementation assays performed with patient A and B isolates, culturing times were carefully adjusted according to growth rate, and data were normalized to culture density.

We found that the diversity of QS phenotypes is high between patients, but also within a given patient. This result suggests that the selective forces that shape the evolution of QS during CF lung infection are also diverse, which is not surprising if one considers the complexity of this infection. Previously, between-patient diversity

has mainly been inferred from the characterization of single isolates from different infections (Fothergill *et al*. 2007, Salunkhe *et al*. 2005, Schaber *et al*. 2004, Tingpej *et al*. 2007). It is thought to result from individual differences in the genetic make-up and virulence potential of the infecting strain(s) (Tümmler 2006), in the co-infection with other microbial species (Harrison 2007), in the severity and context of the genetic defect (Kiesewetter *et al*. 1993), and in host immune function (Miller *et al*. 2005b). Within-patient QS diversity, on the other hand, has been much less well recognized, but is in fact a prerequisite to accurately assessing between-patient diversity. Within-patient diversity may result from the heterogeneity of the lung environment. Given the compartmentalized nature of the lung, and the high viscosity of CF mucus, *P. aeruginosa* cells even within a single infection are exposed to distinct microenvironments. These include steep nutrient and oxygen gradients in the mucus lining the CF airways that impact *P. aeruginosa* metabolism (Worlitzsch *et al*. 2002, Yoon *et al*. 2002), differences in the exposure to host inflammatory responses, and microbial communities of different species composition in different lobes of the lung (Smith *et al*. 1998). Taken together, all these factors may contribute to the diversification of *P. aeruginosa* QS populations in the CF lung. The observed diversity also confirms the notion that conclusions about *P. aeruginosa* populations cannot be determined through the characterization of only a select few isolates. Instantaneous within-patient heterogeneity of *P. aeruginosa* from CF lung infections has been recognized for some time (Tümmler 2006, Zierdt and Schmidt 1964), but has

primarily been associated with distinct morphotypes, most notably mucoid and non-mucoid colony variants.

Two specific mechanisms, social and non-social, have been proposed to explain the frequent emergence of QS variants during infection. Previous studies demonstrated that social cheating can select for QS-mutants *in vitro*, in an animal infection model, and presumably also in acute human infection (Diggle *et al*. 2007, Kohler *et al*. 2009, Rumbaugh *et al*. 2009, Sandoz *et al*. 2007). Here, QS-deficient bacteria benefit from the production of QS-controlled extracellular products by the QS-proficient majority. QS-deficiency may also confer an innate (non-social) physiological advantage under certain growth conditions. For example, *lasR* mutants have a higher growth yield on certain carbon sources, including amino acids, and show increased resistance to alkaline lysis in stationary phase compared to the wild type parent (D'Argenio *et al*. 2007, Heurlier *et al*. 2006). These properties may be relevant *in vivo* as amino acids are abundant in CF sputum (Palmer *et al*. 2007), and amino acid catabolism may lead to localized alkalinization.

Consistent with social cheating, many CF sputum samples contained mixed populations of QS-proficient and QS-deficient isolates; no sample contained 100% fully deficient QS mutants. However, in several cases, the proportion of QS-deficient phenotypes was very high (≥83% for patient A, ≥78% for patient B, and ≥61% for patient H; Table 3.1). If intact QS was indeed required for chronic persistence, then it appears questionable whether the small fraction of QS-proficient cells would be able to sustain the entire *P. aeruginosa* population. Based on recent *in vitro* and *in vivo*

studies, high proportions of QS-deficient variants are predicted to significantly decrease fitness under conditions that require QS (Diggle *et al*. 2007, Rumbaugh *et al*. 2009, Sandoz *et al*. 2007). We also found that predominantly QS-deficient *P. aeruginosa* CF populations persisted and colonized the CF lung to levels similar to those of QS-proficient populations, and that patients harboring these QS-deficient populations had significantly impaired lung function (much of which is generally attributed to *P. aeruginosa*-triggered inflammation). Although more data will be needed to substantiate this finding, one possible interpretation would be that QS is not important for chronic persistence in some individuals, and consequently, that social conflict is not a major selective force. This notion is consistent with a recent longitudinal screen of *P. aeruginosa* CF isolates for mutations in *lasR* suggesting that *lasR* mutants are associated with lung disease progression (Hoffman *et al*. 2009). In addition, acyl-HSL signals are often not detectable in sputum samples from CF patients colonized with *P. aeruginosa*. 3OC12-HSL could not be detected in 22%, 38%, and 46% of all samples, respectively (Chambers *et al.* 2005, Erickson *et al.* 2002, Middleton *et al.* 2002), while C4-HSL could not be detected in 74% of samples (Erickson *et al.* 2002). These data are in accordance with the view that invasive functions (which include many secreted quorum-controlled products and other "traditional" virulence factors) may become dispensable in progressive infections, which often occur in damaged tissues where nutrients are more accessible and immune responses are diminished (Nguyen and Singh 2006). In this case, the efficacy of QS as an anti-virulence drug target appears questionable. It is conceivable that QS, and

hence, social cheating, is more important early in chronic infection, for example for initial colonization of the CF lung. Data from chronic infection models indeed show that QS contributes to lung colonization (Wu *et al.* 2001). Thus, in addition to multiple selective pressures acting on QS evolution in the CF lung at any one time, there may be multiple forces acting at different times during the course of infection.

Many isolates were fully defective for some but not all of the phenotypes tested suggesting either that the responsible mutations are not in central regulatory genes, or that mutations are in central genes but secondary mutations partially restore expression of QS phenotypes. Global QS deficiencies in patient A and B isolates were due to mutations in *lasI* and *rhlI*, as well as impaired *lasR* expression. Complementation analysis of several patient B isolates harboring a presumed loss-of-function *lasR* allele indicated that an upstream regulatory defect, rather than the *lasR* mutation itself, is the underlying cause, emphasizing the importance of experimental verification. While we ruled out a defect in one important regulator of *lasR* expression, Vfr (Albus *et al.* 1997), which is commonly mutated in CF isolates (Smith *et al.* 2006), there are several other candidates (GacAS/RsmAZ, RelA, RpoS) (Schuster and Greenberg 2006), and presumably more that remain to be discovered. Chemical and genetic complementation with acyl-HSL signals and *lasR*, respectively, significantly increased elastase production, although absolute levels were, with one exception, lower than that of the PAO1 reference. This could mean that the genetic capacity for maximal elastase expression is simply lower in the clinical isolates, or that additional, specific mutations contribute to low-level expression. The latter is

plausible since these clinical strains grew significantly slower in rich medium, indicating pleiotrophic defects beyond QS. If social exploitation was the primary selective force in the evolution of QS variants, one might not expect to see *lasI* and *rhlI* mutants as well as other *lasR*-independent QS deficiencies. Mutations in the *las* system could be favored because it is atop the QS hierarchy, although, as recently shown, this regulatory hierarchy is dependent on growth conditions (Dekimpe and Deziel 2009, Medina *et al.* 2003). In particular, *lasR* mutants should have a selective advantage over *lasI* mutants. *lasR* mutants deficient in the expression of hundreds of QS-dependent genes would benefit from their production by others, whereas *lasI* mutants would only benefit from the 3OC12-HSL produced by others.

Although our prediction that global QS deficiencies are due to mutations in central QS genes turned out to be true, the correlation between individual QS phenotypes, acyl-HSL production, and underlying mutation in clinical isolates was not always as straight-forward as one might expect from studies with the PAO1 reference strain. For example, patient A isolates 3, 17 and 18 were partially proficient in expressing QS-controlled factors (Supplemental Table S3.1, Supplemental Figure S3.1, Table 3.3 and Figure 3.3A), although they did not produce detectable levels of acyl-HSL (Figure 3.2A). This suggests that in some cases QS-target genes are expressed independently of a functional QS circuitry, perhaps due to second-site mutations in QS-deficient backgrounds. If this is true, then the restoration of individual QS-regulated functions would in some cases be beneficial for chronic persistence. Similar observations have been made in other studies. In Tingpej *et al.*, a

small proportion of CF isolates produced the QS-controlled factors rhamnolipid and chitinase, although they did not produce detactable acyl-HSL (Tingpej *et al.* 2007). In Schaber *et al.*, one isolate from a urinary tract infection produced high levels of pyocyanin, although it produced less acyl-HSL compared with the PAO1 reference strain (Schaber *et al.* 2004). Cabrol *et al.* reported a lack of correlation between the growth-phase dependent transcription patterns of *lasR* and selected target genes, but there was a reasonable correlation between the presence of a mutation in *lasR* predicted to impair function and QS target gene expression (Cabrol *et al.* 2003).

Deviation in QS regulatory control from the PAO1 paradigm, along with the instantaneous within and between-patient diversity reported here, adds to the complexity of the role of QS in CF lung infection. Our results suggest that any one selective mechanism alone cannot account for the evolution of such diversity. An integrative cross-sectional and longitudinal approach will be needed to fully elucidate the forces driving QS evolution during chronic persistence of *P. aeruginosa*.

Acknowledgements

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Supplemental Tables

Table S3.1: QS phenotypes of all *P. aeruginosa* CF isolates[a]

Patient/Isolate	Mucoidy	Adenosine	Skim milk	Rhamnolipid	Staphylolysis	Elastin Congo Red
Patient A						
1	NM	-	-	-	-	-
2	NM	-	-	-	-	-
3	NM	-	○	○	○	○
4	NM	-	-	NG	-	-
5	NM	-	-	NG	-	-
6	NM	-	-	NG	-	-
7	NM	-	-	-	-	-
8	NM	-	-	-	-	-
9	NM	-	-	-	-	-
10	NM	-	-	-	-	-
11	NM	-	-	-	-	-
12	NM	-	-	-	-	-
13	NM	-	-	NG	-	-
14	NM	-	-	-	-	-
15	NM	-	-	-	-	-
16	NM	-	-	-	-	-
17	NM	+	○	○	○	○
18	NM	+	○	○	○	○
Patient B						
1	M	-	-	NG	-	-
2	M	-	-	NG	-	-
3	M	-	-	NG	-	-
4	M	-	-	NG	-	-
5	M	-	-	NG	-	-
6	M	-	-	NG	-	-
7	M	-	-	NG	-	-
8	M	-	-	NG	-	-
9	M	-	-	NG	-	-
10	M	-	-	NG	-	-
11	M	-	-	NG	-	-
12	M	+	○	○	○	○
13	M	-	-	NG	-	-
14	M	-	-	NG	-	-
15	M	-	-	NG	-	-
16	M	+	○	○	○	○

Continued on next page

Table S3.1-*Continued*

Patient/Isolate	Mucoidy	Adenosine	Skim milk	Rhamnolipid	Staphylolysis	Elastin Congo Red
17	M	+	○	○	○	○
18	M	+	○	○	-	○
Patient C						
1	M	-	○	-	+	○
2	NM	-	○	-	+	○
3	NM	-	-	-	+	○
4	M	-	-	-	+	○
5	M	-	-	-	+	○
6	M	-	-	-	+	○
7	M	-	-	-	○	○
8	M	-	-	-	+	+
9	M	-	-	-	+	+
10	M	-	○	-	+	○
11	NM	-	-	-	+	+
12	M	-	-	-	+	+
13	M	-	○	-	○	○
14	M	-	○	-	+	○
15	M	-	○	-	+	○
16	M	+	+	+	+	○
17	M	-	○	-	+	○
18	M	-	-	-	+	○
19	NM	-	-	NG	-	-
Patient D						
1	NM	+	+	○	+	+
2	NM	+	+	○	+	+
3	NM	+	+	○	+	+
4	NM	+	+	○	+	+
5	NM	+	+	○	+	+
6	NM	+	+	○	+	+
7	NM	+	+	○	+	+
8	NM	+	+	○	+	+
9	NM	+	+	○	+	+
10	NM	+	+	○	+	+
11	NM	+	+	○	+	+
12	NM	+	+	○	+	+
13	NM	+	+	○	+	+
14	NM	+	+	○	+	+
15	NM	+	+	○	+	+

Continued on next page

Table S3.1-*Continued*

Patient/Isolate	Mucoidy	Adenosine	Skim milk	Rhamnolipid	Staphylolysis	Elastin Congo Red
16	NM	+	+	○	+	+
17	NM	+	+	○	+	+
18	NM	+	+	○	+	+
19	NM	+	+	○	+	+
Patient E						
1	M	+	-	-	+	+
2	M	+	-	-	+	-
3	M	+	-	-	-	○
4	M	+	○	○	○	○
5	M	+	-	NG	-	○
6	M	-	-	NG	-	-
7	M	+	○	○	+	○
8	M	-	-	-	-	-
9	M	+	○	○	+	○
10	M	+	-	-	-	-
11	M	+	-	-	○	○
12	M	+	-	-	○	○
13	M	+	○	○	○	○
14	M	+	○	○	+	+
15	M	+	-	-	-	-
16	M	+	-	-	○	○
17	M	+	-	-	○	○
18	M	+	-	-	+	○
19	M	+	○	+	+	○
20	M	-	-	-	○	○
Patient F						
1	NM	+	-	-	+	+
2	NM	+	+	+	+	+
3	NM	+	+	+	+	+
4	NM	+	-	-	+	+
5	NM	+	-	-	-	-
6	NM	+	-	○	-	-
7	NM	+	-	-	-	-
8	NM	+	-	○	-	-
9	NM	+	-	○	-	-
Patient G						
1	M	-	○	NG	-	-
2	M	-	○	-	-	-

Continued on next page

Table S3.1-*Continued*

Patient/Isolate	Mucoidy	Adenosine	Skim milk	Rhamnolipid	Staphylolysis	Elastin Congo Red
3	M	-	○	-	-	-
4	NM	+	○	-	-	-
5	M	-	○	-	-	-
6	NM	+	○	-	-	-
7	M	+	○	+	○	-
8	M	-	○	-	-	-
9	M	+	+	○	+	+
10	NM	-	+	○	+	+
11	M	-	-	-	-	-
12	M	-	+	○	+	+
13	M	-	-	-	-	-
14	M	-	+	○	+	+
Patient H						
1	M	-	-	-	+	+
2	NM	-	-	-	-	-
3	M	-	○	-	-	-
4	M	-	○	-	-	-
5	M	+	+	○	+	+
6	M	-	-	-	○	○
7	M	-	-	-	+	○
8	NM	-	-	○	-	-
9	M	-	-	-	-	-
10	M	-	-	-	-	-
11	M	-	-	-	+	○
12	M	-	-	-	-	-
13	NM	-	-	-	-	-
14	M	+	-	-	-	-
15	M	+	-	-	-	-
16	M	-	-	-	○	-
17	M	-	-	-	-	-
18	M	-	-	-	○	+

[a]NM, non-mucoid; M, mucoid; NG, no growth.
For the adenosine utilization assay, "+" indicates growth, and "- " indicates no growth. For all other assays, "+" indicates a phenotype similar to the PAO1 wild type, "○" indicates a significantly impaired phenotype, and "-" indicates absence. See *Materials and Methods* for further details.

Supplemental Figures

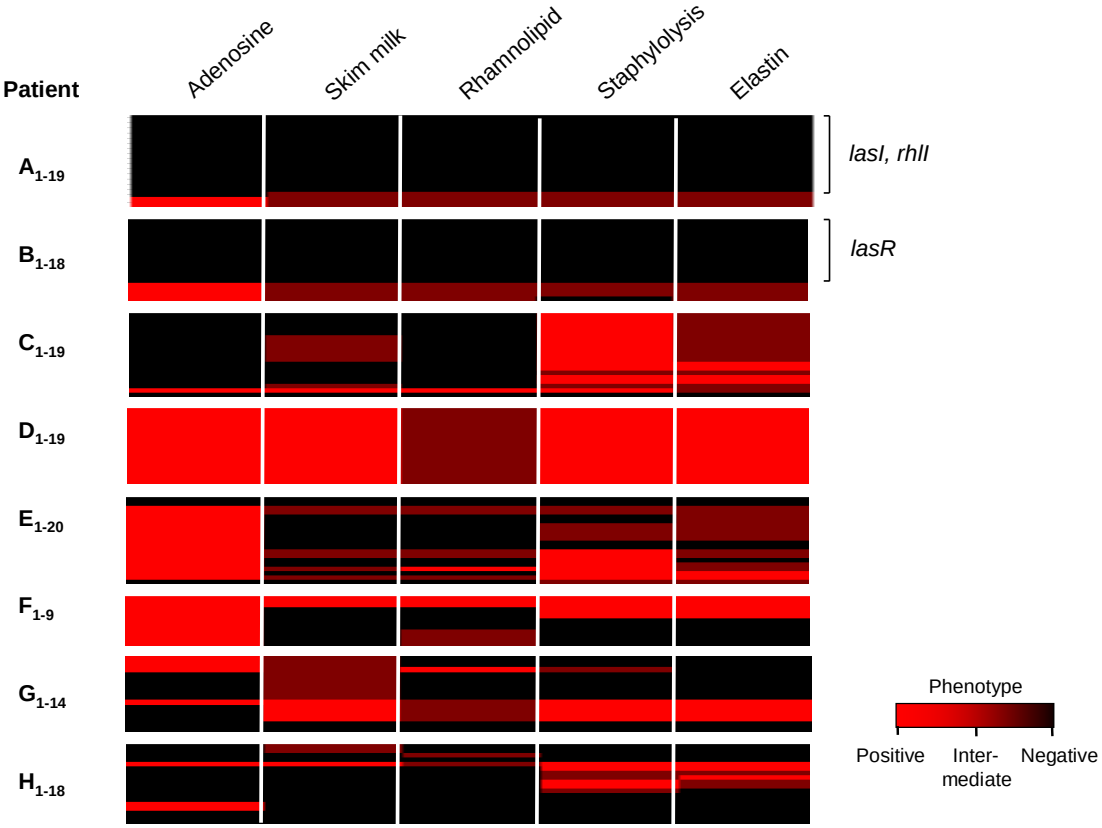

Figure S3.1: QS phenotypes of all *P. aeruginosa* CF isolates. The clustering analysis was created using the program Cluster 3.0; 2: Java Treeview. The parameters were as followed: Cluster, un-centered, complete linkage. This analysis visually represents the data in Supplemental Table 3.1 with color indicative of a QS-proficient or -deficient phenotype.

Within-sample quorum-sensing diversity of not-so-ubiquitous environmental populations of *Pseudomonas aeruginosa*

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Unpublished

Abstract

In *Pseudomonas aeruginosa*, quorum sensing (QS) coordinates cooperative behaviors including the production of extracellular factors required for nutrient acquisition and virulence. Regardless, QS-deficient isolates, particularly those carrying mutations in the central QS regulator *lasR*, are frequently isolated from infections. Both social conflict and non-social, intrinsic fitness benefits have been proposed to explain their emergence. However, very little is known about the abundance of such mutants in the environment. To obtain insight into the instantaneous within-sample QS diversity of environmental *P. aeruginosa* populations, we analyzed soil, sediment, and plant-associated samples from 68 different locations. Surprisingly, the large majority of these samples did not contain any *P. aeruginosa* despite presumed ubiquity. Of the six samples containing *P. aeruginosa*, we assayed a panel of 62 isolates (1-24 isolates per location) for various QS-controlled phenotypes. These populations contained predominantly QS-proficient isolates; few isolates demonstrated partially attenuated QS phenotypes. Sequencing and complementation analysis of selected QS-attenuated variants revealed partial or complete loss of function in the production of C4-HSL and AQ, respectively. Many QS-proficient and -attenuated isolates were clonally related, implying evolution from a common ancestor. Overall, our results indicate that the relatively homogenous QS populations of some natural environments do not appear to be subject to the selective pressures that favor the emergence of QS mutants during infections. Furthermore, this study suggests that *P. aeruginosa* may not be as ubiquitous as commonly assumed.

Introduction

Pseudomonas aeruginosa is a versatile organism found in a multitude of ecological environments throughout the world including soils, plants, and sediment sources (Goldberg 2000, Iglewski 1996, Lyczak *et al.* 2000). In humans, *P. aeruginosa* is an opportunistic pathogen frequently associated with disease in immunocompromised individuals, including those suffering from cystic fibrosis (CF) (Girard and Bloemberg 2008, Wagner and Iglewski 2008). The ability of this bacterium to adapt to a variety of locations and to transition from an environmental microbe to an opportunistic pathogen is thought to be due to its high capacity for genetic regulation (Goodman and Lory 2004, Williams and Camara 2009). Quorum-sensing (QS) is an important global regulatory circuit in this organism that controls gene expression in a cell-density dependent manner (Boyen *et al.* 2009, Williams and Camara 2009).

The *P. aeruginosa* QS circuitry is comprised of three distinct systems, the *las*, *rhl*, and *pqs* systems. These QS pathways are arranged hierarchically with the *las* system positively regulating both the *rhl* (Latifi *et al.* 1996, Pesci *et al.* 1997) and *pqs* (Wade *et al.* 2005) systems. Additionally, the *rhl* system negatively regulates the *pqs* system (McGrath *et al.* 2004, Wade *et al.* 2005). Together, these systems regulate over three-hundred genes, with the *rhl* and *pqs* systems controlling subsets of 112 (≥ 2-fold activation by both signals versus 3OC12-HSL alone) (Schuster *et al.* 2003) and 141 genes (Deziel *et al.* 2005), respectively.

Each of these three systems consists of a diffusible autoinducer signaling molecule and its cognate regulator protein. When population density is high, the autoinducer signaling molecule accumulates and binds its cognate regulator to form a complex that controls gene transcription. Both the *las* and *rhl* systems are acyl-homoserine lactone (acyl-HSL) mediated QS systems, consisting of the autoinducer synthases LasI and RhlI, that generate *N*-(3-oxo-dodecanoyl)-HSL (3OC12-HSL) and *N*-butanoyl-HSL (C4-HSL), respectively (Pearson *et al.* 1994, Pearson *et al.* 1995). The *pqs* system is an alkyl-quinolone (AQ) mediated QS system requiring anthranilate and the products of the *pqsABCD* operon in the synthesis of 2-heptyl-4-quinolone (HHQ). HHQ is subsequently converted into the autoinducer 2-heptyl-3-hydroxy-4-quinolone (PQS) via a FAD-dependent monooxygenase encoded by *pqsH* (Bredenbruch *et al.* 2005). Once a quorum is met, 3OC12-HSL, C4-HSL, and PQS bind to their cognate regulators, LasR, RhlR, and PqsR, respectively, to form a complex that regulates gene transcription (Dubern and Diggle 2008, Juhas *et al.* 2005, Schuster and Greenberg 2006).

Although QS in *P. aeruginosa* is required for virulence and acquisition of certain nutrients, QS-deficient variants have been isolated from numerous infections (D'Argenio *et al.* 2007, Heurlier *et al.* 2006, Smith *et al.* 2006, Tingpej *et al.* 2007, Wilder *et al.* 2009). Frequently, these isolates harbor loss-of-function mutations in the central regulator gene, *lasR*. In CF lung infections, the prevalence of *lasR* mutants has been reported to be as high as 31% (Hoffman *et al.* 2009). One study also characterized 16 environmental *P. aeruginosa* strains isolated from swimming pools

and rivers. Three isolates harbored non-synonymous mutations in *lasR* and showed decreased transcription of *lasR*-dependent genes (Cabrol *et al.* 2003). Previous studies have suggested that QS mutants could arise due to nonsocial mechanisms, such as an intrinsic growth advantage at low oxygen, alkaline pH, or in the presence of certain amino acids (D'Argenio *et al.* 2007, Heurlier *et al.* 2005, Hoffman *et al.* 2010). Alternatively, these isolates may arise via social exploitation (Diggle *et al.* 2007b, Rumbaugh *et al.* 2009, Sandoz *et al.* 2007). Under growth conditions requiring QS, *lasR* mutants evolve from a wild-type ancestor and have a distinct growth advantage when co-cultured with the wild type parent strain (Diggle *et al.* 2007b, Sandoz *et al.* 2007). These mutants also increase in frequency *in vivo* during co-infection of mice (Rumbaugh *et al.* 2009). In particular, a recent study of *P. aeruginosa* populations from the CF lung characterized, both phenotypically and genotypically, the instantaneous within-patient heterogeneity of QS populations (Wilder *et al.* 2009). This study revealed that there are highly diverse populations of QS-proficient and -deficient isolates within and between patients, suggesting that there are diverse selection pressures within the CF lung that may contribute to this heterogeneity via social or nonsocial mechanisms.

To our knowledge, such a study has not been performed for environmental *P. aeruginosa* populations. Several environmental population studies have focused on surveying the biodiversity (Matos *et al.* 2005, Pellett *et al.* 1983, Pirnay *et al.* 2005, Roy and Nair 2007, Wiehlmann *et al.* 2007) of individual *P. aeruginosa* isolates from geographically and ecologically distinct sites. In the present study, we

comprehensively characterized, phenotypically and genotypically, the instantaneous within-sample diversity, which is the heterogeneity among isolates collected from a single sample at any one time, of *P. aeruginosa* QS populations from a diverse selection of ecological sites. Overall, this study further contributes to the understanding of how social conflict affects diversity in *P. aeruginosa* QS populations.

Materials and Methods

Bacterial strains and plasmids

Laboratory strains and plasmids used in this study are shown in Table 4.1.

Table 4.1: Bacterial strains and plasmids

Lab strain or plasmid	Relevant property(ies)	Reference
Strains		
PAO1	*P. aeruginosa* wild-type (Vasil/Ochsner)	(Holloway *et al.* 1979)
PAO1 *lasR*	PAO1 derivative; Δ*lasR*, unmarked in-frame deletion from amino acid 102 to 216	(Wilder *et al.* 2011)
PAO1 *rhlR*	PAO1 derivative; Δ*rhlR*, unmarked in-frame deletion from amino acid 102 to 225	(Wilder *et al.* 2011)
PAO1 *pqsR*	PAO1 derivative; Δ*pqsR*, unmarked in-frame deletion from amino acid 19 to 252.	(Wilder *et al.* 2011)
PfO1	*Pseudomonas fluorescens* wild-type	(Hinsa *et al.* 2003)
Plasmids		

Continued on next page

Table 4.1-*Continued*

Lab strain or plasmid	Relevant property(ies)	Reference
pJN105	*araC*-pBAD cloned in pBBR1 MCS-5; Gm^R	(Newman and Fuqua 1999)
pJN105.*pqsR*-H	*pqsR* coding region in pJN105	(Wilder *et al.* 2011)
pJN105.*pqsR*-N	*pqsR* coding region and upstream regulatory region in pJN105 devoid of *araC*-pBAD	(Wilder *et al.* 2011)

Bacterial isolation and species identification

Environmental samples were collected as previously described from various locations including the soil, sediment, plant rhizospheres, compost, and mushroom surfaces (Supplemental Table S4.1) (Loper *et al.* 1984). These samples were obtained throughout April-September 2010 and 2011 from Oregon, Idaho, Virginia, Germany, and the Czech Republic. Sets of two samples were collected 10 cm apart and processed. For each sample, the following volumes were processed: soil and compost, 5-7 g; sediment, 5-7 g; for plant rhizospheres, 1 rhizosphere system. Samples were incubated in 25 ml of washing buffer (0.1 M phosphate buffer supplemented with 0.1% Bacto-peptone) and shaken (200 rpm) at room temperature for 1 h. Subsequently, samples were plated on X107 media (*Pseudomonas* isolation agar supplemented with 15 µg/ml Nalidixic acid and 200 µg/ml Cetrimide) (Pirnay *et al.* 2005) and incubated for 24 h at 37^{o}C. Colonies were then re-streaked on X107 media. Isolated colonies were grown in LB liquid culture and then frozen as glycerol stocks

as well as analyzed for species type. For each environmental sample, the approximate detection limit is as follows: sediment 25 CFU/ml; soils 25 CFU/g; rhizosphere 250 CFU/rhizosphere; mushroom 250 CFU/mushroom; and compost 2500 CFU/g. This detection limit indicates the minimum number of *P. aeruginosa* colony forming units (CFU) that must be present per unit of sample in order to be detected by our methods. This calculation takes into account the quantity of washing buffer used to resuspend the sample as well as any dilutions made before the sample was plated.

To determine if the environmental isolates were indeed *P. aeruginosa*, we used species-specific 16S ribosomal RNA primers (PA-SS-F and PA-SS-R) in a polymerase chain reaction (PCR) as previously described (Spilker *et al.* 2004). Isolates were also species-typed via PCR using primers oprlF and oprlR to amplify *oprL*, a gene specific to *P. aeruginosa* (Deschaght *et al.* 2009). The resultant PCR samples were analyzed using agarose gel electrophoresis.

Environmental enrichment of P. aeruginosa

To compare our isolation of *P. aeruginosa* to an established enrichment protocol, we gathered soil samples and incubated them in washing buffer as described above. Subsequently, we added aliquots of each sample to acetamide broth (Hedberg 1969, Pellett *et al.* 1983, Smith and Dayton 1972) and incubated up to 4 days at either 37^{o}C or 42^{o}C. Samples were checked daily for UV fluorescence as an indication of the presence of *P. aeruginosa*. Following growth, samples were plated on X107 agar (Pirnay *et al.* 2005), *Pseudomonas* isolation agar (PIA), or Lennox Broth (LB) agar

and incubated 24-48 hours at 37°C. After growth in acetamide broth, the PAO1 wild-type control grows on all three plate types. Samples that did not grow on PIA or X107 media were not considered *P. aeruginosa*.

Production of extracellular proteases, rhamnolipids, nucleoside hydrolase, and alkyl-quinolones (AQ)

These assays were performed in high-throughput format with the appropriate wild-type and various QS-deficient controls (Table 4.1). Skim-milk proteolysis (Sandoz *et al.* 2007), rhamnolipid production (Kohler *et al.* 2000), and growth on adenosine, indicative of nucleoside hydrolase production (Heurlier *et al.* 2005, Sandoz *et al.* 2007), were determined through the use of agar plate assays. AQ production, consisting of both PQS and its precursor HHQ, was detected by bioassay (Fletcher *et al.* 2007). Initially, isolates were grown on Lennox LB agar plates at 37°C for 24 hours. For plating assays, colonies were subsequently replica-plated onto the respective agar test plates. For detection of AQ production, colonies were grown for 18 h in LB broth supplemented with 50 mM 3-(N-morpholino)-propanesulfonic acid (MOPS), pH = 7.0. Culture supernatants were subsequently filtered, and the sterile supernatants were used in a bioassay (Fletcher *et al.* 2007). The scoring scheme was as follows: For AQ production < 20% of the wild-type was considered negative. For adenosine plates, growth was scored as "+", while the absence of growth was scored as "-". For skim-milk proteolysis and rhamnolipid production, the formation of a halo similar to the PAO1 wild-type was scored as "+", the formation of a halo significantly

smaller than that of the PAO1 wild-type was scored as "○", and the absence of a halo was scored as "-".

Random Amplified Polymorphic DNA analysis (RAPD)

RAPD analysis was performed as previously described using 80 ng of genomic DNA per PCR reaction, amplified using primer 208 (Mahenthiralingam *et al.* 1996, Mereghetti *et al.* 1998). Chromosomal DNA was prepared from *P. aeruginosa* liquid cultures using the PURGENE^{TM} DNA Purification Kit (Gentra Systems). Following PCR amplification, samples were visualized using a 1.2% agarose gel run at 85 V for 1 hour 45 min. RAPD analysis was performed and analyzed twice with independently amplified DNA as previously described (Wilder *et al.* 2009). In both cases, very similar results were obtained. Samples were compared using GelCompar II software, with a dendrogram constructed based on densitometric curves (Ranked pearson correlation, 1% optimization, 1% curve smoothing, UPGMA cluster analysis).

DNA sequencing

PCR fragments amplified from isolated *P. aeruginosa* DNA were sequenced at the Center for Genome Research and Biocomputing at Oregon State University. Isolates were amplified and sequenced using appropriate primers as previously described (Wilder *et al.* 2009, Wilder *et al.* 2011).

Acyl-HSL detection

We quantified C4-HSL production in selected strains using an acyl-HSL detection bioassay as previously described (Passador *et al.* 1996, Pearson *et al.* 1995, Pearson *et al.* 1997). Isolates were grown for 18 hours in LB broth supplemented with 50 mM MOPS. Following growth, C4-HSL was extracted from culture supernatants using ethyl acetate. Production of C4-HSL from each sample was then detected and quantified using the reporter strain DH5α/pECP61.5 as previously described (Passador *et al.* 1996, Pearson *et al.* 1995, Pearson *et al.* 1997).

Complementation analysis

For complementation with *pqsR*, we used the low copy-number plasmids pJN105.*pqsR*-H, pJN105.*pqsR*-N, as well as the empty control vector pJN105 (Table 4.1). In pJN105.*pqsR*-H and pJN105.*pqsR*-N, *pqsR* is under the control of the arabinose-inducible *araBAD* promoter (Lee *et al.* 2006b, Newman and Fuqua 1999) and the native *pqsR* promoter, respectively. Plasmids were introduced into environmental strains through electroporation (Choi and Schweizer 2006). Strains were then tested for complementation using an AQ detection bioassay (Fletcher *et al.* 2007). For AQ detection, we grew test strains for 18 h in LB broth supplemented with 50 mM MOPS and 5 mM L-arabinose. We did not use gentamicin for this assay type as it would kill the biosensor strain; we have determined that there is no significant plasmid loss (Wilder *et al.* 2011).

Results

Isolation of P. aeruginosa from the environment

The main objective of this study was to determine the proportion of QS-deficient and QS-proficient ecological isolates from the natural environment in order to further understand the effects of social conflict on QS diversity. Our goal was to characterize numerous concurrent isolates per environmental location to collect sufficient information about the QS diversity of a population. To do so, we used a direct method of isolation where bacterial cells are first separated from soil and plant particles and are then immediately plated on selective media (Pirnay *et al.* 2005). This method is preferable as it preserves the original population diversity and abundance as much as possible. We sampled from a variety of environmental locations within Oregon, Idaho, Virginia, the Czech Republic, and Germany (Supplemental Table S4.1). The majority of our samples were obtained from assorted locations in Oregon, as depicted in Figure 4.1. These samples varied in the type of sample (i.e. sediment, soil, rhizosphere) as well as the consistency of the sample (i.e. variations in soil type). Additionally, we sampled from organic compost piles as an example of nutrient-rich soil. Generally, composting is a decomposition process by which microbes convert decaying organic matter into a nutrient-rich soil-like material (Finstein and Morris 1975, Gray *et al.* 1971). Overall, these sample types were chosen because they are surface-associated environments that would promote the establishment of a bacterial community (Danhorn and Fuqua 2007, Ettema and Wardle 2002).

After sampling over 60 sites using isolation methods as described by Loper *et*

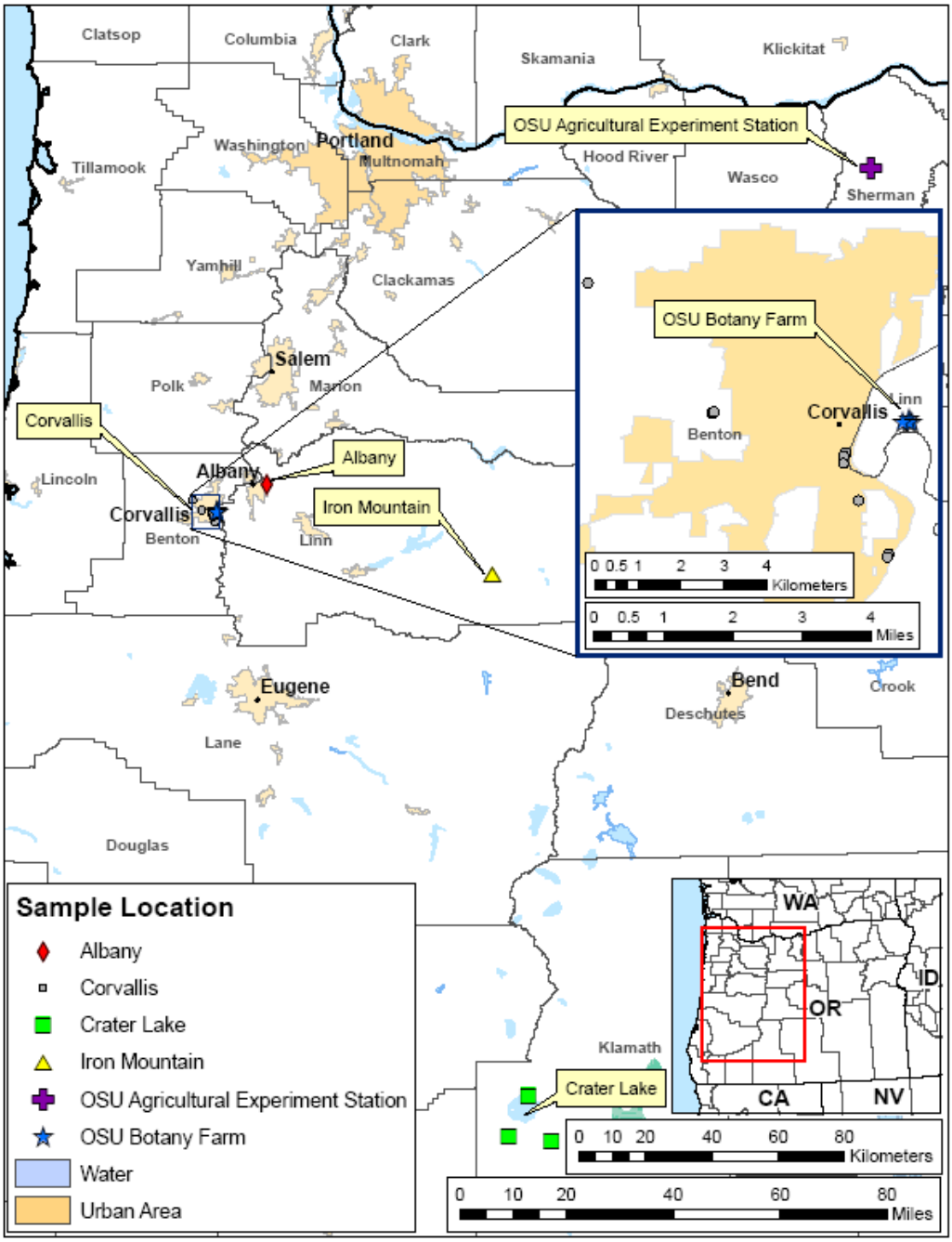

Figure 4.1: Map of environmental samples collected from Oregon. Samples were collected from different locations around Oregon each varying in sample type and consistency. Samples were obtained from various soils, sediment sources, rhizospheres, a mushroom surface, and composts.

al. and Pirnay *et al.*, we obtained only one *P. aeruginosa* isolate from a sediment sample obtained from a stagnant water source found in Albany, Oregon (Sample 6; Table 4.2, Supplemental Table S4.1). Additionally, we found *P. aeruginosa* present in 5 compost samples at bacterial loads of 10^3-10^4 CFU/gram (Supplemental Table S4.1). Species identity was verified using 16S rRNA analysis (Spilker *et al.* 2004). *P. aeruginosa* populations obtained from sediment and compost samples were further tested for QS diversity.

Enrichment of bacteria from the environment

It was surprising that we were able to culture so few *P. aeruginosa* isolates from the environment, as this microbe is often described as ubiquitous (Gregory and Schaffner 1987, Lyczak *et al.* 2000, Pellett *et al.* 1983, Pirnay *et al.* 2005). If *P. aeruginosa* was indeed present in the samples, the bacteria could be in a viable but non-culturable (VBNC) state. In this state, cells are intact and metabolically active, however are unculturable due to a variety of stresses including nutrient starvation, low or high temperatures, or changes in environmental pH (Nystrom 2001, Roszak and Colwell 1987). Therefore, we compared our procedure to an established enrichment protocol. In the latter method, enrichment would allow for the resuscitation of bacteria in the VBNC state. Individual samples (Supplemental Table S4.1; Samples 6, 8, 15, 16) were grown for 4 days at either 37^{o}C or 42^{o}C in minimal media containing acetamide as the sole carbon source (Hedberg 1969, Pellett *et al.* 1983, Smith and Dayton 1972). Generally, acetamide broth has been found to be highly selective for the growth of *P.*

aeruginosa (Hedberg 1969), and has been previously used to enrich for *P. aeruginosa* from riverine ecosystems (Pellett *et al.* 1983) and rectal swabs from burn victims (Smith and Dayton 1972). Following enrichment, bacteria were subsequently plated on both selective (X107, PIA) and nonselective (LB agar) media to determine the presence of *P. aeruginosa*.

Sample sets from each location were unable to grow on selective media whereas our PAO1 wild type control could. These results suggest that *P. aeruginosa* may not be present in the sampling locations. This is consistent with results from our initial method of bacterial isolation.

Environmental samples predominately contain QS-proficient isolates

Our collection of *P. aeruginosa* isolates (Supplemental Table S4.1) was screened for various QS-dependent phenotypes (Table 4.2). All samples were tested for skim milk proteolysis, rhamnolipid and AQ production, and growth on adenosine. In the PAO1 wild-type reference strain, skim milk proteolysis and growth on adenosine are *las*-dependent phenotypes (Sandoz *et al.* 2007); rhamnolipid production is a *rhl*-dependent phenotype (Schuster *et al.* 2003); and AQ production is a *pqs*- dependent phenotype (Deziel *et al.* 2005). All *P. aeruginosa* populations were predominantly QS-proficient. For four out of five QS-dependent phenotypes, the percentage of proficient isolates was above 75% (Tables 4.2 and S4.2). Additionally, several of these populations contained a few isolates that demonstrated partially attenuated QS phenotypes as compared to the PAO1 reference strain.

Table 4.2: Characteristics of *P. aeruginosa* populations from environmental samples

Sample	Sample type	*P. aeruginosa* load (CFU/gram)	Number of isolates	Percentage of QS-proficient isolates in the QS assay indicated[a]			
				Skim milk	Rhamnolipid	Adenosine	AQ
1	Compost	10^3	24	100	100	100	100
2	Compost	10^4	17	76	100	100	88
3	Compost	10^4	4	100	100	100	25
4	Compost	10^3	6	83	100	100	100
5	Compost	10^4	10	80	90	100	90
6	Lake sediment	10^0	1	100	100	100	100

[a] QS-proficient isolates include those with partially attenuated QS phenotypes as designated "○" in Supplemental Table S4.2. For AQ, isolates with > 20% AQ production as compared to the PAO1 wild-type are included.

P. aeruginosa within-sample populations are not clonal

The clonal relatedness of isolates from all samples was assessed by RAPD analysis (Figure 4.2, Supplemental Table S4.2). We considered samples with ≥ 95% similarity to be the same variant type. Within each sample, there were multiple RAPD groupings, indicating fairly diverse populations. Additionally, there were strain variants that were common to more than one sample, suggesting that there are several environmental *P. aeruginosa* strains that are more predominant and robust than others. Lastly, there was no apparent correlation between RAPD profiles and QS phenotypes. There were QS-proficient and -attenuated isolates of the same RAPD type, suggesting that both types evolved rather recently from a common ancestor.

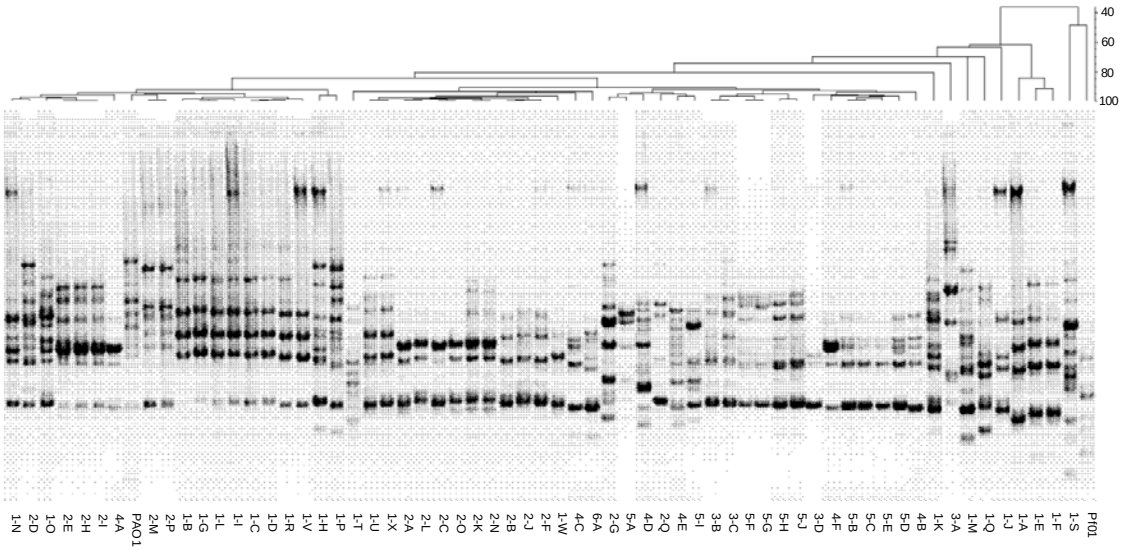

Figure 4.2: RAPD analysis of environmental isolates. Isolates 1A to 1X, 2A to 2Q, 3A to 3D, 4A to 4F, 5A to 5J, and 6A are from sample 1, sample 2, sample 3, sample 4, sample 5, and sample 6, respectively. PAO1 and Pf01 were used as species and non-species controls, respectively. The scale next to the dendrogram indicates relatedness of isolates in percentages. Samples with ≥95% similarity were considered to be the same variant.

Environmental QS-deficient isolates harbor mutations in rhlI and pqsR

Though most *P. aeruginosa* isolates from each sample were predominantly QS-proficient, several isolates demonstrated either a deficiency or attenuation in various QS phenotypes. To obtain further insights into the mutations responsible for these deficient phenotypes, we sequenced QS genes that were believed to be mutated based on their phenotypic profiles (Table 4.2, Supplemental 4.2). For isolates that had minimal rhamnolipid production and enhanced AQ synthesis, we predicted that there would be mutations in either the *rhlR* or *rhlI* gene (Wilder *et al.* 2011). After sequencing, we found that these isolates harbor mutations in the *rhlI*, but not *rhlR*,

Table 4.3: Sequence analysis of selected *P. aeruginosa* environmental isolates

Sample/Isolate[a]	Gene	Non-synonymous mutation[b]	Change[c]
Sample 1			
H	*rhlR*	None	None
H	*rhlI*	A→G (+184), C→A (+249), G→T (+498)	S62G*, D83E, E166D
M	*rhlR*	None	None
M	*rhlI*	A→G (+184), C→A (+249), G→T (+498)	S62G*, D83E, E166D
P	*rhlR*	None	None
P	*rhlI*	A→G (+184), C→A (+249), G→T (+498)	S62G*, D83E, E166D
U	*rhlI*	C→A (+249)	D83E
Sample 2			
B	*pqsR*	A→C (+928), C→T (+941)	I310L*†, A314V*
J	*lasR*	None	None
J	*lasI*	None	None
Sample 3			
B	*pqsR*	A→C (+928), C→T (+941)	I310L*†, A314V*
D	*pqsR*	A→C (+928), C→T (+941)	I310L*†, A314V*
Sample 5			
A	*rhlR*	None	None
A	*rhlI*	A→G (+184), C→A (+249), G→T (+498)	S62G*, D83E, E166D
A	*pqsR*	A→C (+928), C→T (+941)	I310L*†, A314V*
C	*pqsR*	A→C (+928), C→T (+941)	I310L*†, A314V*

[a]Environmental isolates of the respective sample are indicated by a letter.
[b]Nucleotide substitution or insertion at the indicated position relative to translational start site
[c]Amino acid changes relative to the PAO1 protein sequence. *, indicates that the mutation is predicted to be not tolerated by the SIFT algorithm (Ng and Henikoff 2003). †, indicates that the amino acid is conserved.

gene. Isolates 1-H, 1-M, 1-P, and 5-A each had missense mutations in *rhlI*, resulting in a Ser to Gly substitution, an Asp to Glu substitution, and a Glu to Asp substitution (Table 4.3). Only the S62G substitution was predicted to be not tolerated by the SIFT algorithm (Ng and Henikoff 2003). For comparison, we sequenced the QS-proficient isolate 1-U (Table 4.3), which did not harbor the S62G and E166D mutations in *rhlI*.

Compared with the PAO1 reference sequence, the AQ-deficient isolates 2-B, 3-B, 3-D and 5-A each harbored missense mutations in *pqsR*, resulting in an Ile to Leu substitution and a Ala to Val substitution (Table 4.3). Both substitutions of I310L and A314V in *pqsR* were predicted to not be tolerated by the SIFT algorithm (Ng and Henikoff 2003). However, a Val at position 314 was found to naturally occur in *P. aeruginosa* strain PA14 (Lee *et al.* 2006a). Upon further analysis, we found that the AQ-proficient isolate 5-C had the same amino acid changes resultant from identical mutations in the *pqsR* gene, indicating that these mutations are not responsible for the loss of AQ production seen in isolates 2-B, 3-B, 3-D, and 5-A.

Environmental isolates with mutations in rhlI are attenuated, but not fully deficient, in C4-HSL production

Of the four *rhlI* mutants found (Table 4.3), isolates 1-H, 1-M and 1-P each produced pyocyanin (data not shown); a function which is predominantly controlled by the *rhl* system (Gupta *et al.* 2009). This may indicate that the amino acid changes in RhlI may lead to the attenuated, rather than diminished, production of C4-HSL. To further analyze these findings, we quantified C4-HSL production of each isolate. Interestingly, we found that isolates 1-H, 1-M, and 1-P each produce C4-HSL albeit

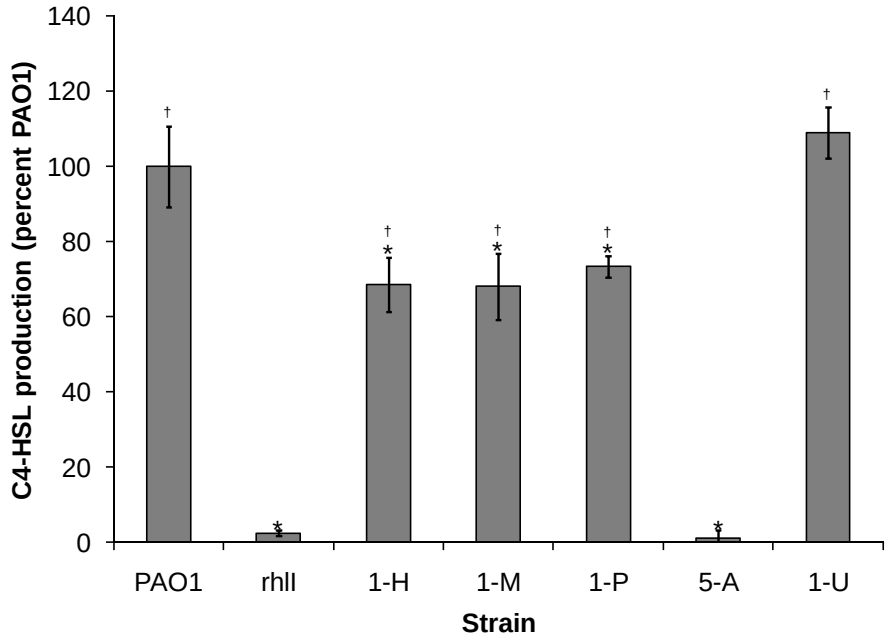

Figure 4.3: Quantification of C4-HSL from selected *P. aeruginosa* isolates. Isolates are from samples 1 and 5. Isolate 1-U is a C4-HSL proficient environmental strain control. Replicates were performed in triplicate and are normalized to optical density. For statistical analysis, values were compared to either the PAO1 wild-type (*) or the *rhlI* mutant (†) using a two-tailed unpaired *t*-test; *p*-values of ≤ 0.05 are statistically significant.

significantly less than the PAO1 wild-type reference (Figure 4.3). In comparison, the QS-proficient isolate 1-U produced wild-type levels of C4-HSL. Overall, these results indicate that the *rhlI* mutations found cause attenuated C4-HSL production.

In contrast, strain 5-A produced little to no C4-HSL, similar to that of the *rhlI* negative control. This result contradicts the findings of the other three *rhlI* mutants, considering all four strains harbor the same amino acid changes in the RhlI protein (Table 4.3). The diminished level of C4-HSL in isolate 5-A may be in part due to the presence of a secondary mutation within the *pqs* system, causing a deficiency in AQ-production (Table 4.3, Supplemental Table S4.2). Under wild-type conditions, the *pqs*

system positively regulates transcription of the *rhlI* gene (McKnight *et al.* 2000). Therefore, it is possible that C4-HSL production in isolate 5-A is fully diminished, rather than attenuated, due to the loss of *rhlI* up-regulation by the *pqs* system.

AQ-deficient environmental isolates likely harbor mutations in genes required for AQ synthesis

Sequence analysis suggested that the AQ-deficient phenotype of isolates 2-B, 3-B, 3-D and 5-A was not caused by the mutations present in the *pqsR* gene. However, a recent study showed that *pqsR* mutants have a growth advantage under conditions that require QS (Wilder *et al.* 2011). Therefore, to determine if this phenotype was due to mutations in an upstream regulator of the *pqs* system, we transformed each strain with a wild-type copy of *pqsR* under the control of either the native promoter (pJN105.*pqsR*-N) or the heterologous pBAD-*araC* promoter (pJN105.*pqsR*-H) (Table 4.1). We analyzed these environmental isolates containing the appropriate plasmids using an AQ-detection bioassay (Fletcher *et al.* 2007). As expected, AQ production was not restored with the addition of a wild-type *pqsR* gene under control of the heterologous pBAD-*araC* promoter (Figure 4.4). Additionally, AQ production was also not restored with the addition of *pqsR* under control of the native promoter (Figure 4.4). These results indicate that the AQ-deficient phenotype is not due to mutations in either the *pqsR* gene, promoter region, or an upstream regulator. Thus, the deficient phenotype is likely a result of aberrant expression of genes required for the synthesis of AQ. Generally, for the generation of the PQS precursor, HHQ, both anthranilate and the products of *pqsABCD* are required. Thus, mutations in genes

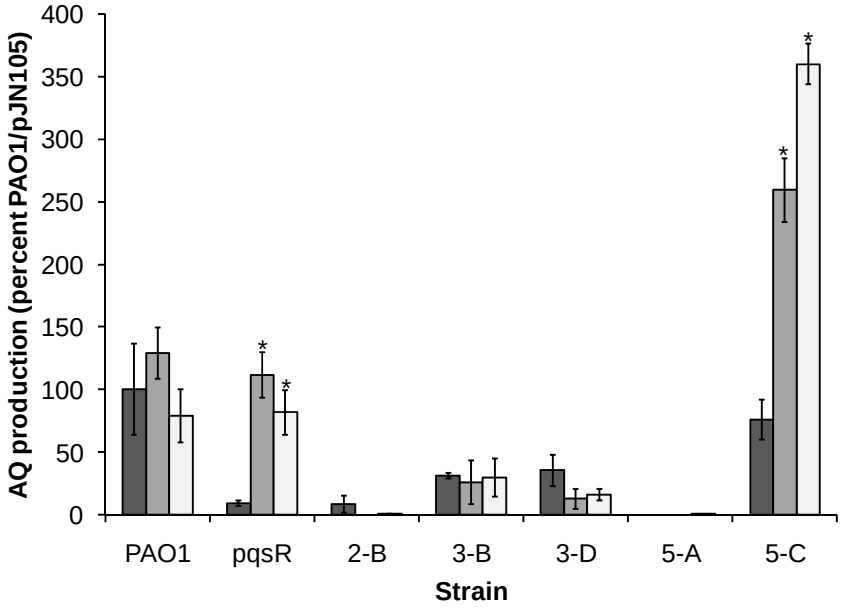

Figure 4.4: *pqsR* complementation of selected environmental isolates. Isolates are from samples 2, 3 and 5 and were transformed with either pJN105 (dark grey), pJN105.*pqsR*-H (medium grey), or pJN105.*pqsR*-N (light grey). Isolate 5-C is an AQ-proficient environmental strain control. Replicates were performed in triplicate and normalized to optical density. Statistical significance of the data was determined using a two-tailed unpaired *t*-test, with "*" indicating *p*-values of ≤ 0.05. Within each isolate, expression with either pJN105.*pqsR*-H or pJN105.*pqsR*-N was compared to that with the vector control.

involved in the synthesis or degradation of anthranilate as well as genes within the *pqsABCD* operon could all lead to an AQ deficient phenotype. Unfortunately, we were unable to locate the site of the mutation. Additionally, we were unable to complement the AQ-deficient phenotype via synthetic AQ signals; there was no detectable difference in the production of pyocyanin or elastase in the presence versus the absence of added signal (data not shown).

Discussion

In the present study, we sought to characterize *P. aeruginosa* populations from the natural environment to obtain further insight into how social conflict affects the diversity and evolution of QS. Current environmental population studies have focused primarily on surveying the biodiversity of *P. aeruginosa* from various ecological sites (Matos *et al.* 2005, Pellett *et al.* 1983, Pirnay *et al.* 2005, Roy and Nair 2007, Wiehlmann *et al.* 2007). Our study is distinct in that we investigated the instantaneous within-sample diversity of *P. aeruginosa* QS populations through the examination of a variety of natural locales. Generally, we analyzed an array of samples including soils, composts, sediment sources, rhizospheres, and a mushroom, using a sampling technique that was successfully used to isolate *P. aeruginosa* from the environment (Loper *et al.* 1984, Pirnay *et al.* 2005).

After sampling 68 locations, we were able to obtain one *P. aeruginosa* isolate only from one sediment sample from a stagnant lake and up to 24 isolates from various composts. The isolation of so few *P. aeruginosa* isolates from the environment was surprising as this bacterium has been isolated from a variety of locations and is often described as a ubiquitous microbe (Gregory and Schaffner 1987, Lyczak *et al.* 2000, Pellett *et al.* 1983, Pirnay *et al.* 2005). It is conceivable that our inability to culture many *P. aeruginosa* isolates from the natural environment is because the bacteria were not in a state ready to tolerate highly selective media. One explanation for this condition is that the bacteria were in a VBNC state (Nystrom 2001, Roszak and Colwell 1987). It is possible that many of the environments we

sampled from were not ideal for active growth of *P. aeruginosa* due to cultural stresses such as low or high temperatures, changes in pH, or nutrient starvation (Nystrom 2001, Roszak and Colwell 1987). In contrast, the *P. aeruginosa* isolates that were obtained for this study may not have been in a VBNC state due to their localization in nutrient-rich environments. Isolate 6-A, for example, was obtained from a sediment sample within a stagnant lake where nutrients may be readily available due to natural or artificial eutrophication (Rodhe 1969). Generally, this process promotes excessive plant growth and decay, thus providing nutrients that can enhance the growth of microorganisms (Raike *et al.* 2003, Rodhe 1969). Additionally, isolates from samples 1 through 5 were obtained from different nutrient rich composts, which contain various decaying organic matter (Finstein and Morris 1975, Gray *et al.* 1971).

To analyze the possibility that the bacteria were in a VBNC state, we compared our method of isolation to an established enrichment method. This latter method allows for the selective growth of *P. aeruginosa* (Hedberg 1969), and has been used in previous studies in the isolation of *P. aeruginosa* from riverine ecosystems (Pellett *et al.* 1983) as well as rectal swabs from burn victims (Smith and Dayton 1972). Interestingly, both methods exhibited comparable results, indicating that *P. aeruginosa* was not present in many of our samples. This trend can be further analyzed by determining either the presence or absence of *P. aeruginosa* DNA via species-specific real-time PCR (Appendix A) (Deschaght *et al.* 2009). Overall, this finding is significant in that it contradicts the current belief that *P. aeruginosa* is a

ubiquitous microbe. Rather, *P. aeruginosa* has the ability to grow in a variety of environments but may not be present in them.

Overall, our panel of environmental *P. aeruginosa* isolates demonstrated predominantly QS-proficient phenotypes (Table 4.2). Isolates that displayed aberrant QS phenotypes as compared to the PAO1 wild type were analyzed for mutations in major QS genes (Table 4.3). Of these strains, isolates harbored mutations in genes required for production of the autoinducer C4-HSL or PQS (Table 4.3, Figures 4.3, 4.4). All isolates attenuated or deficient in the production of C4-HSL were found to harbor identical mutations in *rhlI*. It is likely that isolate 5-A is deficient, rather than attenuated, in the production of C4-HSL due to a secondary mutation resulting in an AQ-deficient phenotype. Under wild-type conditions, the *pqs* system up-regulates the transcription of *rhlI*; therefore, the loss of *pqs*-dependent regulation in isolate 5-A would result in diminished C4-HSL production (McKnight *et al.* 2000). Interestingly, all *rhlI* mutants harbored a mutation, D83E, which has also been found in isolates within cystic fibrosis lung infections; however, this mutation does not appear to play a role in the attenuation of C4-HSL production (Wilder *et al.* 2009).

Through sequencing and complementation analysis with a wild type copy of *pqsR*, we determined that our isolates deficient in AQ production harbor mutations in one or more genes required for AQ synthesis. This may include genes involved in either the synthesis or degradation of the PQS precursor compounds anthranilate and HHQ (Bredenbruch *et al.* 2005, Deziel *et al.* 2005, Gallagher *et al.* 2002). The anthranilate precursor is primarily derived from the degradation of tryptophan within

the kynurenine pathway (*kynA*, *kynB*, and *kynU*) (Farrow and Pesci 2007). In the absence of tryptophan, anthranilate is synthesized through the conversion of chorismate to anthranilate by an anthranilate synthase (*phnAB*) (Deziel *et al.* 2004). Recent studies demonstrate that disruption of these metabolic pathways results in either diminished or attenuated PQS production, respectively (Farrow and Pesci 2007). Anthranilate levels can also be affected by the upregulation of anthranilate dioxygenase (*antABC*) or its cognate regulator (*antR*); these operons are involved in anthranilate degradation (Oglesby *et al.* 2008). Lastly, AQ production can be affected by deleterious mutations in the *pqsABCD* operon. The products of this operon are required for the production of the PQS precursor, HHQ, via the fusion of anthranilate and a 3-oxo-acid (Coleman *et al.* 2008, Gallagher *et al.* 2002, Zhang *et al.* 2008). Overall, we did not determine the source of the AQ-deficient phenotype as it is not within the scope of this study.

Two mechanisms, social and nonsocial, have been proposed to explain the emergence of various QS-deficient isolates within clinical and environmental *P. aeruginosa* populations. Under certain growth conditions, QS deficient strains may have an innate (nonsocial) advantage. Previous studies have shown that *lasR* mutants, for example, confer a growth advantage on certain carbon and nitrogen sources (D'Argenio *et al.* 2007) as well as increased resistance to alkaline conditions (Heurlier *et al.* 2005) as compared to the wild type parent strain. In contrast, QS deficiency may result from selection for social cheaters. Recent studies have demonstrated that social cheating can select for QS mutants *in vitro* (Diggle *et al.* 2007b, Sandoz *et al.* 2007,

Wilder *et al.* 2011) as well as within *in vivo* animal models (Rumbaugh *et al.* 2009). Here, QS-deficient bacteria cease the production of metabolically expensive QS-controlled extracellular products and take advantage of those products produced by the surrounding QS-proficient majority.

A recent study investigating the social behaviors of *P. aeruginosa* strains with either signal-blind (*lasR*, *rhlR*, *pqsR*) or signal-negative (*lasI*, *rhlI*, *pqsA*) mutations determined that *lasR* and *pqsR*, but not *rhlR*, mutant strains behave as social cheaters (Wilder *et al.* 2011). In contrast, all signal-negative strains were found not to behave as social cheaters because the mutant phenotype can be compensated by surrounding autoinducer-producing cells. Additionally, there is a negligible cost of synthesizing autoinducers as compared to the cost of synthesizing hundreds of proteins (Heurlier *et al.* 2006, Wilder *et al.* 2011). In the current study, environmental *P. aeruginosa* populations were found to be predominantly QS-proficient, with several isolates attenuated or deficient in C4-HSL and AQ production, respectively. These results indicate that within these populations, social conflict does not appear to play a significant role in shaping QS diversity. Rather, these autoinducer mutants may have evolved naturally due to selective conditions where only the loss of signal production, and not reception, is beneficial. In addition, each sample was obtained from nutrient-rich environments where small-molecule carbon and nitrogen sources are freely available. Thus, QS-controlled extracellular enzymes would not be necessary to acquire nutrients (Sandoz *et al.* 2007). Under such conditions, *P. aeruginosa* QS

genes are expressed at low levels; thus, one might expect that social cheaters do not have a selective advantage (Sandoz *et al.* 2007).

Lastly, our results provide interesting insight into how social behaviors affect the evolution and diversification of QS in *P. aeruginosa* populations. Generally, QS is believed to have initially evolved to coordinate cooperative behaviors as a means to benefit a population as a whole (Diggle *et al.* 2007a). Despite this, QS is costly to perform and subject to cheating. This presents the question of how QS originally emerged as a stable strategy. Kin selection, which is an evolutionary force that favors the reproductive success of an organism's relatives, provides one theory as to the initial evolution and maintenance of QS (Brown and Johnstone 2001). This theory predicts that individuals are more likely to cooperate when the surrounding population is closely related (Brown and Johnstone 2001, Hamilton 1963). Therefore, it is likely that kin selection may provide the initial evolutionary force that allows clonal populations to cooperate. However, prolonged competition within these populations will eventually lead to social conflict (Sandoz *et al.* 2007). Previous experimental evidence suggests that under growth conditions requiring QS, *lasR* mutants can emerge as social cheaters after ≈100 generations of *in vitro* evolution of the *P. aeruginosa* wild-type strain (Sandoz *et al.* 2007, Wilder *et al.* 2011). This is further apparent in advanced CF lung infections, which often harbor *P. aeruginosa lasR* mutant (Hoffman *et al.* 2009, Tingpej *et al.* 2007, Wilder *et al.* 2009). QS is probably required for the initial establishment of infection; however, competition and limited

dispersal of *P. aeruginosa* may result in the emergence of clonal QS-proficient and -deficient isolates via social conflict and other mechanisms.

In the current study, however, it appears that social conflict may not play a predominant role in shaping QS diversity in some natural populations. We speculate that this may be due to a combination of the following: organic composts are rich in small-molecule sources of nitrogen, carbon, phosphorus, and trace elements (Enwezor 1976, Sharma *et al.* 1997); thus, QS-controlled products would not be necessary for nutrient acquisition (Sandoz *et al.* 2007). Additionally, composts are routinely perturbed to allow for proper aeration, resulting in bacterial dispersal, and consequently reduced competition. Therefore, it is likely that in nutrient-rich environments where QS genes are not expressed, QS-deficient isolates are less likely to arise by social mechanisms.

Overall, our study investigates *P. aeruginosa* QS populations from the natural environment as a means to gain a general understanding of how social behaviors affect these populations. Generally, our results demonstrate that within nutrient-rich natural environments, social conflict may not play an active role in shaping QS populations. However, it is still unknown how social behavior affects QS populations over time as well as under various nutrient availabilities. Thus, the isolation and analysis of additional environmental populations as well as a longitudinal study are needed to fully elucidate the mechanisms behind the evolution and maintenance of QS in *P. aeruginosa*. Furthermore, this study highlights that *P. aeruginosa* may not be as ubiquitous as currently described. Though, additional studies investigating the

presence or absence of *P. aeruginosa* DNA via real-time PCR are necessary to confirm these results.

Acknowledgements

We would like to thank Joyce Loper for her insight into the design of this project as well as Stephanie Hao, Elise Grellman, and Kathryn Sackett for their assistance in the collection of environmental samples. We would also like to thank Keith Moodhe from Advanced Resources International for the construction of Figure 4.1.

Supplementary Tables

Table S4.1: Environmental sampling locations

Sample	Sample type	Location[a]; latitude, longitude	Bacterial density (CFU/unit[b])	Ratio of *P. aeruginosa* (isolates/total screened)	*P. aeruginosa* load (CFU/unit[b])
1	Compost	OR; 44, -123	8.8-18x10^3	24/30	7.0-14x10^3
2	Compost	OR; 44, -123	3.1-6.3x10^4	17/20	2.6-5.4x10^4
3	Compost	OR; 44, -123	9-18 x10^4	4/24	1.5-3.0x10^4
4	Compost	OR; 44, -123	1.3-2.7x10^4	6/30	2.6-5.4x10^3
5	Compost	OR; 44, -123	2.9-5.7x10^4	10/30	9.7-19 x10^3
6	Lake sediment	OR; 44, -123	4	1/10	0-1
7	Lake sediment	OR; 44, -123	8	0/5	0
8	Rhizosphere	OR; 44, -123	2.5x10^3	0/35	0
9	Rhizosphere	OR; 44, -123	5.5x10^3	0/19	0
10	Rhizosphere	OR; 44, -123	1.1x10^3	0/39	0
11	Rhizosphere	OR; 45, -120	1.3x10^5	0/31	0
12	Rhizosphere	OR; 45, -120	1.1x10^5	0/31	0
13	Rhizosphere	OR; 45, -120	1.4x10^5	0/31	0
14	Soil	OR; 44, -123	1.0-1.5x10^2	0/3	0
15	Soil	OR; 44, -123	1.6-2.5x10^1	0/3	0
16	Soil	OR; 44, -123	6.6-10x10^1	0/4	0
17	Soil	OR; 44, -123	NG[c]	0/0	0
18	Rhizosphere	OR; 44, -123	NG	0/0	0
19	Rhizosphere	OR; 44, -123	2.5x10^2	0/1	0
20	Rhizosphere	OR; 44, -123	1.4x10^4	0/113	0
21	Rhizosphere	OR; 44, -123	1.0x10^3	0/11	0
22	River sediment	OR; 44, -123	NG	0/0	0
23	River sediment	OR; 44, -123	1.0x10^1	0/1	0

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Table S4.1-*Continued*

Sample	Sample type	Location[a]; latitude, longitude	Bacterial density (CFU/unit[b])	Ratio of *P. aeruginosa* (isolates/total screened)	*P. aeruginosa* load (CFU/unit[b])
24	River sediment	OR; 44, -123	NG	0/0	0
25	River sediment	OR; 44, -123	$4.0x10^1$	0/4	0
26	Soil	OR; 44, -122	NG	0/0	0
27	Soil	OR; 44, -122	NG	0/0	0
28	Mushroom	OR; 44, -123	NG	0/0	0
29	Soil	OR; 42, -122	NG	0/0	0
30	Soil	OR; 42, -122	NG	0/0	0
31	Compost	OR; 44, -123	$7.0\text{-}14x10^3$	0/16	0
32	Soil	OR; 44, -123	NG	0/0	0
33	Soil	OR; 44, -123	NG	0/0	0
34	Soil	OR; 44, -123	NG	0/0	0
35	Soil	OR; 44, -123	$1.7\text{-}3.5x10^1$	0/20	0
36	Soil	OR; 44, -123	2-3	0/2	0
37	Compost	OR; 44, -123	$6.2\text{-}28x10^2$	0/10	0
38	Lake sediment	ID; 48, -116	$1.5x10^1$	0/2	0
39	Creek sediment	ID; 48, -116	$1.9\text{-}2.5x10^1$	0/9	0
40	Lake sediment	ID; 48, -116	3-4	0/2	0
41	Soil	CR; 50, 15	NG	0/0	0
42	Soil	CR; 50, 15	NG	0/0	0
43	Soil	CR; 50, 15	$1.1\text{-}1.5x10^3$	0/10	0
44	Soil	CR; 50, 15	$1.8\text{-}4.6x10^3$	0/10	0
45	Soil	CR; 50, 15	$0\text{-}1.0x10^3$	0/20	0
46	Soil	CR; 50, 15	NG	0/0	0
47	Soil	CR; 50, 15	NG	0/0	0

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Table S4.1-*Continued*

Sample	Sample type	Location[a]; latitude, longitude	Bacterial density (CFU/unit[b])	Ratio of *P. aeruginosa* (isolates/total screened)	*P. aeruginosa* load (CFU/unit[b])
48	Soil	CR; 50, 15	NG	0/0	0
49	Soil	GM; 51, 9	$3.1\text{-}21\text{x}10^2$	0/10	0
50	Soil	GM; 51, 9	$2.2\text{-}38\text{x}10^1$	0/10	0
51	Soil	GM; 51, 9	$1.1\text{-}6.0\text{x}10^2$	0/10	0
52	Soil	GM; 51, 9	$4.4\text{-}31\text{x}10^2$	0/10	0
53	Soil	GM; 51, 9	0-43	0/1	0
54	Soil	GM; 51, 9	NG	0/0	0
55	Soil	GM; 51, 9	$2.1\text{-}12\text{x}10^2$	0/10	0
56	Soil	GM; 51, 9	$2.6\text{-}24\text{x}10^2$	0/10	0
57	Soil	GM; 51, 9	$3.9\text{-}18\text{x}10^1$	0/16	0
58	Soil	GM; 51, 9	0-94	0/4	0
59	Soil	GM; 51, 9	NG	0/0	0
60	Soil	GM; 51, 9	NG	0/0	0
61	Soil	VA; 38, -77	14	0/28	0
62	Soil	VA; 38, -77	$3.4\text{-}6.4\text{x}10^1$	0/19	0
63	Soil	VA; 38, -77	NG	0/18	0
64	Soil	VA; 38, -77	$5.1\text{-}20\text{x}10^1$	0/39	0
65	Lake sediment	VA; 38, -77	NG	0/0	0
66	Lake sediment	VA; 38, -77	NG	0/0	0
67	Pond sediment	VA; 38, -77	2-12	0/6	0
68	Pond sediment	VA; 38, -77	3-4	0/4	0

[a]Oregon (OR), Idaho (ID), Virginia (VA), Czech Republic (CR), Germany (GM)
[b]Units are grams for soil, compost, and sediment samples, one root system for rhizosphere samples, and one mushroom for mushroom samples.
[c]NG indicates no growth

Table S4.2: QS phenotypes of all *P. aeruginosa* from environmental samples[a]

Sample/Isolate	RAPD group[a]	Skim milk proteolysis[b]	Rhamnolipid production[b]	Adenosine utilization[c]	AQ production[d]
Sample 1					
A	1	+	+	+	110 ± 32
B	2	+	+	+	170 ± 40
C	2	+	+	+	130 ± 30
D	2	+	+	+	140 ± 33
E	3	+	+	+	110 ± 14
F	4	+	+	+	93 ± 6.8
G	2	+	+	+	72 ± 11
H	5	+	○	+	190 ± 38
I	2	+	+	+	100 ± 25
J	6	+	+	+	140 ± 24
K	7	+	+	+	140 ± 31
L	2	+	+	+	240 ± 53
M	8	+	○	+	300 ± 43
N	9	○	+	+	140 ± 52
O	9	+	+	+	143 ± 41
P	5	+	○	+	230 ± 18
Q	10	+	○	+	76 ± 45
R	2	+	+	+	92 ± 17
S	11	+	+	+	80 ± 20
T	12	+	+	+	150 ± 22
U	13	+	+	+	100 ± 32
V	2	+	+	+	130 ± 16
W	13	+	+	+	170 ± 38
X	13	+	+	+	210 ± 32

Continued on next page

Table S4.2-*Continued*

Sample/Isolate	RAPD group[a]	Skim milk proteolysis[b]	Rhamnolipid production[b]	Adenosine utilization[c]	AQ production[d]
Sample 2					
A	13	+	+	+	160 ± 32
B	13	-	+	+	0.56 ± 0.98
C	13	+	+	+	180 ± 71
D	9	+	+	+	84 ± 17
E	9	+	+	+	110 ± 36
F	13	-	+	+	0.0 ± 0.0
G	14	-	+	+	40 ± 7.7
H	9	+	+	+	82 ± 15
I	9	+	+	+	110 ± 26
J	13	-	+	+	34 ± 58
K	13	+	+	+	120 ± 16
L	13	+	+	+	150 ± 17
M	15	+	+	+	130 ± 44
N	13	+	+	+	160 ± 3.5
O	13	+	+	+	160 ± 3.4
P	15	+	+	+	160 ± 35
Q	16	+	+	+	86 ± 23
Sample 3					
A	17	+	+	+	96 ± 45
B	18	+	○	+	9.4 ± 1.9
C	18	+	○	+	11 ± 3.4
D	19	+	○	+	14 ± 4.4
Sample 4					

Continued on next page

Table S4.2-*Continued*

Sample/Isolate	RAPD group[a]	Skim milk proteolysis[b]	Rhamnolipid production[b]	Adenosine utilization[c]	AQ production[d]
A	9	+	+	+	200 ± 13
B	20	+	+	+	240 ± 32
C	21	+	+	+	110 ± 24
D	14	+	+	+	180 ± 40
E	16	-	+	+	54 ± 2.1
F	19	+	+	+	23 ± 7.9
Sample 5					
A	14	-	-	+	5.7 ± 7.3
B	19	+	+	+	53 ± 26
C	19	+	+	+	100 ± 27
D	19	+	+	+	170 ± 48
E	19	+	+	+	110 ± 28
F	18	+	○	+	68 ± 19
G	18	○	○	+	79 ± 28
H	18	○	○	+	66 ± 11
I	16	-	+	+	61 ± 0.68
J	18	+	○	+	74 ± 28
Sample 6					
A	22	+	+	+	96 ± 11

[a] RAPD groupings are based on ≥95% similarity.

[b]Skim milk proteolysis and rhamnolipid production; "+" indicates a phenotype similar to the PAO1 wild type, "○" indicates a significantly impaired phenotype, and "-" indicates absence.

[c]Adenosine utilization; "+" indicates growth, and "- "indicates no growth.

[d]AQ production; data is listed as percent of the PAO1 wild-type. See *Materials and Methods* for further details.

Conclusion

The evolution of QS in *Pseudomonas aeruginosa*

One might predict that altruistic behaviors, which are actions that increase another individual's fitness at a cost to one's own, evolved as a means to benefit a population as a whole. This behavior, however, is difficult to explain as natural selection tends to favor selfish and uncooperative individuals (Hamilton 1963, Hamilton 1964a, Hamilton 1964b). Regardless, many microbes have been found to exhibit social behaviors including communication and the cooperative production of extracellular public goods (Crespi 2001). Bacterial communication, termed quorum sensing (QS), is often considered the paradigm for microbial cooperation as both intra- and interspecies communication has been found in both Gram-negative and -positive bacteria. These communicative organisms use signaling molecules in a density-dependent fashion to coordinate other cooperative behaviors (Swift *et al.* 2001). In *P. aeruginosa*, three QS systems, the *las*, *rhl*, and *pqs* systems, are required to regulate the production of numerous extracellular virulence factors (Venturi 2006).

The problem with cooperative behaviors, such as QS, is that they are often subject to social conflict through invasion by social cheaters (Brown and Johnstone 2001, Diggle *et al.* 2007, Sandoz *et al.* 2007, Wilder *et al.* 2011). These QS-deficient isolates often lack either the ability to produce (signal-negative) or respond (signal-blind) to autoinducer signaling molecules. It is predicted, however, that the signal-blind phenotype is far more likely to arise than the signal-negative phenotype as the

response to the signal in the later can be triggered by the surrounding wild-type majority. Additionally, there is a negligible cost of synthesizing autoinducers as compared to the metabolic cost associated with the regulation and production of hundreds of gene products (Heurlier *et al.* 2006). In *P. aeruginosa* QS, these predictions hold true. All signal-negative strains do not exhibit social cheating behavior (Chapter 2) (Wilder *et al.* 2011). Instead, social cheaters predominantly harbor mutations in the central QS regulator, *lasR* (Chapter 2) (Diggle *et al.* 2007, Sandoz *et al.* 2007, Wilder *et al.* 2011). Furthermore, QS-deficient isolates obtained from environmental and clinical *P. aeruginosa* populations are primarily *lasR* mutants (Chapter 1, Table 1.1, Chapter 3). Surprisingly, the *pqsR* and *rhlR* mutants, however, do not exhibit typical cheater behavior. Rather, they demonstrate complex social behaviors that can be attributed to pleiotropic affects associated with the differential regulation of *pqs* gene expression (Chapter 2) (Wilder *et al.* 2011). Overall, these various social behaviors tend to negatively affect *P. aeruginosa* QS populations.

Drawing from our own data and other studies, we propose a model that incorporates different selective pressures in the emergence of *lasR* mutants during acute and chronic infection (Figure 5.1). During the early stages of infection, environmental conditions are considerably nutrient poor. To survive, *P. aeruginosa* populations must cooperate to regulate the expression of QS-dependent genes (Figure 5.1) (Sandoz *et al.* 2007). However, competition and limited dispersal in an infection may lead to the emergence of *lasR* social cheaters. Recent studies observing the *in vitro* evolution of the *P. aeruginosa* wild-type demonstrated that *lasR* mutants can

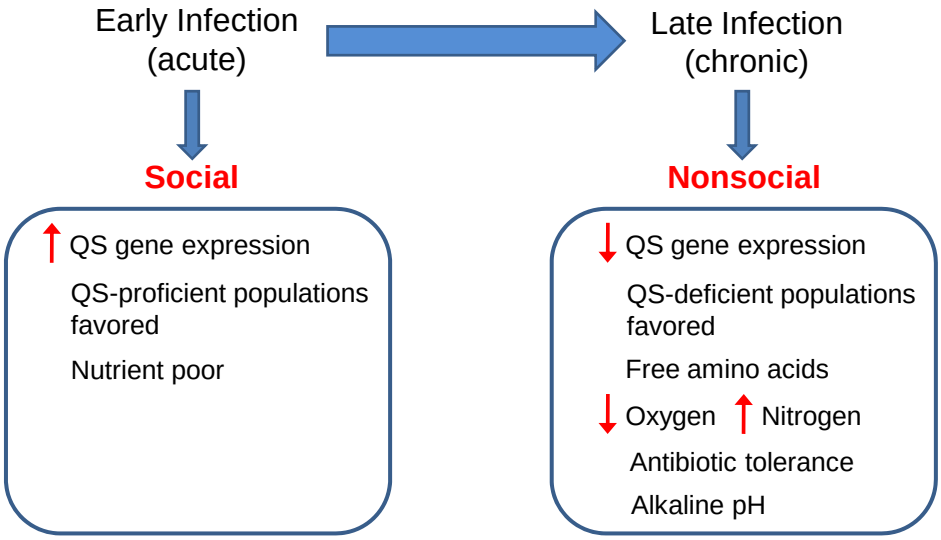

Figure 5.1: The emergence of *lasR* mutations in an infection. This model depicts the different social and nonsocial selective pressures that lead to the emergence of *lasR* mutants in acute and chronic infections, respectively.

emerge as social cheaters in as little as 100 generations (Sandoz *et al.* 2007, Wilder *et al.* 2011). In contrast, during a chronic infection, *lasR* mutants are more likely to arise due to an innate, nonsocial growth advantage as compared to the wild-type (Figure 5.1). Generally, in the late stages of infection, there is severe tissue damage due to the production of QS-controlled proteases as well as host immune-regulated elastases (Voynow *et al.* 2008, Winstanley and Fothergill 2009). This break down of tissue provides an abundance of free amino acids. Under these conditions, QS-controlled extracellular proteases would not be necessary for nutrient acquisition; thus, QS genes are expressed at low levels (D'Argenio *et al.* 2007, Sandoz *et al.* 2007). In chronic cystic fibrosis (CF) lung infections, a combination of CF-associated epithelial abnormalities, advanced microbial infection, and concomitant immune response results in an oxygen-poor, nitrogen-rich environment (Winstanley and Fothergill

2009). Under these anaerobic conditions, *lasR* mutants exhibit a natural metabolic advantage that confers increased fitness as well as decreased susceptibility to several antibiotics commonly used to treat CF lung infections (Hoffman *et al.* 2010). Chronic infections such as CF are a unique habitat and are likely evolutionary impasses for *P. aeruginosa*. In the natural environment, which likely shapes *P. aeruginosa* QS, social mechanisms appear more likely. Despite this emergence of QS-deficient variants, QS has been maintained throughout evolution.

Kin selection theory provides one explanation for the initial emergence of QS between close relatives (Hamilton 1964b). Through helping a close relative reproduce, an individual is able to indirectly pass on its genes to the next generation. Therefore, individuals are more likely to cooperate, and thus communicate, when the surrounding population is closely related (Hamilton 1964a, Hamilton 1964b). Generally, bacteria can often be found in highly related populations due to limited dispersal and clonal reproduction (West *et al.* 2006). It is likely that kin selection may provide the initial evolutionary force that allows clonal populations to cooperate. However, within-species competition also leaves QS populations susceptible to the emergence of social cheaters. To stabilize cooperation, cheating can be constrained via pleiotropy and the re-evolution of cooperators (Foster *et al.* 2007).

Generally, pleiotropic genes, such as QS-regulators, influence multiple phenotypic traits through the regulation of numerous genes. The loss of certain pleiotropic genes may constrain adaptation, thus preventing the origination of cheaters. This mechanism may play a role in preventing the emergence of *rhlR*

mutants. Through *in vitro* studies, we have found that *rhlR* mutants do not have a fitness advantage over the wild-type and are unable to naturally evolve under social conditions (Chapter 2) (Wilder *et al.* 2011). Furthermore, *rhlR* mutants are rarely found in environmental and clinical populations (Table 1.1). It is likely that the negative pleiotropic affects associated with the up-regulation of *pqs* genes prevents the emergence of *rhlR* mutants. In contrast, the loss of other pleiotropic genes, such as *lasR,* can be beneficial in the emergence of social cheaters, at least initially. Cheater strains, however, often harbor hidden costs that are pleiotropically liked to the cheating behavior (Foster *et al.* 2007). These costs can lead to the re-emergence of cooperation through compensatory mutations that reactivate some cooperative functions (Foster *et al.* 2007). Recently, this behavior has been observed in the social development of *Myxococcus xanthus* (Fiegna *et al.* 2006). We have also observed this in *P. aeruginosa* within two separate *in vitro* evolution studies; *lasR* cheaters were converted into cooperators via a secondary site mutation that restored protease production (Chapter 2) (Sandoz *et al.* 2007, Wilder *et al.* 2011). Overall, these results stress the importance of QS in survival.

Understanding social conflict and its effects on QS diversity in laboratory, environmental and clinical *P. aeruginosa* populations

The *P. aeruginosa* signal-blind strains, *lasR*, *pqsR*, and *rhlR*, each demonstrate different complex social behaviors (Chapter 2) (Wilder *et al.* 2011). The variations in these behaviors are primarily due to either positive or negative pleiotropic effects, resulting in differential social phenotypes. In turn, the net fitness benefit of the social

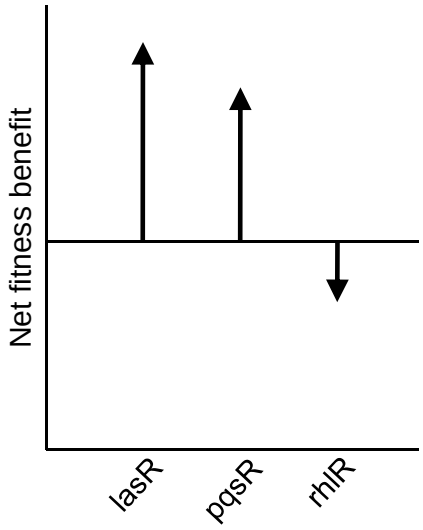

Figure 5.2: The net fitness benefit of the *lasR*, *pqsR*, and *rhlR* mutants in mixed cultures. This graph subjectively takes into account the costs and benefits of each signal-blind strain as compared to the wild-type parent strain and each other. The center line from which the arrows stem from represents no net fitness benefits. Arrows pointing up indicate a positive net fitness benefit, indicating that the benefits of the social phenotype outweigh the costs. Arrows pointing down indicate a negative net fitness benefit, indicating that the costs of the social phenotype outweigh the benefits.

phenotype affects the probability of emergence of the respective signal-blind strain within a QS population. In Figure 5.2, we have depicted the net fitness benefit of each signal-blind strain to visually compare the advantage or disadvantage of the respective social behavior. In this model, we subjectively took into account the costs and benefits of each signal-blind strain as compared to both the wild-type parent and each other. Each social phenotype is further described below.

Under social conditions, the *lasR* mutant strain is a typical social cheater where the benefits of its emergence far outweigh the costs (Figure 5.2, Chapter 2) (Diggle *et al.* 2007, Rumbaugh *et al.* 2009, Sandoz *et al.* 2007, Wilder *et al.* 2011). This is

apparent in both clinical and environmental *P. aeruginosa* populations, as isolated QS-deficient variants predominantly harbor mutations in *lasR* (Chapter 1, Table 1.1, Chapter 3). As described in the previous section, the production of QS-regulated products is required for the establishment of an infection (Popat *et al.* 2008). Therefore, it is likely that *lasR* cheaters will arise during the early stages of infection. However, due to the loss of extracellular protease production, it is probable that the invasion of these cheaters may select for lower virulence. Recent *in vivo* evidence supports this idea. In a mouse model, virulence is significantly reduced when the *P. aeruginosa* population predominantly harbored *lasR* mutants as compared to no mutants (Rumbaugh *et al.* 2009). Similar results were obtained from a recent study analyzing the diversity of QS populations from mechanically ventilated patients. Generally, the onset of pneumonia was significantly delayed in patients that were colonized by populations primarily consisting of *lasR* mutants (Kohler *et al.* 2009). Overall, the *lasR* mutant is an ideal cheater, and will arise readily under social conditions.

In contrast to the *lasR* mutant, the *pqsR* mutant does not exhibit typical social cheating behaviors (Chapter 2) (Wilder *et al.* 2011). Rather, it exhibits a complex social phenotype that can be attributed to pleiotropic gene affects. It is likely that the social phenotype associated with the *pqsR* mutant may result from a combination of factors. The growth advantage of the *pqsR* mutant may be due to the strain saving energy by not producing *pqsR*-dependent factors, in particular, those regulated by *pqsE* (Figure 5.3) (Rampioni *et al.* 2010). Additionally, the loss of the PQS signal as

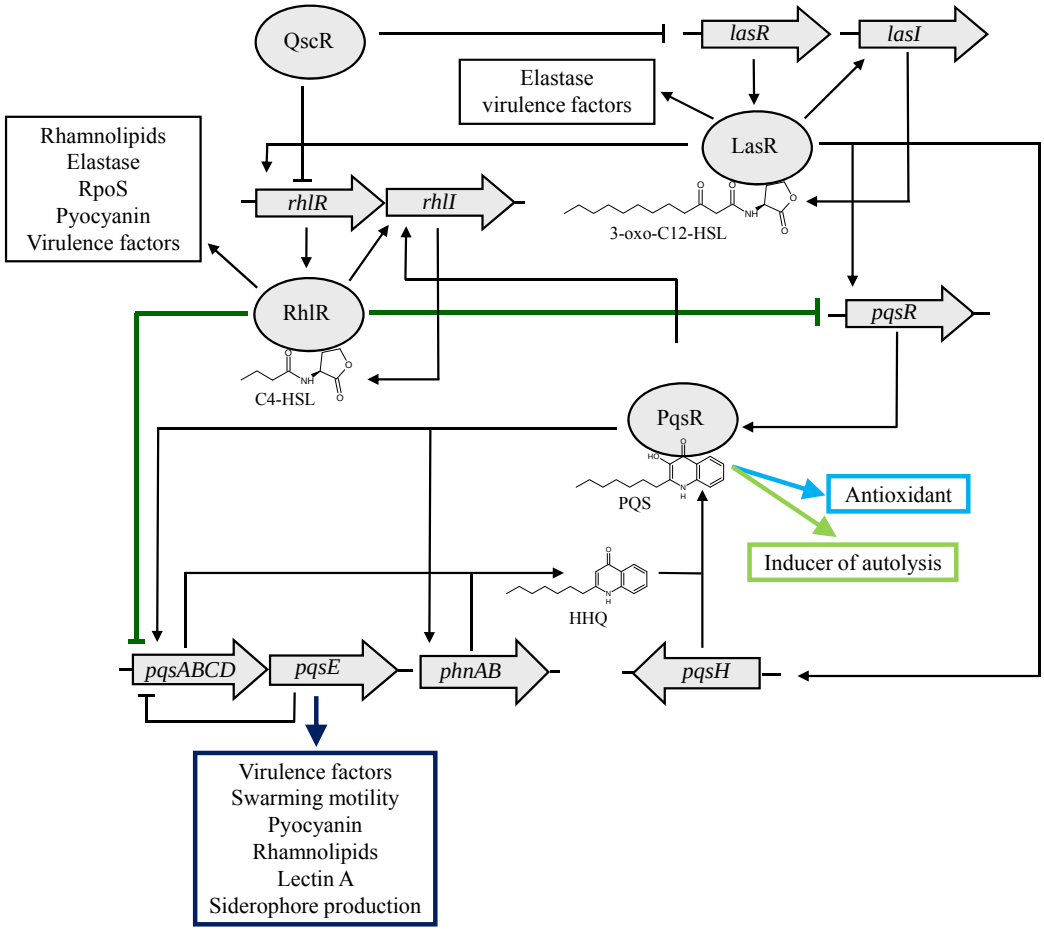

Figure 5.3: Pleiotropic effects associated with the *pqsR* and *rhlR* mutants. The *pqsR* mutation is associated with a growth advantage over the wild-type (dark blue) and negative frequency-dependence (light blue). The *rhlR* mutation is associated with no growth advantage (dark green) and positive followed by negative frequency-dependence (light green).

an antioxidant would incur a fitness cost, thus resulting in the observed negative frequency-dependent relative fitness (Chapter 2, Figure 5.3) (Haussler and Becker 2008, Wilder *et al.* 2011). If the selective forces *in vivo* are similar to that seen in *in vitro* studies, it is likely that *pqsR* mutants will emerge under social conditions. However, they would be subsequently be superseded by *lasR* mutants (Chapter 2) (Wilder *et al.* 2011). Unfortunately, to our knowledge, the prevalence of *pqsR*

mutants in clinical populations has yet to be determined. In contrast, our recent investigation of environmental QS populations was unable to detect the presence of *pqsR* mutant strains (Chapter 4). These populations, however, were obtained from nutrient-rich environments where the expression of QS-controlled products would not be necessary. Therefore, one might expect that *pqsR* mutants would not have a social advantage (Chapter 4) (Enwezor 1976, Sandoz *et al.* 2007, Sharma *et al.* 1997).

The *rhlR* mutant demonstrates a fitness pattern similar to spiteful behavior (Inglis *et al.* 2009). Generally, spiteful behaviors are costly to both the producer and receiver of the behavior. If the social phenotype associated with the *rhlR* mutation is indeed spiteful, it is likely due to the up-regulation of the PQS signaling molecule; when produced in large quantities, PQS acts as an inducer of autolysis (Figure 5.3-5.4, Chapter 2) (D'Argenio *et al.* 2002). If the *rhlR* mutant were to benefit from this process, the wild-type would have to be more sensitive to PQS-mediated killing than the *rhlR* mutant. In addition to possible spiteful behavior, the *rhlR* mutant strain exhibits no growth advantage over the wild-type. This may be in part due to the up-regulation of *pqs*-dependent factors that are not also co-regulated by the *rhl* system (Figure 5.3). Specifically, in the *rhlR* mutant strain, *pqsA* is upregulated (McGrath *et al.* 2004), and under conditions requiring QS for growth, the *rhlR* mutant produces approximately 9-times the amount of AQ as compared to the wild-type strain (Figure 5.4). Therefore, it is likely that the costs associated with the up-regulation of *pqs*-dependent factors may outweigh the benefits of not regulating *rhl*-dependent factors (Figure 5.2). Overall, the social phenotype associated with *rhlR* mutant is more costly

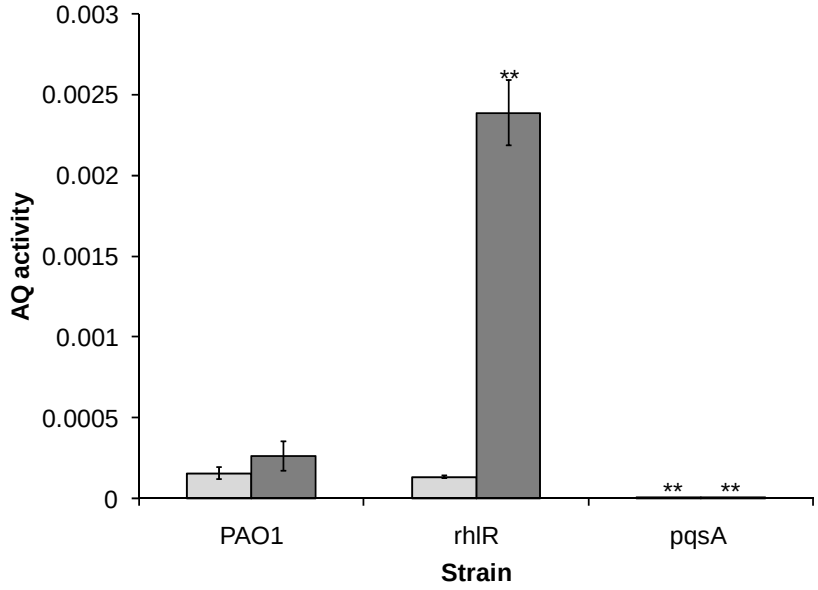

Figure 5.4: AQ production of the *rhlR* mutant strain under conditions requiring QS for growth. Quantification of AQ includes both PQS and HHQ. Strains were grown in either M9-CAA (light grey) or M9-Caseinate (dark grey). Error bars indicate standard deviation of the mean of three replicates. Statistical significance of the data was determined using a two-tailed unpaired *t* test, with "**" indicating *P* values of <0.01 and "*" indicating *P* values of <0.05. For each growth condition, AQ production was compared to that of the wild-type strain.

than it is beneficial (Figure 5.2), which may explain why we were unable to detect it in both clinical and environmental populations (Chapters 1, 3, 4).

Taking into account the costs and benefits of the social phenotypes associated with each *P. aeruginosa* signal-blind strain, it is probable that *lasR* and *pqsR*, but not *rhlR*, mutants are likely to arise socially in both clinical and environmental populations (Chapter 2) (Wilder *et al.* 2011). However, the effects of population viscosity on social conflict must also be considered in the emergence of these signal-blind mutants. Generally, population viscosity refers to limited dispersal, which can be described as the tendency of offspring to disperse slowly from their site of origin

(Mitteldorf and Wilson 2000). Originally, limited dispersal was suggested as a mechanism that might generate high relatedness among interacting individuals, thus promoting cooperation (Hamilton 1964a, Hamilton 1964b). Subsequent theoretical work has demonstrated that limited dispersal can also lead to increased competition between relatives, hence increasing the probability of social conflict (Taylor 1992a, Taylor 1992b, West *et al.* 2002, Wilson *et al.* 1992). Overall, limited dispersal is likely to occur during infections as well as in natural environments (Zhou *et al.* 2002) and may provide a selective force for the evolution of cooperation and subsequent emergence of cheaters.

Recently, the effect of population viscosity on the relative success of cooperation has been investigated *in vitro* (Kummerli *et al.* 2009). In this study, *P. aeruginosa* cooperator and non-cooperator strains were co-cultured in medium with varying degrees of viscosity. For each competition, the fitness consequences for a cooperative trait were examined (Kummerli *et al.* 2009). Surprisingly, as the viscosity of the media increased, both bacterial dispersal and the ability of the non-cooperator to exploit the cooperator decreased. These results are contradictory to the latter theory described above. However, it should be taken into account that *in vitro* conditions are far less complex than *in vivo* conditions. In the CF lung, for example, mucous viscosity varies among patients and over time within patients (App *et al.* 1998, Rubin 2007, Schulz *et al.* 2007). Furthermore, soils can differ in consistency and water content, thus affecting dispersal (Zhou *et al.* 2002). Overall, it is likely that limited dispersal may provide an initial evolutionary force that promotes cooperation within a

population. However additional environmental selection pressures, such as fluctuations in population density, environmental consistency and fluidity, or varying rates of bacterial dispersal and public good diffusion, may result in competition and social conflict (Mitteldorf and Wilson 2000, Taylor 1992a, Taylor 1992b). Though, further experimental evidence is required to support this.

Outlook

Overall, our study investigated the differential social behaviors of inactivated *P. aeruginosa* QS regulators and how these phenotypes affected the QS diversity of clinical and environmental populations. Though our results provide valuable insight into the diversification of QS populations, further analysis is still required to fully understand how social and nonsocial selection pressures drive the evolution of QS-deficient isolates. This can be accomplished through a longitudinal study of QS populations isolated from various environmental and clinical locales. Generally, this study would further contribute to the understanding of how different evolutionary forces shape QS populations over time. In addition to further elucidating the nature of selective pressures, future studies should also focus on clarifying the various facets that contribute to the complex social phenotypes exhibited by *pqsR* and *rhlR* mutants. If these mutants are indeed exhibiting cheating and spiteful behaviors, respectively, further evidence analyzing the role of differential *pqs* gene regulation is required to support this.

Lastly, our investigation into the evolution and diversity of QS populations implicates the disruption of bacterial communication as a potential target for antimicrobial intervention. One strategy currently used is the application of QS inhibitors, such as azithromycin, as a means to inhibit the expression of QS-controlled virulence factors (Tateda *et al.* 2001). However, though azithromycin would potentially reduce the virulence of *P. aeruginosa*, it would also prevent the selection for *lasR* social cheaters as there would no longer be a growth advantage. Consequently, more virulent QS-proficient cells would be maintained (Kohler *et al.* 2010). Therefore, if treatment were to be discontinued, the patient would be at risk for colonization of virulent bacteria (Kohler *et al.* 2010).

Alternatively, the application of *lasR* mutants as a probiotic treatment of *P. aeruginosa* infections should be considered as an antivirulence strategy. In general, the emergence of QS-deficient *lasR* mutants within a population is associated with decreased bacterial fitness (Sandoz *et al.* 2007, Wilder *et al.* 2011) and diminished virulence (Kohler *et al.* 2009, Rumbaugh *et al.* 2009). In addition, *lasR* mutants are able to easily invade QS-proficient populations due to an increased growth rate that is ≈ 60% faster than the wild-type (Sandoz *et al.* 2007, Wilder *et al.* 2011). However, certain considerations must be taken into account when applying this strategy. Generally, *lasR* mutants are relatively resistant to sources of oxidative stress, including the antibiotics ciprofloxacin and tobramycin (Hoffman *et al.* 2010). In addition, *lasR* mutants demonstrate increased resistance to ceftazidime, a β-lactam antibiotic, (Gibson *et al.* 2003) due to increased β-lactamase activity (D'Argenio *et al.* 2007).

These antibiotics are commonly used to treat CF lung infections (Gibson *et al.* 2003). Therefore, for the use of *lasR* as a probiotic to be effective, it should be paired with either a source of reactive nitrogen species or antibiotics to which *lasR* mutants are sensitive. Additionally, the emergence of re-evolved cooperators must also be considered. In predominantly cheater populations, compensatory mutations may arise, thus restoring some QS functions. In *M. xanthus*, for example, re-evolved cooperators were able to resist the cheater mutant as well as surpass the wild-type in spore production (Foster *et al.* 2007, Velicer *et al.* 2006). If the same is true for *P. aeruginosa*, the re-evolved cooperators could be potentially more virulent than the wild-type strain. Overall, the use of *lasR* cheaters as a probiotic could provide an effective strategy to reduce virulence in clinical *P. aeruginosa* populations.

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APPENDICES

Quantification of viable *Pseudomonas aeruginosa* cells from the natural environment

Cara N. Wilder

Rationale

An issue of concern in microbiology is the inability to culture bacterial cells from various environmental locations. These cells are often in a viable but non-culturable (VBNC) state, where they are metabolically active, intact cells that are unculturable due to a variety of environmental stresses including nutrient starvation, high or low temperatures, or changes in salinity or pH (Nystrom 2001, Roszak and Colwell 1987). Many bacteria species including pathogens *Pseudomonas aeruginosa*, *Pseudomonas syringae*, *Escherichia coli*, and *Salmonella enterica* have been found to exist in the VBNC state. This in particular is a public health issue where the detection of viable cells is critical in the prevention of disease (Nystrom 2001, Pyle *et al.* 1999, Roszak and Colwell 1987). Because bacteria in the VBNC state cannot be easily analyzed, novel methods to detect different states of bacteria are required. Below, we describe two methods to detect and quantify environmental *P. aeruginosa* cells in the VBNC state. These methods include quantitative polymerase chain reaction (qPCR) as a means to obtain a RNA/DNA ratio, indicating the presence of viable cells versus all cells. An additional method includes propidium monoazide (PMA)-qPCR, which allows for the selective amplification of DNA from viable cells.

Generally, this protocol is meant to follow up on analyzing the ubiquity and viability of environmental *P. aeruginosa* isolates as described in chapter 4. These methods are assembled based on our preliminary qPCR experiments; however, we have not fully validated this experimentally.

Materials and Methods

Quantification of viable P. aeruginosa cells from the environment using qPCR and Taqman Probes

Primers and probe design

Primers used for qPCR, PAO1S and PAO1A, as well as the appropriate Taqman probe, *oprL* TM, can be designed as previously described (Deschaght *et al.* 2009) (Table A.1, Invitrogen and Integrated DNA technologies, respectively). The probe is labeled on the 3' and 5' ends with a FAM fluorescent label and blackhole quencher, respectively. These primers and probe are specific for the *P. aeruginosa* gene, *oprL*, an outer surface lipoprotein that can be used to differentiate *P. aeruginosa* from other fluorescent pseudomonads (De Vos *et al.* 1997).

Table A.1: Primers used for qPCR

Primer	Sequence (5'→3')	Reference
PAO1S	ACCCGAACGCAGGCTATG	(Deschaght *et al.* 2009)
PAO1A	CAGGTCGGAGCTGTCGTACTC	(Deschaght *et al.* 2009)
oprL TM	AGAAGGTGGTGATCGCACGCAGA	(Deschaght *et al.* 2009)

DNA and RNA isolation

DNA and RNA from environmental soil samples can be extracted using the PowerSoil™ DNA Isolation Kit (MO BIO Cat. 12888-50) and RNA PowerSoil™ Total RNA Isolation Kit (MO BIO Cat. 12866-25), respectively. Samples used for

RNA isolation should be preserved immediately in LifeGuard™ Soil Preservation Solution (MO BIO Cat. 12868-100) prior to extraction. Additionally, RNA samples should be tested for quality control through analyzing the quantity, purity, and integrity of the RNA (Schuster 2011).

qPCR reaction set-up

The Taqman® Universal PCR Master Mix is used when quantifying DNA samples (Invitrogen Cat. 4304437). This is a one-step kit that guarantees an efficient performance with GC-rich sequences, such as those found in *P. aeruginosa*. The reaction mixture is listed in Table A.2. After preparation of the reaction mixture, samples should be analyzed using ABI Prism Sequence Detection System and Sequence Detection System (SDS) software (Applied Biosystems).

Table A.2: Reaction mixture of qPCR using Taqman® Universal PCR Master Mix

Components	Volume (µl)	Final concentration
Taqman® Universal PCR Master Mix	12.5	1x
Forward Primer (5 µM)	1.5	300 nM
Reverse Primer (5 µM)	1.5	300 nM
Probe (1 µM)	2.5	100 nM
DNA template (2 ng/µl)	5	10 ng
dH_2O	to 25	-------

For analysis of RNA sequences, either Taqman® RNA-to-C_T 1-Step Kit (Invitrogen Cat. 4392653) or SuperScript® III Platinum® Two-Step qRT-PCR Kit (Invitrogen Cat. 11734-050) is used. The Taqman® RNA-to-C_T 1-Step Kit is used to perform one-step reverse transcription (RT)-qPCR for quantification of RNA using a real-time PCR system (Table A.3). The SuperScript® III Platinum® Two-Step qRT-PCR Kit is a kit that allows for the conversion of RNA to cDNA in the first step followed by the procession of cDNA by qPCR in a second step (Table A.4). For use of the one-step Taqman® RNA-to-C_T 1-Step Kit, reagents are combined as listed below in Table A.3. Subsequently, the DNA is quantified in the reaction mixture using ABI Prism Sequence Detection System and Sequence Detection System (SDS) software (Applied Biosystems).

Table A.3: Reaction mixture for RT-qPCR using Taqman® RNA-to-C_T 1-Step Kit

Components	Volume (µl)	Final concentration
Taqman® RT-PCR Mix (2x)	12.5	1x
Taqman® Gene Expression Assay (20x)	1.25	1x
Taqman® RT Enzyme Mix (40x)	0.625	1x
Forward Primer (5 µM)	1.5	300 nM
Reverse Primer (5 µM)	1.5	300 nM
Probe (1 µM)	2.5	100 nM
DNA template (2 ng/µl)	5	10 ng
dH_2O	to 25	-------

Table A.4: Reaction mixture for RT-qPCR using SuperScript® III Platinum® Two-Step qRT-PCR Kit

Components	Volume (µl)	Final concentration
cDNA synthesis		
RT Reaction Mix (2x)	10	1x
RT Enzyme Mix	2	-------
RNA template	X	10 pg to 1 µg
DEPC-treated water	to 20	-------
qPCR reaction mixture		
Platinum® Quantitative PCR Supermix-UDG	12.5	1x
Forward Primer (5 µM)	1.5	300 nM
Reverse Primer (5 µM)	1.5	300 nM
Probe (1 µM)	2.5	100 nM
DNA template (2 ng/µl)	5	10 ng
DEPC-treated water	to 25	-------

When using the SuperScript® III Platinum® Two-Step qRT-PCR Kit, the cDNA should be synthesized as described below (Invitrogen manuel, Table A.4), and subsequently used in a qPCR reaction. The qPCR reaction mixture should be prepared as described in Table A.4 and then analyzed using ABI Prism Sequence Detection System and Sequence Detection System (SDS) software (Applied Biosystems).
In the qPCR reaction described below, either annealing temperature can be used; 55^{o}C is preferred for this primer set, though 60 ^{o}C increases specificity.

Hold 50°C – 2 min.
Hold 95 °C – 10 min
95 °C –15 sec
60 °C or 55 °C – 1 min } 40 cycles

cDNA synthesis

To synthesize cDNA, all cDNA preparation components are combined in a tube on ice (Table A.4). The tube contents are then gently mixed and incubate at 25°C for 10 minutes. Subsequently, sample tubes are incubated at 42°C for 50 minutes. To terminate the reaction, each reaction mixture is placed at 85°C at 5 minutes, followed by chilling on ice. Next, add 1 µl (2U) of *E. coil* RNase H to each sample and incubate at 37°C for 20 minutes. The cDNA can be stored -20°C until use. In the qPCR protocol, use 5 µl of the cDNA.

Controls for qPCR

DNA analysis

Various controls are required concomitantly with DNA quantification. During the extraction of DNA from environmental samples, it is important to have an internal amplification control. This control functions dually as a means to quantify isolation efficiency as well as to determine if the qPCR reaction has been inhibited by materials found in the environment or from reagents used in nucleic acid extraction. For this control, you will need a genetically modified Gram-negative bacterial strain that is not *P. aeruginosa*. This strain must be unique and not naturally found in the environment; for example, a genetically engineered bacterial strain expressing a foreign gene. You

will need to add known amounts of genomes or cells of this strain to each sample prior to DNA extraction. Thus, for each sample you will either quantify the control strain or any present *P. aeruginosa* (Table A.1). For quantification of the control strain, you will need to design primers that span the genetic mutation.

In addition to an internal amplification control, it is important to ensure that the reaction has not been compromised with contaminating DNA. For this control, add water in place of a DNA template. Lastly, to test for non-specific amplification, analyze primer sets using DNA from *Pseudomonas fluorescens* or *Pseudomonas putida*.

RNA analysis

For the analysis of RNA from environmental samples, the same controls as described above for DNA analysis can be used. However, for the internal amplification control, known amounts of RNA, rather than DNA, should be added to each sample type. Additionally, when analyzing RNA via a one-step reaction, a no-reverse-transcriptase control should be used as a means to test for the presence of contaminating DNA.

Quantification of viable P. aeruginosa cells from the environment using PMA-qPCR

PMA is a membrane-impermeable dye that selectively binds to extracellular DNA as well as DNA within compromised cells, which are considered dead. PMA intercalates into DNA via covalent cross-linkage, which in turn inhibits PCR amplification (Nocker *et al.* 2007). By using qPCR with *oprL* specific primers after treatment with

PMA, one will be able to quantify the number of viable *P. aeruginosa* cells within each environmental sample type. This procedure is an alternative to the protocol described above. *P. aeruginosa* cells will be quantified using PMA-qPCR as previously described with several modifications (Nocker *et al.* 2007).

Soil samples or sediment with water samples are collected in sterile 50 ml tubes. To separate bacterial cells from soil particles, each sample should be mixed with washing buffer and incubated for 1 hour shaking at room temperature. Subsequently, 1 ml of the turbid supernatant should be harvested and transferred to a clean tube. Cells are then be pelletted by centrifugation at 5000 x g for 5 min, and resuspended in 1 ml 1xM9. Subsequently, 500 µl aliquots of each sample are transferred into two light-transparent tubes. The first aliquot is treated with Propidium monoazide (PMA) and the other left untreated. To prepare the PMA mixture, the PMA stock is dissolved in 20% dimethyl sulfoxide to create a stock concentration of 20 mM. This can be stored at -20^{o}C in the dark.

To PMA-treated cells, 1.25 µl of PMA is added to the sample with a final concentration of 50 µM. Following an incubation period of 5 min in the dark with occasional mixing, samples are exposed to light for 2 minutes using a 650-W halogen light source (Sealed beam lamp, CW 120 V, 3200 K; GE Lighting, General Electric.). Each sample tube should be laid horrizontally 20 cm from the light source on ice to avoid excessive heating. Additionally, the samples are occasionally shaken to allow for homogenous exposure to light.

Once the cells are prepared either with or without PMA, genomic DNA is extracted using PowerSoil™ DNA Isolation Kit (MO BIO Cat. 12888-50). Viable *P. aeruginosa* cells are then quantified using qPCR, with *oprL* specific primers and Taqman probe using ABI Prism Sequence Detection System and Sequence Detection System (SDS) software (Applied Biosystems).

Extraction efficiency control preparation

The extraction efficiency control for qPCR will be prepared as previously described (Silkie and Nelson 2009). For this control, an engineered strain that is not found naturally within the environment is used. This strain must be Gram-negative, similar to *P. aeruginosa*, however must be a different species. To prepare this control, the control strain is grown in liquid media until logarithmic phase. Next, the fresh cells are centrifuged until a pellet forms. The supernatant is then discarded and the cells are rinsed three times using 1xM9 salts. These cells can be frozen at -80^{o}C in stocks containing 15% glycerol-0.01 M $MgSO_4$. Lastly, an aliquot of these cells is fixed with an equal amount of 4% paraformaldehyde in 1xM9 and gently vortexed.

These fixed cells can be enumerated using fluorescent microscopy. Firstly, cells must be applied to a black filter using a vacuum mainifold. Afterward, the cells are stained using 100 µg/ml 4'-6-diamidino-2-phenylindole (DAPI); these cells should be incubated with DAPI at room temperature for 20 min. Generally, DAPI is a fluorescent stain that binds strongly to DNA. Following this incubation, the filter is placed onto a glass slide and overlay with oil and a coverslip. Subsequently, cells are

counted using a fluorescent microscope. The remainder of the resuspended cells can be frozen into single-use aliquots.

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Mycobacterium avium genes MAV_5138 and MAV_3679 are transcriptional regulators that play a role in invasion of epithelial cells, in part by their regulation of CipA, a putative surface protein interacting with host cell signaling pathways

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Abstract

The *Mycobacterium avium* complex (MAC) is an important group of opportunistic pathogens for birds, cattle, swine, and immune-suppressed humans. Although invasion of epithelial cells lining the intestine is the chief point of entry for these organisms, little is known about the mechanisms by which members of the MAC are taken up by these cells. Studies with *M. avium* have shown that cytoskeletal rearrangement via activation of the small G-protein Cdc42 is involved, and that this activation is regulated in part by the *M. avium fadD2* gene. The *fadD2* gene indirectly regulates a number of genes upon exposure to HEp-2 cells, including transcriptional regulators, membrane proteins, and secreted proteins. Over-expression of two fadD2-associated regulators (MAV_5138 and MAV_3679) led to increased invasion of HEp-2 cells, as well as altered expression of other genes. The protein product of one of the regulated genes, named CipA, has domains that resemble the PXXP motif of human Piccolo proteins, which bind SH3 domains in proteins involved in the scaffold complex formed during cytoskeletal rearrangement. Although CipA was not detected in the cytoplasm of HEp-2 cells exposed to *M. avium*, the recombinant protein was shown to be potentially expressed on the surface of *Mycobacterium smegmatis* incubated with HEp-2 cells and, possibly, to interact with human Cdc42. The interaction was then confirmed by showing that CipA activates Cdc42. These results suggest that members of the *M. avium* complex have a novel mechanism for activating cytoskeletal rearrangement, prompting uptake by host epithelial cells, and that this mechanism is regulated in part by *fadD2*, MAV_5138, and MAV_3679.

Introduction

Environmentally encountered organisms of the *Mycobacterium avium* complex are known for being pathogens of birds and swine, and are a common cause of opportunistic infection. In non-AIDS patients, *M. avium* can be isolated as the etiologic agent of lung infections; while, in AIDS patients, *M. avium* enters the intestinal mucosa primarily through the epithelial lining of the small intestine (Damsker and Bottone 1985). After translocation across the epithelium cell, the bacterium is taken up by submucosal macrophages and disseminates.

Understanding of the invasion of host mucosal epithelial cells by *M. avium* has been slow due to difficulties in genetic manipulation of these organisms. It is known, however, that other intracellular pathogens impact host signaling pathways triggering cytoskeletal rearrangement as a means to achieve invasion of non-phagocytic epithelial cells. For example, *Cryptosporidium parvum* has been shown to affect actin polymerization in host cells during invasion (Elliott *et al.* 2001), while *Bordetella* (Masuda *et al.* 2002) and *Salmonella* (Stender *et al.* 2000) can modify small GTPases such as Rac and Cdc42 through activities similar to eukaryotic guanine nucleotide exchange factors and GTPase-activating proteins.

A transposon mutant library of *M. avium* serovar 109 (MAC109) was screened in our laboratory for clones with impaired ability to enter human laryngeal epithelial (HEp-2) cells. A number of genes were found to be important for invasion of these cells, including the *fad*D2 gene (Dam *et al.* 2006). This gene encodes a fatty acyl CoA synthetase involved in fatty acid degradation. In *Salmonella*, the *fad*D gene has been

established as a regulator of invasion through *hil*A expression (Lucas *et al.* 2000).

Further analysis of the Δ*fad*D2 mutant strain of MAC strain 109 (MAC109: Δ*fad*D2) showed that this strain did not activate the Cdc42 pathway leading to cytoskeletal reorganization (Dam *et al.* 2006). Previous studies have shown that Cdc42 activates N-WASp indirectly through phosphorylation, and that N-WASp subsequently binds and activates the Arp2/3 complex, leading to actin polymerization (Rohatgi *et al.* 1999). Previous studies indicated that invasion by the Δ*fad*D2 mutant was delayed by at least 15 min and did not result in N-WASp phosphorylation or binding to and activation of the Arp2/3 complex. The Δ*fad*D2 mutant invasion efficiency could be partially restored by addition of supernatant from HEp-2 cells infected with the wild-type MAC109 strain (Dam *et al.* 2006), suggesting the presence of secreted proteins and secretory systems associated with this mechanism of invasion.

Very little is known about secretory systems and surface proteins of mycobacteria involved in epithelial cell invasion. In *Mycobacterium tuberculosis* and *Mycobacterium avium* subsp. *paratuberculosis*, a number of secreted or surface proteins have been shown to be involved in macrophage or epithelial cell entry, including the mycobacterial cell entry (Mce) family of proteins (Gioffre *et al.* 2005), the ESAT-6 family of proteins (Brodin *et al.* 2004), a tyrosine phosphatase (PtpA) (Bach *et al.* 2006), and the heparin-binding hemagglutinin protein (HbhA) (Reddy and Hayworth 2002, Sechi *et al.* 2006), but the mechanisms by which these proteins function in invasion are unknown. Kitaura and colleagues (Kitaura *et al.* 2000) found five *M. avium* proteins that bind fibronectin, including Ag85 and Mpb51. Fibronectin

is expressed on the surface of M cells rather than enterocytes, while *M. avium* preferentially enters enterocytes (Sangari *et al.* 2000), suggesting that these proteins are not primarily important for epithelial cell invasion. A recent study identifying secreted proteins of *M. tuberculosis* by proteomic methods indicated that a large portion of the secreted proteins were previously unknown, and that almost 40% of the proteins were secreted by a mechanism other than the general secretory pathway (Malen *et al.* 2007), indicating there are also likely to be many surface and secreted proteins and systems by which these proteins are secreted by *M. avium* that are not yet identified.

In the present study, the role of the *fad*D2 gene in regulation of invasion was further examined and putative surface or secreted proteins that could be responsible for the effect on the Cdc42 signaling pathway were identified. The results suggest that *M. avium* invasion of epithelial cells is regulated in part by *fad*D2 and other downstream transcriptional regulators, and that the mechanism of invasion involves the activation of actin polymerization through interaction of a bacterial structure putatively expressed on the surface with the host cell membrane and Cdc42.

Materials and Methods

Cell Culture

Laryngeal cells (HEp-2 cells) were obtained from the American Type Culture Collection (ATCC, Catalog #CCL-23) and cultured in RPMI 1640 medium (Invitrogen, Carlsbad CA) supplemented with 10% heat-inactivated fetal bovine serum

(GIBCO, Grand Island NY). Cells were cultured in 25 cm^2 or 75 cm^2 flasks (Corning).

Bacteria

M. avium strain 109 (MAC109), serovar 4, and *M. avium* strain 104 (MAC104), serovar 1, are virulent clinical isolates obtained from the blood of AIDS patients. *Mycobacterium smegmatis* mc^2 155 was a gift from Dr. William Jacobs Jr. (Albert Einstein School of Medicine, NY). All mycobacterial strains were cultured on 7H11 Middlebrook agar or in 7H9 Middlebrook broth (Difco Laboratories, Detroit MI) with 10% oleic acid, albumin, dextrose, and catalase (OADC, Hardy Diagnostics). The *fad*D2 transposon mutant (MAC109: Δ*fadD2*) was generated as described by Dam *et al.* (2006) and was grown on media supplemented with 400 μg/ml kanamycin. Other recombinant strains were generated as described below and cultured in broth or media supplemented with either 400 μg/ml or 50 μg/ml kanamycin. For infection, bacteria were grown at 37°C to log phase in broth prior to inoculation. Luria-Bertani broth and agar (Difco Laboratories, Detroit MI) with indicated antibiotics were used to culture all *Escherichia coli* strains.

HEp-2 cell invasion assays

Assays were performed as described previously by Sangari and colleagues (Sangari *et al.* 2000). Briefly, MAC109, MAC109:MAV_5138 and MAC109:MAC109:MAV_3679 (two clones overexpressing the transcription

regulator, Table B.2) were adjusted to 10^8/ml by McFarland standards and verified by plating serial dilutions. For each strain, 100 μl of this inoculum was added to 4 wells of a 24-well culture plate containing HEp-2 cells. After 30 min or a 1-hour infection time, the supernatant was removed, and the wells were washed 3 times with HBSS to remove extracellular bacteria. Sterile water containing a 1:5 dilution of 0.025% sodium dodecyl sulfate (SDS) was added to the wells to lyse the cells. The lysate was diluted serially and plated onto 7H11 agar to determine the colony forming units per ml (CFU/ml). Assays were performed in replicate, and the resulting CFUs from all assays were analyzed compared to the inoculum to determine the percent invasion after 30 min and 1 h for the three strains.

RNA extraction from mycobacteria

RNA was isolated and purified from mycobacterial strains as follows. Thirty ml mid-log culture of the wild-type and mutant were each divided into two parts, then centrifuged at 3000 *g* at 4°C. The pellets were washed with Hank's balanced salt solution (HBSS, Invitrogen, Carlsbad CA) and re-suspended in HBSS at a concentration of 3×10^8/ml. HEp-2 cells that were first washed with HBSS were exposed to the wild-type and recombinant MAC109 strains for 15 min at 37°C. Extracellular bacteria were then recovered from the flasks, and centrifuged at 3600 rpm at 4°C, in addition to the bacteria resuspended in HBSS that were not exposed to cells. The pellets were suspended in 1 ml Trizol (Invitrogen, Carlsbad CA) and transferred to 2 ml screw cap tubes with 0.4 ml glass beads. The samples were shaken

three times for 30 sec at maximum speed in a bead beater, and periodically inverted. RNA was extracted from the aqueous Trizol solution with Chloroform:isoamyl alcohol (24:1) and isopropanol and washed with 75% ethanol. Resuspended RNA was treated with TurboDNase (Ambion, Austin TX) for 30m at 37°C, and precipitated with 100% ethanol and 3M sodium acetate (pH 5.2). Purity and quantity were analyzed by spectrophotometer.

DNA-microarray

In order to gain some leads in the genes influenced by *fad*D2, we performed a DNA microarray using the *Mycobacterium tuberculosis* template (*M. avium* array was not available at that time). Each oligo was 70 mers, and the array hybridization was performed using the Sigma Genosys Panorama cDNA Labeling and Hybridization kit (Sigma-Genosys, St. Louis MO) according to the manufacturer's protocol. The DNA array was repeated twice and the significance of the fold difference was calculated. Because using the *M. tuberculosis* array can result in false hybridization or no hybridization if the oligo nucleotide sequences are species-specific, we use the results only to provide initial guidance.

Real-time PCR

To confirm the DNA-microarray data, HEp-2 cells were exposed to wild-type MAC109 and the MAC109: Δ*fad*D2. In additional studies, epithelial cells were also exposed to MAC109:pMH6 or MAC109:pMH7 transcription factors over-expression

strains (see below). After no exposure or a 15-minute exposure to epithelial cells, RNA was extracted from the bacteria as described above. By video microscopy, it was determined as the period of time for bacterial internalization to start happening (Sangari *et al.* 2000). cDNA was generated from the RNA using the SuperScript III First Strand Synthesis for RT-PCR Kit (Invitrogen, Carlsbad CA) per the manufacturer's instructions. Briefly, 3 μg of each RNA was combined with 50 ng/μl random hexamers and a 10 mM dNTP mix and incubated at 65°C for 5 min. 10X RT Buffer, 25 mM $MgCl_2$, 0.1 M DTT, and 40 U RNaseOUT were added to the primed RNAs and incubated at room temperature for 2 min. The 200 U SuperScript III Reverse Transcriptase was added to each sample, and incubated for 10 min at room temperature, followed by 50 min at 50°C. The reactions were terminated for 15 min at 70°C, and then treated with RNase H for 20 min at 37°C. Selected genes were amplified by Real-time PCR in a BioRad iQ iCycler, using SybrGreen (BioRad, Hercules CA) and the primers listed in Table B.1. Fold-change in gene expression after exposure to HEp-2 cells was determined by the following formula:

$$\Delta Ct_{exp} = Ct_{exp/gene} - Ct_{exp/16S}$$

$$\Delta Ct_{cont} = Ct_{cont/gene} - Ct_{cont/16S}$$

$$\Delta(\Delta Ct) = \Delta Ct_{exp} - \Delta Ct_{cont}$$

$$\text{Fold change} = 2^{-\Delta(\Delta Ct)}$$

where "exp" refers to RNA samples from bacteria after exposure to HEp-2 cells, and "cont" refers RNA samples from bacteria in HBSS.

Table B.1: Primer sequences used for Real-time PCR [a].

Gene[b]	Forward primer	Reverse primer
16S rDNA	GAGTAACACGTGGGCAATCTG	GTCTGGGCCGTATCTCA
CipA	CCTGCGTGACCCGGTCTC	GCCGGACGTCATTGATATTGA
MAV_5138	GGTGCGGCCGTCTACTTCCAG	GGTGGTGCGGATCTCCTCGTC
MAV_3679	CCTCCGAGTCGATCCGCAAAC	CGTCGGTGAAATAGCGCAGCA
MAV_4139	CAGCGAGATCCTGACGTTCCT	GCACCCAGCCCTCGATCA
MAV_1190	CCGAGTGGTTGACCCGCATGA	GCCTGGGCGATGTCGATGGTG
MAV_3436	GTTCGGCTGGGCGTGGAA	CTCAGCCCGATCAGCGTGGAG
MAV_4666	GCTGCGGCTGAAGTACCAG	AGACGGTGCGGTCGGAGTTGA
MAV_4360	GCCGGGGGACAGGGACGTTAC	CGAGTTGGCGCAGGAACGA

[a] All PCR reactions were performed with an annealing temperature of 60°C, with an extension time of 30 s.
[b] The forward and reverse primers amplify fragments of the indicated genes ranging in size from 200 - 400 base pairs.

Transcription regulators

The transcription regulators MAV_5138 and MAV_3679 were chosen for additional studies because of the up-regulation (2.5-fold and 3.6-fold respectively) and the fact that homologous regulators have been associated with invasion of other bacteria. Strains of MAC109 over-expressing the MAV_5138 and MAV_3679 transcriptional regulator genes were constructed as follows: Using the primers indicated in Table B.2, the gene sequences were amplified from MAC109 genomic DNA, and the amplicons were ligated into pLDG13, a *Mycobacterium/E. coli* shuttle vector containing the strong G13 constitutive promoter from *Mycobacterium marinum* (Danelishvili *et al.* 2005), at the HinDIII and EcoRI restriction sites. After screening for insertion in *E. coli*, the recombinant plasmids, pLDG13:MAV_5138 and

pLDG13:MAV_3679 (named pMH7 and pMH6, respectively) were transformed to competent MAC109, resulting in MAC109:pMH6 and MAC109:pMH7.

Two-hybrid system

The inactivation of fadD2 gene resulted in the inability to activate Cdc42 when the bacterium interacted with epithelial cells. We then hypothesized that a *M. avium* protein might bind to Cdc42 and activate the host cell protein. In an attempt to identify the *M. avium* protein, we performed a two-hybrid system using the Cdc42 as bait and a *M. avium* genomic library. Primers indicated in Table B.2 were used to amplify human *cdc*42 placental isoform from the recombinant pcDNA3.1 plasmid provided by the Guthrie Research Institute (Sayre, PA). This fragment was then cloned into the pBT plasmid (Stratagene, LaJolla, CA) in frame with the λcI repressor gene at the EcoRI restriction site. Transformants were plated on LB agar containing 34.5 μg/ml chloramphenicol and screened by digestion and PCR. MAC104 genomic DNA was partially digested with Sau3A, and cloned into the pTRG plasmid (Stratagene, LaJolla CA) at the BamHI restriction site. Transformants were plated on LB agar containing 12.5 μg/ml tetracycline. Seventy thousand colonies were selected from 10 ligations plated onto 100 plates and combined to create an *M. avium* library downstream of the RNAP-α gene fragment (pTRG:MAClib). The *M. avium* MAV_4671 gene (termed *cipA* for "Cdc42 interacting protein") was amplified from MAC104 genomic DNA using the primers listed in Table B.2. The PCR product was cloned into the pBT plasmid at the EcoRI restriction site. The human *cdc*42 placental

Table B.2: Bacterial strains constructed for this study.

Bacterial strain name	Vector Name	Bacterial host	Use	Parent vector	Gene insert	Forward primer sequence	Reverse primer sequence
MAC109: MAV_5138	pLDG13: MAV_5138	*M. avium*	Over-expression of MAV_3679	pLDG13[a]	MAV_5138	TTTAAGCTTGCACCC GGATGCACGACGCTG	TTTGAATTCCTACGACG ACGAGAGGGTCAGGGG
MAC109: MAV_3579	pLDG13: MAV_3679	*M. avium*	Over-expression of MAV_5138	pLDG13[a]	MAV_3679	TTTAAGCTTGTGAGG GCTGACGAAGAGCGT	TTTGAATTCTTATGCCGT TACCCAGATCGC
E. coli: pBT:*cdc42*	pBT:*cdc42*	*E. coli*	Bacteriomatch 2-hybrid screen	pBT	*cdc42*[b]	GTGGAATTCCACCAT GCAGACAATTAAGTG	GTGGAATTCAACGGGCC CTCTAGACTCGAG
E. coli: pBT:*cipA*	pBT:*cipA*	*E. coli*	Bacteriomatch 2-hybrid screen	pBT	*cipA*	GAATTCCAACGTGAC GAACCCACAAGGACC	GATGAATTCGAGGTTGG CGAAGCAGGGGTCGTT
E. coli: pTRG:*cdc42*	pTRG: *cdc42*	*E. coli*	Bacteriomatch 2-hybrid screen	pTRG	*cdc42*	GTGGAATTCCACCAT GCAGACAATTAAGTG	GTGGAATTCAACGGGCC CTCTAGACTCGAG
MAC104: pMH4	pMH4	*M. avium*	MAV_4671: *Bordetella pertussis cyaA* fusion protein	pLDG13[a]	*cyaA*[c]	TTTCTGCAGCAATCG CATGAGGCTGGTTAC	TTTCTGCAGCTAGCGTT CCATCGCGCCCAG
MAC104: pMH5	pMH5	*M. avium*	MAV_4671: *Bordetella pertussis cyaA* fusion protein	pMH4	*cipA*	GAATTCCAACGTGAC GAACCCACAAGGACC	GATGAATTCGAGGTTGG CGAAGCAGGGGTCGTT
M. smeg:GFP	pGFP	*M. smegmatis*	Fluorescence microscopy	pMV261	GFPmut2[d]	GCTGGATCCTTGCTC AGGTTGGTAGGGC	GAGAATTCCGAAGCAG GGGTCGTTGTTG
M. smeg: CipA: GFP	pGFP:CipA	*M. smegmatis*	Fluorescence microscopy	pGFP	*cipA*	TCTGAATTCAAGAAG GAGATATACATAT	CAGGTAACTTATTTATT TTGTATAGTTC

[a] Danelishvili *et al.* (Danelishvili *et al.* 2005)
[b] Amplified from the pcDNA3.1:*cdc42* plasmid, obtained from the Guthrie Research Institute, Sayre PA
[c] Amino acids 2-400, amplified from pACYA (Day *et al*. (Day *et al.* 2003))
[d] Obtained from Rafael Valdivia and Stanley Falkow, Stanford University

isoform gene product was amplified as indicated above and cloned into the pTRG plasmid at the EcoRI restriction site.

The recombinant pBT:*cdc*42 and pTRG:MAClib were co-transformed into the Bacteriomatch Two-Hybrid System (Stratagene, LaJolla CA) reporter strain *E. coli*. Transformants were plated onto LB agar containing 400 μg/ml carbenicillin, 12.5 μg/ml tetracycline, 34.5 μg/ml chloramphenicol, and 50 μg/ml kanamycin. Colonies that grew after 30 h at 30°C were picked from these plates and transferred to LB agar containing the same antibiotics except carbenicillin and 80 μg/ml X-Gal and 200 μM phenylethyl β-D-thio galactoside (an X-Gal inhibitor), prepared in dimethyl formamide. Colonies that became blue in color in the presence of X-Gal and the inhibitor after 17 h at 30°C were further analyzed to determine the *M. avium* sequence of the insert in the pTRG vector. Co-transformation of the pBT:*cipA* vector with pTRG:*cdc*42 vectors and pBT:*cipA* with the pTRG:MAClib, and screening of the resulting transformants was repeated as described above.

Investigation for intracellular delivery of CipA

To determine whether CipA is delivered to the intracellular environment prior to bacterial invasion, we constructed a CipA:CyaA fusion protein, using primers listed in Table B.2 to amplify the DNA encoding amino acids 2-400 of the *Bordetella pertussis cyaA* gene from pACYA, a kind gift from Gregory V. Plano (University of Miami, FL). The *cyaA* PCR product was ligated into pLDG13 at the PstI restriction site and screened in *E. coli*, resulting in pMH4. The *cipA* PCR product was amplified from

MAC104 using the primers listed in Table B.2. The pMH4 and the CipA PCR product were digested with EcoRI, ligated, and screened in *E. coli*, resulting in pMH5, a plasmid containing an in-frame fusion of *cipA* and *cyaA* behind the strong G13 promoter. The completed pMH5 construct was transformed by electroporation to MAC104 competent cells, and the resulting colonies were screened by PCR to confirm the presence of the plasmid. Resulting transformants were called MAC104:pMH5.

Protein extraction and Western blot analysis

MAC104 and MAC104:pMH5 were grown to log phase in 7H9 media without OADC. After centrifugation, the pellets were resuspended in HBSS and the resulting inocula were used to infect HEp-2 cells in 6-well tissue culture plates or 75 cm^2 flasks at an MOI of 100:1. All steps post-infection were completed at 4°C or on ice. At 30 min, 1 h, and 2 h time points, the extracellular bacteria were removed from the wells and pelleted by centrifugation. The infected HEp-2 cells were lysed in water containing a protease inhibitor cocktail (Sigma), and then centrifuged to remove the cellular debris and intracellular bacteria. The contents of the HEp-2 cells after lysis and centrifugation were incubated with mouse monoclonal α-CyaA [Santa Cruz Biotech (SCBT), CA] and agarose-conjugate α-IgG beads (SCBT, CA) overnight at 4°C. The beads were washed four times with PBS and resuspended in Laemmli sample loading buffer (BioRad, Hercules CA). After denaturation by boiling for 5 min, the protein samples were run on a 12% Tris-HCl protein gel (Bio-Rad, Hercules, CA) for 1 h at 150 V. The proteins were transferred to a nitrocellulose membrane

using a semi-dry transfer apparatus with a constant current of 15 V for 1 h.

Western blotting was performed using the Odyssey Western Blotting system by Li-Cor (Lincoln NE) per the manufacturer's instructions. Briefly, the membrane was blocked in a 1:1 dilution of TBS and Odyssey blocking buffer overnight at 4°C. The membrane was then incubated with mouse α-cyaA antibody (1:300 in TBS containing 0.1% Tween-20) (Santa Cruz Biotech, CA) for 3 hrs. Following washes with TBS-0.1% Tween-20, the membrane was incubated with anti-mouse IgG linked to AlexaFluor680 (1:2000 in TBS containing 0.1% Tween and 0.01% SDS) (Li-Cor, Lincoln NE). After additional washes the membrane was scanned using the Odyssey Infrared Imager (Li-Cor, Lincoln NE).

Assay for cAMP in infected HEp-2 cultures

cAMP was assayed in uninfected HEp-2 cells and at 30 min, 1 h, 2 h, and 4 h after contact between the bacteria and HEp-2 cells using the Direct cAMP Enzyme Immunoassay Kit (Sigma, St. Louis MO) per manufacturer's instructions. Briefly, MAC104 and MAC104:pMH5 were used to infect the cells at an MOI of 100:1. At each time point, extracellular bacteria were removed from the wells, and the infected cells were washed with HBSS, and then lysed in 0.1M HCl. After centrifugation of the lysate at 600 × *g*, the supernatants were acetylated and neutralized in a 96 well plate. Each sample was then incubated with a cAMP conjugate and cAMP antibody for 2 h at room temperature and then washed. The plate was read at 405 nm after incubation with the p-NPP substrate for 1 h and addition of the stop solution. The

levels of cAMP in the samples were calculated based on the standard curve generated from serial dilutions of known cAMP concentrations.

To investigate the possibility that CipA is a putative outer cell wall protein, we constructed a *M. smegmatis* strain expressing CipA (*M. smegmatis* genome does not contain a CipA homologue). A promoterless GFPmut2 gene (obtained from Rafael Valdivia and Stanley Falkow, Stanford University) was inserted in the HinDIII and EcoRI sites of the pMV261 vector containing the Hsp60 promoter, resulting in pMV261:GFP (Miltner *et al.* 2005). *CipA* was amplified, purified and inserted between the promoter and the GFPmut2 gene at BamHI and EcoRI sites, in frame with GFP. Colonies were screened in *E. coli*, and the resulting pMV261:CipA:GFP was transformed to competent *M. smegmatis* mc^2155. PCR was used to screen *M. smegmatis* expressing GFP for the presence of the CipA:GFP sequence.

HEp-2 cells were infected with wild-type *M. smegmatis* or bacterium containing pMV261:CipA:GFP plasmid over-expressing CipA protein. After 15 min infection, cells were washed with TBS and lysed with lysis/binding/wash buffer containing protease inhibitor cocktail (Sigma) as previously described (Alonso-Hearn *et al.* 2008). For a positive control uninfected cell lysates were treated with GTPγS to activate Cdc42 pathway. Activated Cdc42 were captured using EZ-Detect Cdc42 Activation kit according to the manufacturer's instructions (Pierce, Rockford, IL). Eluted proteins were resolved on a 12% SDS-PAGE gel, transferred to a membrane and blocked overnight with blocking buffer (Li-Cor) in TBS. Proteins were probed with anti-Cdc42 antibody (Pierce, Rockford, IL) and visualized with goat anti-mouse

secondary antibody (Li-Cor). Membranes were scanned using Odyssey Imager (Li-Cor, Lincoln, NE).

Fluorescence microscopy and immuno-histochemistry

M. smegmatis strains containing either the pMV261:GFP or pMV261:CipA:GFP vector, expressing GFP or GFP fused to the 3' end of the CipA protein, and *M. avium* stained with fluorescein were resuspended in RPMI at 10^7/ml, estimated by McFarland's standards. 100 μl of this inoculum was added to a monolayer of HEp-2 cells in each chamber of an 8-chamber slide with 100 μl fresh RPMI or 100 μl fresh RPMI containing 10 μl of a 10 mg/ml solution of cytochalasin D. Following a 15 min, 30 min, or 1 h incubation time, the media was removed, and the wells were incubated with a 4% paraformaldehyde solution for 1 h at room temperature. After washing, monolayers were examined by fluorescence microscopy, or further prepared for immuno-histochemistry as follows.

Cells were permeabilized with a 0.1% Triton X-100, 0.1% sodium citrate solution for 2 min on ice. After blocking overnight in 10% Bovine Serum Albumin (BSA), wells were incubated with rabbit α-Cdc42 (Santa Cruz Biotech, CA), diluted 1:1000 in 10% BSA for 1 h at room temperature, and then anti-rabbit IgG-Texas Red conjugate (Santa Cruz Biotech, CA), diluted 1:200 in 10% BSA for 1 h. Monolayers were examined by fluorescence microscopy.

Bioinformatics

The *M. avium* 104 genome sequence is posted on The Institute for Genome Research (TIGR) website (www.tigr.org). Sequences obtained from the microarray and positive pTRG vectors in the bacterial 2-hybrid screen were analyzed by the Basic Local Alignment Search Tool (BLAST) to find similarity to the published MAC104 genome and for putative conserved domains. Once *M. avium* gene sequences were obtained, the protein-specific iterated BLAST (PSI-BLAST) and the SignalP 3.0 server (Bendtsen *et al.* 2004) were utilized to further characterize the gene sequences.

Statistical Analysis

Statistical analysis was based on the mean of three experiments +/- the standard deviation. Student's t-test was used to compare values for each strain. P-values < 0.05 were considered significant.

Results

The M. avium fadD2 gene is a regulator of gene expression upon exposure to HEp-2 cells

To explore whether FadD2 was playing a potential role in gene regulation upon epithelial cell invasion, a heterologous microarray was performed by hybridizing *M. avium* RNA from wild-type and the Δ*fad*D2 mutant strains against a Panaroma *M. tuberculosis* microarray obtained from Sigma Genosys (data not shown). The microarray was strictly to gain leads and among the genes upregulated in the array, several encoded for proteins of unknown function.

Using the preliminary results of the microarray and also from the preliminary bacterial 2-hybrid screen described later, we performed real-time PCR to analyze the expression of a subset of genes appearing in one or both of the exploratory experiments. Five genes were selected with varied responses to HEp-2 exposure based on the results of the microarray: MAV_5138, MAV_3679, and *cipA* were all up-regulated in the wild-type (at least 2-fold increase over control), but not the mutant upon exposure to HEp-2 cells; MAV_4139 and MAV_1190 showed no up-regulation in either the wild-type or mutant. CipA, MAV_4139, and MAV_1190 also putatively interacted with human Cdc42, based on a two-hybrid screen. By real-time PCR analysis, the MAV_5138 and MAV_3679 and MAV_1190 were shown to be up-regulated in the wild-type, but not the mutant, after exposure to HEp-2 cells (Figure B.1, $p < 0.05$). The *cipA* gene did not increase in expression upon exposure, but had higher expression compared to the *fad*D2 mutant under the same conditions (although this difference was not significant) (data not shown). *Cip*A and MAV_4139 were not upregulated in the wild-type or mutant strains after exposure to HEp-2 cells.

MAV_5138 and MAV_3679 are transcriptional regulators involved in invasion of HEp-2 cells

MAV_5138 and MAV_3679 are homologous to transcriptional regulator families found in other species that have been shown to play a role in invasion. Based on their up-regulation in the wild-type *M. avium,* but not the *fad*D2 mutant strain upon exposure to HEp-2 cells, MAV_5138 and MAV_3679 were over-expressed in *M. avium* behind the G13 promoter. Strains over-expressing either transcriptional

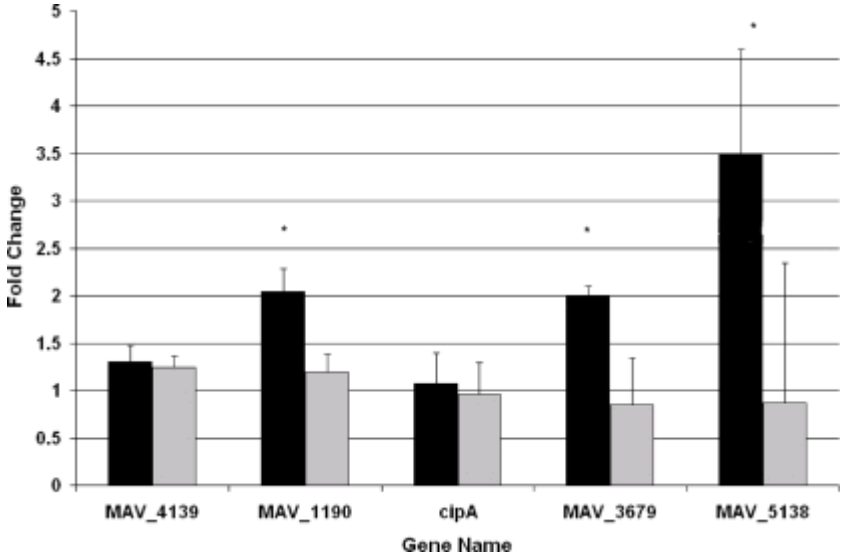

Figure B.1: Real-time PCR results comparing the fold-change in gene expression upon 15 minute exposure to HEp-2 cells between the MAC109 and MAC109Δ*fadD2* strains. The y-axis represents fold-change between broth grown bacteria and bacteria incubated for 15 min with HEp-2 cells, using the 16S rDNA as an internal control. * indicates significant differences between the two strains, based on three independent experiments ($p < 0.05$).
■ MAC109; ▨ MAC109: Δ*fadD2*

regulator had an increased percent invasion of HEp-2 cells compared to MAC109 (Figure B.2). The MAC109:MAV_5138 strain had significantly higher invasion after a 30 min incubation, while the MAC109:MAV_3679 had significantly higher invasion after both a 30 min and 1 h incubation ($p < 0.05$).

Because FadD2 is likely not a direct regulator of downstream gene expression, we hypothesized that the MAV_5138 and MAV_3679 gene products may play some role in regulating the genes involved in cytoskeletal rearrangement seen upon epithelial cell invasion by wild-type *M. avium*, but not in the Δ*fad*D2 strain. We selected five genes from the preliminary microarray and two-hybrid results, and analyzed their expression in strains of *M. avium* overexpressing either of the putative

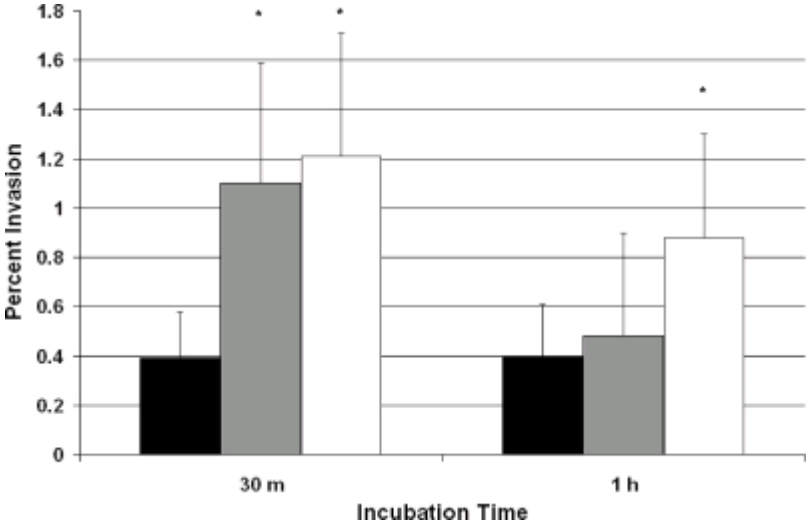

Figure B.2**:** Percent invasion of HEp-2 cells by strains of *M. avium* over-expressing MAV_3679 or MAV_5138 after a 30 minute or 1 h incubation time. Diagonal bars represent MAC109; checks represent MAC109:pMH6; and vertical bars represent MAC109:pMH7. Both recombinant strains have significantly higher invasion than MAC109 after 30 min, and MAC109:pMH7 also has a significantly higher invasion after 1 h. * indicates significant differences from baseline, based on three independent experiments ($p < 0.05$).
■ MAC109; ▩ MAC109:MAV_5138; □ MAC109:MAV_3679.

transcriptional regulators. Over-expression of the MAV_3679 repressor regulator led to up-regulation of all 7 genes in broth-grown bacteria (Figure B.3A). Similarly, all 7 genes were up-regulated in broth-grown bacteria in the MAC109:MAV_5138 strain compared to the wild-type (Figure B.3B).

M. avium expresses proteins that putatively interact with host cell Cdc42

To connect the bacterial genes being expressed upon invasion of HEp-2 cells with the subsequent Cdc42 activation and cytoskeletal rearrangement in host cells, we wanted to identify bacterial proteins interacting with Cdc42. In an exploratory 2-hybrid screen, we co-transformed pBT:*cdc*42 and pTRG:MAClib to the reporter strain *E. coli*

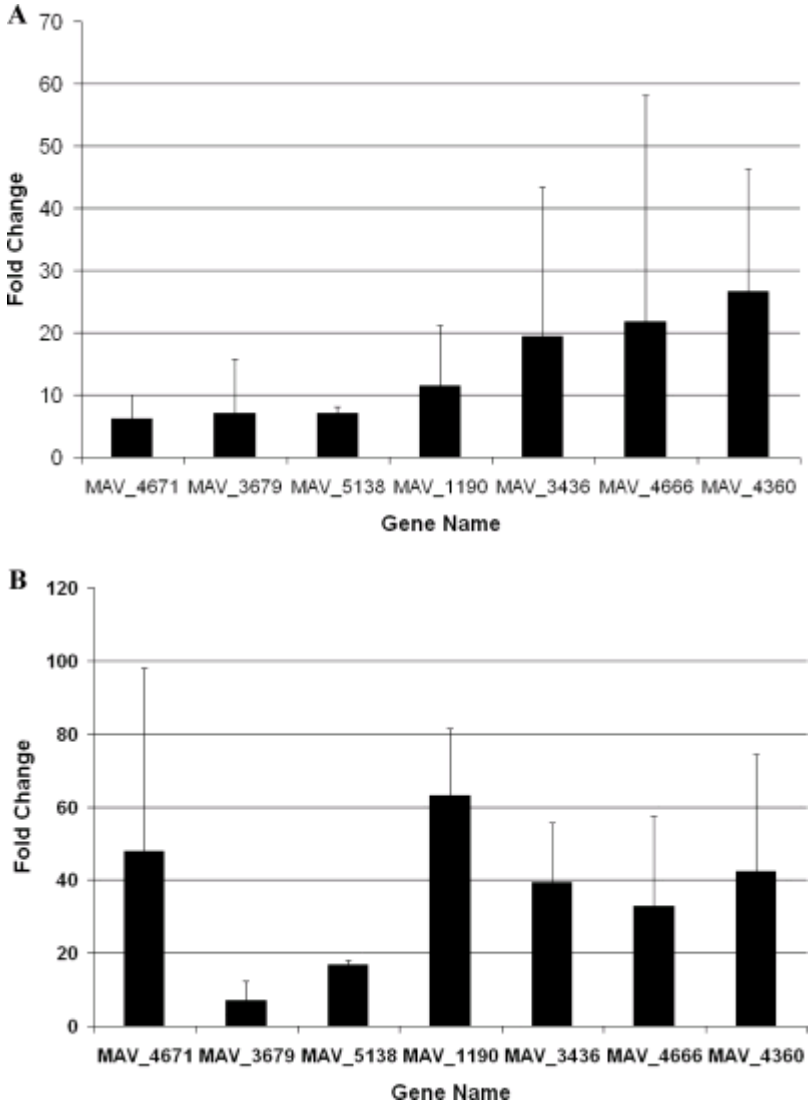

Figure B.3**:** Fold-change in expression of genes in *M. avium* over-expressing A) MAV_3679, or B) MAV_5138, compared to wild-type MAC109 grown in culture medium. Results shown are the average and standard error of three experiments.

and obtained three proteins that potentially interact with Cdc42: CipA, MAV_1190, and MAV_4139 (Table B.3). As described previously, real-time PCR indicated that the MAV_1190 transcript is upregulated with exposure to HEp-2 cells in wild-type bacteria, but not the Δ*fad*D2 strain. The MAV_4139 and *cip*A genes showed no

Table B.3: Results of 2-hybrid screen for *M. avium* proteins interacting with human Cdc42 or full-length *M. avium* CipA.

Bait	*M. avium* gene	Putative function
Cdc42		
	CipA	Hypothetical protein
	MAV_1190	Patatin, putative esterase
	MAV_4139	Hypothetical protein
CipA		
	MAV_4952	Acyltransferase
	MAV_1043	Hypothetical protein, phage-related protein
	MAV_1300	Hypothetical protein
	MAV_3034	Oxidoreductase
	MAV_4319	Transposase, mutator family protein
	MAV_3595	Sigma factor, *mysA*
	MAV_0876	Transcriptional regulator, *iclR* family

difference in regulation between the wild-type and Δ*fad*D2 strain. All three genes, however, were upregulated when MAV_5138 and MAV_3679 were over-expressed.

The CipA amino acid sequence suggests it is a secreted or transmembrane protein with domains for interaction with eukaryotic proteins

Although *cip*A transcripts were not affected by deletion in the *fad*D2 gene, the increase in expression when MAV_5138 and MAV_3679 were over-expressed and its amino acid sequence led us to further explore the role of this protein in epithelial cell invasion. The sequence of CipA has a number of interesting characteristics that lend support to this idea. Prior to the recent annotation of *M. avium*, an amino acid sequence corresponding to the *cipA* gene was listed as a putative multicopper oxidase

in the NCBI database. The coding sequence listed in this entry is shorter than annotated homologues in other mycobacterial species, and there is no sequence or biochemical evidence to support this putative function. The current annotation lists this protein as a hypothetical protein. There are very few homologues to CipA, and their predicted functions are diverse, including the following: a putative membrane protein in *M. tuberculosis* (Rv0479c) and other sequenced mycobacteria, a hypothetical protein in *M. avium* subsp. *paratuberculosis* (MAP3972c), and putative secreted proteins in *Mycobacterium leprae, Corynebacterium diphtheriae, Rhodococcus* sp. RHA-1, and *Burkholderia cepacia*. According to the database, the first 100 amino acids are not found in any bacteria besides other mycobacterial species. A PSI-BLAST of this sequence reveals that there are segments in the first 100 amino acids similar to the PXXP domains of the Piccolo domain proteins in human cells, which have been implicated to play a role in scaffolding proteins involved in actin dynamics (Fenster *et al.* 2003). These domains are present in only *M. avium* and *M. avium* subsp. *paratuberculosis*, and not other characterized mycobacteria, such as *M. tuberculosis*. The remaining 252 amino acids correspond to putative secreted proteins, and this region also contains a putative signal peptide, predicted by SignalP 3.0 (Bendtsen *et al.* 2004). The region of this gene sequenced from the pTRG plasmid corresponds to the C-terminal 50 amino acids (Figure B.4).

CipA is not secreted into the cytoplasm of HEp-2 cells

If CipA interacts with Cdc42, it must be secreted into host cells or inserted into the

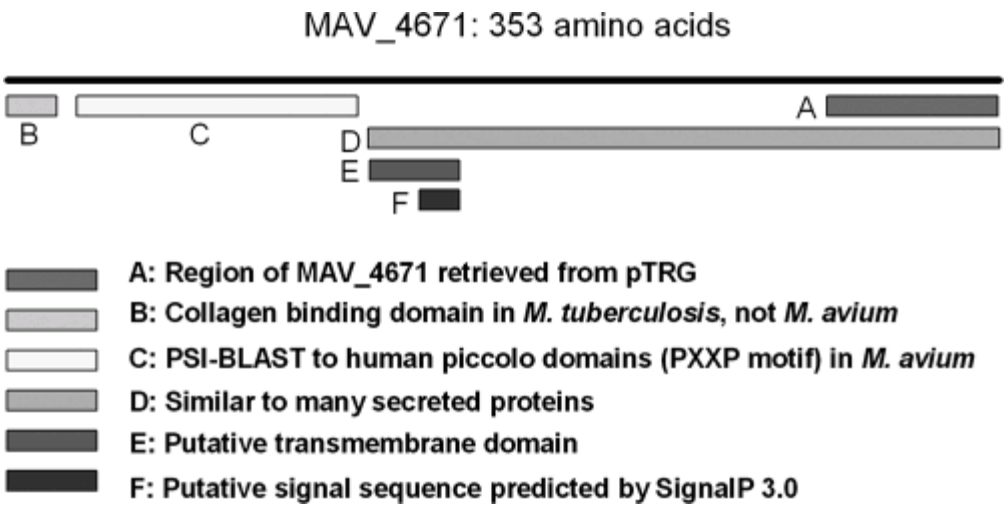

Figure B.4: Domains/regions present in the CipA amino acid sequence.

membrane of these cells. To determine if CipA is a secreted protein, it was fused to amino acids 2-400 of the *B. pertussis* adenylate cyclase protein. Using antibodies against the *B. pertussis cyaA* tag, expression of the CipA:CyaA fusion protein was detected in *M. avium* by Coomassie stain of whole cell lysates (Figure B.5A). After incubating HEp-2 cells with the MAC104:pMH5 strain, the fusion protein could not be detected in the cytoplasm or insoluble fraction of the lysed HEp-2 cells by western blot (Figure B.5B). When in the cytoplasm of eukaryotic cells, the fragment of the adenylate cyclase protein leads to an increase in cAMP that can be assayed by ELISA. After incubation with MAC104:pMH5 for 1, 2, and 3 h, there was no significant increase in cAMP levels compared to the wild-type MAC104 (data not shown) There was an initial increase at 2 h, but it was not significant, and the levels of cAMP in the cells incubated with the wild-type increased to nearly the same levels after 3 h.

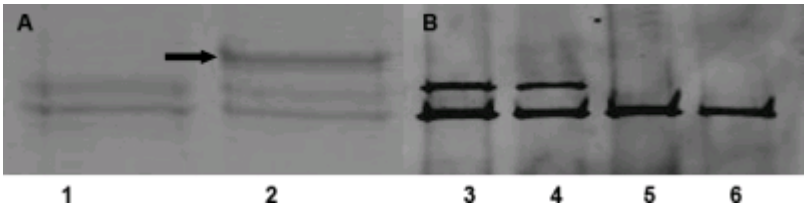

Figure B.5: CipA expressed as a fusion protein with the *Bordetella pertussis* adenylate cyclase gene is not detected in the cytosolic or insoluble fractions of HEp-2 cells infected with *Mycobacterium avium*. Proteins were extracted from broth-grown *M. avium*, or from HEp-2 cells infected with strains of *M. avium*. An α-CyaA antibody and agarose-conjugate α-IgG beads were used to precipitate the CipA:CyaA fusion protein from the lysates. A) Protein gel stained with coomassie blue showing the expression of the 80 kDa CipA:CyaA fusion protein (arrow) in broth-grown *M. avium*. Lane 1: MAC104, Lane 2: MAC104:pMH5. B) Western blot showing proteins immunoprecipitated from HEp-2 cells infected with strains of *M. avium.* Lane 3: Soluble lysate fraction incubated with MAC104; Lane 4: Soluble lysate fraction incubated with MAC104:pMH5; Lane 5: Insoluble lysate fraction incubated with MAC104; Lane 6: Insoluble lysate fraction incubated with MAC104:pMH5.

CipA apparently localizes to the membrane of M. smegmatis when exposed to HEp-2 cells, with an associated accumulation of Cdc42 near M. smegmatis and M. avium

Because we could not identify CipA in the cytoplasm of host cells, we explored whether this protein is potentially expressed on the surface of the bacterium. A strain of *M. smegmatis* expressing CipA fused to GFP was used to infect HEp-2 cells. Construction of recombinant strains of *M. avium* expressing this fluorescent fusion protein was unsuccessful. After an incubation time of 1 h with the recombinant strains of *M. smegmatis*, a structure could be observed on the tip of bacteria near HEp-2 cells (Figure B.6A). The HEp-2 cells had been treated with cytochalasin D to prevent uptake of the bacteria. This structure was not present on wild-type *M. smegmatis* expressing GFP alone (Figure B.6B).

To determine if Cdc42 was accumulating near the invading bacterium,

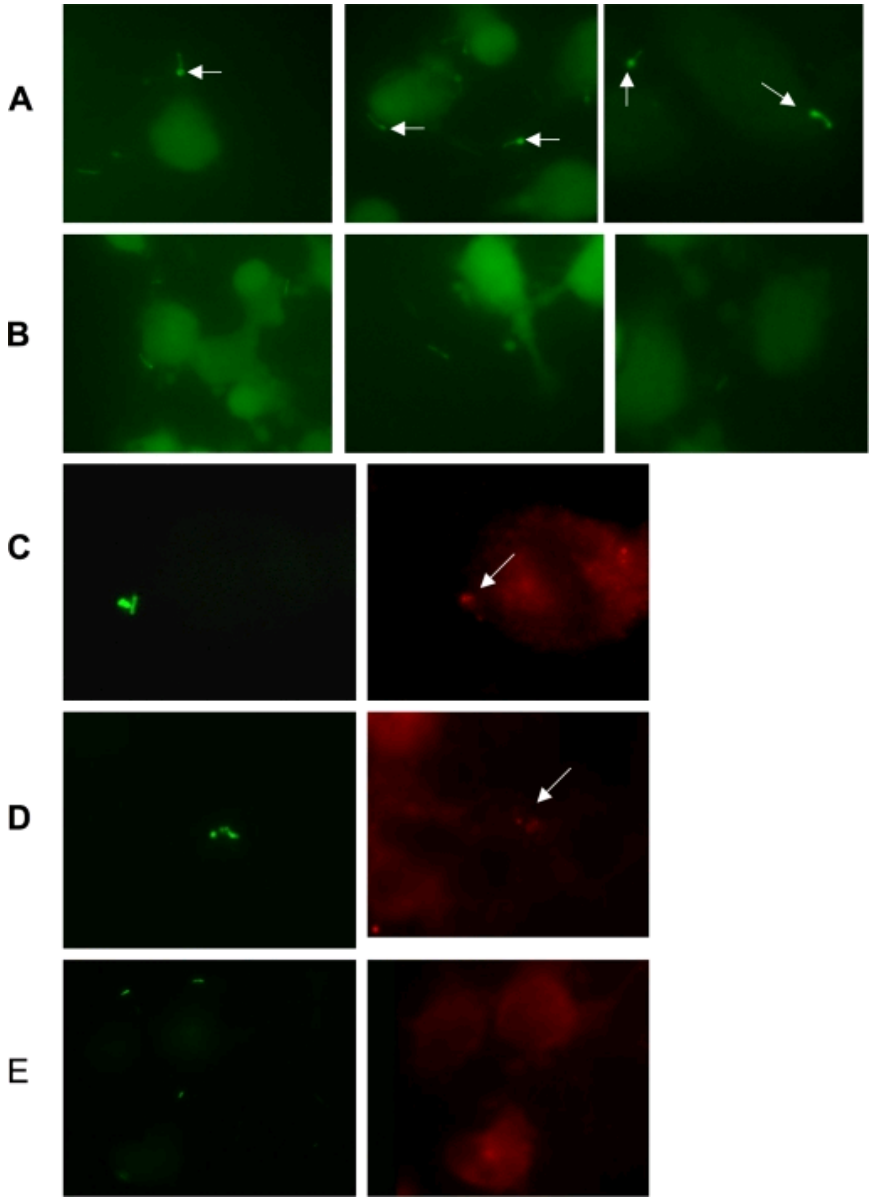

Figure B.6: A-B) HEp-2 cells exposed to *M. smegmatis* expressing CipA fused to GFP, or GFP alone. There is a polar accumulation of GFP/CipA. A) Representative images of individual bacterial cells, expressing the fusion protein, in contact with cytochalasin D-treated HEp-2 cells. B) Representative images of individual bacterial cells expressing GFP only, in contact with cytochalasin D-treated HEp-2 cells. C-F) Cells infected with either strain of *M. smegmatis*, or fluroescein-labeled *M. avium* were incubated with α-Cdc42 antibodies, and a Texas-red conjugated secondary. C) cells infected with *M. avium* for 15 min; D) cells infected with *M. avium* for 30 min; and E) cells infected with *M. smegmatis* for 1 h. The figure shows that, while *M. avium* appears to interact with Cdc42 on the cell, *M. smegmatis* does not.

immuno-histochemistry was performed after infection of HEp-2 cells with the recombinant *M. smegmatis* strains, as well as fluorescein-labeled *M. avium.* After fixation, infected cells were incubated with antibodies against Cdc42 at 15 min, 30 min, and 1 h after infection. In cells infected with fluorescein-labeled *M. avium*, Cdc42 was observed to accumulate near mycobacteria at 15- and 30-min post-infection (Figures B.6C and D), but not in cells infected for 1 h with *M. smegmatis* control (Figure B.6E).

Pull-down assay for activated Cdc42 protein followed by a Western blot analysis with an anti-Cdc42 revealed that HEp-2 cells infected with wild type *M. smegmatis* failed activation of Cdc42 at 15 min after infection. However, the bacterium expressing the CipA protein activates Cdc42 at the same time point of infection. Cdc42 is not activated in uninfected HEp-2 cells (Figure B.7).

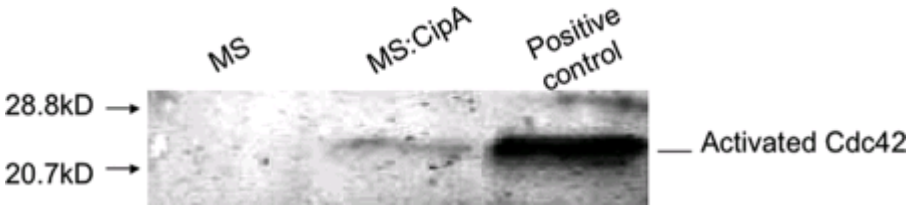

Figure B.7: Western blot showing that CipA is capable of activating host cell Cdc42. HEp-2 cells were incubated either with *M. smegmatis* wild-type or *M. smegmatis* expressing CipA in the membrane. HEp-2 cells were lysed and positive control were treated with GTPγδ to activate Cdc42. Activated Cdc42 was captured, as described in Materials and Methods. Proteins were eluted and resolved in SDS-PAGE gel and transferred to a membrane and probed with anti-Cdc42 antibody. Lane 1, *M. smegmatis* not expressing CipA; Lane 2, positive control; Lane 3, *M. smegmatis* expressing CipA.

CipA interacts with M. avium and HEp-2 cell proteins

If CipA is on the surface of the bacteria upon contact with epithelial cell, it may be

part of a secretory complex, or interact a chaperone in the membrane. To identify putative *M. avium* proteins interacting with CipA, the *M. avium* protein library in the pTRG vector was screened with the CipA protein in the pBT vector, by co-transformation in the 2-hybrid reporter strain. In five separate co-transformations, 87 colonies grew on plates containing the selective antibiotic. Of these colonies, 23 were further analyzed after becoming blue in the second screen. Sequences retrieved from the pTRG vector indicated that 7 proteins putatively interact with CipA (Table B.3), including MAV_3034, an oxidoreductase, and MAV_1300, a hypothetical protein with some loose similarity to intracellular transport proteins (Figure B.8)

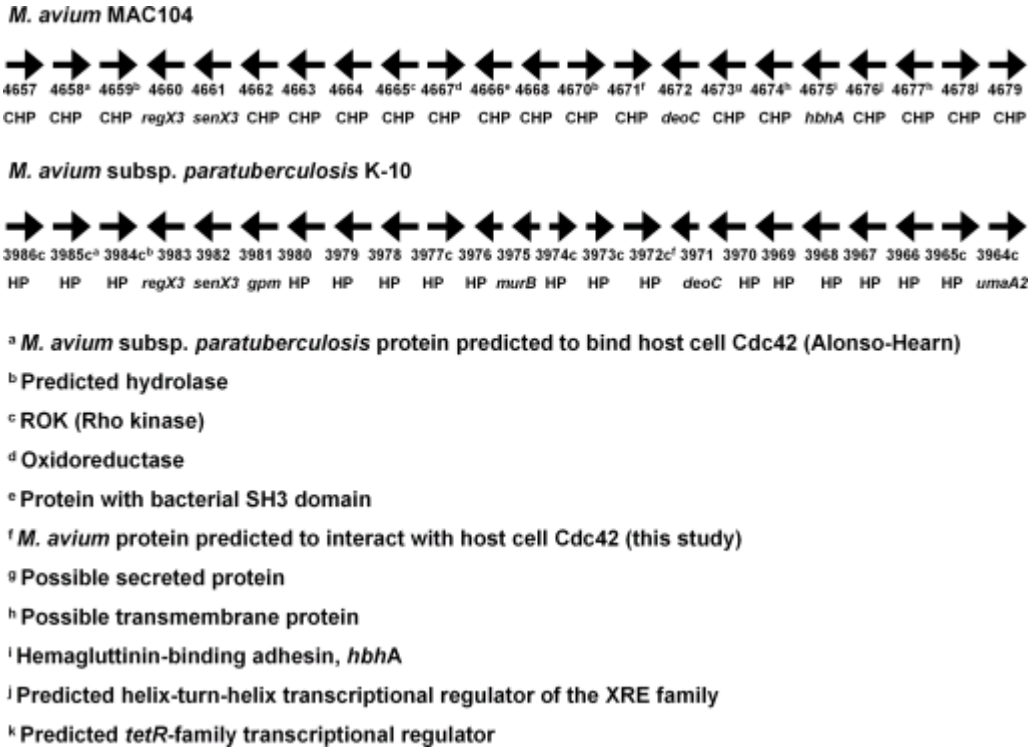

Figure B.8**:** The *M. avium* (numbers represent MAV annotation) and *M. avium* subsp. *paratuberculosis* (numbers represent MAP annotation) chromosomal regions encompassing the CipA gene discussed in this study. CHP: Conserved hypothetical protein; HP: Hypothetical protein.

Discussion

Mycobacterium avium is similar to other intracellular pathogens, such as *Salmonella* sp., *Yersinia* sp., and *Shigella* sp., in its ability to enter the cells of the intestinal epithelium by an active process involving cytoskeletal reorganization. There is little known, however, about the molecular mechanism of this process in *M. avium*. Unlike these other intracellular pathogens, *M. avium* does not have genes encoding proteins similar to surface invasins or internalins, as in the case of *Yersinia* (Isberg and Leong 1988, Isberg and Leong 1990) or *Listeria* (Cossart *et al.* 2003), or those that comprise type III secretion systems of *Salmonella* (Zhou and Galan 2001) or *Shigella* (Sansonetti 2001). These secretion systems are commonly involved in the secretion of proteins effecting cytoskeletal rearrangement and subsequent epithelial cell invasion. It is plausible, however, to hypothesize that *M. avium* uses mechanisms, which the end result is similar.

There is little doubt that, regardless of the pathogen, the genes involved in epithelial cell invasion are regulated in response to environmental cues such as pH, oxygen and osmolarity. A study by Bermudez and colleagues (Bermudez *et al.* 1997) showed that exposure to low O_2 tension and hyperosmolarity led to a significant increase of invasion of epithelial cells by *M. avium*. In *Salmonella*, Lucas *et al.* (Lucas *et al.* 2000) identified a number of genes that work independently to activate *hilA*, which in turn activates genes in the pathogenicity island SP-1, encoding the TTSS involved in epithelial cell invasion. The regulators identified by this group included two-component response regulators similar to those involved in

mycobacterial invasion of macrophages, as well as *fadD*. The FadD protein is involved in the breakdown of endogenous and long-chain fatty acids, although the mechanism of regulation by this protein is yet unknown (Lucas *et al.* 2000).

In two separate screens of mutant libraries of *M. avium*, mutations in the *fadD2* (Dam *et al.* 2006) and *fadE20* (Miltner *et al.* 2005) genes were associated with reduced invasion of epithelial cells. Further studies with the *fadD2* mutant strain revealed that it was deficient in the ability to activate the host cell Cdc42 signaling pathway, leading to actin polymerization via N-Wasp and the Arp2/3 complex (Dam *et al.* 2006). This signaling pathway, from *fadD* in the bacterium to actin polymerization in the host, is involved in cell entry by other intracellular pathogens of both plants and animals (Barber *et al.* 1997, Lucas *et al.* 2000, Soto *et al.* 2002). We hypothesized that the *M. avium fadD2* gene may also be involved in regulation of genes that effect epithelial cell invasion, and conducted experiments to determine the role of *fadD2* in this process.

In an exploratory heterologous microarray and real-time PCR comparing the up-regulation of genes in a wild-type *M. avium* strain upon exposure to HEp-2 cells to the *fadD2* mutant strain, we identified genes possibly regulated by *fadD2*. A number of homologues to transcriptional regulators were also identified, including MAV_3679 and MAV_5138. Real-time PCR confirmed that MAV_3679 and MAV_5138 are regulated by *fadD2* upon invasion of epithelial cells, as they were not up-regulated in the *fadD2* mutant (Figure B.1). MAV_3679 encodes an ion-dependent regulator, similar to *sirR* from *Staphylococcus epidermidis*, *dtxR* from *Corynebacterium*

diptheriae, mntR from *Staphylococcus aureus*, and both *ideR* and Rv2788 from *M. tuberculosis*. Many of these ion-dependent regulators, which bind the operators of and influence ABC transporters, have been shown to be involved in virulence (Ando *et al.* 2003, Manabe *et al.* 1999). Interestingly, constitutive expression of an ion-independent mutant of *dtxR* in both *S. aureus* and *M. tuberculosis* exhibited attenuation in mice (Ando *et al.* 2003). In this study, constitutive over-expression of this gene behind a non-native strong promoter (G13) led to increased invasion of HEp-2 cells. Although it is possible that MAV_3679 is involved in the regulation of the *mntH* gene (regulated by *mntR* in *S. aureus*) also identified in the microarray (data not shown), the *M. tuberculosis* homolog of *mntH* is not involved in virulence (Domenech *et al.* 2002), and the connection between expression of MAV_3679 and *M. avium mntH* was not further explored.

For its genome size, *M. avium* has a very large number of *tetR*-like transcriptional regulators, and more than almost all other sequenced bacteria (Ramos *et al.* 2005). The family of *tetR* regulators has many members, and the MAV_5138 gene is most similar to a homolog in *M. tuberculosis* of the AcrR family member. Proteins in the AcrR family have not been shown to play a role in virulence, but other TetR-family regulators, such as HapR of *Vibrio cholera* (Kovacikova and Skorupski 2002) and TvrR of *Pseudomonas syringae* (Preiter *et al.* 2005) have been implicated in virulence. In general, the TetR-family is important to regulation of genes in response to the environment. *M. avium* has more than twice as many *tetR*-like genes than *M. tuberculosis*, so it is possible that there are as yet uncharacterized members in

this large family involved in *M. avium* virulence. Similar to the *sirR*-like MAV_3679, over-expression of the MAV_5138 protein led to an increase in invasion of HEp-2 cells. TetR-like regulators are thought to influence their own expression (Ramos *et al.* 2005).

Concurrently with the analysis of bacterial genes that may be regulated by MAV_3679 or MAV_5138, and in turn, by FadD2, we used a bacterial 2-hybrid screen, with human Cdc42 as the bait, was used to explore this pathway from the host side. Fragments of three genes were retrieved from target plasmid after showing interaction with Cdc42: CipA, MAV_1190, and MAV_4139. These three genes were also up-regulated in the strains of *M. avium* over-expressing both the MAV_3679 and MAV_5138 regulators.

The CipA protein sequence also does not contain any domains collected in the conserved domain database, but does have similarity to domains by BLAST, as described in the results section (Figure B.4). The PXXP piccolo domains in the first 100 amino acids of CipA, and the putative interaction with Cdc42, prompted us to analyze whether this protein is secreted or potentially expressed on the surface of *M. avium* in such a way that it is a part of a putative protein scaffold complex used to activate Cdc42 transmit signals to downstream proteins, such as N-WASp. Using a construct expressing a CipA:CyaA fusion protein, the presence of this protein could not be detected in the host cell cytoplasm by western blot, or increased levels of cAMP (Figure B.5). In addition, we looked for cleavage of the protein into two fragments using tags at the N- and C-terminus, using shorter tag sequences (Day *et al.*

2003), but could detect neither fragment in the cytoplasm by western blot (data not shown). It is possible the protein is present in the host cells, but at levels below an ability to detect it. Together, these results suggest, however, that the protein is not secreted into the cytoplasm of the host cells. A study by Cain and colleagues (Cain *et al.* 2004) showed that the secreted effectors of *Salmonella* are also not secreted into the host cell cytoplasm, but localize to the cell membrane instead, where they induce their effects on Cdc42 and downstream actin reorganization.

Because CipA was not detectable in the cytosol or the insoluble cell fractions, we hypothesized that this *M. avium* protein might be expressed on the surface of the bacterium upon invasion of epithelial cells, forming part of a structure which interacts with or inserts into the host cell membrane. Morphological changes in the recombinant strain of *M. avium* expressing the CipA:CyaA fusion protein lent support to the temporary localization of this protein to the bacterial membrane or cell wall (data not shown). Both CipA and MAP3985c (Cdc42 binding protein) are in small operons containing a hydrolase. MAP3985c was identified based on its interaction with an oxidoreductase shown to be involved in *M. avium* subsp. *paratuberculosis* invasion of epithelial cells (Alonso-Hearn *et al.* 2008). In this study, CipA was also shown to putatively interact with an oxidoreductase (Table B.3).

There are many hypothetical proteins in this region, corresponding to putative transmembrane proteins, a Rho-kinase, and proteins with bacterial SH3 domains. Also present in this region are both characterized and uncharacterized transcriptional regulators, including *regX3/senX3* and MAV_4676, a regulator that was shown by

microarray to be up-regulated upon exposure to HEp-2 cells, but not in the *fadD2* mutant strain, and the *hbhA* gene, encoding the surface heparin-binding hemagglutinin protein shown to adhere to epithelial cells (Reddy and Hayworth 2002, Sechi *et al.* 2006). This region is currently being further analyzed for its role in the invasion of epithelial cells.

M. avium does not have a TTSS, but may have an analogous mechanism for getting proteins into the host cell where they can interact with host cell signaling pathways. Because the CipA protein has domains suggesting its binding to host cell proteins, but could not be shown to be secreted, we expressed this protein in *M. smegmatis,* fused to GFP, and were capable fo observing a structure at the end of *M. smegmatis* near host cells.

Our results lead us to hypothesize that the region of the genome including *cip*A is important to the invasion of epithelial cells by *M. avium*. Our model suggests that in the presence of host-specific environmental cues, various regulators including *fadD2* lead to activation of additional transcriptional regulators, through yet unknown mechanisms. These regulators, including MAV_3679 and MAV_5138, directly or indirectly activate the expression of proteins from this region, and likely other regions, that make up the components of a mechanism for altering host cell signaling. Observed putative protein-protein interactions and other data from our lab and others suggest that oxidoreductases may play a role in the regulation of this mechanism as well, perhaps acting as chaperones. As the bacterium comes into contact with the host cells, these proteins are inserted into the host cell membrane, where they form a

complex with Cdc42 and other scaffolding proteins that are present leading to actin polymerization, and subsequent uptake of the bacterium. Future work will address the identification and characterization of the proteins involved in this mechanism of epithelial cell invasion.

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