ORIGINAL ARTICLE

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

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Pseudomonas aeruginosa coordinates the transcription of hundreds of genes, including many virulence genes, through three hierarchically arranged quorum-sensing (QS) systems, namely *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 ($\Delta lasR$, $\Delta rhlR$, $\Delta pqsR$) and signal-negative ($\Delta lasI$, $\Delta rhlI$, $\Delta pqsA$) mutants from each QS system. As 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 reported previously. However, the *pqsR* and *rhlR* mutants exhibited more complex behaviors, which 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 adopted here may account for the prevalence of naturally occurring QS mutants.

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Introduction

Cooperation can be defined as a behavior that benefits another individual and that is maintained because of its beneficial effects on the recipient (West *et al.*, 2006, 2007). In microbes, cooperation generally involves the production of public goods in the form of extracellular products (such as toxins, proteases, surfactants and siderophores), which 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 (Williams *et al.*, 2007; Ng and Bassler, 2009).

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*, namely *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. However, under certain conditions, the *rhl* and *pqs* systems can also be activated in the absence of *las* QS (Diggle *et al.*, 2003; Medina *et al.*, 2003; Dekimpe and Deziel, 2009). In addition, the *rhl* system negatively regulates the *pqs* system (McGrath *et al.*, 2004; Wade *et al.*, 2005). These three QS systems each comprise 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* systems,

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autoinducer synthases LasI and RhII produce the acyl-homoserine lactone (acyl-HSL) signals *N*-(3-oxododecanoyl)-HSL and *N*-butyryl-HSL (C4-HSL), respectively. These signals interact with their cognate receptors LasR and RhIR, 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 ~112 genes (≥ 2 -fold activation by both signals versus *N*-(3-oxododecanoyl)-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 (AQs) (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. Therefore, QS populations are 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 (Sandoz *et al.*, 2007; Diggle *et al.*, 2007b) 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 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 (Heurlier *et al.*, 2006; Smith *et al.*, 2006; D'Argenio *et al.*, 2007; Fothergill *et al.*, 2007; Tingpej *et al.*, 2007; Hoffman *et al.*, 2009; Kohler *et al.*, 2009), 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 this study, we investigated the social behavior of signal-blind (*rhlR*, *pqsR*) and signal-negative (*rhlI*, *pqsA*) mutants from the *rhl* and *pqs* systems and compared them with *lasR* and *lasI*

mutant strains. These experiments were performed under defined conditions, in a growth medium requiring and not requiring QS-regulated extracellular proteases, resembling 'social' and 'non-social' conditions, respectively. Although 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 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 the *rhlR* and *pqsR* mutants invade wild-type populations less than does the *lasR* mutant 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. Finally, 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 1. For general liquid culture, we used Lennox LB broth buffered with 50 mM MOPS (3-(*N*-morpholino)-propanesulfonic acid, 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 using true biological replicates on different days with different inocula. Further details are described in Supplementary Materials and Methods and in Supplementary Table S1.

In vitro evolution and analysis of QS mutant phenotypes

In vitro evolution of the *P. aeruginosa* PAO1 wild type in liquid batch cultures containing M9-caseinate medium was performed as described previously (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) per milliliter, as well as the types of mutants arising, aliquots were appropriately diluted in $1 \times$ M9 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 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),

Table 1 Bacterial strains and plasmids

Lab strain or plasmid	Relevant property	Reference or origin
Strains		
PAO1	<i>P. aeruginosa</i> wild-type (obtained from M Vasil and U Ochsner)	(Holloway <i>et al.</i> , 1979)
PAO1 <i>lasR</i>	PAO1 derivative; $\Delta lasR$, unmarked in-frame deletion from amino acid 102 to 216	This study
PAO1 <i>rhIR</i>	PAO1 derivative; $\Delta rhIR$, unmarked in-frame deletion from amino acid 102 to 225	This study
PAO1 <i>pqsR</i>	PAO1 derivative; $\Delta pqsR$, unmarked in-frame deletion from amino acid 19 to 252	This study
PAO1 <i>lasI</i>	PAO1 derivative; $\Delta lasI$, unmarked in-frame deletion from amino acid 12 to 176	This study
PAO1 <i>rhII</i>	PAO1 derivative; $\Delta rhII$, unmarked in-frame deletion from amino acid 13 to 185	This study
PAO1 <i>pqsA</i>	PAO1 derivative; $\Delta pqsA$, unmarked in-frame deletion from amino acid 32 to 419	This study
PAO1 $\Delta pqsA$ CTX- <i>lux::pqsA</i>	AQ biosensor strain; chromosomal deletion of the <i>pqsA</i> gene in PAO1 containing mini CTX- <i>lux::pqsA</i>	(Diggle <i>et al.</i> , 2007b)
Plasmids		
pJN105	<i>araC</i> -pBAD cloned in pBBR1 MCS-5; Gm ^R	(Newman and Fuqua, 1999)
pJN105. <i>pqsR</i> -H	<i>pqsR</i> coding region in pJN105	This study
pJN105. <i>pqsR</i> -N	<i>pqsR</i> coding region and upstream regulatory region in pJN105 devoid of <i>araC</i> -pBAD	This study
pJN105. <i>lasR</i> -H	<i>lasR</i> coding region in pJN105	(Lee <i>et al.</i> , 2006)
pJN105. <i>lasR</i> -N	<i>lasR</i> coding region and upstream regulatory region in pJN105 devoid of <i>araC</i> -pBAD	This study
pJN105. <i>rhIR</i> -H	<i>rhIR</i> coding region in pJN105	(Schuster and Greenberg, 2007)
pJN105. <i>rhIR</i> -N	<i>rhIR</i> coding region and upstream regulatory region in pJN105 devoid of <i>araC</i> -pBAD	This study
pEX18Tc	Tc ^R ; <i>oriT</i> , <i>sacB</i> , gene replacement vector with MCS from pUC18	(Hoang <i>et al.</i> , 1998)
pEX18Tc. $\Delta pqsR$	pEX18Tc with the <i>pqsR</i> gene containing an in-frame deletion from residue 19 to 252	This study
pEX18Tc. $\Delta lasR$	pEX18Tc with the <i>lasR</i> gene containing an in-frame deletion from residue 102 to 216	This study
pEX18Tc. $\Delta rhIR$	pEX18Tc with the <i>rhIR</i> gene containing an in-frame deletion from residue 102 to 225	This study
pEX18Tc. $\Delta pqsA$	pEX18Tc with the <i>pqsA</i> gene containing an in-frame deletion from residue 32 to 419	This study
pEX18Tc. $\Delta lasI$	pEX18Tc with the <i>lasI</i> gene containing an in-frame deletion from residue 12 to 176	This study
pEX18Tc. $\Delta rhII$	pEX18Tc with the <i>rhII</i> 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

Abbreviations: AQ, alkyl quinolone; MCS, multiple cloning site; *P. aeruginosa*, *Pseudomonas aeruginosa*.

using a 96-well microplate format. The AQ bioassay detects both PQS and its precursor 2-heptyl-4-quinolone. AQ levels were normalized to the optical density at 600 nm (OD₆₀₀) 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, whereas 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 scored as positive and the absence of a halo was scored as negative. These assays were performed in the same manner with all control strains shown in Table 2.

Table 2 Analysis of *P. aeruginosa* QS mutant phenotypes

Strain	Skim milk ^a	Rhamnolipid ^a	Adenosine ^b	AQ ^c
Wild type	+	+	+	100 ± 12
<i>lasR</i>	—	+	—	43 ± 21
<i>lasI</i>	—	+	—	24 ± 11
<i>rhIR</i>	+	—	+	230 ± 21
<i>rhII</i>	+	+	+	310 ± 60
<i>pqsR</i>	+	+	+	6.7 ± 2.0
<i>pqsA</i>	+	+	+	0.99 ± 1.8

Abbreviations: AQ, alkyl quinolone; *P. aeruginosa*, *Pseudomonas aeruginosa*; QS, quorum sensing.

^a‘+’ indicates the presence and ‘—’ indicates the absence of a halo on indicator plates.

^b‘+’ indicates growth and ‘—’ indicates no growth.

^cThe percentage of the wild type is shown.

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 OD₆₀₀ 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⁻¹ of each culture was determined at 0, 2, 5, 8, 12, 18, 24 and 30 h after inoculation. For co-culturing experiments, M9-CAA and M9-caseinate cultures were harvested after 12 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 Supplementary Materials and Methods.

The relative fitness (v) of each mutant in co-culture was first calculated by comparing its initial and final frequencies over the duration of the experiment (Ross-Gillespie *et al.*, 2007a; Diggle *et al.*, 2007b). In particular, $v = (x_1(1-x_0))/(x_0(1-x_1))$, where x_0 and x_1 are the initial and final mutant frequencies, respectively. Therefore, values of v signify 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 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⁻¹, and Y_0 and Y_1 the initial and final wild-type CFU ml⁻¹, respectively.

Complementation analysis, DNA sequencing and fluorescein isothiocyanate (FITC) casein assay

These procedures are described in Supplementary 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 (Sandoz *et al.*, 2007; Diggle *et al.*, 2007b; Rumbaugh *et al.*, 2009). The main objective of this study was to further analyze the social behavior of signal-blind and signal-negative mutants from the other two major QS systems in *P. aeruginosa*, namely *rhl* and *pqs*. We previously showed that *lasR* mutants emerge during *in vitro* evolution in a 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

Figure 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 circles), rhamnolipid production (solid triangles) and AQ production (solid diamonds) are shown. CFU ml⁻¹ (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.

growth experiment and screened for mutants in all three QS systems. We grew the PAO1 wild type in M9-caseinate medium for 20 24-h cycles, subculturing 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 (Van Delden *et al.*, 1998; Sandoz *et al.*, 2007). Under standard growth conditions (LB liquid culture), *las*, *rhl* and *pqs* mutants all show reduced expression of LasB elastase (Brint and Ohman, 1995; Pearson *et al.*, 1997; Diggle *et al.*, 2003; Schuster *et al.*, 2003; Deziel *et al.*, 2005).

We used a screening scheme for distinct QS phenotypes that allowed us to distinguish between the different *las*-, *rhl*- and *pqs*-deficient variants (Table 2). After 8 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 1, Supplementary Table S2). Our previous work had shown that all isolates that did not grow on adenosine were *lasR* mutants (Sandoz *et al.*, 2007). Therefore, we 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). Indeed,

sequencing analysis revealed that seven isolates harbor an insertion, deletion or single-nucleotide change within the *pqsR* gene (Table 3).

Figure 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 gray) or pJN105.*pqsR*-N controlling *pqsR* from the native *P. aeruginosa* promoter (light gray). 'PQS' and 'HHQ' are synthetic signal controls. Data are given as percentage of PAO1/pJN105. Error bars indicate s.d. of the mean of three replicates.

Table 3 *pqsR* sequence analysis of selected AQ-deficient *in vitro* evolution isolates of *P. aeruginosa*

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

Abbreviations: AQ, alkyl quinolone; *P. aeruginosa*, *Pseudomonas aeruginosa*.

^aNucleotide 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 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 4). Sequencing analysis of the *rhlR* gene from both rhamnolipid-deficient isolates further confirmed that these isolates are not *rhlR* mutants (Table 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).

The pqsR mutant, but not the lasR and rhlR mutants, grows well in QS medium because of 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 of approximately 30–40-fold less than that of the PAO1 wild type after 24 h, indicating a severe growth deficiency (Figure 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 did the wild-type strain over the course of 24 h, indicating that the *pqsR* mutant has high intrinsic fitness in QS medium (Figure 3). All strains grew similarly in M9-CAA, reaching maximum density after 12 h.

Table 4 Analysis of rhamnolipid-deficient *in vitro* evolution isolates of *P. aeruginosa*

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

Abbreviations: N/A, not applicable; *P. aeruginosa*, *Pseudomonas aeruginosa*.

^a‘+’ indicates the presence and ‘—’ indicates the absence of a halo on indicator plates.

^bThe percentage of the wild type is shown.

^cLab strains were not sequenced.

Figure 3 Single-culture growth of signal-blind *P. aeruginosa* strains. Growth of the wild-type (black), *lasR* (dark gray), *rhIR* (light gray) and *pqsR* (open) strains in M9-CAA and in M9-caseinate media (circle and square symbols, respectively). Error bars indicate s.d. of the mean of three replicates, and are too small to be visible in some cases.

Figure 4 Caseinolytic activity of signal-blind *P. aeruginosa* strains. Degradation of FITC-casein by the indicated strains grown in either M9-CAA (light gray bars) or M9-caseinate (dark gray bars). Data are given as the percentage of the wild type grown in M9-caseinate. Error bars indicate s.d. of the mean of three replicates. Statistical significance of the data was determined using a two-tailed unpaired *t*-test with ‘*’ indicating *P*-values < 0.05. Protease production of each mutant was compared with that of the wild type in the respective media.

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 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 than did the wild type, consistent with previous findings (Brint and Ohman, 1995; Pearson et al., 1997; Diggle et al., 2003; Schuster et al., 2003; Deziel et al., 2005). After 24 h of growth in M9-caseinate, the *pqsR* mutant showed caseinolytic activity that was indistinguishable from the wild

type, whereas the *lasR* and *rhIR* mutants showed a much lower activity. Thus, only the *las* and *rhI* systems but not the *pqs* system is required for the production of proteases to digest caseinate in QS medium.

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 *rhIR* mutant, which showed high caseinolytic activity on plates (Table 2) but low activity in liquid culture (Figure 4). This difference may be due to upregulation of *lasR* or *lasI* on solid media, permitting enhanced exoprotease expression that would otherwise require both *las* and *rhI* QS.

In QS co-culture, both lasR and pqsR mutants have a growth advantage over the wild-type cooperator, but rhIR 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 ~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 5a and b), but the *rhIR* mutant did not (Figure 5c). To confirm that the *rhIR* mutant does not exhibit a subtle growth advantage that cannot be captured after a single 24-h incubation period, we co-cultured an unmarked *rhIR* mutant with the wild type for 3 consecutive 24 h cycles. This *rhIR* mutant also did not enrich (data not shown). The three signal-negative mutants, namely *lasI*, *pqsA* and *rhII*, showed little to no enrichment in M9-caseinate (Figures 5a–c). The same held true for all signal-negative and 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 5d). Interestingly, both the *lasR* and *pqsR* mutants enriched, whereas the *rhIR* mutant did not. Overall, these results show that *lasR* and *pqsR* mutants can invade PAO1 wild type populations 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

Figure 5 Enrichment of defined QS mutant strains in co-culture. Cultures were grown in M9-CAA (light gray bars) and M9-caseinate (dark gray bars) media with initial mutant frequencies of ~1%. Values above each bar indicate fold enrichment (the ratio of final frequency versus initial frequency). (a) The *lasR* or *lasI* mutants in wild-type co-culture. (b) The *pqsR* or *pqsA* mutants in wild-type co-culture. (c) The *rhIR* or *rhII* mutants in wild-type co-culture. (d) The *lasR*, *pqsR* and *rhIR* mutants combined (each at an initial frequency of 1%) in wild-type co-culture. (e) The *lasR* or *rhIR* mutants (each at an initial frequency of 1%) in *pqsR* mutant co-culture. Error bars indicate s.d. of the mean of three replicates. Statistical significance of the data was determined using a two-tailed unpaired *t*-test, with ‘***’ indicating *P*-values <0.05 and ‘**’ indicating *P*-values <0.1. For each individual condition, the initial frequency was compared with 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.

M9-caseinate, the *lasR* mutant enriched almost two-fold (Figure 5e). For comparison, the *rhIR* mutant showed no enrichment (Figure 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 co-cultured each mutant and its PAO1 parent in M9-caseinate for 24 h at starting mutant frequencies of ~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 observed previously, the *lasR* mutant exhibited negative frequency dependence (Diggle *et al.*, 2007b) (Figure 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 6b). This confirms that the

relative dominance of the *pqsR* mutant is entirely dependent on social interactions with the wild type. In contrast, the *rhIR* mutant seemed to exhibit positive frequency dependence (Figure 6c), indicating that its relative fitness increases with increasing abundance in a population (Molofsky *et al.*, 2001). This result was surprising in light of the observation that the *rhIR* mutant displays low intrinsic fitness similar to the *lasR* mutant (Figure 3). Therefore, we further analyzed the fitness of the *rhIR* mutant in wild-type co-culture at even higher mutant frequencies of 90% and 99% (Figure 6d) and observed a decline to levels similar to those at low frequencies. Taken together, the relative fitness of the *rhIR* 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 low viscosity growth conditions that do and do not require QS. In our previous long-term evolution study, we focused on the identification and characterization of the *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) allowed us to detect variants

Figure 6 Frequency-dependent relative fitness of signal-blind strains. Relative fitness of (a) the *lasR* mutant, (b) the *pqsR* mutant and (c and d) the *rhIR* mutant calculated as the comparison of initial and final mutant frequencies (v , left panels) or as the ratio of mutant and wild-type Malthusian growth parameters (w , right panels). Data in the left panels a–c are on a double logarithmic scale and are fitted with either a power regression line (panels a and c) or an exponential regression line (panel b). Data in the right panels a–c are on a semi-logarithmic scale and are fitted with either a logarithmic regression line (panels a and c) or an exponential regression line (panel b). Panel d includes initial *rhIR* 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 performed in quadruplicate.

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 1 and 2, Tables 3,4). Of the nine complemented AQ-deficient variants, seven showed a mutation in *pqsR* (Table 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). 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) 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 because of 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 the *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 5d). Indicative of social cheating and consistent with previous studies (Sandoz et al., 2007; Diggle et al., 2007b; Rumbaugh et al., 2009), our defined *lasR* mutant demonstrated substantial enrichment in wild-type co-culture (Figure 5a), had negative frequency dependence (Figure 6a) and displayed a growth deficiency when grown individually (Figure 3). 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 (for example, 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 and other signal-negative strains showed virtually no enrichment in co-culture (Figures 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 with the cost of synthesizing hundreds of proteins. However, in previous studies by Diggle et al. (2007b) and Rumbaugh et al. (2009), *lasI* mutants displayed

significantly higher relative fitness than did the wild type, similar to that observed for *lasR* mutants. *In vivo* competition experiments by Rumbaugh et al. (2009) were performed using an animal model mimicking either a chronic or an acute burn wound. Diggle et al. (2007b) used minimal medium supplemented with 1% bovine serum albumin as the sole carbon source and a starting inoculum that was ~20-fold higher than ours. 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 under alkaline conditions during prolonged stationary phase (Heurlier et al., 2005). However, these benefits 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. Although our work with the *lasR* mutant was consistent with previous studies and our hypothesis (Sandoz et al., 2007; Diggle et al., 2007b; Rumbaugh et al., 2009), the social behaviors of the *pqsR* and *rhlR* mutants were more complex. The *pqsR* mutant enriched substantially in co-culture (Figure 5b) and showed overall negative frequency dependence (Figure 6b), a pattern characteristic of social cheating. Individually, however, the *pqsR* mutant grew as well as did the wild-type parent (Figure 3). The production of wild-type levels of protease accounts for its high intrinsic fitness (Figure 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 5b). Therefore, we 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 with 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 1 and 5e). To our knowledge, a systematic analysis on the prevalence of natural *pqs* mutants has not been published.

Similar to the *pqsR* mutant, the behavior of the *rhlR* mutant did not conform to our prediction of social cheating. Although it displayed a similar individual growth pattern as did the *lasR* mutant in QS medium, the *rhlR* mutant was unable to invade a wild-type population (Figures 3 and 5c). The non-invasive nature of the *rhlR* mutant could be due to a large cost associated with the upregulation of *pqs*-dependent factors (those not also co-regulated by the *rhl* system) that outweighs the benefits from not producing *rhl*-dependent factors (Table 2, Figure 4) (McGrath *et al.*, 2004). In particular, synthesis of PQS itself and transcription of *pqsA* have been shown to be upregulated sixfold and sevenfold, respectively, in *rhl* mutant strains (McGrath *et al.*, 2004). In addition, 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 6d). Such a pattern could result from a frequency-dependent effect of the wild type on the upregulation 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 (West and Buckling, 2003; Inglis *et al.*, 2009). If the behavior of the *rhlR* mutant was indeed spiteful, it could be in relation to the upregulation of PQS (McGrath *et al.*, 2004). In addition to its beneficial effects at low concentration, PQS can be deleterious and can promote autolysis when overproduced (D'Argenio *et al.*, 2002; Haussler and Becker, 2008). If the *rhlR* mutant was to gain an advantage through this process, the wild type would have to be more sensitive to PQS-mediated killing than the *rhlR* mutant. However, regardless of the mechanism, 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 have a role in modulating the behaviors of the *pqsR* and *rhlR* mutant strains.

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