The ISME Journal (2013) 7, 2159–2168

www.nature.com/ismej

ORIGINAL ARTICLE

Negative regulation of bacterial quorum sensing tunes public goods cooperation

Rashmi Gupta[1,2] and Martin Schuster[1]
[1]*Department of Microbiology, Oregon State University, Corvallis, OR, USA*

Bacterial quorum sensing (QS) often coordinates the expression of other, generally more costly public goods involved in virulence and nutrient acquisition. In many Proteobacteria, the basic QS circuitry consists of a synthase that produces a diffusible acyl-homoserine lactone and a cognate receptor that activates public goods expression. In some species, the circuitry also contains negative regulators that have the potential to modulate the timing and magnitude of activation. In this study, we experimentally investigated the contribution of this regulatory function to the evolutionary stability of public goods cooperation in the opportunistic pathogen *Pseudomonas aeruginosa*. We compared fitness and public goods expression rates of strains lacking either *qteE* or *qscR*, each encoding a distinct negative regulator, with those of the wild-type parent and a signal-blind receptor mutant under defined growth conditions. We found that (1) *qteE* and *qscR* mutations behave virtually identically and have a stronger effect on the magnitude than on the timing of expression, (2) high expression in *qteE* and *qscR* mutants imposes a metabolic burden under nutrient conditions that advance induction and (3) high expression in *qteE* and *qscR* mutants increases population growth when QS is required, but also permits invasion by both wild-type and receptor mutant strains. Our data indicate that negative regulation of QS balances the costs and benefits of public goods by attenuating expression after transition to the induced state. As the cells cannot accurately assess the amount of cooperation needed, such bet-hedging would be advantageous in changing parasitic and nonparasitic environments.
The ISME Journal (2013) **7,** 2159–2168; doi:10.1038/ismej.2013.109; published online 4 July 2013
Subject Category: Microbe-microbe and microbe-host interactions
Keywords: bet-hedging; cooperation; negative regulation; *Pseudomonas aeruginosa*; public goods; quorum sensing

Introduction

Bacterial quorum sensing (QS) regulates multiple cooperative behaviors like production of extracellular proteases, siderophores, exopolysaccharides, antibiotics and biosurfactants (Miller and Bassler, 2001; Fuqua and Greenberg, 2002; Williams *et al.,* 2007). The opportunistic pathogen *Pseudomonas aeruginosa* provides a particularly well-understood example of QS-regulated virulence gene expression (Juhas *et al.,* 2005; Schuster and Greenberg, 2006). *P. aeruginosa* infects immunocompromised individuals such as those suffering from cystic fibrosis and burns. It possesses two complete QS systems, *las* and *rhl*, that each produce and respond to a distinct diffusible acyl-homoserine lactone (acyl-HSL) signal molecule. The *las* system consists of the signal synthase LasI that produces 3-oxo-dodecanoyl-HSL (3OC12-HSL), and the cognate transcriptional regulator LasR. The *rhl* system consists of the signal synthase RhlI that produces butanoyl-HSL (C4-HSL), and the cognate transcriptional regulator RhlR. LasR and RhlR, in complex with their respective acyl-HSL signals, control the expression of over 300 genes (Hentzer *et al.,* 2003; Schuster *et al.,* 2003; Wagner *et al.,* 2003). The *las* system controls activation of the *rhl* system (Latifi *et al.,* 1996; Pesci *et al.,* 1997), but this regulatory hierarchy is nutritionally conditional (Medina *et al.,* 2003; Dekimpe and Deziel, 2009).

The production of QS-controlled 'public goods' is subject to exploitation by social cheaters that avoid the cost of cooperation but reap the benefits (Keller and Surette, 2006; West *et al.,* 2007a). This has been demonstrated both *in vitro* (Diggle *et al.,* 2007; Sandoz *et al.,* 2007) and *in vivo* (Kohler *et al.,* 2009; Rumbaugh *et al.,* 2009). Thus, explaining the stability of cooperative behaviors has been a major challenge to evolutionary biology. A common explanation is kin selection, according to which cooperative behaviors are favored if they increase the reproductive potential of related individuals

Correspondence: M Schuster, Department of Microbiology, 220 Nash Hall, Oregon State University, Corvallis, OR 97331, USA.
E-mail: martin.schuster@oregonstate.edu
[2]Current address: Department of Biomedical Sciences, College of Veterinary Medicine, 105 Magruder Hall, Oregon State University, Corvallis, OR 97331-4801, USA.
Received 30 March 2013; accepted 17 May 2013; published online 4 July 2013

(Hamilton, 1964). In microbes, kin selection is thought to be achieved primarily through limited dispersal, which keeps related individuals together (West *et al.,* 2007a). Pleiotropic constraints, repetitive population fragmentation and prudent metabolic regulation of public goods may also contribute to the stability of cooperation (Cremer *et al.,* 2012; Foster *et al.,* 2004; Kummerli and Brown, 2010; West *et al.,* 2007b; Xavier and Foster, 2007).

It is generally assumed that QS further optimizes cooperative behaviors by restricting the expression of public goods to when they are beneficial, such as at high population density or limited diffusion. A recent study experimentally confirmed that QS may indeed provide a density-dependent fitness benefit (Darch *et al.,* 2012). Other modeling studies and experimental work with a synthetic QS pathway generally indicated that QS-controlled public goods production is a trade off between the cost of constitutive or very early exoenzyme activation and the loss of attainable benefit from no or very late activation (Czaran and Hoekstra, 2009; Pai and You, 2009; Pai *et al.,* 2012).

In many bacteria, the basic QS circuitry is subject to regulation by other pathways that have the potential to affect the quorum threshold (Mellbye and Schuster, 2011). In *P. aeruginosa*, negative regulation by two proteins, QscR and QteE, is intimately linked to the basic QS circuitry. QscR is an orphan LuxR-type protein (Chugani *et al.,* 2001) that responds to the LasI-generated signal 3OC12-HSL and other long-chain acyl-HSLs (Lee *et al.,* 2006). It primarily represses the expression of *las* and *rhl*-dependent genes, including *lasI* and *rhlI,* possibly by forming inactive heterodimers with LasR and RhlR (Chugani *et al.,* 2001; Ledgham *et al.,* 2003; Lequette *et al.,* 2006). QscR also functions as a transcriptional activator of at least two genes (Lee *et al.,* 2006).

QteE is a unique regulator without close homologs in other species (Siehnel *et al.,* 2010; Liang *et al.,* 2011). Its transcription appears to be activated by LasR (Gilbert *et al.,* 2009), and it is thought to function analogously to the QS antiactivator TraM in *Agrobacterium tumefaciens* (Fuqua *et al.,* 1995; Piper and Farrand, 2000). QteE represses the expression of several *las* and *rhl*-dependent target genes by independently reducing LasR and RhlR protein stability, probably through direct protein–protein interaction (Siehnel *et al.,* 2010). Both QscR and QteE therefore have the capacity to modulate the quorum threshold and maximum gene expression levels through analogous mechanisms, namely their interaction with LasR and RhlR. Some of the outstanding questions concern the evolutionary purpose of these regulators: Why is it that in some bacterial species the basic QS circuitry of diffusible signal and cognate receptor is not sufficient to control target gene expression, and how does the presumed mechanism help optimize QS-controlled gene expression?

Here, we investigate the functional significance of the precise timing and level of QS-controlled public goods production under defined growth conditions, including those that favor QS. We compared QS gene expression kinetics, associated metabolic burden and fitness consequences of high, normal and no cooperation by *qscR/qteE*, wild-type and *lasR* strains, respectively, in individual and mixed culture.

Materials and methods

Bacterial strains and plasmids

Bacterial strains used in this study are listed in Table 1. Mutant strains of *P. aeruginosa* PAO1 were obtained from a nonredundant transposon library at the University of Washington (Jacobs *et al.,* 2003). The insertions are in individual genes rather than in operons eliminating the possibility of polar effects, they contain a suitable antibiotic resistance marker to distinguish strains in coculture, eliminating the need for an additional genetic manipulation, and they are out-of-frame such that translational *lacZ* fusions are not generated. A defined *qteE* deletion mutant was obtained from Richard Siehnel, University of Washington (Siehnel *et al.,* 2010).

Luria broth (LB) medium (Lennox) buffered with 3-(N-morpholino)propanesulfonic acid, pH 7.0, was used for routine culturing unless specified. Antibiotics tetracycline ($50\,\mu g\,ml^{-1}$) and carbenicillin ($200\,\mu g\,ml^{-1}$) for *P. aeruginosa,* and ampicillin ($100\,\mu g\,ml^{-1}$) for *Escherichia coli* were used where appropriate.

Strain construction

To distinguish strains in some of the cocultures, the wild-type and the *lasR* mutant were tagged with a carbenicillin resistance gene using site-specific integration (Becher and Schweizer, 2000). A 1200-bp ampicillin resistance gene with its promoter was PCR-amplified from pUCP18 (Schweizer, 1991) using primers pRG12-F (5′-N_6AAGCTTTTCTTAGACGTC AGGTGGCAC-3′) and pRG12-R (5′-N_6CTCCAGTGTT TGCAAGCAGCAGATTACG-3′) and digested with *Hin*dIII and *Xho*I (restriction sites underlined). This fragment was ligated into the equally digested miniCTX3a vector (Hoang *et al.,* 2000). The resulting construct, pRG12, was confirmed by sequencing. Plasmid pRG12 was introduced into the wild-type and the *lasR* mutant by electroporation and selected for integration into the *P. aeruginosa* genome as described (Hoang *et al.,* 2000).

To construct the *lasB'-gfp* transcriptional fusion, a 240-bp *lasB* promoter region was PCR-amplified from the PAO1 genome using the primers pRG13-F (5′-N_6AAGCTTGGCCTACAAGCTCGACG TCA-3′) and pRG13-R (5′-N_6GAACTTGTTTTCGAC GGTGCTTTCGT-3′). The PCR product was digested with *Hin*dIII and *Eco*RI (restriction sites underlined) and ligated with an equally digested promoter probe vector, pProbeAT (Miller *et al.,* 2000). The resulting construct pRG13 was confirmed by sequencing.

Table 1 Bacterial strains and plasmids

Strain or plasmid	*Relevant property*	*Reference*
Strains		
P. aeruginosa		
PAO1	Wild-type, PAO1 UW library strain	(Jacobs *et al.*, 2003)
PW3598	*lasR-C01*::IS*lacZ*/hah, UW strain, Tc^R; lack of signal reception results in reduced target gene expression	(Jacobs *et al.,* 2003)
PW4325	*qscR-B10*::IS*lacZ*/hah, UW strain, Tc^R; lack of QS repression results in increased target gene expression	(Jacobs *et al.*, 2003)
PW5355	*qteE-C10*::IS*lacZ*/hah, UW strain, Tc^R; lack of QS repression results in increased target gene expression	(Jacobs *et al.,* 2003)
PAO1 Δ*qteE*	Markerless *qteE* deletion mutant, in PAO1 UW background	(Siehnel *et al.*, 2010)
PAO1 Cb	PAO1 tagged with a carbenicillin resistance gene, Cb^R	This study
PAO1 *lasR*-Cb	PW3598 tagged with a carbenicillin resistance gene, Cb^R	This study
E. coli		
DH5α	F^- φ80d*lacZ*ΔM15 Δ (*lacZYA-argF*) U169 *deoR recA1 endA1 hsdR17*($r_K^- m_K^+$) *phoA supE44* λ^- *thi-1 gyrA96 relA1*	Invitrogen
SM10	Mobilizing strain, RP4 *tra* genes integrated in the chromosome, *thi-1 thr leu tonA lacY supE recA*::RP4-2-Tc::Mu Km^R	(Simon *et al.,* 1983)
Plasmids		
pminiCTX3a	Suicide vector permitting site-specific integration at *attB* in *P. aeruginosa*, Tc^R	(Hoang *et al.,* 2000)
pProbeAT	Broad-host-range vector with a promoterless *gfp*, Cb^R	(Miller *et al.,* 2000)
pRG12	1200 bp ampicillin/carbenicillin resistance gene cloned with its native promoter in pminiCTX3a	This study
pRG13	240 bp *lasB* promoter cloned into pProbeAT	This study

Growth assays

Cultures of wild-type, *qscR*, *qteE* and *lasR* strains were grown in M9 minimal medium with 1% (w/v) sodium caseinate, 0.5% (w/v) casamino acids (CAA) or 0.5% (w/v) monosodium glutamate as the sole carbon (C-) sources (Sandoz *et al.*, 2007; Wilder *et al.*, 2011). Caseinate medium represents a condition that requires cooperation, whereas CAA and glutamate media represent conditions that do not require cooperation. Experimental cultures were started from overnight (18 h) LB cultures that had been inoculated with a freshly grown single colony of the respective strain. The starting optical density (OD_{600}) of the experimental cultures was 0.05 (based on a 1-cm pathlength), corresponding to $\sim 10^8$ colony forming units (CFU) per ml. Cells from the overnight cultures used for inoculation were not washed, as washing resulted in greatly delayed growth in caseinate medium and in poor reproducibility (Sandoz *et al.,* 2007; Wilder *et al.,* 2011). All cultures were grown at 37 °C with shaking. The growth of individual strains in caseinate medium was assessed in glass culture tubes containing 4 ml of medium. At 0, 12, 24, 36 and 48 h, an aliquot was removed and appropriate dilutions were plated onto LB plates to determine CFU per ml. This scheme is different from our previous studies (Sandoz *et al.,* 2007; Wilder *et al.,* 2011) and was chosen because the PAO1 strain (University of Washington) used here grows more slowly on caseinate medium than the PAO1 strain (Mike Vasil, University of Colorado) used previously.

To measure the growth of individual strains in CAA and glutamate media, 200-μl cultures were grown in transparent 96-well plates (Greiner bio-one, Monroe, NC, USA; Cat. No. 655185) in a multifunction plate reader (Tecan Infinite M200, San Jose, CA, USA). OD_{600} was measured every 15 min for up to 12 h. The reported OD_{600} values are directly from these microtiter plate cultures and were not converted to a 1-cm pathlength. Growth rates were quantified during exponential phase between 120 and 210 min in CAA medium and between 240 and 360 min in glutamate medium. To confirm that the presence of an antibiotic tag did not affect growth, cultures of the tagged wild-type and *lasR* mutant strains were compared with their respective untagged parents in a similar manner.

To verify that the presence of a transposon in the negative regulator mutants did not affect their phenotype, we compared individual growth and gene expression of the *qteE* transposon mutant with that of the defined *qteE* deletion mutant (Supplementary Figure). To confirm that the enhanced growth of the negative regulator mutants in caseinate medium was not caused by higher amounts of exoproducts present in the inoculum source (LB overnight cultures), we performed the following experiment. LB overnight cultures of the *qteE* mutant and the wild-type were centrifuged to separate the supernatant from the cells. The cells were washed and resuspended in either their own or in the other strain's filter-sterilized supernatant. Specifically, *qteE* mutant cells were resuspended in *qteE* mutant or wild-type supernatant, and wild-type cells were resuspended in *qteE* mutant or wild-type supernatant. Individual caseinate cultures were inoculated with these cell suspensions and growth was assessed as described above.

Cocultures were initiated from LB overnight cultures such that the frequency of the rare strain was 1% based on OD_{600}. The rare strain carried an antibiotic resistance marker. At the indicated times, culture aliquots were removed, appropriately

diluted and plated to determine CFU per ml. Strains were distinguished by plating onto LB plates with and without the appropriate antibiotic. Percent enrichment was calculated by dividing the final ratio by the initial ratio.

Relative fitness was also calculated as the ratio of Malthusian growth parameters (w), which is essentially the ratio of the number of doublings by two competitor populations (Lenski *et al.,* 1991). Here, $w = \ln(X_1/X_0)/\ln(Y_1/Y_0)$, where X_0 and X_1 are the initial and final CFU per ml of the *lasR* mutant, and Y_0 and Y_1 are the initial and final CFU per ml of the wild-type or *qteE*/*qscR* mutants, respectively.

Statistical significance was determined by either a two-sample *t*-test (two-tailed, equal variance) for pairwise comparison of experimental data or by a one-sample *t*-test (two-tailed) for comparison of experimental data to a fixed value (that is, no enrichment in coculture).

Measurement of lasB'-gfp expression

lasB promoter activity was quantified by fluorescence spectroscopy in the wild-type, *lasR*, *qscR* and *qteE* strains containing plasmid pRG13. Experimental cultures were started from overnight LB cultures that had been inoculated with a freshly grown single colony. The cultures were washed once in M9 salts and inoculated into fresh LB at an OD_{600} of 0.002 (based on a 1-cm pathlength). The cultures were grown to an OD_{600} of 0.2, washed again and finally diluted to an OD_{600} of 0.01 in CAA and glutamate medium in 96-well plates (Greiner bio-one, Cat. No. 655090). This washing and culturing scheme was done as previously reported to reduce preinduction of *lasB'-gfp* in the *qteE* and *qscR* strains (Siehnel *et al.,* 2010). Absorbance at 600 nm and GFP fluorescence at excitation and emission wavelengths of 480 and 535 nm, respectively, were measured using a multifunction plate reader (Tecan Infinite M200). The GFP protein encoded by pProbeAT is GFPmut1, a fast-folding and very stable variant (Cormack *et al.,* 1996). Thus, fluorescence measurements at any given time represent cumulative expression levels. Background fluorescence of a strain without the reporter fusion, normalized to its OD, was subtracted from the fluorescence signal of the strain carrying the reporter fusion, also normalized to its OD, at the same time point. To determine the rate of *lasB* expression, we differentiated the fluorescent signal over time as described (Zaslaver *et al.,* 2006). The change in GFP/OD within a 15-min timespan, corresponding to two successive measurements, was calculated (dGFP/dt/OD). The data were smoothed by averaging three consecutive measurements.

Results

Effect of QS dysregulation on public goods expression and growth

We first assessed the fitness characteristics of wild-type, *lasR, qscR* and *qteE* strains by comparing their culture growth in a minimal medium with either CAA or glutamate as the sole C-source. Both C-sources do not favor QS; they can be taken up and metabolized without prior processing by QS-dependent extracellular enzymes. This allowed us to investigate QS target gene expression and associated metabolic burden independent from a potential growth benefit afforded by QS.

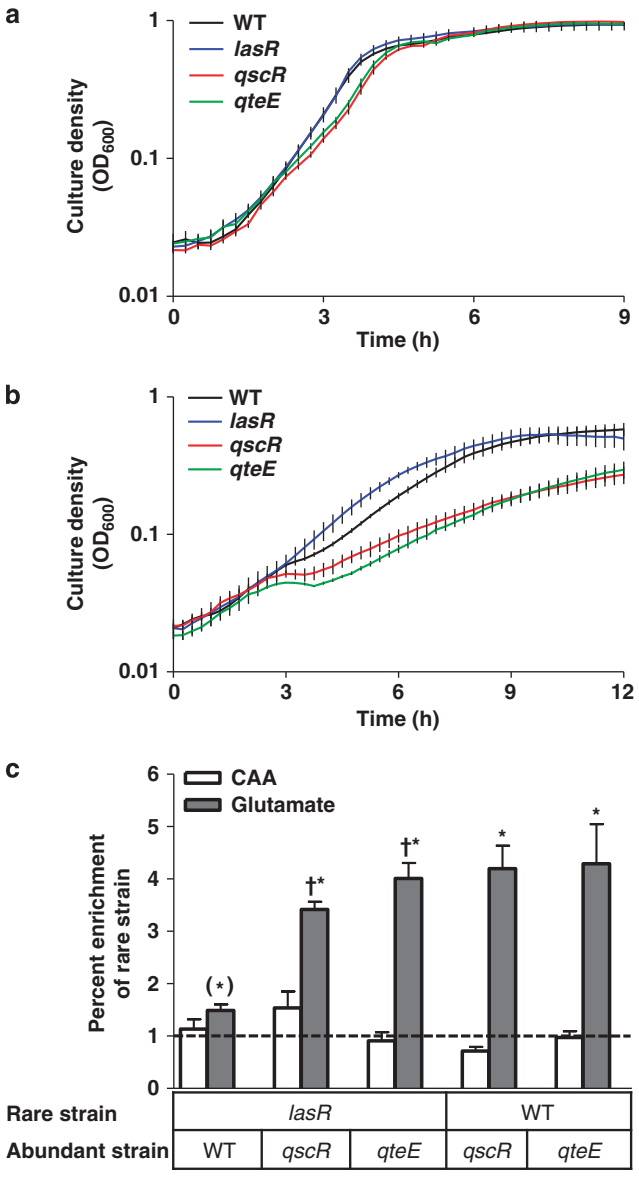

Figure 1 *P. aeruginosa* fitness under conditions that do not favor QS. Wild-type, *lasR, qscR* and *qteE* strains were grown in CAA and glutamate medium. (**a**) Culture density (OD_{600}) of individual strains grown in CAA medium. (**b**) Culture density (OD_{600}) of individual strains grown in glutamate medium. (**c**) Coculturing of different strain combinations in CAA and glutamate medium. Two-strain cultures were initiated with 1% initial frequency of the rare strain as indicated and grown for 12 h. The error bars indicate s.d. of the mean of three independent biological replicates. The asterisk indicates statistically significant difference from the initial inoculum (dashed horizontal line; $P<0.05$). The asterisk in parentheses indicates borderline significance ($P=0.052$). For the *lasR* cocultures, the dagger indicates statistically significant difference from the wild-type ($P< 0.05$). s.d., standard deviation.

P. aeruginosa grows faster in CAA than in glutamate medium, as growth in the latter necessitates *de novo* synthesis of amino acids. Amino-acid starvation and concomitant reduction in growth rate prematurely activate QS gene expression via the stringent response (van Delden *et al.,* 2001). Thus, growth with the two different C-sources permitted modulation of the quorum threshold.

Table 2 Growth rates in CAA and glutamate media

Strain	*Doubling times (t_d) with the respective C-source*			
	CAA		*Glutamate*	
	t_d *(min)*	P *(vs wild-type)*	t_d *(min)*	P *(vs wild-type)*
WT	34.7 ± 2.4	NA	91.2 ± 1.2	NA
lasR	33.7 ± 7.3	0.83	71.0 ± 6.3	0.0055
qscR	41.7 ± 5.9	0.22	149 ± 4	0.000033
qteE	39.5 ± 6.4	0.26	152 ± 4	0.000025

Abbreviations: WT, wild-type; NA, not applicable.

In individual culture, the growth rates of the *qscR* and *qteE* mutants were very similar to those of the wild-type and the *lasR* mutant in CAA medium, but substantially slower in glutamate medium (Figures 1a and b and Table 2). Relative fitness of strains in coculture reflected their growth in individual culture (Figure 1c). The *lasR* mutant and the wild-type strain, at 1% initial frequency, invaded *qteE* and *qscR* mutant populations in glutamate but not in CAA medium.

The observed growth characteristics of each of the strains correlated with individual differences in the timing and magnitude of QS-controlled public goods expression. We chose LasB elastase, a *las* and *rhl*-responsive extracellular virulence factor (Pearson *et al.,* 1997) and major casein protease in *P. aeruginosa* (Galloway, 1991), as a relevant public

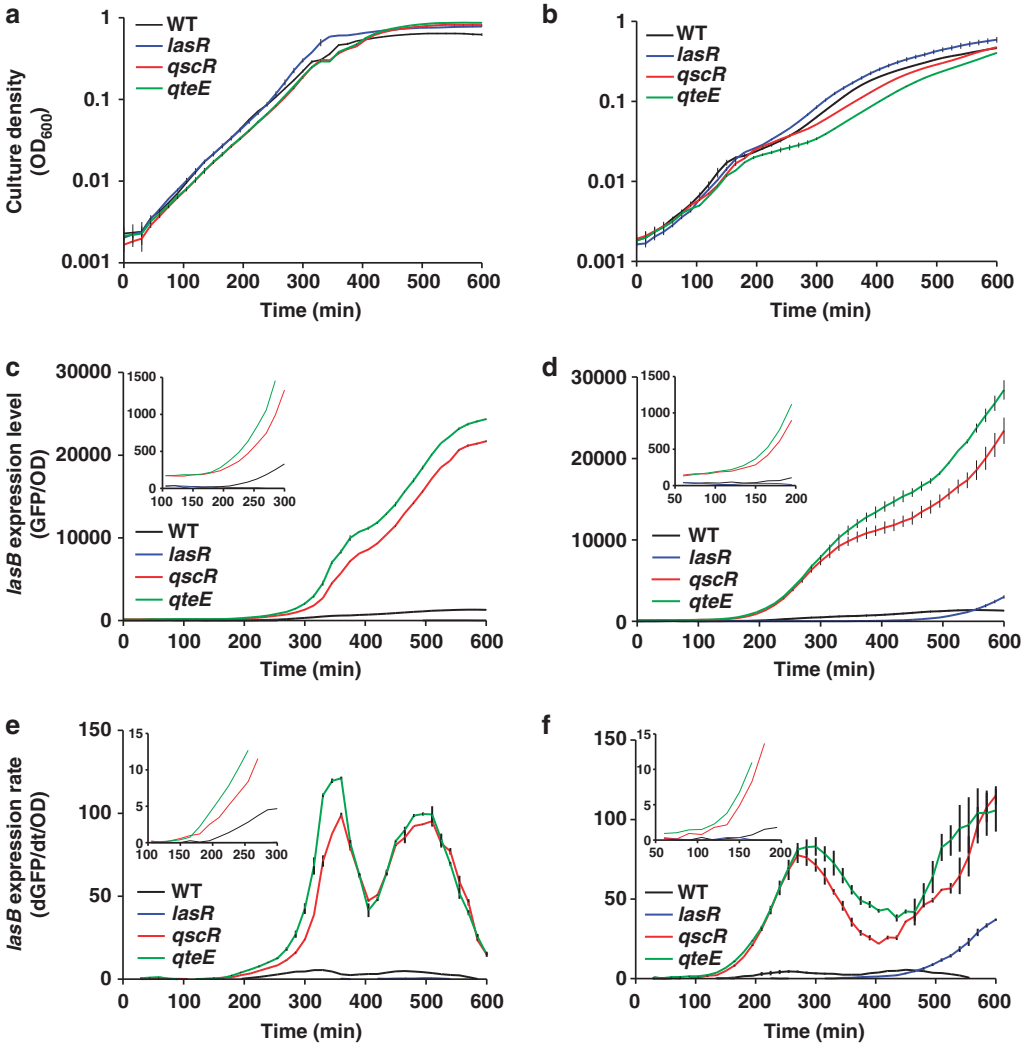

Figure 2 *P. aeruginosa lasB* expression in individual cultures. Wild-type, *lasR, qteE* and *qscR* strains containing the *lasB'-gfp* reporter plasmid pRG13 were grown in CAA medium (left panels **a**, **c**, **e**) and in glutamate medium (right panels **b**, **d**, **f**). (**a, b**) Culture density (OD_{600}). (**c**, **d**) Cumulative *lasB* expression (GFP/OD). (**e**, **f**) Rate of *lasB* expression (dGFP/dt/OD). The insets are a magnification of the gene expression early in growth. The error bars indicate s.d. of the mean of three independent biological replicates.

good. We quantified *lasB* promoter activity in the wild-type, *lasR*, *qscR* and *qteE* strains using a *gfp* transcriptional reporter fusion (Figure 2). For reasons that are unclear, the growth of the *qscR* and *qteE* mutants in glutamate medium was less distinct from that of the wild-type and *lasR* mutant when the strains harbored the *lasB'-gfp* reporter plasmid (Figure 2b vs Figure 1b). We measured both the cumulative levels as well as the rates of *lasB* expression (see Materials and Methods for technical details). Interestingly, we obtained two distinct rate peaks (Figures 2e and f), the potential significance of which is discussed below. The *qscR* and *qteE* mutants showed higher *lasB* expression as compared with the wild-type and the *lasR* mutant in both CAA and glutamate media (Figures 2c–f). However, in CAA, the induction was observed at the transition from exponential to stationary phase (onset $\sim$300 min, rate peak 350 min; Figures 2a, c and e), whereas in glutamate medium it was observed earlier in growth at lower cell density (onset $\sim$200 min, rate peak 270 min; Figures 2b, d and f). Induction upon entry into stationary phase had little impact on bacterial growth rate, whereas induction during active growth did. The transient decrease in growth rate in glutamate medium around 200 min correlated with the sharp increase in the rate of *lasB* expression. The timing of *lasB* induction was slightly advanced in the *qteE* and *qscR* mutants compared with the wild-type (Figures 2c–f insets). Both mutants also exhibited a low basal level of *lasB* expression (Figures 2c and d insets). However, in contrast with the high expression rate post induction, the low expression rate early in growth had a negligible impact on fitness.

Effect of QS dysregulation on bacterial fitness under conditions that favor QS

Next, we assessed *P. aeruginosa* growth in defined medium with caseinate as the sole C-source. Caseinate utilization requires QS-dependent extracellular proteases, including LasB (Iglewski and van Delden, 1998; Sandoz *et al.,* 2007). We found that both *qscR* and *qteE* mutants grew substantially faster than the wild-type in caseinate medium (Figure 3a). This is to be expected if growth is limited by exoenzyme concentration. The *qscR* and *qteE* mutants highly express LasB and other exoenzymes, and therefore degrade caseinate faster than the wild-type. We verified that the observed differences in growth were not caused by the presumably different amounts of exoenzymes present in the culture inoculum (Figure 3b). The *lasR* mutant grew to a density about 30-fold lower than that of the other three strains (Figure 3a).

To investigate how a QS-deficient cheater would benefit from varied levels of public goods production, we cocultured the *lasR* mutant, at 1% initial frequency, with the wild-type, the *qscR* or the *qteE* mutant. Enrichment was quantified after 24 h of competition, because in an individual culture, the *qscR* and *qteE* mutants reached stationary phase at this time (Figure 3a). This allowed us to restrict competition to the period of active growth, and keep the duration of competition identical for all cocultures. We found that enrichment of the *lasR* cheater was significantly higher in *qscR* or *qteE* mutant coculture than in the wild-type coculture (Figure 3c). However, this measure of relative fitness based on initial and final frequencies does not consider the density dependence of cooperation (Ross-Gillespie *et al.,* 2009). During the 24-h timespan, the *qteE* and *qscR* mutant populations reached a higher population density and underwent more rounds of replication, in principle affording cheaters increased opportunity to enrich. To address this point, we calculated the relative fitness of the *lasR* mutant based on the ratio of the number of doublings (Malthusian parameter) and found no difference in wild-type coculture compare with *qteE* or *qscR* coculture ($w_{lasR\ vs\ WT} = 1.67 \pm 0.09$; $w_{lasR\ vs\ qteE} = 1.69 \pm 0.09$; $w_{lasR\ vs\ qscR} = 1.64 \pm 0.11$; $P > 0.7$ for both pairwise comparisons).

To investigate whether the wild-type with a normally functioning QS system can invade *qscR* and *qteE* mutant populations, we cocultured the wild-type at 1% initial frequency with either the *qscR* or the *qteE* mutant for 24 h. The wild-type enriched significantly ($P < 0.05$; Figure 3c). Conversely, the *qscR* or *qteE* mutant, when rare, did not enrich in wild-type coculture (Figure 3c).

Discussion

We have shown that negative regulation of QS by QteE and QscR has a major impact on absolute and relative fitness in the opportunistic bacterial pathogen *P. aeruginosa*. Inactivation of each of the regulators had very similar effects on QS-controlled public goods expression and QS-dependent growth, suggesting that it is indeed the negative regulation of QS common to both that causes the observed behaviors rather than potential less well-characterized pleiotropic effects. Almost identical phenotypes of both mutants point to a similar—albeit not completely redundant—mechanism of action, presumably heterodimerization with LasR and RhlR (Ledgham *et al.,* 2003; Siehnel *et al.,* 2010).

First, we demonstrated that both regulators mainly affect the magnitude and to a lesser extent the timing (threshold) of expression of a representative public goods gene, *lasB*. The effect on the quorum threshold appears less pronounced than that reported previously (Chugani *et al.,* 2001; Siehnel *et al.,* 2010). Both previous studies used a complex rather than a defined growth medium, but they used plasmid-borne reporter constructs similar to ours. Siehnel *et al.* (2010) used the same *qteE* transposon insertion mutant and Chugani *et al.* (2001) used a *qscR* insertion mutant that, although

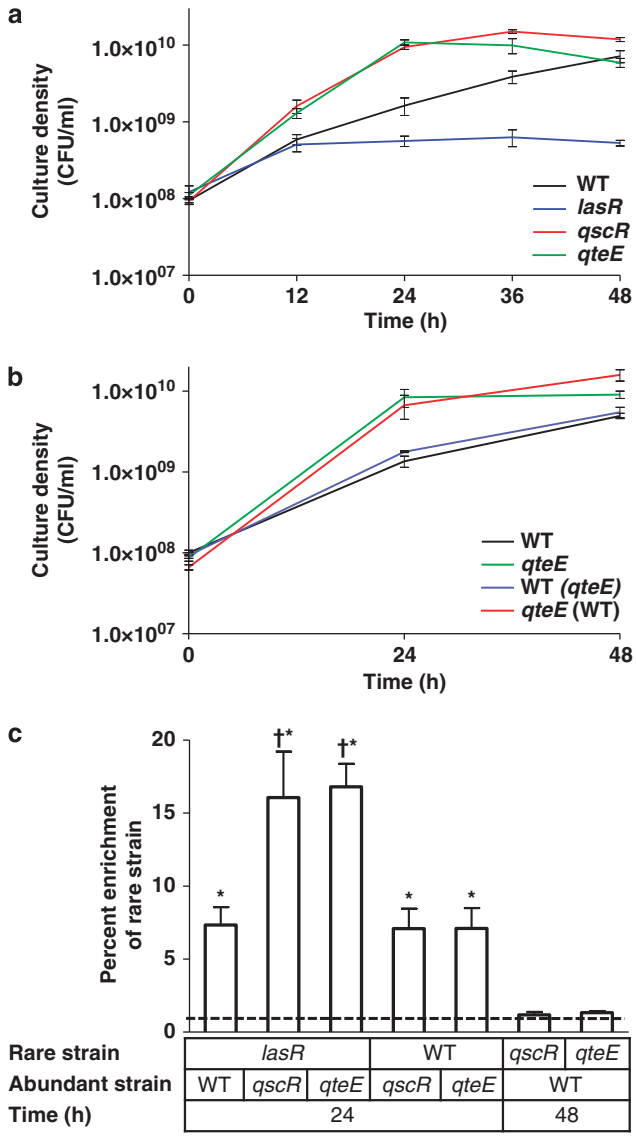

Figure 3 *P. aeruginosa* fitness under conditions that favor QS. Wild-type, *lasR, qteE* and *qscR* strains were grown in caseinate medium. (**a**) Density (CFU per ml) of individual cultures. (**b**) Density (CFU per ml) of *qteE* and wild-type cultures when inoculated with their own supernatant and with the supernatant from the respective other seed culture. Supernatant swapping had no statistically significant effect on growth ($P<0.05$). (**c**) Coculturing of different strain combinations. Two-strain cultures were initiated with 1% initial frequency of the rare strain as indicated and grown for the time specified. The error bars indicate s.d. of the mean of three independent biological replicates. The asterisk indicates statistically significant difference from the initial inoculum (dashed horizontal line; $P<0.05$). For the *lasR* cocultures, the dagger indicates statistically significant difference from the wild-type ($P<0.05$).

differently constructed, was at least in the same PAO1 strain background. However, Siehnel *et al.* (2010) also conceded that their measurement of the cumulative expression levels of *lasB* and other genes could not discern the contribution of early induction to maximum expression in stationary phase. This is where our rate measurements can provide further insight. It is clear from our data that there is a major induction of *lasB* expression in the *qteE* and *qscR* mutants that largely overlaps with the induction kinetics seen in the wild-type and that is largely responsible for the cumulative expression levels seen in stationary phase.

It is possible that a more prominent role of QteE and QscR in controlling the QS threshold may be uncovered by simultaneous inactivation of both (and perhaps other) regulators. Nevertheless, we did observe a low basal level of *lasB* expression in *qscR* and *qteE* mutants at very low cell densities. This could represent truly constitutive expression, presumably caused by self-induction or 'short-circuiting' of the QS pathway in individual cells (Pai and You, 2009), although we cannot exclude the possibility that this is residual GFP from previous induction. We attempted to minimize this effect by diluting and pre-growing *P. aeruginosa* strains at low density.

Next, we showed that the magnitude and timing of gene expression correlates with absolute and relative fitness. Early induction of QS in glutamate medium, but not late induction in CAA medium, significantly slowed the growth of the *qteE* and *qscR* mutants, allowing invasion by *lasR* mutant and wild-type strains. The congruence between growth and gene expression kinetics implies that the response of the chosen gene, *lasB*, is representative for the QS regulon as a whole, even though the responses of other individual genes might differ (Chugani *et al.,* 2001; Siehnel *et al.,* 2010). QteE and QscR dampened the expression of QS target genes after transition to the 'on' state, providing homeostatic regulation. A similar mechanism balances 3OC12-HSL production in *P. aeruginosa*: RsaL, another QS repressor, is activated by LasR and directly represses *lasI* transcription (Rampioni *et al.,* 2006; Rampioni *et al.,* 2007). Thus, the initial positive feedback mechanism in QS, autoinduction, is tightly coupled with and immediately followed by multiple negative feedback mechanisms that confine the metabolic cost of public goods production. The latter regulatory principle is analogous to the well-established endproduct inhibition of biosynthetic pathways.

Interestingly, the expression rate graphs revealed two distinct peaks (Figures 2e and f), possibly signifying the relative contribution of *las* vs *rhl*-dependent induction. The second peak could represent *rhl*-dependent expression as it coincides with the *lasR*-independent *lasB* activation of the *lasR* mutant in glutamate medium. Additional mutant experiments would be required to substantiate these findings.

Under growth conditions that strictly favor QS, *qteE* and *qscR* mutants overproducing public goods have an absolute fitness advantage in individual culture. This is consistent with a previous study reporting enhanced virulence of a *qscR* mutant in an insect infection model (Chugani *et al.,* 2001). However, the relative fitness of *qteE* and *qscR* mutants in mixed culture is low as they are not

only invaded by *lasR*-deficient cheaters but even by the QS-proficient wild-type. On the basis of growth rate ratios, the relative fitness of the *lasR* mutant is actually no higher in *qteE* and *qscR* mutant coculture than it is in wild-type coculture (the *lasR* mutant does grow faster in *qteE* and *qscR* mutant coculture, but so do the *qteE* and *qscR* mutants themselves). This appears puzzling initially as the increased metabolic burden from public goods overproduction by the *qteE* and *qscR* mutants should afford the *lasR* mutant a larger growth advantage. We surmise that the maximum growth rate of the *lasR* mutant in *qteE* and *qscR* coculture is limited by the rate of uptake or intracellular processing of the digested protein substrate rather than by the rate of extracellular proteolysis.

More broadly, our assessment of the costs and benefits of negative regulation permits inferences about the predominant adaptive environment of *P. aeruginosa*. Conditions that strictly require QS, including certain infections, do not appear to be the dominant growth environment for clonal, cooperating populations of *P. aeruginosa*. Otherwise, negative regulation would be lost. Maintenance of negative regulation would require substantial residence in environments that favor QS less or not at all, or would require significant mixing of producing and nonproducing strains. It is evident from our experiments (Figures 1 and 2) and numerous others that *P. aeruginosa* is unable to restrict QS-controlled public goods expression, through appropriate co-regulation, to those nutritional environments that require QS. For example, the constraints of transmembrane signaling may simply prevent the direct sensing of a generic extracellular protein substrate for the purpose of exoprotease expression. The tuning of public goods expression by negative regulation can therefore be considered a bet-hedging strategy that optimizes cost and benefits for life in alternating environments that do and do not favor QS. This scenario is of particular relevance to opportunistic pathogens such as *P. aeruginosa* that are thought to maintain their 'virulence' factors and associated control mechanisms primarily due to advantages in nonparasitic contexts (Brown *et al.,* 2012). Here, tuning could be important because QS-controlled products may sometimes—but not always—confer a fitness advantage by nonspecifically inhibiting competing microbes (Hibbing *et al.,* 2010). As a bet-hedging strategy, tuning may complement cell-to-cell variability of QS gene expression in isogenic populations (Anetzberger *et al.,* 2009; Garmyn *et al.,* 2011; Perez and Hagen, 2010). Such phenotypic heterogeneity can be caused by random fluctuations in the levels of regulatory proteins, and may also serve to enhance population fitness in dynamic and unpredictable environments (Veening *et al.,* 2008).

QscR may integrate additional information in natural multispecies environments; it may attenuate QS gene expression by eavesdropping on acyl-HSL signals produced by other species (Lee *et al.,* 2006). Taken together, 'normal' QS with functional QscR and QteE appears to be the strategy best suited to balance absolute and relative fitness. The tuning of QS-controlled gene expression through feedback inhibition can thereby help stabilize public goods cooperation in bacteria.

Acknowledgements

We thank Kevin Foster for his suggestions, and Richard Siehnel and Pradeep Singh for providing the *P. aeruginosa qteE* deletion mutant. This study was funded by the National Science Foundation grant 1158553 (to MS). Several *P. aeruginosa* strains used in this study were from the University of Washington transposon mutant library, generated with funds from the National Institutes of Health grant P30 DK089507.

References

Anetzberger C, Pirch T, Jung K. (2009). Heterogeneity in quorum sensing-regulated bioluminescence of *Vibrio harveyi*. *Mol Microbiol* **73**: 267–277.

Becher A, Schweizer HP. (2000). Integration-proficient *Pseudomonas aeruginosa* vectors for isolation of single-copy chromosomal *lacZ* and *lux* gene fusions. *Biotechniques* **29**: 948–950. 952.

Brown SP, Cornforth DM, Mideo N. (2012). Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. *Trends Microbiol* **20**: 336–342.

Chugani SA, Whiteley M, Lee KM, D'Argenio D, Manoil C, Greenberg EP. (2001). QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **98**: 2752–2757.

Cormack BP, Valdivia RH, Falkow S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). *Gene* **173**: 33–38.

Cremer J, Melbinger A, Frey E. (2012). Growth dynamics and the evolution of cooperation in microbial populations. *Sci Rep* **2**: 281.

Czaran T, Hoekstra RF. (2009). Microbial communication, cooperation and cheating: quorum sensing drives the evolution of cooperation in bacteria. *PloS One* **4**: e6655.

Darch SE, West SA, Winzer K, Diggle SP. (2012). Density-dependent fitness benefits in quorum-sensing bacterial populations. *Proc Natl Acad Sci USA* **109**: 8259–8263.

Dekimpe V, Deziel E. (2009). Revisiting the quorum-sensing hierarchy in *Pseudomonas aeruginosa*: the transcriptional regulator RhlR regulates LasR-specific factors. *Microbiology* **155**: 712–723.

Diggle SP, Griffin AS, Campbell GS, West SA. (2007). Cooperation and conflict in quorum-sensing bacterial populations. *Nature* **450**: 411–414.

Foster KR, Shaulsky G, Strassmann JE, Queller DC, Thompson CRL. (2004). Pleiotropy as a mechanism to stabilize cooperation. *Nature* **431**: 693–696.

Fuqua C, Burbea M, Winans SC. (1995). Activity of the *Agrobacterium* Ti plasmid conjugal transfer regulator

TraR is inhibited by the product of the *traM* gene. *J Bacteriol* **177**: 1367–1373.

Fuqua C, Greenberg EP. (2002). Listening in on bacteria: acyl-homoserine lactone signalling. *Nat Rev Mol Cell Biol* **3**: 685–695.

Galloway DR. (1991). *Pseudomonas aeruginosa* elastase and elastolysis revisited: recent developments. *Mol Microbiol* **5**: 2315–2321.

Garmyn D, Gal L, Briandet R, Guilbaud M, Lemaitre JP, Hartmann A *et al.* (2011). Evidence of autoinduction heterogeneity via expression of the Agr system of *Listeria monocytogenes* at the single-cell level. *Appl Environ Microbiol* **77**: 6286–6289.

Gilbert KB, Kim TH, Gupta R, Greenberg EP, Schuster M. (2009). Global position analysis of the *Pseudomonas aeruginosa* quorum-sensing transcription factor LasR. *Mol Microbiol* **73**: 1072–1085.

Hamilton WD. (1964). The genetical evolution of social behaviour, I & II. *J Theor Biol* **7**: 1–52.

Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N *et al.* (2003). Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors. *EMBO J* **22**: 3803–3815.

Hibbing ME, Fuqua C, Parsek MR, Peterson SB. (2010). Bacterial competition: surviving and thriving in the microbial jungle. *Nat Rev Microbiol* **8**: 15–25.

Hoang TT, Kutchma AJ, Becher A, Schweizer HP. (2000). Integration-proficient plasmids for *Pseudomonas aeruginosa*: Site-specific integration and use for engineering of reporter and expression strains. *Plasmid* **43**: 59–72.

Iglewski BH, Van Delden C. (1998). Cell-to-cell signaling and *Pseudomonas aeruginosa* infections. *Emerg Infect Dis* **4**: 551–560.

Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S *et al.* (2003). Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **100**: 14339–14344.

Juhas M, Eberl L, Tummler B. (2005). Quorum sensing: the power of cooperation in the world of *Pseudomonas*. *Environ Microbiol* **7**: 459–471.

Keller L, Surette MG. (2006). Communication in bacteria: an ecological and evolutionary perspective. *Nat Rev Microbiol* **4**: 249–258.

Kohler T, Buckling A, van Delden C. (2009). Cooperation and virulence of clinical *Pseudomonas aeruginos*a populations. *Proc Natl Acad Sci USA* **106**: 6339–6344.

Kummerli R, Brown SP. (2010). Molecular and regulatory properties of a public good shape the evolution of cooperation. *Proc Natl Acad Sci USA* **107**: 18921–18926.

Latifi A, Foglino M, Tanaka K, Williams P, Lazdunski A. (1996). A hierarchical quorum-sensing cascade in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhIR (VsmR) to expression of the stationary-phase sigma factor RpoS. *Mol Microbiol* **21**: 1137–1146.

Ledgham F, Ventre I, Soscia C, Foglino M, Sturgis JN, Lazdunski A. (2003). Interactions of the quorum sensing regulator QscR: interaction with itself and the other regulators of *Pseudomonas aeruginosa* LasR and RhlR. *Mol Microbiol* **48**: 199–210.

Lee JH, Lequette Y, Greenberg EP. (2006). Activity of purified QscR, a *Pseudomonas aeruginosa* orphan quorum-sensing transcription factor. *Mol Microbiol* **59**: 602–609.

Lenski RE, Rose MR, Simpson SC, Tadler SC. (1991). Long-term experimental evolution in *Escherichia coli*. I. adaptation and divergence during 2,000 generations. *Am Nat* **138**: 1315–1341.

Lequette Y, Lee JH, Ledgham F, Lazdunski A, Greenberg EP. (2006). A distinct QscR regulon in the *Pseudomonas aeruginosa* quorum-sensing circuit. *J Bacteriol* **188**: 3365–3370.

Liang H, Duan J, Sibley CD, Surette MG, Duan K. (2011). Identification of mutants with altered phenazine production in *Pseudomonas aeruginosa*. *J Med Microbiol* **60**: 22–34.

Medina G, Juarez K, Diaz R, Soberon-Chavez G (2003). Transcriptional regulation of *Pseudomonas aeruginosa rhlR*, encoding a quorum-sensing regulatory protein. *Microbiology* **149**: 3073–3081.

Mellbye B, Schuster M. (2011). More than just a quorum: Integration of stress and other environmental cues in acyl-homoserine lactone signaling. In: Storz G, Hengge R (eds) *Bacterial stress responses*. ASM Press: Washington, DC.

Miller MB, Bassler BL. (2001). Quorum sensing in bacteria. *Annu Rev Microbiol* **55**: 165–199.

Miller WG, Leveau JH, Lindow SE. (2000). Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. *Mol Plant Microbe Interact* **13**: 1243–1250.

Pai A, Tanouchi Y, You L. (2012). Optimality and robustness in quorum sensing-mediated regulation of a costly public good enzyme. *Proc Natl Acad Sci USA* **109**: 19810–19815.

Pai A, You L. (2009). Optimal tuning of bacterial sensing potential. *Mol Syst Biol* **5**: 286.

Pearson JP, Pesci EC, Iglewski BH. (1997). Roles of *Pseudomonas aeruginosa las* and *rhl* quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J Bacteriol* **179**: 5756–5767.

Perez PD, Hagen SJ. (2010). Heterogeneous response to a quorum-sensing signal in the luminescence of individual *Vibrio fischeri*. *PloS One* **5**: e15473.

Pesci EC, Pearson JP, Seed PC, Iglewski BH. (1997). Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **179**: 3127–3132.

Piper KR, Farrand SK. (2000). Quorum sensing but not autoinduction of Ti plasmid conjugal transfer requires control by the opine regulon and the antiactivator TraM. *J Bacteriol* **182**: 1080–1088.

Rampioni G, Bertani I, Zennaro E, Polticelli F, Venturi V, Leoni L. (2006). The quorum-sensing negative regulator RsaL of *Pseudomonas aeruginosa* binds to the lasI promoter. *J Bacteriol* **188**: 815–819.

Rampioni G, Schuster M, Greenberg EP, Bertani I, Grasso M, Venturi V *et al.* (2007). RsaL provides quorum sensing homeostasis and functions as a global regulator of gene expression in *Pseudomonas aeruginosa*. *Mol Microbiol* **66**: 1557–1565.

Ross-Gillespie A, Gardner A, Buckling A, West SA, Griffin AS. (2009). Density dependence and cooperation: theory and a test with bacteria. *Evolution* **63**: 2315–2325.

Rumbaugh KP, Diggle SP, Watters CM, Ross-Gillespie A, Griffin AS, West SA. (2009). Quorum sensing and the social evolution of bacterial virulence. *Curr Biol* **19**: 341–345.

Sandoz KM, Mitzimberg SM, Schuster M. (2007). Social cheating in *Pseudomonas aeruginosa* quorum sensing. *Proc Natl Acad Sci USA* **104**: 15876–15881.

Schuster M, Greenberg EP. (2006). A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. *Int J Med Microbiol* **296**: 73–81.

Schuster M, Lohstroh CP, Ogi T, Greenberg EP. (2003). Identification, timing and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol* **185**: 2066–2079.

Schweizer HP. (1991). *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19. *Gene* **97**: 109–121.

Siehnel R, Traxler B, An DD, Parsek MR, Schaefer AL, Singh PK. (2010). A unique regulator controls the activation threshold of quorum-regulated genes in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci USA* **107**: 7916–7921.

Simon R, Priefer U, Pühler A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Nature Biotechnology* **1**: 784–791.

van Delden C, Comte R, Bally AM. (2001). Stringent response activates quorum sensing and modulates cell density-dependent gene expression in *Pseudomonas aeruginosa*. *J Bacteriol* **183**: 5376–5384.

Veening JW, Smits WK, Kuipers OP. (2008). Bistability, epigenetics, and bet-hedging in bacteria. *Annu Rev Microbiol* **62**: 193–210.

Wagner VE, Bushnell D, Passador L, Brooks AI, Iglewski BH. (2003). Microarray analysis of *Pseudomonas aeruginosa* quorum-sensing regulons: effects of growth phase and environment. *J Bacteriol* **185**: 2080–2095.

West SA, Diggle SP, Buckling A, Gardner A, Griffins AS. (2007a). The social lives of microbes. *Annu Rev Ecol Evol S* **38**: 53–77.

West SA, Griffin AS, Gardner A. (2007b). Social semantics: altruism, cooperation, mutualism, strong reciprocity and group selection. *J Evol Biol* **20**: 415–432.

Wilder CN, Diggle SP, Schuster M. (2011). Cooperation and cheating in *Pseudomonas aeruginosa*: the roles of the *las*, *rhl* and *pqs* quorum-sensing systems. *ISME J* **5**: 1332–1343.

Williams P, Winzer K, Chan WC, Camara M. (2007). Look who's talking: communication and quorum sensing in the bacterial world. *Philos Trans R Soc Lond B Biol Sci* **362**: 1119–1134.

Xavier JB, Foster KR. (2007). Cooperation and conflict in microbial biofilms. *Proc Natl Acad Sci USA* **104**: 876–881.

Zaslaver A, Bren A, Ronen M, Itzkovitz S, Kikoin I, Shavit S *et al*. (2006). A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli*. *Nat Methods* **3**: 623–628.

Supplementary Information accompanies this paper on The ISME Journal website (http://www.nature.com/ismej)