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Gene dosage compensation calibrates four regulatory RNAs to control *Vibrio cholerae* quorum sensing

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Quorum sensing is a mechanism of cell-to-cell communication that allows bacteria to coordinately regulate gene expression in response to changes in cell-population density. At the core of the *Vibrio cholerae* quorum-sensing signal transduction pathway reside four homologous small RNAs (sRNAs), named the quorum regulatory RNAs 1–4 (Qrr1–4). The four Qrr sRNAs are functionally redundant. That is, expression of any one of them is sufficient for wild-type quorum-sensing behaviour. Here, we show that the combined action of two feedback loops, one involving the sRNA-activator LuxO and one involving the sRNA-target HapR, promotes gene dosage compensation between the four *qrr* genes. Gene dosage compensation adjusts the total Qrr1–4 sRNA pool and provides the molecular mechanism underlying sRNA redundancy. The dosage compensation mechanism is exquisitely sensitive to small perturbations in Qrr levels. Precisely maintained Qrr levels are required to direct the proper timing and correct patterns of expression of quorum-sensing-regulated target genes.

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Introduction

Chemical communication allows groups of bacteria to monitor and synchronously alter gene expression in response to changes in cell number and species-composition of the surrounding bacterial community. Communication is accomplished through the synthesis, secretion, and subsequent detection of signalling molecules called auto-inducers (AIs). This process, known as quorum sensing, is used by many bacterial species to coordinately control a battery of behaviours (Waters and Bassler, 2005; Bassler and Losick, 2006). In the human pathogen *Vibrio cholerae*, quorum sensing regulates progression through the infectious cycle, controls genes encoding virulence factors, and regulates biofilm formation (Zhu *et al*, 2002; Hammer and Bassler, 2003; Zhu and Mekalanos, 2003).

Vibrio cholerae makes and responds to two AIs that function synergistically to control group behaviours (Miller *et al*, 2002). At low cell-population density (LCD), when the extracellular AI concentration is low, membrane-bound AI-receptors function as kinases and phosphorylate a shared phosphotransfer protein called LuxU, which subsequently transfers the phosphate to the response regulator LuxO. LuxO-P, together with the alternative sigma factor σ^{54}, activates transcription of four genes encoding small non-coding RNAs (sRNAs), called the quorum regulatory RNAs (Qrr1–4) (Figure 1, and Miller *et al*, 2002; Lenz *et al*, 2004). When transcribed, the Qrr sRNAs function together with the RNA chaperone Hfq to control translation of target mRNAs. One target mRNA, which is destabilized by the Qrrs at LCD, encodes the major quorum-sensing transcription factor, HapR.

At high cell-population density (HCD), AIs accumulate extracellularly and bind their respective receptors. This event switches the receptors' enzymatic activity from kinase to phosphatase, ultimately resulting in dephosphorylation of LuxO-P. Dephosphorylated LuxO cannot activate *qrr* transcription. Existing sRNAs are rapidly turned over, as Hfq-dependent sRNAs are degraded stoichiometrically with their target mRNAs (Masse *et al*, 2003). In the absence of Qrr sRNAs, *hapR* mRNA is translated and HapR protein accumulates and activates or represses its target genes. In summary, *V. cholerae* cells at LCD are characterized by the presence of Qrr sRNAs and the absence of HapR, whereas *V. cholerae* cells at HCD are characterized by the absence of Qrr sRNAs and the presence of HapR.

Small RNAs are widely used as key regulators of stress responses, virulence, and central metabolic pathways in bacteria (Romeo, 1998; Gottesman, 2004; Majdalani *et al*, 2005; Storz *et al*, 2005). In many cases, multiple homologous sRNAs exist, and often they appear to carry out identical functions (Weilbacher *et al*, 2003; Wilderman *et al*, 2004; Guillier and Gottesman, 2006). In the case of *V. cholerae* quorum sensing, the Qrr sRNAs are encoded by four unlinked loci. They are ~80% identical in sequence and predicted to have similar secondary structures (Lenz *et al*, 2004). Previous analyses of single, double, triple, and quadruple *qrr* deletions in *V. cholerae* showed that the four Qrr sRNAs function redundantly to control quorum sensing (Lenz *et al*, 2004). That is, if any one of the four Qrr sRNAs is present, *V. cholerae* expresses quorum-sensing target genes in a den-

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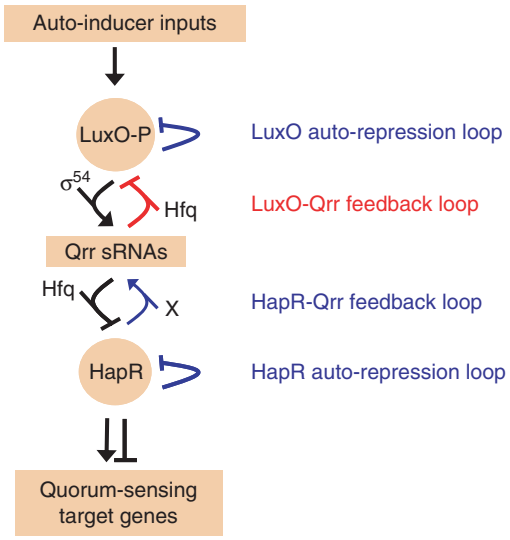

Figure 1 Model of the core of the *V. cholerae* quorum-sensing circuit. The backbone of the quorum-sensing signalling pathway is depicted in black. Auto-inducer inputs are ultimately transmitted to LuxO. At LCD, LuxO-P functions together with σ^{54} to activate transcription of the genes encoding the four Qrr sRNAs. The Qrr sRNAs, in conjunction with Hfq, repress translation of *hapR* mRNA. When *hapR* translation is derepressed, HapR controls downstream target genes. The previously defined feedback loops are shown in blue. HapR and LuxO auto-repress the *hapR* and *luxO* promoters, respectively (see discussion for details on the LuxO auto-repression loop). HapR also enhances *qrr* transcription through an unknown factor, denoted by 'X'. The feedback loop between the Qrr sRNAs and LuxO identified in this work is shown in red. Arrows indicate positive interactions, T-bars indicate negative interactions.

sity-dependent manner similar to the wild-type strain. By contrast, in the closely related bacterium *Vibrio harveyi*, the analogous multiple Qrr sRNAs contribute additively to control quorum sensing (Tu and Bassler, 2007).

Two feedback loops have been described in the *V. cholerae* regulatory network, which appear, first, to ensure the network's exquisite responsiveness to changes in extracellular AI concentrations, and second, to set the AI concentration thresholds at which quorum-sensing-regulated behaviours are initiated or terminated. The two feedback loops are as follows.

HapR auto-repression loop

The HapR protein binds to a site immediately downstream of the *hapR* transcriptional start site and, in this capacity, represses its own transcription (Figure 1, HapR auto-repression loop) (Lin *et al*, 2005). At HCD, HapR accumulates to a level sufficient to regulate its target genes, but because it also binds to its own promoter, it prevents additional *hapR* transcription, and thereby prevents excessive accumulation of HapR. HapR auto-repression is essential for the proper timing of the quorum-sensing response because the HapR pool must be maintained at a low enough level that HapR can be efficiently eliminated when *V. cholerae* switches from the HCD to the LCD gene expression pattern (Lin *et al*, 2005; Svenningsen *et al*, 2008).

HapR-Qrr feedback loop

HapR enhances transcription of the four *qrr* genes (Figure 1, HapR-Qrr feedback loop). However, because there is also an absolute requirement for LuxO-P to initiate *qrr* transcription, the HapR-Qrr feedback loop only functions when *V. cholerae* cells shift from the HCD to the LCD condition (Svenningsen *et al*, 2008). At this transition, the HapR-Qrr feedback provides a surge in *qrr* transcription, which accelerates the alterations in gene expression required for the *V. cholerae* LCD lifestyle.

Here, we investigate the mechanism underlying Qrr redundancy and we find that the Qrr sRNAs compensate for one another. Specifically, in the absence of any one Qrr, the other Qrrs are upregulated. The combination of two feedback loops, the HapR-Qrr feedback loop described above, and a new feedback loop described in this work, the LuxO-Qrr feedback loop, underlies Qrr dosage compensation. Together, these feedback loops provide a mechanism for adjusting *qrr* transcription on the basis of the total activity of the Qrr sRNAs present in a cell at any given time. Remarkably, the Qrr dosage compensation mechanism is able to respond to modest, that is, physiologically relevant, alterations in Qrr levels. Calibration of the Qrr sRNA levels through dosage compensation ensures precise timing of the activation and termination of quorum-sensing-controlled behaviours.

Results

The four Qrr sRNAs compensate for one another

Our previous results showed that all four *qrr* sRNAs have redundant functions in quorum sensing: any one of them is sufficient for cell-density-dependent expression of HapR-controlled target genes (Lenz *et al*, 2004). We wondered how any one Qrr sRNA could be sufficient for an approximately wild-type quorum-sensing response. One possibility is that, in the absence of a particular sRNA, the levels of the remaining sRNAs increase. To test this possibility, we used northern blots to measure the levels of each individual Qrr sRNA in the wild-type strain and in triple *qrr* deletion strains lacking the other three *qrr* genes (Figure 2A). Each row shows a blot probed specifically for the Qrr sRNA indicated on the right. For example, results for Qrr1 are shown in the top row. Lane 1 contains total RNA from the wild-type strain, and lane 2 contains the same amount of total RNA from the triple Δ*qrr*2,3,4 deletion strain. It is evident that greater Qrr1 is present in the absence of the other three Qrr sRNAs, than in their presence. The same pattern holds true for Qrr2, Qrr3, and Qrr4. As a control, lane 3 of each row contains total RNA from a *V. cholerae* mutant deleted for only the Qrr sRNA being probed. This lane shows that the Qrr1, Qrr2, and Qrr4 probes are specific for their particular sRNAs and do not cross-hybridize. Weak cross-hybridization occurs with the Qrr3 probe; however, this low level of cross-hybridization does not affect the interpretation of the results.

Dosage compensation functions at the level of qrr transcription

The increased abundance of one Qrr sRNA in the absence of the other Qrr sRNAs could be the result of increased transcription of the *qrr* gene in question, increased stability of the Qrr sRNA, or both. If transcription of one *qrr* gene increases in the absence of the other Qrr sRNAs, we reasoned that a transcriptional reporter fusion would reflect this. By contrast, regulation at the level of sRNA stability would not be

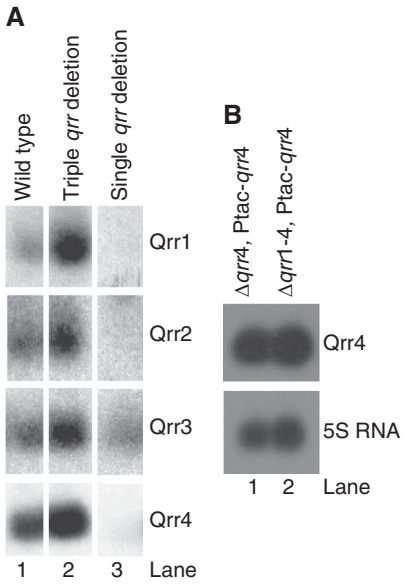

Figure 2 Qrr sRNA levels in wild-type and triple *qrr* deletion strains. (**A**) Northern blots showing Qrr levels in wild-type *V. cholerae* (lane 1), *V. cholerae qrr* triple deletion strains, possessing only the *qrr* gene encoding the sRNA indicated on the right (lane 2), and *V. cholerae qrr* single deletion strains, lacking only the *qrr* gene encoding the Qrr sRNA indicated on the right (lane 3). Total RNA was visualized with ethidium bromide as the loading control (not shown). (**B**) Northern blot showing Qrr4 levels in a *V. cholerae qrr*4 single deletion strain expressing *qrr*4 from the Ptac promoter (lane 1) and a *V. cholerae* Δ*qrr*1–4 quadruple deletion expressing *qrr*4 from the Ptac promoter (lane 2). 5S RNA is shown as a loading control. Total RNA was collected from the indicated strains at $OD_{600} = 0.1$.

Table I Dosage compensation acts at the level of transcription of the *qrr* genes

	qrr1-lux[a]	*qrr2-lux*[a]	*qrr3-lux*[a]	*qrr4-lux*[a]
Wild type	56 (3)	140 (20)	7 (3)	74 (15)
Δ*qrr*1-4	149 (3)	373 (28)	159 (5)	378 (26)
Fold repression[b]	2.7 (0.05)	2.7 (0.16)	24 (0.39)	5.1 (0.22)
Δ*hapR*	28 (1)	154 (28)	6 (1)	47 (30)
Δ*hapR* Δ*qrr*1–4	34 (1)	226 (55)	29 (10)	179 (52)
Fold repression[b]	1.2 (0.04)	1.5 (0.30)	4.8 (0.35)	3.8 (0.69)

[a]Light production from the indicated *qrr–lux* construct was measured at $OD_{600} = 0.1$ in the indicated *V. cholerae* strains. The average relative light units ($RLU/10^8$) from three independent cultures is reported. The standard error from the mean ($RLU/10^8$) is indicated in parentheses.
[b]Fold repression is calculated as the light produced by the Δ*qrr*1–4 mutant divided by the light produced by the isogenic *qrr*1–4$^+$ strain.

manifested using a transcriptional reporter fusion. We engineered *lux* reporter fusions to the +1 transcriptional start sites of each *qrr* gene (Svenningsen *et al*, 2008). Expression of the *lux* fusions in the wild-type and the Δ*qrr*1–4 strains was measured at $OD_{600} = 0.1$, the cell density at which the Qrr sRNAs are maximally produced (Svenningsen *et al*, 2008), and the results are shown in Table I. Expression of each *qrr* gene is higher in the Δ*qrr*1–4 mutant than in the wild type. Thus, dosage compensation occurs at the level of *qrr* transcription. We note that dosage compensation affects the four *qrr* promoters to different extents (see 'Fold Repression', Table I). We return to this point later.

In addition to transcriptional control, dosage compensation could also be a consequence of regulation of sRNA stability. To examine this possibility, we needed to uncouple regulation at the transcriptional level from regulation at the post-transcriptional level. To do this, we expressed the *qrr*4 gene from an exogenous Ptac promoter in Δ*qrr*4 and Δ*qrr*1–4 strains and measured Qrr4 levels by Northern blot (Figure 2B). Qrr4 driven by the Ptac promoter accumulates to identical levels in the presence and absence of the other *qrr* genes, indicating that the Ptac-*qrr*4 construct is not sensitive to alterations in sRNA levels. Thus, we conclude that, at least for *qrr*4, and presume for the other *qrr* genes, dosage compensation stems from transcriptional control, and not from the regulation of sRNA stability.

Dosage compensation is independent of the origin of the Qrr sRNAs

We considered two possible mechanisms that could give rise to the Qrr dosage compensation observed above. First, Qrr dosage compensation could be a regulatory element wired into the quorum-sensing network, that is, a Qrr-responsive negative feedback loop that represses the *qrr* promoters could exist. In this scenario, any shortage in Qrr sRNAs would result in reduced repression of the *qrr* promoters, leading to a compensatory increase in Qrr sRNA production. Second, dosage compensation could be an incidental consequence of titration of a transcription factor(s) required for expression of the *qrr* promoters. In this scenario, in the absence of one or more *qrr* genes, increased levels of this putative transcription factor(s) would be available to bind and activate the expression of the remaining *qrr* promoters. In the first case, an exogenously provided source of Qrr sRNA would cause repression of *qrr* transcription. In the second case, only Qrr sRNAs made from endogenous *qrr* promoters would cause repression of *qrr* transcription.

To test which mechanism is correct, we measured light production from the *qrr–lux* promoter fusions in the absence of Qrr sRNAs (Figure 3, white bars), in the presence of Qrr sRNAs produced from their endogenous promoters (Figure 3, black bars), Qrr4 sRNA produced from a plasmid-borne endogenous *qrr*4 promoter (Figure 3, striped bars), and Qrr4 sRNA produced from a plasmid carrying the exogenous Ptac promoter, which, besides core RNA polymerase, shares no transcription factors with those required for native *qrr* expression (Figure 3, dotted bars). The figure shows that Qrr sRNAs produced from any source cause repression of the *qrr–lux* promoter fusions. Thus, dosage compensation must be a result of negative feedback control of *qrr* expression by the Qrr sRNAs themselves, and not due to titration of factors required for *qrr* transcription.

The HapR-Qrr feedback loop is partially responsible for Qrr dosage compensation

On the basis of the above results, we hypothesize that the Qrr sRNAs compensate for one another by controlling the translation of a transcription factor, which in turn feeds back to regulate *qrr* gene expression. As described in the Introduction, one obvious candidate is the HapR-Qrr feedback loop identified previously (Svenningsen *et al*, 2008, Figure 1; HapR-Qrr feedback loop). We reason that if there is a shortage of Qrr sRNAs, increased HapR could be

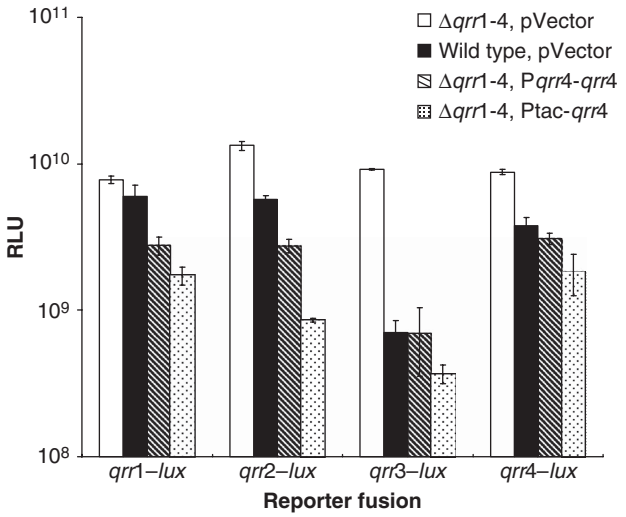

Figure 3 Dosage compensation is insensitive to the origin of the Qrr sRNAs. Light production from the indicated *qrr–lux* constructs was measured at $OD_{600} = 0.1$ in a *V. cholerae* Δ*qrr*1–4 mutant carrying the vector (white bars), *V. cholerae* wild type carrying the vector (black bars), a *V. cholerae* Δ*qrr*1–4 mutant expressing *qrr*4 under control of the endogenous *qrr*4 promoter on the vector (striped bars), and a Δ*qrr*1–4 mutant expressing *qrr*4 under control of the Ptac promoter on the same vector (dotted bars). Each bar shows the average light production from three independent cultures. Error bars indicate one standard deviation from the mean. RLU: relative light units.

produced, which in turn could feed back to increase synthesis of Qrr sRNAs, resulting in Qrr dosage compensation.

To test if the HapR-Qrr feedback loop is required for Qrr dosage compensation, we compared *qrr–lux* light production in a Δ*hapR V. cholerae* strain with that in a Δ*hapR*, Δ*qrr*1–4 strain (Table I). Our rationale is that if the HapR-Qrr feedback loop is responsible for Qrr dosage compensation, dosage compensation will not occur in the Δ*hapR* strains because any feedback loop requiring HapR will not be functioning in the Δ*hapR* strains. Indeed, when compared with the wild-type strain background, the extent of dosage compensation is reduced for all four *qrr* promoters in the Δ*hapR* strain backgrounds (Table I, 'Fold Repression'), suggesting that the HapR-Qrr feedback loop is involved in dosage compensation. However, whereas removal of the HapR-Qrr feedback loop nearly eliminated dosage compensation for *qrr*1 and *qrr*2, dosage compensation at *qrr*3 and *qrr*4 continued to occur in the Δ*hapR* strains. Thus, *qrr*1 and *qrr*2, which are the least subject to dosage compensation in wild-type *V. cholerae* (Table I), require the HapR-Qrr feedback loop for dosage compensation. By contrast, *qrr*3 and *qrr*4, which show a greater degree of dosage compensation, although obviously regulated by the HapR-Qrr feedback loop, must also respond to an additional regulatory component(s) for dosage compensation.

luxOU mRNA is a target of Qrr sRNA regulation

To identify the additional regulatory component involved in *qrr*3 and *qrr*4 dosage compensation, we relied on our findings in *V. harveyi*, which is closely related to *V. cholerae* and has a similar quorum-sensing circuit. In *V. harveyi*, the Qrr sRNAs repress translation of LuxO (Tu *et al*, manuscript in preparation). Thus, we wondered if the Qrr sRNAs might feed back to regulate *luxO* translation as part of the dosage compensation mechanism in *V. cholerae*. Alignment of the 5′-untranslated region (5′-UTR) of *V. cholerae luxO* and the two known targets of Qrr1–4, *hapR* and *vca0939*, showed that the 5′-UTR of the poly-cistronic *luxOU* mRNA contains a region of complementarity to the Qrr sRNAs similar to that predicted in the *hapR* and *vca0939* 5′-UTRs (Figure 4A, and Tu *et al*, manuscript in preparation, Lenz *et al*, 2004; Hammer and Bassler, 2007).

To test if the Qrr sRNAs feed back to regulate *luxOU* mRNA in *V. cholerae*, we assayed the stability of *luxOU* mRNA using northern blots. Rifampicin was added to LCD *V. cholerae* cultures to terminate transcription, after which the level of *luxOU* mRNA transcript was monitored over time (Figure 4B). In wild-type cells (denoted by WT), *luxOU* mRNA is degraded with a half-life of ∼94 s following termination of transcription. In the Δ*qrr*1–4 strain, the stability of the *luxOU* mRNA is increased, (half-life = ∼115 s). By contrast, in a *V. cholerae* strain that overexpresses Qrr4 (denoted by Δ*qrr*1–4 Ptac-*qrr*4), the half-life of *luxOU* mRNA is reduced to ∼35 s, supporting the idea that Qrr1–4 destabilize *luxOU* mRNA.

To measure the consequence of Qrr sRNA-mediated degradation of the *luxOU* mRNA on LuxO levels, we engineered a translational fusion of the *luxO* 5′-UTR including the first 10 amino acids of the LuxO ORF to green fluorescent protein (GFP). We introduced the plasmid-borne LuxO–GFP fusion into *Escherichia coli* strain SLS1277, which expresses *V. cholerae qrr*4 from the chromosome, under control of the P_{BAD} promoter. Figure 4C (left bars) shows the production of LuxO–GFP in SLS1277 without or with induction of Qrr4 synthesis by the addition of arabinose. LuxO–GFP expression is repressed ∼4-fold by Qrr4, suggesting that the Qrr sRNAs repress translation of *luxOU* mRNA.

The LuxO-Qrr feedback loop is partially responsible for Qrr dosage compensation

The results presented in Figure 4 suggest that Qrr repression of *luxO* could aid in Qrr dosage compensation because reduced Qrr sRNA levels could lead to increased LuxO production, which in turn could result in increased Qrr sRNA production (Figure 1, LuxO-Qrr feedback loop). To explore this idea, we engineered mutations in the *luxO* 5′-UTR that prevent pairing between the *luxOU* mRNA and the Qrr sRNAs. The predicted region of pairing overlaps the ribosome binding site of *luxO*, so most nucleotide changes in this region alter the basal level of *luxO* expression (data not shown). One mutation, however, $luxO^{AUCC}$, nearly eliminates Qrr-mediated repression of *luxO* (Figure 4C, right pair of bars), without significantly changing the basal expression level of *luxO* (Figure 4C, compare the two black bars). In this mutant, nucleotides −6 to −3 (TAGG) with respect to the first nucleotide in the *luxO* start codon were mutated to the complementary sequence (ATCC). The mutated sequence is underlined in Figure 4A. In Figure 5, we compare the extent of Qrr dosage compensation in the wild-type (black bars), the Δ*hapR* strain lacking the HapR-Qrr feedback loop (white bars), the $luxO^{AUCC}$ strain, which lacks the LuxO-Qrr feedback loop (grey bars), and the Δ*hapR*, $luxO^{AUCC}$ double mutant, which lacks both feedback loops (striped bars). Qrr dosage compensation was measured as the fold repression of

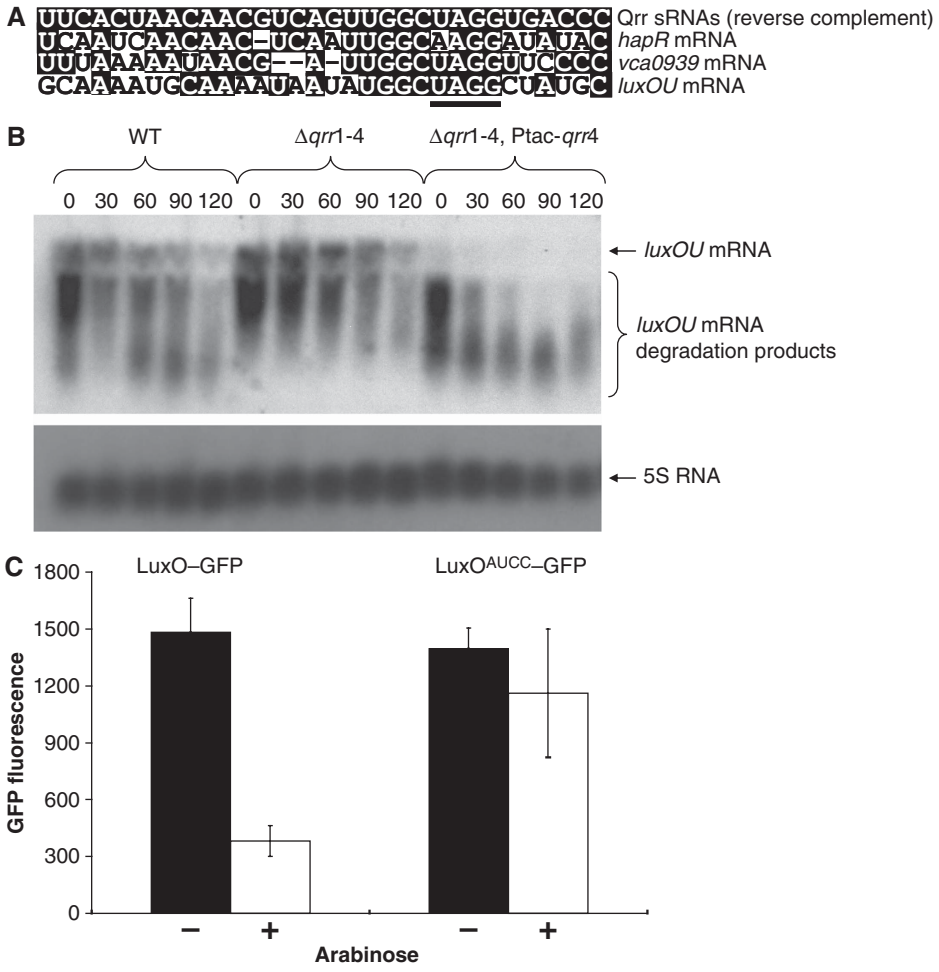

Figure 4 *luxOU* mRNA is a target of Qrr sRNA translational repression. (**A**) Alignment of the reverse complement of the conserved pairing region of the Qrr sRNAs with the 5′-UTR of two known target mRNAs, *hapR* and *vca0939*, and the 5′-UTR of *luxOU* mRNA. The region of the Qrr sRNAs that pair with the *hapR* and *vca0939* 5′-UTRs is completely conserved among the four Qrrs. Nucleotides in the target mRNAs that are complementary to the Qrr sRNAs are highlighted in white on black background. The underlined sequence (UAGG) of *luxOU* mRNA is mutated to AUCC in the $luxO^{AUCC}$ mutant. (**B**) Degradation of the *luxOU* mRNA was measured by northern blot in *V. cholerae* wild-type, Δ*qrr*1–4 and Δ*qrr*1–4, Ptac-*qrr*4 following transcription termination. The indicated times are seconds after addition of rifampicin. 5S RNA is shown as a loading control. (**C**) *E. coli* SLS1277 carrying plasmids harbouring either LuxO–GFP (pSLS146) or $LuxO^{AUCC}$–GFP (pSLS152) protein fusions were grown overnight in either LB (black bars) or LB supplemented with 0.4% arabinose (white bars). The experiment was performed in duplicate on three separate occasions. Error bars indicate one standard deviation from the mean of all six measurements.

each *qrr–lux* transcriptional fusion in the *qrr*1–4$^+$ strain compared with that in the isogenic Δ*qrr*1–4 strain.

For reference, we show again that the HapR-Qrr feedback loop is involved in dosage compensation (compare white bars with black bars). The $luxO^{AUCC}$ mutation partially eliminates dosage compensation for each *qrr* gene (compare grey bars with black bars), showing that indeed the LuxO-Qrr feedback loop contributes to Qrr dosage compensation. However, we note that the two feedback loops contribute distinctly to dosage compensation of each *qrr* gene. Dosage compensation in the case of *qrr*1 and *qrr*2 is largely due to the HapR-Qrr feedback loop. By contrast, the Qrr-LuxO feedback loop is the major source of dosage compensation for *qrr*4. In all three of these cases, simultaneous disruption of the HapR-Qrr and LuxO-Qrr feedback loops completely eliminates dosage compensation (compare striped bars with black bars). These results show that for *qrr*1, *qrr*2, and *qrr*4, the two feedback loops are sufficient to account for dosage compensation. Remarkably, *qrr*3, although clearly regulated by the two feedback loops, remains responsive to dosage compensation in the absence of both the HapR-Qrr and the LuxO-Qrr feedback loops. We interpret this to mean that an additional feedback loop, which is involved in dosage compensation, exists that has yet to be identified. This feedback loop is apparently specific to *qrr*3.

Determining the boundaries of Qrr dosage compensation

The Qrr sRNAs constitute the core of the quorum-sensing regulatory cascade, and regulation by them ultimately dictates the expression patterns of all downstream quorum-sensing target genes. Thus, we predict that keeping Qrr levels tightly constrained is a priority for this regulatory network. To investigate this idea, we examined the accuracy of Qrr dosage compensation in the quorum-sensing circuit. We made one assumption; that the four Qrr sRNAs are equally effective in pairing with their target mRNAs. If so, accurate dosage compensation should result in an identical total Qrr sRNA

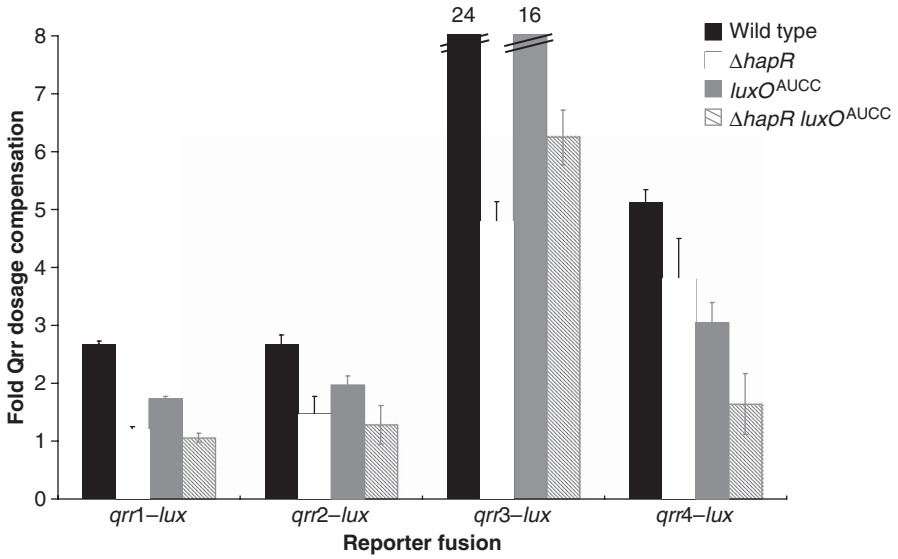

Figure 5 The LuxO-Qrr feedback loop contributes to Qrr dosage compensation. Light production from the indicated *qrr–lux* fusions was measured at $OD_{600} = 0.1$ in *V. cholerae* wild-type (black bars), Δ*hapR* (white bars), $luxO^{AUCC}$ (grey bars) and Δ*hapR*, $luxO^{AUCC}$ (striped bars) strains containing or lacking the four chromosomal *qrr* genes. In each case, Qrr dosage compensation is calculated as the light produced from the Δ*qrr*1–4 mutant divided by the light produced from the isogenic *qrr*1–4$^+$ strain. Regarding the *qrr*3–*lux* data, the two slashes indicate that the bars extend beyond the scale of the *y* axis. The fold-dosage-compensation value is indicated above the corresponding bar. Light production from each strain was measured in triplicate. Error bars indicate one standard deviation from the mean. The standard deviation is ± 0.39 for *qrr*3–*lux* in wild type, and ± 0.09 for *qrr*3–*lux* in the $luxO^{AUCC}$ mutant.

pool size in each of the *qrr* mutant strains because the loss of the contribution of a particular sRNA following deletion should be compensated for by overexpression of the remaining Qrr sRNAs. To survey a range of altered Qrr levels, we examined Qrr dosage compensation accuracy in response to a large perturbation in *qrr* gene dosage by deleting all combinations of three *qrr* genes, as well as more modest changes in gene dosage by sequentially deleting individual *qrr* genes.

Dosage compensation is inaccurate in triple qrr mutant strains. To measure the total Qrr sRNA pool, we performed northern blots with a probe complementary to the 32 bp region that is 100% conserved among the four Qrr sRNAs. This probe binds the four Qrr sRNAs indiscriminately as confirmed using known concentrations of each Qrr transcribed *in vitro* (data not shown). Figure 6A shows the total Qrr sRNAs in wild type and the four *qrr* triple mutant strains. All the triple mutant strains, especially the *qrr*1$^+$ mutant, contain markedly less total Qrr sRNAs than does the wild-type strain. This finding indicates that Qrr dosage compensation is not accurate in the *qrr* triple mutants.

We were surprised that dosage compensation is not exact in the *qrr* triple mutants, because all four *qrr* triple mutants appear to have wild-type quorum-sensing behaviour, as measured by the cell-density-dependent expression of quorum-sensing reporters (Lenz *et al*, 2004). We wondered whether our earlier measurements of the final quorum-sensing output behaviour are too far downstream in the quorum-sensing cascade to accurately reflect Qrr activities. To examine the effect of the depletion of the Qrr sRNAs on their immediate quorum-sensing target, we measured *hapR* mRNA levels in wild type and the same triple *qrr* mutants shown in Figure 6A by quantitative real-time PCR. The level of *hapR* mRNA in wild-type cells is set to 1. Figure 6B shows that, in the *qrr*4$^+$ triple-mutant strain, *hapR* mRNA levels are as low as in wild type; however, in the three other triple mutant

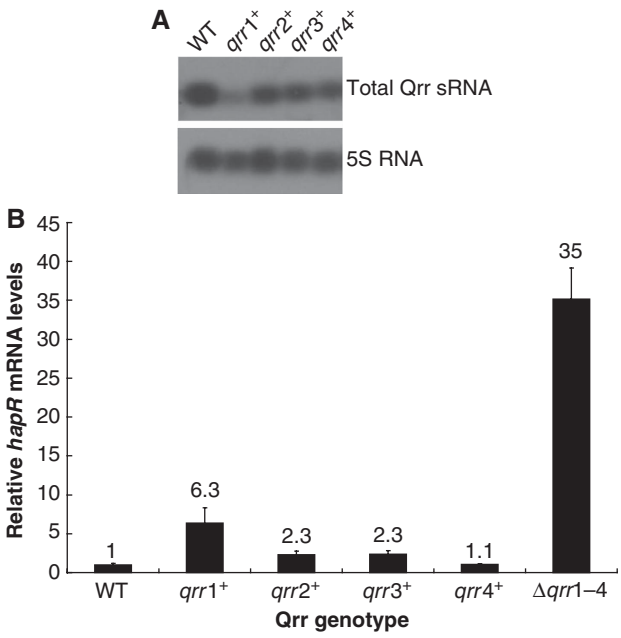

Figure 6 Qrr dosage compensation is not accurate in the *qrr* triple deletion mutants. (**A**) The total level of Qrr sRNAs in wild-type (denoted WT), Δ*qrr*2,3,4 (denoted *qrr*1$^+$), Δ*qrr*1,3,4 (denoted *qrr*2$^+$), Δ*qrr*1,2,4 (denoted *qrr*3$^+$), and Δ*qrr*1,2,3 (denoted *qrr*4$^+$) *V. cholerae* strains grown to $OD_{600} = 0.1$ was measured by northern blot with a probe for the 32-bp region that is completely conserved among Qrr1–4 sRNAs. 5S RNA is shown as a loading control. (**B**) The level of *hapR* mRNA in the same strains shown in (A) and in a Δ*qrr*1–4 *V. cholerae* strain grown to $OD_{600} = 0.1$ was measured by RT–PCR. Error bars indicate one standard deviation from the mean of triplicate measurements. The experiment was repeated three times with similar results.

strains, in which only *qrr*1, *qrr*2, or *qrr*3 is present, *hapR* mRNA levels are higher than in wild type. For comparison, we show that in the Δ*qrr*1–4 mutant strain, derepressed *hapR*

mRNA levels are 35-times higher than in the wild type. Qrr4 is the most abundant of the Qrrs (Lenz *et al*, 2004; Lenz and Bassler, 2007), and apparently it alone is sufficient to correctly regulate *hapR* mRNA levels (Figure 6B). This is not the case, however, for the less abundant Qrrs 1, 2, and 3. We conclude that there is an upper limit to the extent to which the quorum-sensing cascade can tolerate and compensate for changes that deplete the Qrr sRNA pool in *V. cholerae*. Even so, we note that *hapR* mRNA is repressed extensively in all the triple *qrr* mutants compared with the Δ*qrr*1–4 mutant. This finding apparently explains their wild-type-like quorum-sensing phenotypes.

Dosage compensation is sensitive to small perturbations in Qrr levels. The above results show that, in a *qrr* triple deletion mutant, although production of the remaining Qrr sRNA is increased compared with its expression in wild-type *V. cholerae* (Figure 2A), dosage compensation is not especially accurate except in the case of Qrr4 (Figure 6B). We hypothesize that the variations in Qrr levels that *V. cholerae* experiences in its natural habitats, and thus the variations that the quorum-sensing dosage compensation mechanism is tuned to detect and respond to, are more modest than the extreme variations in gene dose caused by triple deletion of the *qrr* genes. To test if this is so, we assayed dosage compensation following small alterations in Qrr levels, by sequential removal of only one of the four *qrr* genes. Specifically, we measured Qrr4 levels in wild-type (WT), single (Δ*qrr*3), double (Δ*qrr*2,3), and triple (Δ*qrr*1,2,3) *qrr* mutants by northern blot. We engineered the set of mutants to retain *qrr*4 because, as mentioned, Qrr4 is the most abundant Qrr sRNA in wild-type *V. cholerae*, and hence sequential deletion of *qrr*1, *qrr*2, and *qrr*3 while keeping *qrr*4 intact causes the least perturbation to the Qrr pool.

Figure 7A shows that indeed Qrr4 levels increase with increasing deletions of *qrr* genes, indicating that *qrr*4 dosage compensation is sensitive to the loss of any one of the other *qrr* genes. To examine the sensitivity of dosage compensation at the other *qrr* promoters, we quantified the level of each of the individual Qrr sRNAs in the wild-type, single, double, and triple *qrr* mutant strains (Figure 7B). The level of each RNA species in the wild type is set to 1 (green bars). In the Δ*qrr*3 single mutant strain, each of the remaining Qrr sRNA levels is higher than in the wild type (compare pink bars with green bars). In the Δ*qrr*2,3 double-mutant strain, levels of the two remaining Qrrs are even higher (compare yellow bars with pink bars). Finally, in the Δ*qrr*1,2,3 triple-mutant strain, Qrr4 is at the highest level (compare blue bar with yellow bar). Thus, each *qrr* promoter is sensitive to the deletion of any single *qrr* gene.

The figure shows that sequential deletion of *qrr* genes apparently triggers the production of sufficient remaining Qrr sRNA to provide wild-type-like repression of *hapR* mRNA levels (right group of bars). This result could indicate that the dosage compensation mechanism is exquisitely accurate, that is, that the exact amount of the remaining Qrr sRNAs is synthesized to repress *hapR* mRNA to exactly wild-type levels. Alternatively, an additional factor required for *hapR* mRNA degradation could be limiting. In this scenario, the *hapR* mRNA level present in wild-type cells at LCD is the lowest *hapR* level achievable by excess production of the Qrr sRNAs. To test this latter possibility, we examined *hapR* mRNA levels in a *V. cholerae* Δ*qrr*1–4 strain overexpressing *qrr*4 from the Ptac promoter. This construct is not sensitive to dosage compensation (see Figure 2B). We reasoned that, if Qrr-mediated repression of *hapR* mRNA is not limited by some other factor, *hapR* mRNA levels should decrease in the Qrr4 overexpression strain. Figure 7B shows that Qrr4 levels in this strain are ~250-fold higher than in wild type (see Qrr4 and compare black bar with green bar). Nonetheless, *hapR* mRNA levels in this strain are essentially identical to wild type (see *hapR*, compare black bar with green bar). Thus, Qrr-mediated *hapR* mRNA degradation operates at its full capacity in wild-type *V. cholerae* cells at LCD, and production of additional Qrr sRNAs does not increase *hapR* mRNA degradation.

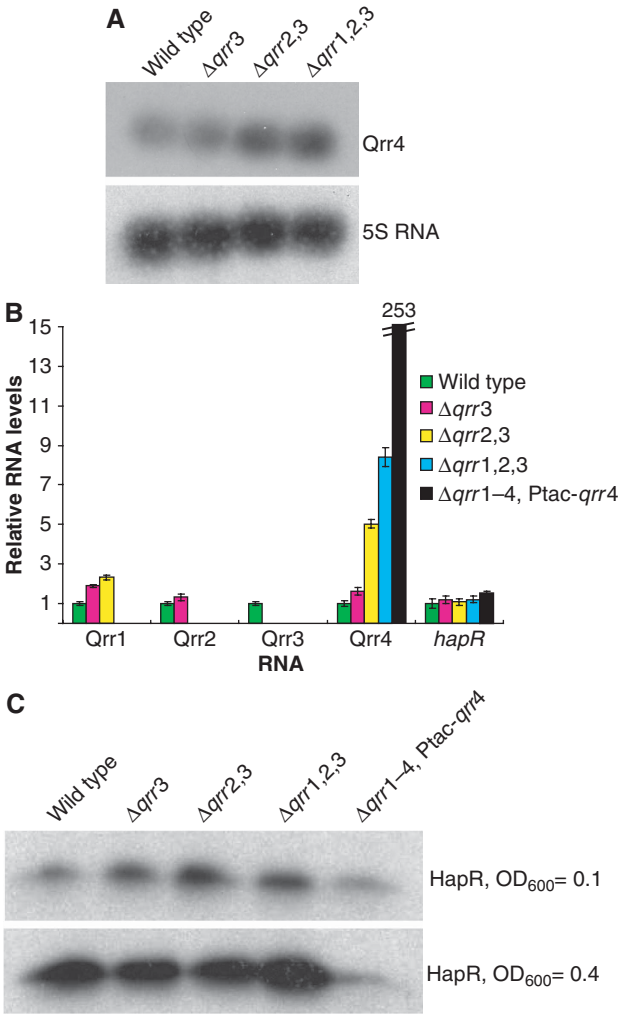

Figure 7 Dosage compensation is sensitive to the loss of any individual *qrr* gene. (**A**) Northern blot showing Qrr4 levels in *V. cholerae* wild-type, single (Δ*qrr*3), double (Δ*qrr*2,3), and triple (Δ*qrr*1,2,3) *qrr* mutants grown to $OD_{600} = 0.1$. 5S RNA is shown as a loading control. (**B**) Relative levels of Qrr1–4 and *hapR* mRNA were measured by RT–PCR in the same strains shown in (A) as well as in a Δ*qrr*1,2,3,4 mutant strain carrying a plasmid-borne Ptac-*qrr*4 overexpression construct (Δ*qrr*1–4 Ptac-*qrr*4). Error bars indicate one standard deviation from the mean of triplicate measurements. The standard deviation is ± 25.1 for Qrr4 levels in the Δ*qrr*1–4 Ptac-*qrr*4 *V. cholerae* strain. (**C**) Western blot showing HapR protein levels in the same strains as (B) at LCD (top panel, $OD_{600} = 0.1$) and at intermediate cell density (bottom panel, $OD_{600} = 0.4$).

Figure 7B shows that at LCD, even with vastly more Qrr present, the *hapR* mRNA level cannot decrease to below that present in wild-type cells. We wondered what happens to HapR protein levels following overproduction of Qrr sRNAs. To examine this, we determined HapR levels in the same *V. cholerae* strains shown in Figure 7B. At LCD ($OD_{600} = 0.1$, Figure 7C top panel), HapR levels are only slightly elevated in the three *qrr* mutants compared with wild type. This result suggests that sufficient Qrr sRNAs are produced by dosage compensation in the *qrr* mutants to promote wild-type-like repression of HapR synthesis. Importantly, HapR protein levels are at the lowest level in both the *V. cholerae* wild-type (WT) and the Ptac-*qrr*4 overexpression strain (Δ*qrr*1–4, Ptac-*qrr*4) confirming that HapR synthesis is maximally repressed in the *V. cholerae* wild-type strain at LCD, and that additional Qrr sRNAs do not cause further repression of HapR synthesis. Thus, provided that the Qrr levels are not limiting for repression, exact compensation of the Qrr sRNA pool is not required for wild-type-like *hapR* repression at LCD.

Precise calibration of total Qrr levels, although apparently not important at LCD because HapR is fully repressed, could be critical for the timely termination of individual behaviours and initiation of group behaviours as *V. cholerae* transitions from LCD to HCD conditions. To address this hypothesis, we examined HapR levels in the same set of *V. cholerae* strains shown in Figure 7B, at an intermediate cell density ($OD_{600} = 0.4$, Figure 7C, bottom panel). HapR levels are similar in the wild-type and the dosage-compensated single (Δ*qrr*3), double (Δ*qrr*2,3), and triple (Δ*qrr*1,2,3) *qrr* mutant strains at intermediate cell density. Notably, this level of HapR protein is higher than that in the Δ*qrr*1–4, Ptac-*qrr*4 strain, showing that HapR remains fully repressed in the non-dosage-compensated *qrr*4 overexpression strain (Δ*qrr*1–4, Ptac-*qrr*4). Thus, during the quorum-sensing transition, sequential deletion of *qrr* genes triggers the production of almost exactly the amount of the remaining Qrr sRNAs required to provide wild-type, that is, accurate, repression of HapR. We conclude that, in response to small perturbations in Qrr levels, the Qrr sRNAs are precisely controlled by dosage compensation in *V. cholerae*, and thus calibrated to provide quite accurate quorum-sensing behaviour.

Discussion

Cell-population density, which is monitored through quorum sensing, is among the key parameters that regulate progression through the *V. cholerae* infectious cycle (Zhu and Mekalanos, 2003). Among the known targets of quorum sensing are cholera toxin (*ctxA*) and the toxin-co-regulated pilus (*tcpP*), virulence factors required for colonization of the host intestinal lining and induction of the severe diarrhoea characteristic of *V. cholerae* infection (Miller *et al*, 2002; Zhu *et al*, 2002), the *vps* operon required for biofilm formation (Hammer and Bassler, 2003), and *hapA*, a protease needed for detachment of individual cells from HCD biofilms (Jobling and Holmes, 1997). Thus, not surprisingly, a Δ*luxO V. cholerae* strain, which is incapable of quorum sensing, is avirulent in an infant mouse model (Miller *et al*, 2002). Four homologous sRNAs, Qrr1–4, constitute the signalling-hub of the quorum-sensing network. Input information about the surrounding microbial community and the metabolic state of the cell is combined to control the expression of *qrr*1–4, and, in response, quorum-sensing-regulated behaviours are initiated or terminated by Qrr sRNA regulation of target mRNAs (Lenz *et al*, 2004). Hence, the expression patterns of *qrr*1–4 determine the precise cell-population densities at which quorum-sensing target genes are activated or repressed. Therefore, expression of *qrr*1–4 must be tightly controlled to obtain proper timing of quorum-sensing transitions.

Here, we report the identification of a new target of the Qrr sRNAs and a negative feedback loop that assists in the regulation of Qrr levels. The new sRNA target, *luxOU* mRNA, and a previously described Qrr sRNA target, *hapR* mRNA, are destabilized upon pairing with the Qrr sRNAs, and thus LuxO and HapR production are repressed by the Qrr sRNAs. Additionally, LuxO and HapR are both activators of *qrr* transcription. This regulatory arrangement generates two feedback loops, the LuxO-Qrr feedback loop and the HapR-Qrr feedback loop, which together enable fine-tuning of Qrr levels (Figure 1). The abundance of the Qrr sRNAs in wild-type *V. cholerae* is Qrr4 > Qrr2 ≈ Qrr3 > Qrr1 (Lenz *et al*, 2004; Lenz and Bassler, 2007). We show that deletion of any one of the three least abundant Qrr sRNAs (*qrr*1, 2, or 3) elicits a compensatory increase in the remaining sRNAs that is sufficient to maintain wild-type-like repression of a target (*hapR*) mRNA at LCD. Interestingly, dosage compensation promotes the identical, wild-type-like, degradation of *hapR* mRNA in these mutants, but slight differences among the mutants can be observed at the level of HapR protein accumulation. This finding indicates that, beyond promoting degradation of the *hapR* mRNA, the Qrrs could have an additional function in blocking *hapR* translation. This is consistent with the observation that other Hfq-dependent sRNAs function primarily to inhibit translation of target mRNAs, with the destabilization of the mRNA being a secondary effect of the inhibition of translation (Morita *et al*, 2006; Aiba, 2007).

Overexpression of a Qrr sRNA at LCD does not facilitate degradation of *hapR* mRNA beyond what occurs in wild-type cells at LCD, suggesting that in wild-type *V. cholerae*, the Qrr sRNAs are not the limiting component for *hapR* mRNA degradation at LCD. Rather, we hypothesize that an additional factor required for sRNA-mediated *hapR* mRNA decay limits the rate of *hapR* mRNA degradation. Most likely candidates for this limiting factor are the RNA chaperone Hfq, which is required for Qrr repression of *hapR* mRNA, or the endonuclease RNase E, which is often involved in sRNA-mediated mRNA decay (Masse *et al*, 2003; Aiba, 2007). This result implies that above a certain Qrr-threshold, precise regulation of the Qrr pool is not required for wild-type-like *hapR* repression at LCD. By contrast, we show that Qrr levels must be kept precisely in balance to relieve repression of HapR synthesis in a timely manner, as *V. cholerae* transitions from LCD to HCD. This allows HapR to direct the appropriate pattern of expression of quorum-sensing-regulated target genes.

The best-known example of gene dosage compensation in bacteria involves the ribosomal RNA (rRNA) operons of *E. coli*. *E. coli* contains seven copies of the rRNA operon. Increasing or decreasing the number of these operons does not alter the total level of cellular rRNA, due to gene dosage compensation (Jinks-Robertson *et al*, 1983; Condon *et al*, 1993). The exact mechanism of dosage compensation is not

understood in this system, but it is known that rRNA must be assembled into translation-capable ribosomes to feed back to the rRNA genes (Cole *et al*, 1987). Similarly, we show here that Qrr dosage compensation requires functional Qrr sRNAs because the dosage compensation mechanism relies on regulation of target mRNAs by the Qrr sRNAs. This ensures that dosage compensation is based on sRNA activity, and, as a consequence, only functional copies of the sRNAs are accounted for by the dosage compensation mechanism. An additional consequence of activity-based dosage compensation is that it allows for differences in the potency of the four Qrr sRNAs. If, for example, Qrr1 is the least potent sRNA repressor (i.e., Qrr1 binds target mRNAs with the lowest affinity), then a higher concentration of Qrr1 than the other Qrr sRNAs is required to repress a particular target mRNA pool. Therefore, a *qrr*1 deletion should be compensated for by a sub-stoichiometric increase in the remaining Qrrs. We suspect that differences in sRNA repressor potency explain why the total levels of Qrr sRNAs in the *qrr* triple mutants in Figure 6A do not correspond directly to the degree of repression of *hapR* mRNA shown in Figure 6B.

What advantage do two, rather than one, negative feedback loops provide to Qrr dosage compensation? We suggest that the individual feedback loops operate under different regimes, as LuxO-P and HapR are maximally produced at different cell densities. HapR is produced only at HCD and therefore affects only *qrr* transcription following the transition from HCD to LCD conditions (Svenningsen *et al*, 2008). Hence, the HapR-Qrr feedback loop most likely does not contribute to Qrr dosage compensation under conditions in which *V. cholerae* is consistently at LCD. LuxO-P, on the other hand, is present only at LCD and could mediate Qrr dosage compensation under this condition. Thus, using two negative feedback loops increases the adaptability of the dosage compensation mechanism to different conditions. The putative third negative feedback loop (Figure 5) could increase the plasticity of the dosage compensation mechanism even further.

Interestingly, the wiring of the regulatory feedback loops described here results in calibration of the total level of Qrr activity, rather than calibration of a specific level of the individual Qrr sRNAs. This design suggests that the combined activity of the Qrr sRNAs, and not their individual contributions, is the critical parameter that *V. cholerae* monitors to ensure proper timing of quorum-sensing-regulated behaviours. Supporting this observation is our finding that all four Qrr sRNAs each regulate the three known Qrr targets—*luxO*, *hapR*, and *vca0939* (encoding a GGDEF enzyme)—and no functions exclusive to one or a subset of the four Qrr sRNAs have been identified (Lenz *et al*, 2004; Hammer and Bassler, 2007). However, our finding that the four *qrr* promoters are affected to different extents by each individual feedback loop (Figure 5) could indicate that there exist conditions in which it is beneficial for *V. cholerae* to exclusively increase the expression of one particular Qrr sRNA.

There exists an upper bound to the functioning of the dosage compensation mechanism: in triple *qrr* mutant strains where *qrr*4 is among the deleted *qrr* genes, whereas the remaining *qrr* gene is upregulated, its promoter is not activated strongly enough to completely compensate for the lack of the other three *qrr* genes. Consistent with this, we find that *hapR* mRNA is not fully repressed under this condition. We reason that this constraint on the functioning of the dosage compensation mechanism is due to LuxO auto-repression. In *V. harveyi*, LuxO represses its own expression, irrespective of its phosphorylation state, by binding to a site overlapping the −35 sequence of the *luxO* promoter, thereby preventing RNA polymerase from initiating transcription (Tu *et al*, manuscript in preparation). The LuxO-binding site and the −35 box are completely conserved in *V. cholerae*, suggesting that LuxO auto-repression functions equivalently in this organism (Figure 1, LuxO auto-repression loop). As LuxO represses its own promoter, LuxO can only accumulate to within a confined range even in the absence of Qrr-mediated repression. Therefore, the *qrr* promoters for which LuxO has the lowest affinity do not become fully activated in the triple *qrr* mutants. We suspect that the limits to dosage compensation measured by deletion of three of the four *qrr* genes are not relevant for wild-type *V. cholerae*. Rather, our measurements of Qrr levels following more minor alterations in gene dosage (Figure 7) suggest that under relatively physiological conditions, the dosage compensation mechanism is quite accurate, and results in target gene expression identical to that observed in wild-type *V. cholerae*.

The analogous quorum-sensing circuit in *V. harveyi* possesses five homologous Qrr sRNAs, which function additively to control quorum sensing. Specifically, deletion of one or more of the *qrr* genes in *V. harveyi* results in intermediate expression of quorum-sensing behaviours (Tu and Bassler, 2007). The quorum-sensing circuit of *V. harveyi* contains all four feedback loops described in this work (Figure 1) (Chatterjee *et al*, 1996; Tu *et al*, 2008), and hence it seems paradoxical that the Qrrs function additively in one system and redundantly in the other. Using the logic outlined in the above section, we hypothesize that different degrees of LuxO auto-repression in *V. harveyi* and *V. cholerae* could explain this finding: If LuxO auto-repression is stronger in *V. harveyi* than in *V. cholerae*, and thus, LuxO is confined to a more restricted concentration range in *V. harveyi* than in *V. cholerae*, then dosage compensation in *V. harveyi* may only be capable of accurately calibrating the Qrr sRNA levels in response to very small fluctuations in Qrrs. Thus, in *V. harveyi*, accurate compensation does not occur even in the absence of a single *qrr* gene, which would manifest in *qrr* mutant phenotypes that appear additive.

The continued discovery of bacterial sRNAs now provides many examples of multiple redundant sRNAs (Rudd, 1999; Weilbacher *et al*, 2003; Wilderman *et al*, 2004; Guillier and Gottesman, 2006; Kay *et al*, 2006; Urban and Vogel, 2008). A few examples of sRNA dosage compensation have been reported (Weilbacher *et al*, 2003; Kay *et al*, 2006), but in most cases of redundant sRNAs, the issue of dosage compensation has not been addressed. Hence, it is possible that dosage compensation among homologous sRNAs is a common phenomenon. We propose that the apparent requirement for multiple, redundant sRNAs in many bacterial regulatory circuits is coupled to their stoichiometric mode of action. Coupled degradation of an sRNA with its mRNA target is predicted to provide ultrasensitivity to sensory circuits and also to enable prioritization of expression of multiple mRNA targets (Lenz *et al*, 2004; Mitarai *et al*, 2007). This is because if the rate of synthesis of an sRNA is even slightly higher than the rate of synthesis of its mRNA partner, the sRNA can

accumulate and the mRNA pool can be effectively eliminated. Reciprocally, if the rate of synthesis of an mRNA exceeds that of the partner sRNA, then the mRNA can accumulate and the sRNA disappears (Lenz *et al*, 2004). Similarly, if two mRNAs, m1 and m2, have different affinities for a shared sRNA regulator, the mRNA with the highest affinity for the sRNA, say m1, will be degraded first. This can effectively protect m2 from sRNA repression if all the sRNA is degraded along with the m1 mRNA (Mitarai *et al*, 2007). These characteristics of sRNA-mediated regulation make it crucial that sRNA production is tightly controlled because a small change in the production rate of the sRNA can dramatically affect the expression of target mRNAs.

Variations in the amount of RNA produced from a single gene over time or from cell to cell are caused by fluctuations in the amount, location, and activity of the necessary transcription factors (extrinsic noise) as well as by inherent stochasticity in gene transcription (intrinsic noise) (Elowitz *et al*, 2002). We propose that dosage compensation with multiple redundant sRNAs keeps variations in sRNA levels to a minimum. First, negative feedback regulation of any gene tends to reduce fluctuations in the gene product and maintain homoeostasis (Seshasayee *et al*, 2006). Second, the inclusion of multiple redundant genes in a negative feedback loop (i.e., dosage compensation) has been shown theoretically to buffer downstream processes from variations arising from extrinsic noise (Kafri *et al*, 2006). This can be understood intuitively in the case of the Qrr sRNAs, as fluctuations in the synthesis of one *qrr* gene caused by variations in local concentrations of LuxO and σ^{54}-RNA polymerase holoenzyme will be counteracted by altered expression of the additional *qrr* loci. If correct, the increased accuracy in downstream gene expression stemming from Qrr dosage compensation could explain how multiple copies of the *qrr* genes have been selected and maintained throughout the Vibrios.

Materials and methods

Bacterial strains and culture conditions

Vibrio cholerae strains used in this study are derivatives of El Tor strain C6706str2 (Thelin and Taylor, 1996). *E. coli* strains S17-1λ*pir* (de Lorenzo and Timmis, 1994) and ElectroMAX DH10B (Invitrogen) were used for cloning and plasmid propagation. All strains were grown in LB broth with aeration or on LB agar at 30°C. Antibiotics were used at the following concentrations (μg/ml): ampicillin 200, kanamycin 100, chloramphenicol 10, polymyxin B 50, streptomycin 1000, and tetracycline 10.

DNA manipulations

All bacterial strains and plasmids used in this study are listed in Supplementary Table S1. DNA manipulations were performed according to Sambrook *et al* (Sambrook *et al*, 1989) unless otherwise noted. Herculase polymerase (Stratagene) was used for PCRs in cloning procedures, and Taq polymerase (Roche) was used for all other PCRs. *V. cholerae* in-frame deletions were constructed by the method of Skorupski and Taylor (1996). The *lux* transcriptional fusion plasmids were constructed as reported (Lenz *et al*, 2004) and introduced into *V. cholerae* by conjugation. The LuxO–GFP protein fusion was constructed by cloning the *luxO* promoter sequence including the first 10 codons of the *luxO* ORF immediately upstream of the *gfp* gene encoded on pCMW1 (Waters and Bassler, 2006) using the *Spe*I and *Nhe*I restriction sites. This strategy resulted in plasmid pSLS146. The *luxO*^{AUCC} mutation was introduced into pSLS146 by Quickchange mutagenesis (Invitrogen) to generate pSLS152. An oligonucleotide containing the −35 to +1 sequence of the Ptac promoter as well as the first 20 nucleotides of the *V. cholerae qrr*4 gene was used to amplify *qrr*4 from *V. cholerae* C6706str2 chromosomal DNA in a PCR with a downstream primer complementary to the 3′-end of *qrr*4. This Ptac-*qrr*4 construct was subsequently cloned into pEVS141 (Dunn *et al*, 2006) using *Eco*RI and *Bam*HI restriction sites to generate pSLS155. *E. coli* strain SLS1277, which carries *V. cholerae qrr*4 under the control of the chromosomal P_{BAD} promoter, was obtained by recombineering (Court *et al*, 2002). Specifically, the *araBAD* genes of *E. coli* MG1655, which are controlled by the P_{BAD} promoter, were replaced by *V. cholerae qrr*4 linked to a kanamycin resistance cassette, and the desired recombinant was obtained by selection for kanamycin resistance.

Bioluminescence assays

Bioluminescence was measured as described previously (Lenz *et al*, 2004). In all assays, overnight cultures were diluted 1000-fold and grown to $OD_{600} = 0.1$, at which point light production was measured. Relative light units (RLU) are defined as counts per min per ml per OD_{600}^{-1}.

Northern blot analysis

Northern blots were performed as described (Martin *et al*, 1989; Svenningsen *et al*, 2008) except that single-stranded DNA probes were designed to hybridize to the entire length of the sRNAs and were prepared by asymmetric PCRs. A common Qrr probe was made to hybridize to the 32-bp region that is 100% conserved among the four Qrr sRNAs. This probe was a radioactive-labelled Starfire™ (Integrated DNA Technologies) oligonucleotide with the sequence 5′-ACTAACAACGTCAGTTGGCTAGGTGACCCT-3′. For single time-point northern blots, overnight cultures were diluted 1000-fold and grown to $OD_{600} = 0.1$, at which point total RNA was collected as described (Svenningsen *et al*, 2008; Tu *et al*, 2008). For Figure 4B, cultures were grown as described above, and rifampicin was added at 100 μg/ml when the cells reached $OD_{600} = 0.1$. Aliquots were collected every 30 s after rifampicin addition, combined with 0.2 volumes of stop solution (Papenfort *et al*, 2008), and snap-frozen in liquid nitrogen. Signal intensities were quantified using an Alpha Innotech FluorChem image analysis system.

Western blot analysis

Overnight cultures of the indicated *V. cholerae* strains were diluted 1000-fold in fresh LB medium. At $OD_{600} = 0.1$ and 0.4, cells were collected and resuspended in loading buffer (Henke and Bassler, 2004). Immunoblotting was performed as described (Henke and Bassler, 2004). Membranes were exposed to polyclonal HapR antiserum (Lenz *et al*, 2004).

Flow cytometry

Fluorescence of individual *E. coli* SLS1277 cells carrying pSLS146 or pSLS152 that had been grown overnight with or without 0.4% arabinose was measured on a Becton Dickinson FACS Aria cell sorter. Data were analysed using the Becton Dickinson FACSDiva software. GFP fluorescence values reported here represent the mean of 10 000 individual cells.

Quantitative real-time PCR analysis

V. cholerae overnight cultures were diluted 1000-fold and grown to $OD_{600} = 0.1$ at which point total RNA was collected as described above for northern blot analysis. Samples were treated with DNAse I (Ambion). Purified RNA was quantified by triplicate readings on a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies). cDNA synthesis and real-time PCR analysis were carried out as described previously (Tu and Bassler, 2007). *hfq* was used as the endogenous control. Primer sequences are available upon request.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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