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 coordinate 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 beha-

viours (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 σ54, 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. D 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 (Weibacher 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-
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 Δqrr2,3,4 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
Dosage compensation acts at the level of transcription of the qrr genes

| Wild type | qrr1-lux<sup>a</sup> | qrr2-lux<sup>a</sup> | qrr3-lux<sup>a</sup> | qrr4-lux<sup>a</sup> |
|-----------|-----------------|-----------------|-----------------|-----------------|
| 56 (3)    | 140 (20)        | 7 (3)           | 74 (15)         |
| Δqrr1-4   | 149 (3)         | 373 (28)        | 159 (5)         | 378 (26)        |
| ΔhapR     | 28 (1)          | 154 (28)        | 6 (1)           | 47 (30)         |
| ΔhapR Δqrr1-4 | 34 (1) | 226 (55)        | 29 (10)         | 179 (52)        |
| Fold repression<sup>b</sup> | 2.7 (0.05) | 2.7 (0.16) | 24 (0.39) | 5.1 (0.22) |
| ΔhapR Δqrr1-4 | 1.2 (0.04) | 1.5 (0.30) | 4.8 (0.35) | 3.8 (0.69) |

<sup>a</sup>Light production from the indicated qrr-lux construct was measured at OD<sub>600</sub> = 1.0 in the indicated V. cholerae strains. The average relative light units (RLU/10<sup>6</sup>) from three independent cultures is reported. The standard error from the mean (RLU/10<sup>6</sup>) is indicated in parentheses.

<sup>b</sup>Fold repression is calculated as the light produced by the Δqrr1–4 mutant divided by the light produced by the isogenic qrr1–4<sup>+</sup> strain.

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 qrr4 gene from an exogenous Ptac promoter in Δqrr4 and Δqrr1–4 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-qrr4 construct is not sensitive to alterations in sRNA levels. Thus, we conclude that, at least for qrr4, 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 exogenous 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 qrr4 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...
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 constructs in a ΔhapR V. cholerae strain with that in a ΔhapR, Δqrr1–4 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 ΔhapR strains because any feedback loop requiring HapR will not be functioning in the ΔhapR strains. Indeed, when compared with the wild-type strain background, the extent of dosage compensation is reduced for all four qrr promoters in the ΔhapR 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 qrr1 and qrr2, dosage compensation at qrr3 and qrr4 continued to occur in the ΔhapR strains. Thus, qrr1 and qrr2, 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, qrr3 and qrr4, 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 qrr3 and qrr4 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 Δqrr1–4 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 Δqrr1–4, Ptac-qrr4), 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 qrr4 from the chromosome, under control of the PBAD 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<sup>ATCC</sup>, 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 ΔhapR strain lacking the HapR-Qrr feedback loop (white bars), the luxO<sup>ATCC</sup> strain, which lacks the LuxO-Qrr feedback loop (grey bars), and the ΔhapR, luxO<sup>ATCC</sup> double mutant, which lacks both feedback loops (striped bars). Qrr dosage compensation was measured as the fold repression of...
each qrr-lux transcriptional fusion in the qrr1-4+ strain compared with that in the isogenic Δqrr1-4 strain.

For reference, we show again that the HapR-Qrr feedback loop is involved in dosage compensation (compare white bars with black bars). The luxOAUCC 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 qrr1 and qrr2 is largely due to the HapR-Qrr feedback loop. By contrast, the Qrr-LuxO feedback loop is the major source of dosage compensation for qrr4. 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 qrr1, qrr2, and qrr4, the two feedback loops are sufficient to account for dosage compensation. Remarkably, qrr3, 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 qrr3.

### 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.

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**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 luxOUAUCC mutant. (B) Degradation of the luxOU mRNA was measured by northern blot in V. cholerae wild-type, Δqrr1-4 and Δqrr1-4, Ptac-qrr4 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 LuxOAUCC–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.

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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), ΔhapR (white bars), luxO$_{AUCC}$ (grey bars) and ΔhapR, 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 Δqrr1–4 mutant divided by the light produced from the isogenic qrr1–4$^{+}$ strain. Regarding the qrr3–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 ± 0.09 for qrr3–lux in wild type, and ± 0.09 for qrr3–lux in the luxO$_{AUCC}$ 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), Δqrr2,3,4 (denoted qrr1$^{+}$), Δqrr1,3,4 (denoted qrr2$^{+}$), Δqrr1,2,4 (denoted qrr3$^{+}$), and Δqrr1,2,3 (denoted qrr4$^{+}$) _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 Δqrr1–4 _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.

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 qrr1$^{+}$ 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 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 qrr4$^{+}$ triple-mutant strain, hapR mRNA levels are as low as in wild type; however, in the three other triple mutant strains, in which only qrr1, qrr2, or qrr3 is present, hapR mRNA levels are higher than in wild type. For comparison, we show that in the Δqrr1–4 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 Δqrr1–4 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 (Δqrr3), double (Δqrr2,3), and triple (Δqrr1,2,3) qrr mutants by northern blot. We engineered the set of mutants to retain qrr4 because, as mentioned, Qrr4 is the most abundant Qrr sRNA in wild-type V. cholerae, and hence sequential deletion of qrr1, qrr2, and qrr3 while keeping qrr4 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 qrr4 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 Δqrr3 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 Δqrr2,3 double-mutant strain, levels of the two remaining Qrrs are even higher (compare yellow bars with green bars). Finally, in the Δqrr1,2,3 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 Δqrr1–4 strain overexpressing qrr4 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 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\textsubscript{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-qrr4 overexpression strain (Δqrr1–4, Ptac-qrr4) 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\textsubscript{600} = 0.4, Figure 7C, bottom panel). HapR levels are similar in the wild-type and the dosage-compensated single (Δqrr3), double (Δqrr2,3), and triple (Δqrr1,2,3) qrr mutant strains at intermediate cell density. Notably, this level of HapR protein is higher than that in the Δqrr1–4, Ptac-qrr4 strain, showing that HapR remains fully repressed in the non-dosage-compensated qrr4 overexpression strain (Δqrr1–4, Ptac-qrr4). 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 HapR. Therefore, expression of qrr1–4 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, luxO/U 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 (qrr1, 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 tRNA 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 qrr1 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 qrr4 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; Wellbacher 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 (Wellbacher 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 (intrinsinc 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 homeostasis (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 σ44-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**

_V. cholerae_ strains used in this study are derivatives of El Tor strain C6706str2 (Thelin and Taylor, 1996). _E. coli_ strains S17-1, K12 (Kafri et al., 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 SpeI and NheI restriction sites. This strategy resulted in plasmid pSLS146. The luxO MutG mutation was introduced into pSLS146 by Quickchange mutagenesis (Invitrogen) to generate pSLS152. An oligonucleotide containing the −35 to +1 sequence of the Tet promoter as well as the first 20 nucleotides of the _V. cholerae_ qrr4 gene was used to amplify qrr4 from _V. cholerae_ C6706str2 chromosomal DNA in a PCR with a downstream primer complementary to the 3′-end of qrr4. This Tet-qr4 construct was subsequently cloned into pEVSA141 (Dunn et al., 2006) using EcoRI and BamHI restriction sites to generate pSLS155. _E. coli_ strain SLS1277, which carries _V. cholerae_ qrr4 under the control of the chromosomal P_BAD promoter, was obtained by recombining (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_ qrr4 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 OD600 = 0.1, at which point light production was measured. Relative light units (RLU) are defined as counts per min per ml per OD600.

**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′-ACTAACAAGCTAGGTGACCCT-3′. For single time-point northern blots, overnight cultures were diluted 1000-fold and grown to OD600 = 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 OD600 = 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 OD600 = 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 FACS Diva 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 OD600 = 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). hqg 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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