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A single *qrr* gene is necessary and sufficient for LuxO-mediated regulation in *Vibrio fischeri*

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Summary

All members of the *Vibrionaceae* harbor LuxO, a response regulator that integrates outputs from various signaling systems, ultimately controlling specific traits that are crucial to the distinct biology of each species. LuxO is phosphorylated in response to low cell density, activating the transcription of a family of small RNAs called Qrrs, which in turn, control the levels of a global regulatory protein conserved within the *Vibrionaceae*. Although the function of each Qrr is similar, the number of *qrr* genes varies among the different species. Using a bioinformatics approach, we have determined the number of *qrr* genes in fully-sequenced *Vibrionaceae* members. Phylogenetic analysis suggests the most recent common ancestor of all *Vibrionaceae* shared a single, ancestral *qrr* gene, which duplicated and diverged into multiple *qrr* genes in some present-day vibrio lineages. To demonstrate that a single *qrr* gene is sufficient to mediate repression of LitR, the global regulator in *Vibrio fischeri*, we have performed a series of genetic and phenotypic analyses of the LuxO pathway and its output. Our studies contribute to a better understanding of the ancestral state of these pathways in vibrios, as well as to the evolution and divergence of other sRNAs within different bacterial lineages.

Introduction

The natural symbiosis between the bacterium *Vibrio fischeri* and the Hawaiian squid *Euprymna scolopes* has become a model system to explore the mechanisms underlying the specific interactions between bacteria and host epithelial tissue (Visick and Ruby, 2006; Nyholm and McFall-Ngai, 2004). *V. fischeri* is a Gram-negative bacterium that exclusively colonizes a specialized light-emitting organ within the squid. Because juvenile squid hatch uncolonized and acquire their bacterial symbionts from the surrounding seawater (Nyholm *et al.*, 2000), the early stages of colonization have been under thorough investigation and are now well-characterized. Initially, *V. fischeri* cells associate with mucus secreted by ciliated cells on the surface of the light organ. After 2–3 hours, the bacteria migrate through pores on the surface of the light organ that lead to interior crypts that house and nourish the bacteria.

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Author Contributions

T.M. and M.S.W. performed the bioinformatics and phylogenetic analyses. T.M. and X.C. performed the qRT-PCR experiments. T.M., X.C., and D.O. performed the competition experiments. T.M. performed all other experiments. T.M., M.S.W., and E.G.R. wrote the paper.

Within these crypts, *V. fischeri* cells establish a beneficial infection that persists throughout the life of the squid.

One of the hallmarks of this symbiotic association between *V. fischeri* and *E. scolopes* is the bioluminescence produced by the bacteria for the benefit of the host. Bioluminescence is necessary for success of the symbiosis; that is, non-luminescent mutants of *V. fischeri* are unable to persist normally within the host (Visick *et al.*, 2000). The genes involved in light production are commonly referred to as the *lux* genes and are located in two divergent operons, one encoding *luxR*, and the other consisting of *luxICDABEG* (Meighen, 1993). Expression of this locus is necessary and sufficient for light production in *V. fischeri*. The *luxI* gene encodes a protein that synthesizes a small molecule involved in the regulation of the *lux* genes (discussed below). The luciferase enzyme ultimately responsible for the emission of light is comprised of α and β subunits that are encoded by *luxA* and *luxB*, respectively. Luciferase catalyzes the oxidation of a long-chain aliphatic aldehyde and reduced flavin mononucleotide, which results in the emission of light. The *luxC*, *luxD*, and *luxE* genes are responsible for recycling the aliphatic acid produced by the luciferase reaction back into the aliphatic aldehyde substrate. Recently, the *luxG* gene of *Photobacterium leiognathi* was shown to encode a flavin reductase involved in supplying the reduced flavin mononucleotide for the luciferase reaction (Nijvipakul *et al.*, 2008).

As mentioned above, LuxI synthesizes a small molecule, namely *N*-3-oxo-hexanoyl homoserine lactone (3-oxo-C6), which freely diffuses across cell membranes (Kaplan and Greenberg, 1985). When isolated from culture extracts, this autoinducer molecule exhibits biological activity, inducing luminescence in various isolates of *V. fischeri* (Eberhard *et al.*, 1981). Subsequent studies demonstrated that the mechanism underlying this autoinduction of luminescence is the binding of 3-oxo-C6 to LuxR, a transcriptional regulator that activates the expression of the *lux* operon (Stevens and Greenberg, 1997). The LuxR-LuxI regulatory module of *V. fischeri* represents the first characterized example of quorum sensing, a term that describes the general process by which bacteria synthesize, secrete, and detect small-molecule autoinducers to coordinate bacterial behaviors at the population level. Since this initial discovery, quorum sensing has been shown to regulate a remarkable number of bacterial processes, including motility, biofilm formation, host colonization, and virulence (Waters and Bassler, 2005).

In *V. fischeri*, two additional quorum-sensing systems, AinS-AinR and LuxS-LuxP/Q, regulate bioluminescence and host colonization (Lupp and Ruby, 2004). AinR detects the autoinducer octanoyl homoserine lactone (C8), which is produced by AinS (Gilson *et al.*, 1995). Studies of the LuxP and LuxQ homologues in *V. harveyi* have shown that the LuxP/Q complex detects the autoinducer AI-2, which is produced by LuxS (Surette *et al.*, 1999). In the absence of their respective autoinducer ligands, AinR and LuxP/Q independently phosphorylate the phosphotransfer protein LuxU, which in turn phosphorylates LuxO, activating it as a transcription factor. In both *V. harveyi* and *V. cholerae*, phosphorylated LuxO activates multiple genes that encode several small RNAs (Lenz *et al.*, 2004). These quorum regulatory RNAs (Qrr) lead to the degradation of target mRNAs (for example, the *hapR* transcript in *V. cholerae*) in a manner dependent on the RNA chaperone Hfq. In *V. fischeri*, the HapR homologue is called LitR, and has been shown to positively regulate the expression of *luxR* (Fidopiastis *et al.*, 2002; Lupp and Ruby, 2005). Homologous, but distinct, signaling systems have been described in several other *Vibrio* species (Milton, 2006).

Given the high similarity among the components comprising the quorum-sensing systems in the *Vibrionaceae*, we were curious to learn whether LuxO regulates *litR*, and hence

bioluminescence, in *V. fischeri* via one or more sRNAs. As shown below, our results demonstrate that *V. fischeri* uses a single *qrr* gene to mediate regulation by LuxO.

Results

A single *qrr* locus is conserved among all *Vibrionaceae*

Multiple *qrr* genes have been identified in the genomes of *V. cholerae*, *V. harveyi*, *V. parahaemolyticus*, and *V. vulnificus* (Lenz *et al.*, 2004; Tu and Bassler, 2007). To determine the number of *qrr* genes within *V. fischeri* strain ES114, we performed two bioinformatics searches. The first approach consisted of a BLAST search using a 32-nucleotide motif conserved among all identified *qrr* sequences as bait. The second approach used a software program (Infernal 1.0.2) designed to identify members of canonical small RNA families by their sequence and secondary structure. The results of both approaches were the same: the genome of the *V. fischeri* strain ES114 contains only a single *qrr* family member, *qrr1* (VF_2597).

Analogous searches of other vibrio genomes using BLAST and Infernal identified all of the previously reported *qrr* loci of both *V. cholerae* and *V. harveyi* (Lenz *et al.*, 2004; Tu and Bassler, 2007), supporting the robustness of this approach. A phylogenetic analysis revealed a pattern relating the number of putative *qrr* family members and the evolutionary history of the *Vibrionaceae*. Specifically, while *Photobacterium* species and *Vibrio fischeri*-group members contain a single putative *qrr* locus, all other 'core' vibrios contain 4 or 5 *qrr* loci. Phylogenetic reconstructions of the *luxO* and *litR* gene trees of 13 fully-sequenced, representative *Vibrionaceae* demonstrated a pattern of descent similar to that reported in studies of either concatenated housekeeping genes (Urbanczyk *et al.*, 2007) or whole genomes (Thompson *et al.*, 2009). The more basal taxa in these reconstructions (*Photobacterium* species and the *V. fischeri*-group) possess a single *qrr* family member per genome, while the more distal *Vibrio* species have multiple *qrr* sequences (Fig. 1). This evolutionary pattern among the *Vibrionaceae* is supported by the observation that all species with a single copy of a *qrr* family member possess this copy upstream of the *luxO* locus on the large chromosome, while all *Vibrionaceae* with multiple *qrr* loci possess one copy upstream of *luxO* on the large chromosome, and the additional 3–4 loci spread across the small chromosome. From this result, we hypothesized that (i) *luxO* and *qrr1* form a genetic module (the LuxO module) in *V. fischeri*, and (ii) *qrr1* is both necessary and sufficient for LuxO-mediated regulation in this species.

LuxO positively regulates *qrr1*

In *V. cholerae* and *V. harveyi*, LuxO activates the transcription of multiple *qrr* genes dispersed throughout their chromosomes (Lenz *et al.*, 2004; Tu and Bassler, 2007). To determine whether LuxO regulates the transcription of *qrr1* in *V. fischeri*, a two-color fluorescent-reporter plasmid was constructed by cloning the *qrr1* promoter upstream of *gfp*, the gene encoding green fluorescent protein (GFP). The reporter plasmid also contains *mCherry*, which encodes the red fluorescent protein, mCherry, under the control of the *tetA* promoter, which is constitutively expressed in the absence of TetR (Hillen and Berens, 1994). In cells harboring this reporter plasmid, the GFP/mCherry fluorescence ratio is a quantitative measure of *qrr1* transcription that accounts for potential variations in the copy number of the reporter plasmid and differences in cellular growth rates.

For wild-type *V. fischeri*, the GFP/mCherry fluorescence ratio was higher in cultures of low cell density than in cultures of high cell density (Fig. 2), suggesting that quorum sensing regulates *qrr1* expression. This response of *qrr1* expression to cell density depends on LuxO, as *qrr1* transcription in a $\Delta luxO$ strain was both lower and insensitive to cell density

in comparison to wild-type cells. In contrast, no significant difference in the regulation of *qrr1* transcription was observed between $\Delta qrr1$ and wild-type strains, indicating that there is no feedback on the *qrr1* promoter by the signaling components downstream of the LuxO module. Together, these results show that *qrr1* is controlled by the LuxO signaling system in *V. fischeri*.

The LuxO module negatively regulates LitR levels

Based on studies of *V. harveyi* and *V. cholerae*, we hypothesized that the putative regulatory role of Qrr1 in *V. fischeri* is to post-transcriptionally repress LitR levels. To test the effect of a disruption of signaling by the LuxO module on LitR levels, we performed quantitative reverse-transcriptase PCR (qRT-PCR) on RNA extracted from various mutants of *V. fischeri*. The level of *litR* transcript was elevated in both $\Delta luxO$ and $\Delta qrr1$ strains compared to the wild-type strain (Fig. 3A). We also performed Western blot analysis on whole-cell lysates using sera raised against HapR, the LitR homologue (45% amino-acid identity), found in *V. cholerae* C6706 (Liu *et al.*, 2008). These antibodies are able to detect both affinity-purified LitR, as well as LitR from whole-cell lysates (Fig. 3B). LitR was not detected in whole-cell lysates from either the $\Delta litR$ mutant generated in this study (TIM358) or a previously reported antibiotic-marked *litR* mutant (PMF8). Analysis by densitometry showed that the levels of LitR in either $\Delta luxO$ or $\Delta qrr1$ strains are approximately 1.5-fold higher than those in wild-type strains grown under conditions of high cell density (Fig. 3B), suggesting that the disruption of the LuxO module results in the de-repression of LitR levels. Furthermore, at low cell density, the level of LitR was further repressed in wild-type strains (Fig. S1). These results demonstrate that the LuxO module controls the level of LitR in *V. fischeri*.

Elevated LitR levels prime cells for bioluminescence

Previous work demonstrated that LitR binds upstream of the *luxR* promoter and enhances *luxR* transcription (Fidopiastis *et al.*, 2002). To test the transcriptional response of *luxR* to different levels of LitR, we constructed a two-color fluorescent reporter of *luxR* promoter activity. As predicted, in $\Delta luxO$ and $\Delta qrr1$ strains, the level of *luxR* transcription was significantly higher than that in the wild-type strain (Fig. 4A). In contrast, *luxR* transcription was lower in a *litR* mutant strain than in wild-type. It is interesting to note that there is significant transcription of *luxR* independent of *litR* expression levels, which suggests that the LuxO module regulates bioluminescence by finely tuning the level of LuxR. Together, these data show that the LuxO module represses *luxR* transcription by suppressing LitR levels.

In *V. fischeri*, transcription of the bioluminescence (*lux*) genes requires the activation of LuxR as a transcription factor through its binding of the autoinducer *N*-3-oxo-hexanoyl-homoserine lactone (3-oxo-C6) (Stevens and Greenberg, 1997; Urbanowski *et al.*, 2004). In other words, the level of bioluminescence emitted from cultures depends on both the level of LuxR and the concentration of 3-oxo-C6. In light of these observations, we predicted that regulation of bioluminescence by the LuxO module is not detectable unless the level of autoinducer is sufficient to activate the *lux* genes.

To test this prediction, we measured the specific luminescence of mid-log cultures grown in the presence of 120 nM 3-oxo-C6, a concentration sufficient to induce bioluminescence in *V. fischeri* (Lupp and Ruby, 2004). In wild-type cells, we observed a 45-fold increase in luminescence in response to this addition (Fig. 4B). Compared to wild type, *litR* mutants showed a smaller bioluminescence response to 3-oxo-C6. Consistent with our prediction above, strains with a deletion in either *luxO* or *qrr1* exhibited higher levels of bioluminescence than wild-type cells in response to 3-oxo-C6. In $\Delta luxO$ and $\Delta qrr1$ mutants,

the wild-type bioluminescence response to 3-oxo-C6 was restored by complementation *in trans* with an intact copy of the corresponding gene (Fig. S2). Overall, we attribute these differences in the bioluminescence emitted by the various mutants to corresponding changes in *luxI* transcription (Fig. S3) and, thus, in the concentration of transcriptionally active LuxR-autoinducer conjugate. Together, these results demonstrate that strains containing a disrupted LuxO module are 'primed' for bioluminescence due to their enhanced LitR levels.

***qrr1* is necessary for LuxO-mediated regulation of bioluminescence**

The $\Delta qrr1$ and $\Delta luxO$ mutants exhibited similarly enhanced bioluminescence levels when compared to wild type (Fig. 5), suggesting that *qrr1* is necessary for the repression of bioluminescence by LuxO. To determine whether the effects on bioluminescence by these two mutants are synergistic, we constructed a strain that lacks both *qrr1* and *luxO*. The bioluminescence emitted by this double mutant in response to 120 nM 3-oxo-C6 was comparable to that of either single mutant (Fig. 5). This result demonstrates that the effects of *qrr1* and *luxO* on bioluminescence are not synergistic, and that *qrr1* is necessary for the LuxO-mediated repression of bioluminescence.

To test the role of LitR on the bioluminescence response to 3-oxo-C6, we also generated double and triple mutants by introducing the $\Delta litR$ allele into the $\Delta qrr1$ - and $\Delta luxO$ -single and $\Delta (qrr1 luxO)$ -double mutants described above. As expected, the specific luminescence levels of the resulting mutants were lower than of the parental strains that possess a wild-type copy of *litR* (Fig. 5). Interestingly, these specific luminescence levels were also consistently higher than a $\Delta litR$ single mutant, suggesting that a *qrr1*-regulated factor other than *litR* may also control cellular luminescence.

***qrr1* is sufficient for LuxO-mediated regulation of bioluminescence**

To determine whether expression of *qrr1* is sufficient for LuxO-mediated regulation, we introduced a plasmid that constitutively expresses *qrr1* from the *tetA* promoter (pTM312) into a $\Delta (qrr1 luxO)$ double mutant of *V. fischeri*. Compared to vector controls, the double mutant harboring pTM312 displayed repressed levels of *litR* transcript (Fig. 6A). Furthermore, the bioluminescence response resulting from the addition of 120 nM 3-oxo-C6 to the double mutant containing pTM312 was lower than the response by either wild-type or the double mutant containing an empty vector (Fig. 6B), consistent with a reduced level of *litR* transcript and, consequently, LitR-induced LuxR. Taken together, these results demonstrate that expression of *qrr1* is sufficient for LuxO-mediated regulation of *litR* transcript levels and, therefore, of bioluminescence.

The LuxO module is necessary for normal colonization in the host

The data presented above provide evidence that the LuxO module of *V. fischeri* is functional and controls the capacity for bacterial bioluminescence via regulation of *luxR* expression. Previous work using strains containing an antibiotic marker inserted either in *luxO* or in *litR* showed that the overall population size of these mutants within the light organ of *E. scolopes* are indistinguishable from that of wild type (Lupp *et al.*, 2003; Fidopiastis *et al.*, 2002). We have obtained similar results using strains containing the $\Delta luxO$, $\Delta qrr1$, and $\Delta litR$ alleles constructed in this study (data not shown). Furthermore, we have found that squid colonized by $\Delta luxO$, $\Delta qrr1$, or $\Delta litR$ mutants emit comparable levels of light (data not shown), confirming published results involving the mutants containing antibiotic markers linked to *luxO* and *litR* that are described above.

We also tested the ability of each mutant to colonize the squid in the presence of a wild-type strain. By 48 h, the $\Delta litR$ mutant TIM358 had out-competed the wild-type strain (Fig. 7), confirming the result previously observed using the antibiotic-marked mutant PMF8

(Fidopiastis *et al.*, 2002). In contrast, both the $\Delta qrr1$ and $\Delta luxO$ mutants displayed colonization defects when competed against wild type (Fig. 7). This defect was abrogated by the complementation of the respective gene *in trans* for either mutant (data not shown). We hypothesized that the colonization defect was due to the enhanced levels of LitR in the $\Delta qrr1$ and $\Delta luxO$ mutants. To test this hypothesis, we conducted a series of epistasis experiments using $\Delta qrr1 \Delta litR$ and $\Delta luxO \Delta litR$ double mutants. Although the $\Delta litR$ allele returned the $\Delta qrr1$ and $\Delta luxO$ mutants to colonization capacity on par with wild type, surprisingly, neither double mutant displayed the competitive advantage that was observed with the $\Delta litR$ single mutant (Fig. 7), suggesting Qrr1 regulates a colonization factor that is independent of LitR. Together, these experiments highlight the complex regulatory roles of the LuxO module during colonization of *E. scolopes* by *V. fischeri*.

Discussion

Despite having markedly different lifestyles, the various members of the *Vibrionaceae* have in common the quorum-sensing regulatory module consisting of LuxU, LuxO and at least one *qrr* gene (see Fig. 1, and (Milton, 2006)). The mechanism underlying the activation of the LuxO module also appears to be similar among the *Vibrionaceae* species: histidine kinases phosphorylate LuxU, which in turn transfers the phosphate to LuxO. Phosphorylated LuxO activates the expression of *qrr* genes that specifically target degradation of the mRNAs of a global regulator belonging to the HapR family.

In this study, we provide both evolutionary and genetic evidence that *qrr1* is the only *qrr* gene present in *V. fischeri*. This result is in stark contrast to other *Vibrionaceae* species examined to date, which have multiple *qrr* genes whose products apparently function in concert to post-transcriptionally regulate the mRNA levels of the *litR* homologue. For example, in *V. cholerae*, four Qrr sRNAs lead to degradation of the *hapR* transcript in a redundant fashion, requiring the deletion of all four *qrr* genes to fully stabilize *hapR* transcript (Lenz *et al.*, 2004; Svenningsen *et al.*, 2009). In contrast, the five Qrr sRNAs of *V. harveyi* act synergistically to degrade the transcript of its *litR* homologue (Tu and Bassler, 2007). Although the selective pressures to evolve and maintain multiple *qrr* genes remain unclear (Ng and Bassler, 2009), our studies demonstrate that the simpler network architecture involving a single *qrr* gene appears to be the ancestral state of the LuxO module, and is functional at least in *V. fischeri*.

Our phylogenetic analysis provides a useful organizational structure for framing future studies of the evolution and regulatory roles of the LuxO module within the *Vibrionaceae*. For instance, in the genome of the bioluminescent symbiont *Photorhabdus luminescens*, a member of the *Enterobacteriaceae*, we were able to identify homologues of *luxOU* and *litR* but not *qrr*. In addition, the Qrr-binding motif is absent from the region upstream of the *litR*-like sequence in *P. luminescens*. Together, these observations suggest that LuxO functions in a manner independent of both LitR and Qrr1 in this distantly related bacterial lineage. Furthermore, this notion raises the question: are small RNAs a necessary component in all LuxO modules, or are there alternative methods to achieve similar control within the overall regulatory network? Another intriguing observation was that the *qrr1-luxO* locus of *Photobacterium damsela* does not have a *luxU* paralog, suggesting that a LuxU-independent means to phosphorylate LuxO is likely to be possible within this circuitry.

As depicted in Fig. 8, the regulation of the *lux* genes by the LuxO module in *V. fischeri* is indirect; *i.e.*, Qrr1 negatively regulates the level of LitR, which positively regulates the level of LuxR. Because LuxR must bind the autoinducer 3-oxo-C6 to activate the *lux* genes, we developed a bioluminescence assay for LuxR levels based on the exogenous addition of saturating levels of the LuxR-specific autoinducer 3-oxo-C6. Earlier work has demonstrated

that cellular bioluminescence may be used as a bioassay for autoinducer (Kaplan and Greenberg, 1985). In the experiments presented in this study, addition of 120 nM 3-oxo-C6 was sufficient to induce bioluminescence to levels that enable the detection of different levels of *luxR* expression (Fig. 4B). Using this assay, we demonstrated that strains lacking a functional LuxO module are primed for bioluminescence due to enhanced LuxR levels.

For several decades bacterial bioluminescence has been a powerful tool for exploring the mechanisms underlying quorum sensing. However, there is growing evidence that in *V. fischeri* these signaling systems regulate other biological processes in addition to bioluminescence. For example, LuxR was shown to regulate the transcription of several genes that encode proteases and peptidases, which may assist symbiotic *V. fischeri* in acquiring amino acids within the squid light organ (Antunes *et al.*, 2007; Graf and Ruby, 1998). In addition to bioluminescence and squid colonization, LitR was recently shown to control the acetate switch by regulating *acs*, the gene that encodes acetyl coenzyme A synthetase, a key regulatory point in central metabolism (Studer *et al.*, 2008). Finally, LuxO has been shown to regulate multiple genes involved in flagellar assembly (Lupp and Ruby, 2005), suggesting that the LuxO module also modulates motility. Together, these reports highlight the vast array of cellular processes controlled by various branches of the quorum-sensing network in *V. fischeri*.

While much is now known about quorum sensing in *V. fischeri*, at this time we have not determined the underlying cause for the colonization defect in strains lacking a functional LuxO module. Previous work has demonstrated that LuxO and LitR regulate motility on soft-agar in opposite manners in *V. fischeri* (Lupp and Ruby, 2005). We are currently exploring this link between quorum sensing and motility to further our understanding of the steps involved in establishing a successful infection of its host by a symbiotic bacterium.

Experimental Procedures

Growth and media

Liquid cultures of *V. fischeri* were grown at 28°C with aeration in LBS medium (Graf *et al.*, 1994). When necessary, chloramphenicol and erythromycin were used at 2.5 µg/ml and 5.0 µg/ml, respectively. *N*-3-oxo-hexanoyl homoserine lactone (3-oxo-C6), which was synthesized as previously described (Geske *et al.*, 2007), was kindly provided by H. E. Blackwell.

Strains and plasmids

Strains and plasmids used in this study are summarized in Table 1, and additional details and oligonucleotide sequences used in their construction are located in the supporting information text and table S1, respectively. All *V. fischeri* strains were derived from the sequenced isolate ES114 (Ruby *et al.*, 2005; Mandel *et al.*, 2008).

***V. fischeri* mutants**—Strains with mutations in *luxO* (VF_0937), *qrr1* (VF_2597), or *litR* (VF_2177) were constructed using allelic exchange based on pEVS79 (Stabb and Ruby, 2002). Briefly, a 1.0–1.5 kb fragment of DNA upstream of each target was amplified by PCR and ligated to a 1.0–1.5 kb fragment of DNA downstream of each target using an engineered restriction site. To generate each mutant, the relevant allelic-exchange vector was conjugated into the appropriate parent strain of *V. fischeri* via pEVS104 (Stabb and Ruby, 2002). Stable conjugants were grown to stationary phase in the absence of antibiotic selection, and plated non-selectively. Antibiotic-sensitive colonies were purified and screened for the presence of the desired mutation by colony PCR. All mutations were verified by sequencing.

Two-color fluorescent-reporter plasmids—The plasmid-based reporter pTM267 was constructed in two steps. First, *mCherry* was amplified by PCR and cloned downstream of an engineered *tetA* promoter in pZEP08 (Hautefort *et al.*, 2003), which contains a bright variant of *gfp* called *gfp+*, to yield pTM146. Second, the cassette containing *gfp+* and *mCherry* was cloned into pVSV105 (Dunn *et al.*, 2006), which contains the pES213 origin of replication and is maintained at approximately 10 copies per chromosome. To generate the reporter plasmids used in this study, the promoter regions of *qrr1*, *luxR* (VF_A0925), and *luxI* (VF_A0924) were amplified by PCR and cloned upstream of *gfp+*.

Chromosomal integration vectors—To generate the wild-type control strain used in Fig. 7, the *tetA* promoter and *gfp+* were individually amplified by PCR and cloned into pEVS107 (McCann *et al.*, 2003), which is a site-specific mini-Tn7 vector. To generate the complementation vectors pTM250 and pTM251, *luxO* and *qrr1* were separately cloned with their native promoters into pEVS107. Integration of pEVS107-derived plasmids into the Tn7 site was performed using the helper plasmids pEVS104 and pUX-BF13 as described elsewhere (McCann *et al.*, 2003).

Phylogenetic analyses

For full details and model parameters used to construct the cladogram in Fig. 1, see supporting information and Table S2. Briefly, the *luxO* and *litR* nucleotide sequences of *V. fischeri* ES114 were used to query GenBank using the tblastx version of BLAST. To estimate the number of *qrr*-like sequences existing in each of the 14 bacterial genomes, we compared the results of two search methods: (i) a BLAST search of nucleotide databases in PubMed using a 32-nucleotide query (GGGTCACCTAKCCCAACTGACGTTGTTAGTGAA, where K is G or T), and (ii) a search for putative *qrr* family members as listed within version 10.0 of the Rfam database (Gardner *et al.*, 2009) or as identified by searching unfinished genomes using Infernal 1.0.2 (Nawrocki *et al.*, 2009).

Fluorescence assay

Overnight cultures were diluted 1:100 (high cell density) or 1:10,000 (low cell density) and grown aerobically in LBS broth at 28°C. After 3 h, each culture was serially diluted and spread onto LBS agar to determine colony-forming units (CFU) levels. The remaining cultures were cooled quickly using an ice-slurry mix, and 250- μ l (high cell density) and 25-ml (low cell density) samples were centrifuged at 4°C for 15 min at 4,000 $\times$ g. Cell pellets were resuspended in 100 μ l of minimal media without a supplemented carbon source. The optical density at 600 nm, GFP fluorescence, and mCherry fluorescence of each sample was measured using a Tecan Genios Pro plate reader (Tecan Group Ltd., Männedorf, Switzerland). A 485-nm excitation and 535-nm emission filter set was used for GFP fluorescence measurements, and a 535-nm excitation and 612-nm emission filter set was used for mCherry fluorescence measurements. A non-fluorescent strain of *V. fischeri* was used to subtract out any autofluorescence.

Quantitative Reverse Transcriptase PCR (qRT-PCR)

Total RNA from *V. fischeri* cultures was isolated from cells grown in LBS broth to the indicated cell densities. RNA was isolated using a QuickExtract RNA Extraction Kit and DNaseI (Epicentre Biotechnologies, Madison, WI), and used to make cDNA with random hexamer primers and AMV Reverse Transcriptase (Promega, Madison, WI). Reactions for qRT-PCR were performed in triplicate in 25 μ l volumes with iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA), cDNA template and appropriate primers at 0.25 μ M. Reactions were carried out with a two-step cycling protocol in an iCycler Thermal Cycler

(Bio-Rad Laboratories). Amplification was performed under the following conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 30 sec. As a negative control, DNase-free RNA without RT treatment was used as template. Primer sequences to probe *litR* and the normalizing constitutive locus *rpoD* (*VF_2254*) are listed in Table S1.

6xHis-LitR expression and purification

To purify 6xHis-LitR, 100 ml LB/carbinicillin (100 µg/ml) cultures of BL21(DE3) harboring pTM304 were grown at 37°C to OD₆₀₀ 0.5. IPTG was added to a final concentration of 1 mM, and the cultures were grown for an additional 2 h. At that time, cells were harvested, lysed by sonication, and spun at 4°C for 15 min at 30,000 × g. The soluble fraction (approximately 5 ml) was incubated with 1.25 ml of 50% Ni-NTA agarose (Qiagen Inc., Valencia, CA) at 4°C for 1 h. The agarose bed was washed twice and eluted into four 0.5-ml fractions. The purity of the fractions was determined after electrophoresis through 12% SDS-polyacrylamide gels, and visualization by nonspecific staining of protein with Coomassie. Protein levels were determined by BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL). The fraction containing the highest level of 6xHis-LitR was dialyzed into storage buffer (50 mM NaH₂PO₄ [pH 8.0]; 300 mM NaCl; 10% glycerol; 0.1 M EDTA; 1 mM DTT) and stored at 4°C.

Western-blot assay

Overnight cultures were diluted 1:100 into 2 ml LBS broth, and grown aerobically at 28°C. After 2 h, cultures were quickly cooled using an ice-slurry mix and centrifuged. Whole-cell lysates were separated by electrophoresis through 12% Tris-HCl ReadyGel (Bio-Rad Laboratories) according to the manufacturer's instructions, and transferred to Immun-Blot PVDF Membrane (Bio-Rad Laboratories). Serum isolated from rabbits inoculated with purified HapR (Liu *et al.*, 2008) was used at a 1:1000 dilution as the primary antibody. Bands were visualized using an ECF Western Blotting Reagent Pack (GE Healthcare Bio-Sciences Corp., Piscataway, NJ), and were imaged using a Typhoon Imager 8600 (GE Healthcare Bio-Sciences Corp.). Image analysis was performed using ImageJ software (NIH).

Bioluminescence assay—Overnight cultures were diluted 1:100 into LBS broth and grown aerobically at 28°C. After 2 h, cultures were diluted 1:10 into LBS broth containing 3-oxo-C6 at a final concentration of 120 nM, and grown aerobically at 28°C. When cultures reached OD₆₀₀ = 0.5, the luminescence of 100 µl of each sample was measured using a TD 20/20 luminometer (Turner Design, Sunnyvale, Calif.). To determine CFU levels, cultures were serially diluted and spread onto LBS agar. In this study, one RLU corresponds to 1.9 quanta s⁻¹ cell⁻¹.

Squid colonization experiments

Overnight cultures of *V. fischeri* strains were diluted 1:100 into LBS broth and grown aerobically at 28°C to OD₆₀₀ = 1.0. Cultures were diluted to 2000–5000 CFU/ml in filtered-sterilized Instant Ocean (Spectrum Brands Inc., Atlanta, Georgia) containing newly hatched squid. After 48 hrs, animals were homogenized. Homogenates were diluted and plated onto LBS to calculate total CFU levels for each animal. For competition experiments, colonies were also scored for GFP fluorescence using a Leica MZFLIII fluorescence dissecting microscope (Leica Microsystems, Wetzlar, Germany) equipped with a GFP2 filter set.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Number of identified *qrr* loci present in strains belonging to the *Vibrionaceae* lineage mapped onto phylogenetic reconstructions of two quorum-sensing loci
 Bayesian majority-rule consensus trees are depicted for both *luxO*- and *litR*-like loci among 13 strains of *Vibrionaceae*. For each depicted node, statistical support is given as Bayesian posterior probability (top left), maximum likelihood bootstrap percentage of 1000 pseudoreplicates (top right), or maximum parsimony bootstrap percentage of pseudoreplicates (bottom center); ‘-’ indicates bootstrap support less than 50%. Colored boxes highlight the clusters of vibrio strains that contain the same number of *qrr*-like sequences. No *qrr*-like sequences were detected for the outgroup, *Photobacterium luminescens* TT01. Bars represent 0.1 substitutions per site.

Fig. 2. Expression of *qrr1* in various strains of *V. fischeri* grown to different cell densities
 A. Relative transcriptional activity of *qrr1*. MJM1100 (WT), TIM306 ($\Delta luxO$), and TIM305 ($\Delta qrr1$), harboring the two-color fluorescent-reporter plasmid pTM268, were grown to low cell density (white bars) or high cell density (gray bars) in LBS. Transcription of *qrr1* was determined from the fluorescence ratio GFP/mCherry.
 B. Cell densities of cultures used in 'A'.
 Graphical and error bars indicate the average and standard deviation of three independent experiments, respectively.

Fig. 3. Regulation of LitR levels by the LuxO module

A. qRT-PCR analysis of *litR* expression. Wild-type MJM1100 (WT), TIM305 ($\Delta qrr1$), and TIM306 ($\Delta luxO$) were grown in LBS broth to approximately 1.1×10^8 CFU/ml. The transcript levels of *rpoD* were used to normalize *litR* levels. Graphical and error bars indicate the average and standard deviation of three independent experiments, respectively.

B. Western-blot analysis of LitR levels. Samples were either affinity-purified His-tagged LitR or whole-cell lysates from LBS cultures of MJM1100 (WT), TIM305 ($\Delta qrr1$), TIM306 ($\Delta luxO$), TIM358 ($\Delta litR$), and PMF8 (*litR::kan*) grown to approximately 7.0×10^7 CFU/ml. The primary antibodies used to probe for LitR were raised against purified *V. cholerae* HapR (Liu *et al.*, 2008). Nonspecific bands in lanes containing whole-cell lysates are denoted by 'ns'. The amount of His-tagged LitR loaded is indicated above the blot image. Numbers below the blot image are signal intensities of corresponding bands normalized to the lowest level of His-tagged LitR. Due to the overlapping bands of LitR and ns2 in lanes containing whole-cell lysates, the corresponding signal intensities have been combined. The experiment was repeated three times with similar results.

Fig. 4. The impact of LitR levels on the bioluminescence response of *V. fischeri* to 3-oxo-C6

A. Relative transcriptional activity of *luxR*. MJM1100 (WT), TIM305 ($\Delta qrr1$), TIM306 ($\Delta luxO$), and PMF8 (*litR::kan*) harboring the two-color fluorescent-reporter plasmid pTM279, were grown to mid-log phase in LBS broth. Relative expression of *luxR* was determined from the fluorescence ratio GFP/mCherry. Graphical and error bars indicate the average and standard deviation of three independent experiments, respectively. An asterisk indicates a significant difference from wild type ($P < 0.005$) as determined by a t-test.

B. Bioluminescence activity. MJM1100 (WT), TIM305 ($\Delta qrr1$), TIM306 ($\Delta luxO$), TIM358 ($\Delta litR$), and PMF8 (*litR::kan*) were grown in LBS broth in the presence (gray bars) or absence (white bars) of a 120-nM addition of 3-oxo-C6. Culture luminescence was measured and normalized to relative luminescence units (RLU) by the corresponding CFU level. Graphical and error bars indicate the average and standard deviation of three independent experiments, respectively.

Fig. 5. Bioluminescence response of various *V. fischeri* mutants to 3-oxo-C6
 Strains MJM1100 (column 1), TIM358 (column 2), TIM305 (column 3), TIM306 (column 4), TIM325 (column 5), TIM354 (column 6), TIM355 (column 7), and TIM357 (column 8) were grown in LBS broth in the presence of a 120-nM addition of 3-oxo-C6. Culture luminescence was measured and normalized to relative luminescence units (RLU) by the corresponding CFU level. Graphical and error bars indicate the average and standard deviation of three independent experiments, respectively. The presence of the corresponding mutations in each strain is indicated below the graph.

Fig. 6. The impact of overexpressing *qrr1* on *litR* transcript and bioluminescence levels
 A. Analysis of *litR* expression by qRT-PCR. pVSV105/MJM1100 (WT vector), pVSV105/TIM325 ($\Delta(qrr1\ luxO)$ vector), and pTM312/TIM325 ($\Delta(qrr1\ luxO)$ P_{tetA} -*qrr1*) were grown in LBS to approximately 1.5×10^8 CFU/ml. For each sample, the level of *litR* expression was normalized by the level of *rpoD* expression.
 B. Analysis of bioluminescence. Strains described in 'A' were grown in LBS broth in the presence of 120 nM 3-oxo-C6. Culture luminescence was measured and normalized to relative luminescence units (RLU) by the corresponding CFU level.
 In each figure, graphical and error bars indicate the average and standard deviation of three independent experiments, respectively.

Fig. 7. Competition experiments comparing pairs of *V. fischeri* strains during colonization of *E. scolopes*

Newly hatched squid were exposed to a mixed inoculum containing a GFP-labeled wild-type control strain (TIM302), and one of the test strains listed along the right axis. After 48 hrs, the squid were homogenized, and dilutions were plated onto LBS agar medium. Colonies were screened for GFP fluorescence. The relative competitive index (RCI) is defined as the ratio of test strain/control strain CFUs within the homogenate normalized by this ratio within the inoculum. The top panel indicates that there was no significant difference between the wild-type strain (MJM1100) and the GFP-labeled wild-type control strain (TIM302). White and gray symbols represent those test strains that either carried or did not carry the wild-type allele of *litR*, respectively. Circles represent test strains with wild-type alleles of *qrr1* and *luxO*. Triangles and diamonds represent test strains containing $\Delta qrr1$ and $\Delta luxO$ alleles, respectively. Black symbol and error bars represent the mean and 95% confidence intervals of each data set, respectively. Animals colonized by only a single strain were excluded from the analysis. Each competition experiment was performed two times with similar results.

Fig. 8. Model for regulation of bioluminescence by the LuxO module in *V. fischeri*

In the absence of the autoinducers octanoyl homoserine lactone (C8; diamonds) and AI-2 (squares), respectively, AinR and the LuxP/Q complex phosphorylate LuxU. Phosphorylated LuxU phosphorylates LuxO, which is a transcriptional activator of *qrr1*. Qrr1 post-transcriptionally represses *litR* via the sRNA chaperone Hfq. In the presence of C8 or AI-2, the signaling cascade is inactivated, resulting in stable LitR production. LitR transcriptionally activates *luxR*, which encodes the transcriptional regulator LuxR. LuxI synthesizes the autoinducer *N*-3-oxo-hexanoyl homoserine lactone (3-oxo-C6; triangles), which freely diffuses across cell membranes. In the presence of 3-oxo-C6, LuxR activates several genes, including the *luxCDABEG* locus that is responsible for light production. Phosphotransfer reactions within the network are indicated by dashed arrows.

Table 1

Strains and plasmids used in this study.

Strain	Relevant Genotype	Source
BL21(DE3)	protein expression <i>E. coli</i> host	Invitrogen
MJM1100	sequenced wild-type <i>V. fischeri</i> ES114	(Ruby <i>et al.</i> , 2005; Mandel <i>et al.</i> , 2008)
PMF8	ES114 <i>litR::kan</i>	(Fidopiastis <i>et al.</i> , 2002)
TIM302	ES114 Tn7::pTM242[<i>P_{tetA}-gfp+ erm</i>]	This work
TIM305	ES114 $\Delta qrr1$	This work
TIM306	ES114 $\Delta luxO$	This work
TIM313	ES114 Tn7::pEVS107[<i>erm</i>]	This work
TIM314	ES114 $\Delta qrr1$ Tn7::pEVS107[<i>erm</i>]	This work
TIM315	ES114 $\Delta luxO$ Tn7::pEVS107[<i>erm</i>]	This work
TIM318	ES114 $\Delta luxO$ Tn7::pTM250[<i>luxO erm</i>]	This work
TIM319	ES114 $\Delta qrr1$ Tn7::pTM251[<i>qrr1 erm</i>]	This work
TIM325	ES114 $\Delta(qrr1 luxO)$	This work
TIM354	ES114 $\Delta qrr1 \Delta litR$	This work
TIM355	ES114 $\Delta luxO \Delta litR$	This work
TIM357	ES114 $\Delta(qrr1 luxO) \Delta litR$	This work
TIM358	ES114 $\Delta litR$	This work

Plasmid	Relevant Genotype	Source
pET-15b	Protein expression vector	Novagen
pEVS79	pBC SK (+) <i>oriT cat</i>	(Stabb and Ruby, 2002)
pEVS107	R6Kori <i>oriT mini-Tn7 mob erm kan</i>	(McCann <i>et al.</i> , 2003)
pRSETB-mCherry	<i>mCherry</i> expression vector	(Shaner <i>et al.</i> , 2004)
pTM146	pZEP08 <i>P_{tetA}-mCherry</i>	This work
pTM235	pEVS79 $\Delta luxO$	This work
pTM238	pEVS79 $\Delta qrr1$	This work
pTM242	pEVS107 <i>P_{tetA}-gfp+</i>	This work
pTM250	pEVS107 <i>luxO</i>	This work
pTM251	pEVS107 <i>qrr1</i>	This work
pTM267	pVSV105 <i>kan gfp+ P_{tetA}-mCherry</i>	This work
pTM268	pVSV105 <i>P_{qrr1}-gfp+ P_{tetA}-mCherry</i>	This work
pTM273	pEVS79 $\Delta(qrr1 luxO)$	This work
pTM279	pVSV105 <i>P_{luxR}-gfp+ P_{tetA}-mCherry</i>	This work
pTM280	pVSV105 <i>P_{luxI}-gfp+ P_{tetA}-mCherry</i>	This work
pTM301	pEVS79 $\Delta litR$	This work
pTM304	pET-15b <i>6xhis-litR</i>	This work
pTM312	pVSV105 <i>P_{tetA}-qrr1</i>	This work
pUX-BF13	R6Kori <i>tns bla</i>	(Bao <i>et al.</i> , 1991)
pVSV105	R6Kori <i>ori</i> (pES213) RP4 <i>oriT cat</i>	(Dunn <i>et al.</i> , 2006)
pZEP08	ColE1ori <i>bla cat kan gfp+</i>	(Hautefort <i>et al.</i> , 2003)