Analysis of Activator and Repressor Functions Reveals the Requirements for Transcriptional Control by LuxR, the Master Regulator of Quorum Sensing in *Vibrio harveyi*

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ABSTRACT LuxR-type transcription factors are the master regulators of quorum sensing in vibrios. LuxR proteins are unique members of the TetR superfamily of transcription factors because they activate and repress large regulons of genes. Here, we used chromatin immunoprecipitation and nucleotide sequencing (ChIP-seq) to identify LuxR binding sites in the *Vibrio harveyi* genome. Bioinformatics analyses showed that the LuxR consensus binding site at repressed promoters is a symmetric palindrome, whereas at activated promoters it is asymmetric and contains only half of the palindrome. Using a genetic screen, we isolated LuxR mutants that separated activation and repression functions at representative promoters. These LuxR mutants exhibit sequence-specific DNA binding defects that restrict activation or repression activity to subsets of target promoters. Altering the LuxR DNA binding site sequence to one more closely resembling the ideal LuxR consensus motif can restore *in vivo* function to a LuxR mutant. This study provides a mechanistic understanding of how a single protein can recognize a variety of binding sites to differentially regulate gene expression.

IMPORTANCE Bacteria use the cell-cell communication process called quorum sensing to regulate collective behaviors. In vibrios, LuxR-type transcription factors control the quorum-sensing gene expression cascade. LuxR-type proteins are structural homologs of TetR-type transcription factors. LuxR proteins were assumed to function analogously to TetR proteins, which typically bind to a single conserved binding site to repress transcription of one or two genes. We find here that unlike TetR proteins, LuxR acts as a global regulator, directly binding upstream of and controlling more than 100 genes. Again unlike TetR, LuxR functions as both an activator and a repressor, and these two activities can be separated by mutagenesis. Finally, the consensus binding motifs driving LuxR-activated and -repressed genes are distinct. This work shows that LuxR, although structurally similar to TetR, has evolved unique features enabling it to differentially control a large regulon of genes in response to quorum-sensing cues.
bound to DNA (2, 4, 5). No ligand has been identified for any LuxR-type protein.

Although LuxR possesses the HTH motif that places it in the TetR family, it is unique. First, LuxR can act as an activator and a repressor (6, 7), whereas TetR-type proteins are typically repressors (3). Second, LuxR directly and indirectly controls the expression of 625 genes (7). TetR-type proteins, by contrast, generally control only their own expression and that of the adjacent gene. Third, LuxR can bind to multiple binding sites in its target promoters (8). Typically, only one binding site is present in TetR-regulated promoters. Finally, unlike the conserved TetR binding motif, the LuxR, SmcR, and HapR binding site consensus sequences are degenerate, indicating that this is a general attribute of LuxR transcription factors (9, 10). Together, these features allow LuxR and its homologs to positively and negatively control large regulons of genes (7, 9).

Here, we used chromatin immunoprecipitation and nucleotide sequencing (ChIP-seq) to define the set of LuxR binding sites in the V. harveyi genome. We showed that LuxR directly activates 35 genes and represses 80 genes. We characterized LuxR binding and how that impinges on gene regulation at two representative promoters: one that is activated and one that is repressed. We isolated mutations in the DNA binding domain of LuxR that confer sequence-specific DNA binding defects. The binding defect of a repression-defective LuxR mutant can be suppressed by altering the LuxR DNA binding site sequence to one that more closely matches the ideal LuxR binding site. Our findings demonstrate that LuxR-type proteins recognize subtle variations in binding sites to distinguish between activated and repressed promoters.

RESULTS

LuxR directly regulates 115 genes. Previous in vitro experiments coupled with bioinformatics analyses predicted 36 LuxR binding sites in the V. harveyi genome (11). However, LuxR is known to regulate 625 genes (7). We considered two possibilities to explain this discrepancy: (i) most LuxR-controlled genes are regulated indirectly, or (ii) the bioinformatic predictions underestimated the number of LuxR binding sites. To distinguish between these possibilities, we performed chromatin immunoprecipitation (ChIP) assays using FLAG-tagged LuxR. As a control, we verified that FLAG-LuxR behaves similarly to wild-type LuxR in vivo (see Fig. S1A in the supplemental material). We first measured FLAG-LuxR occupancy at promoter regions containing known LuxR binding sites (6, 11, 12). Our ChIP analyses revealed 10- to 100-fold enrichment of LuxR binding at the promoters of apha, luxC, and qrr but not at promoters of genes known not to be bound by LuxR (qrr1 and qrr5) (Fig. 1A). As a second control, we showed that a DNA binding-defective LuxR mutant (LuxR R17C) (13, 14) did not immunoprecipitate any promoter regions (Fig. 1A).

LuxR binding was assessed globally by high-throughput sequencing of the FLAG-LuxR-bound DNA (ChIP-seq). We identified 1,165 LuxR binding peaks spanning 582 genomic regions. Quantitative Western blot analyses showed that there are approximately 6,500 dimers of LuxR in wild-type V. harveyi, and thus there is sufficient LuxR present to bind at 1,165 sites (see Fig. S1B in the supplemental material). MEME (multiple EM for motif elicitation) (15) analysis of the DNA sequences surrounding the LuxR binding peaks revealed a 20-bp consensus binding motif (Fig. 1B). This motif is nearly identical to the 21-bp LuxR consensus motif previously determined in vitro (11) and closely resembles the consensus motifs determined for other LuxR proteins (9, 10, 16). Although this motif exhibits dyad symmetry, LuxR has a stronger preference for nucleotide sequences on one side of the palindrome (Fig. 1B, left). Studies of other LuxR homologs identified motifs of 16 bp (HapR) to 22 bp (SmcR) (9, 10). We investigated the DNA substrate length required for LuxR binding using electrophoretic mobility shift assays (EMSAs) with DNA fragments of various lengths. LuxR binds to a 28-bp substrate with the highest affinity, and LuxR cannot bind to substrates shorter than 23 bp (see Fig. S1C in the supplemental material).

To determine which LuxR binding sites contribute to LuxR regulation of gene expression, we analyzed the locations of LuxR binding peaks relative to genes in the LuxR regulon, which was defined previously by microarray analyses (7). We identified 227 LuxR peaks in promoter regions upstream of 115 LuxR-regulated genes (35 of these genes are activated by LuxR, and 80 genes are repressed by LuxR) (see Tables S1 and S2 in the supplemental material). Because LuxR both activates and represses transcription, we hypothesized that differences could exist in the consensus sequences for activated and repressed genes. To explore this, we separately analyzed peaks in the promoters of activated genes and repressed genes (see Tables S1 and S2). The LuxR binding motif in repressed promoters has clear dyad symmetry (Fig. 1C), whereas the motif present in activated promoters has a strong preference...
for one side, and the palindrome is incomplete on the other side (Fig. 1D). Thus, the combination of motifs from activated and repressed genes (see Fig. S1D) results in the overall consensus motif derived from the ChIP-seq (Fig. 1B).

LuxR: examples of activation and repression. To examine how differences in LuxR binding motifs contribute to activation and repression, we characterized LuxR activity at one representative repressed promoter and one representative activated promoter. A typical case of a gene repressed by LuxR is represented by VIBHAR_05222, encoding a putative thioesterase. According to ChIP-seq analyses, the VIBHAR_05222 promoter (P_05222) contains five LuxR binding peaks, although one peak was significantly stronger than the other four (Fig. 2A). To determine the precise locations of LuxR binding sites that are relevant for regulation, we used the position weight matrix (PWM) generated by our ChIP-seq data to scan for binding sites at the promoter region (A) or the luxC promoter region (B). Sites determined in vitro (black boxes) or by MAST are shown relative to the putative translation start codons (denoted as +1) of VIBHAR_05222 (A) or luxC (B). The transcription start site of luxC is shown with an arrow (12). DNA binding curves determined by quantitative EMSAs are shown for LuxR binding at the P_05222 binding site (A) or P_luxC binding sites 1 to 3 (B). Dissociation constants (K_d) are shown for each binding site. Error bars represent the standard deviations of three independent measurements.

LuxR binds to activated and repressed promoters. Diagrams illustrating the locations and relative strengths of LuxR binding (ChIP-seq peaks) at the VIBHAR_05222 promoter region (A) or the luxC promoter region (B). Sites determined in vitro (black boxes) or by MAST are shown relative to the putative translation start codons (denoted as +1) of VIBHAR_05222 (A) or luxC (B). The transcription start site of luxC is shown with an arrow (12). DNA binding curves determined by quantitative EMSAs are shown for LuxR binding at the P_05222 binding site (A) or P_luxC binding sites 1 to 3 (B). Dissociation constants (K_d) are shown for each binding site. Error bars represent the standard deviations of three independent measurements.

First, we isolated activation-defective mutants; these mutants exhibit decreased P_luxC activation (low GFP levels) but maintain wild-type P_05222 repression (low mCherry levels, Fig. 3A). We obtained five mutants with substitutions at the N55 position, and of
these, LuxR N55I and LuxR N55K exhibited the most severe phe-
notypes (see Fig. S2 in the supplemental material). Four mutants
were partially defective for activation of P_{luxC}, and they have the
substitutions L139P, L139R, N55Y, and N142D (Fig. 3A) (see also
Fig. S2). A reciprocal screen was used to identify repression-
defective mutants; these variants exhibit decreased repression of
P_{05222} (high mCherry levels) but maintain wild-type activation of
P_{luxC} (high GFP levels). We obtained two mutants: LuxR T52M/I24V is completely
defective for repression, while LuxR A51T is partially defective (Fig. 3A). We
separated the LuxR T52M/I24V muta-
tions by constructing LuxR T52M and LuxR I24V. The LuxR T52M mutant was
inactive at both the activated and re-
pressed promoters, and the LuxR I24V
mutant resembled wild-type LuxR (see
Fig. S2), so both mutations are required
for the phenotype. We showed that the
phenotypes of the mutant proteins were
not due to altered protein production
(see Fig. S2). The amino acid substitu-
tions are shown in an alignment of the
DNA binding domains of LuxR ho-

![FIG 3] LuxR mutants defective for activation or repression. (A) LuxR mutants with decreased activation of P_{luxC} (activation-defective mutants) and decreased repression of P_{05222} (repression-defective mutants). E. coli strains containing an empty vector (pJV036, denoted as “No Protein”), expressing luxR (pJV239), or expressing luxR mutants (N55I [pJV240], L139P [pJV242], N142D [pJV261], T52M/I24V [pJV241], and A51T [pJV247]) were assayed for transcriptional activation of P_{luxC} and repression of P_{05222}. Fluorescence from each strain was measured using a reporter construct (pJV064) harboring P_{05222}-mCherry and P_{luxC}-gfp. Error bars represent the standard deviations of measurements for three biological samples. (B) Alignment of the predicted DNA binding domains of LuxR homologs from V. harveyi (LuxR, AAA27539), V. vulnificus (SmcR, AAF72582), V. cholerae (HapR; ABD24298), V. parahaemolyticus (OpaR, NP_798895), and V. fisheri (LitR, YP_205560) aligned to E. coli TetR (P0ACT4). Conserved residues (including in TetR) are shown in black, and similar residues are shown in gray. The black triangles indicate the locations of amino acid substitutions in activation- and repression-defective mutant LuxR proteins. Sequence alignments were assembled using the ClustalW software program (45) and viewed using the ESPript (46) and Boxshade programs.

![FIG 4] LuxR mutants are defective for regulating specific promoters in V. harveyi. Transcript levels of genes regulated by LuxR were assayed by qRT-PCR from a V. harveyi ΔluxR strain (KM669) containing an empty vector (pJV036, denoted as “No Protein”) or expressing luxR (pJV239), luxR N55I (pJV240), luxR L139P (pJV242), or luxR T52M/I24V (pJV241). Error bars represent the standard deviations of measurements of three biological samples. See also Fig. S2 in the supplemental material for all tested promoters and LuxR mutants.
tants. The LuxR T52M/I24V substitution abolished repression at all promoters (Fig. 4; see also Fig. S2 in the supplemental material), and it showed a severe decrease in activation except at P_{luxC} (Fig. 4), the promoter used in the screen. LuxR A51T, which was partially defective at repression of P_{luxC}, was also partially defective for repression of other promoters (see Fig. S2 ). Collectively, these results suggest that defects associated with mutations in the DNA binding domain of luxR (e.g., LuxR N55I, T52M/I24V, and A51T) are not specific for activation or repression but rather are specific to each promoter. Mutations residing outside the DNA binding domain (LuxR L139P and LuxR N142D), in contrast, are generally defective only for activation.

**LuxR mutant proteins are defective for DNA binding at specific sequences.** The majority of the mutations we obtained in luxR reside in the DNA binding domain. Therefore, we hypothesized that the phenotypes of the LuxR activation- and repression-defective mutants could be due to changes in DNA binding capabilities at specific promoters. We examined DNA binding with a focus on the activation-defective mutants LuxR N55I and LuxR L139P and the repression-defective mutant LuxR T52M/I24V because those substitutions resulted in the strongest phenotypes in vivo. First, we examined DNA binding at our representative repressed promoter P_{luxC} using EMSAs with purified proteins. The activation-defective mutants LuxR N55I and LuxR L139P both bound the P_{luxC} binding site with only slightly lower affinity than wild-type LuxR (Fig. 5A), which is consistent with the observation that these substitutions did not affect repression of P_{luxC}. However, the repression-defective mutant LuxR T52M/I24V showed severely decreased binding affinity (K_d = 56.6 nM) compared to that of wild-type LuxR (K_d = 0.7 nM) (Fig. 5A). Thus, LuxR T52M/I24V is defective for repression at P_{luxC} because it is impaired for DNA binding at this particular promoter.

We next tested DNA binding at sites 1 and 2 in P_{luxC} of our representative activated promoter. The activation-defective mutant LuxR N55I bound site 1 with affinity similar to that of wild-type LuxR (Fig. 5B) and showed no binding to site 2 (Fig. 5C; see also Fig. S3 in the supplemental material). Thus, LuxR N55I is defective for activation of P_{luxC} because it is unable to bind site 2, the site that is critical for activation of luxC. LuxR L139P, which is partially defective for activation at P_{luxC}, has wild-type binding affinities at both sites 1 and 2 (Fig. 5B and C). Thus, the LuxR L139P phenotype is not due to a defect in DNA binding. The repression-defective mutant LuxR T52M/I24V did not bind site 1 (see Fig. S3) but bound site 2 with weak affinity (Fig. 5C; see also Fig. S3). Because strains containing LuxR T52M/I24V are capable of activating P_{luxC}, similar to strains with wild-type LuxR, this result suggests that even modest binding at site 2 is necessary and sufficient for LuxR activation.

**Mutagenesis of the 05222 promoter suppresses the LuxR T52M/I24V defect.** The above biochemical analyses of the LuxR mutants suggest that the activation- and repression-defective phenotypes of LuxR N55I and LuxR T52M/I24V stem from defects in binding to specific DNA sequences. We hypothesized that changing the binding sites in the corresponding promoters could suppress the mutant phenotypes. We first examined P_{05222}. We randomly mutagenized the LuxR binding site at P_{05222} and screened for substitutions that suppressed the LuxR T52M/I24V repression defect but did not affect basal transcription in the absence of LuxR (Fig. 6A). Two substitutions restored repression to the level of that for wild-type LuxR (Fig. 6B). Importantly, these two substitutions (P_{05222} G → T and P_{05222} T → C) converted the P_{05222} sequence to one more closely resembling the ideal LuxR repressed gene consensus motif (Fig. 6A). Individually, each mutation partially increased LuxR T52M/I24V repression, whereas combining both mutations further increased LuxR T52M/I24V binding affinity and fully restored repression (Fig. 6B and C). The two mutations also increased the DNA binding affinity and repression activity of wild-type LuxR (Fig. 6B and C). Thus, suppression of the repression-defective phenotype occurs by increasing LuxR DNA binding affinity at the P_{05222} site. In a parallel screen, we attempted to identify substitutions in P_{luxC} that suppressed the activation-defective phenotype of LuxR N55I. However, we were unsuccessful, perhaps because the LuxR binding site at this promoter could not be altered to suppress the N55I phenotype without affecting basal transcription rates.

**DISCUSSION**

The master transcription factor LuxR precisely controls the expression of more than 600 genes in the V. harveyi quorum-sensing regulon. LuxR directly regulates approximately one-fifth of these genes (115 genes). Quorum-sensing regulons of similar sizes are known in other vibrios, such as V. vulnificus, in which the LuxR homolog SmcR binds to 121 promoters (9). Four of the genes...
directly controlled by LuxR are predicted to be transcription factors, which likely control second-tier genes in the LuxR regulon.

Our ChIP-seq studies provide a global view of LuxR binding. We identified a significant number of LuxR binding sites in promoters of genes for which we do not observe regulation by LuxR. We propose that LuxR regulates these genes during growth under conditions that are not mimicked by our laboratory experiments. Previous studies of transcription factors in other organisms, such as Drosophila melanogaster and Candida albicans, also found that the number of protein binding sites is significantly larger than the number of genes displaying regulation (19–22). More than half of the LuxR binding peaks were present within ORFs (671 out of 1,165 LuxR binding peaks), another feature consistent with findings of these earlier studies. For example, LuxR bound within the ORFs of VIBHAR_05222 and luxC, the two representative promoters we characterized, although none of these sites is necessary for LuxR regulation of the gene in vivo. It is noteworthy that among the 75 LuxR-regulated genes with binding peaks within the ORF, 50 of these also harbor additional binding peaks upstream of their start codons. It is therefore possible that binding sites within ORFs paired with binding sites in the promoter are important for LuxR transcriptional regulation under some conditions.

Unlike canonical TetR proteins with stringent consensus motifs, the consensus motif of LuxR-type proteins is degenerate and asymmetric (9–11, 16). One-half of the dyad is more strongly conserved than the other half, as observed from 1,165 binding site sequences. However, we discovered how the unusual asymmetric nature of the LuxR consensus motif is generated. As with other TetR-type proteins, LuxR binding sites in repressed promoters contain a palindrome of roughly equal symmetry. In contrast, the LuxR binding sequences present in activated promoters are non-palindromic, containing only the left half of the repressed promoter site. Thus, the combination of these two distinct sites results in a skewed palindrome in vitro and in vivo (11). We propose that LuxR has evolved the flexibility to tolerate minor changes to the DNA binding sequence at activated and repressed promoters, which could underpin why LuxR proteins have the capability to control the expression of hundreds of quorum-sensing genes. A model showing these ideas is presented in Fig. 7.

To study the mechanism that drives LuxR activation and repression activity, we performed a screen to identify LuxR variants that only activate or only repress. None of the resulting LuxR mutants was specifically defective for either activation or repression. Two activation-defective mutants, LuxR L139P and LuxR N142D, were defective only at activating luxC and VIBHAR_p08175. However, LuxR 139P did not exhibit any defects in
DNA binding. We predict that the region of LuxR containing amino acids L139 and N142 may be critical for RNA polymerase interaction and thus required for activation of luxC. Several of our LuxR mutants exhibited defects in DNA binding only at specific LuxR binding sites, and each of these mutant LuxR proteins harbored substitutions in the DNA binding domain. This finding suggests that modifications to the amino acids in the conserved HTH motif restrict DNA sequence recognition to specific sequences. Two such LuxR mutants, carrying N55I and T52M/I24V, exhibited the strongest defects in gene regulation, suggesting that these amino acids likely play the most important roles in DNA sequence recognition, at least at the two promoters we examined.

A common theme that we observe is that when repression is considered, LuxR functions similarly to TetR. First, the LuxR consensus motif at repressed promoters is a symmetrical palindromic. Second, LuxR bound a single site at the example repressed promoter P_luxC, which is reminiscent of TetR proteins that bind a single operator at a promoter. Third, similar to what we show for LuxR, altering the conserved residues in the DNA binding domain of TetR residues decreases its repression activity (23). For example, the TetR T40M substitution (compare with LuxR T52M) reduced TetR repression activity 153-fold in vivo. Finally, we found that the phenotype of a repression-defective mutant, LuxR T52M/I24V, could be suppressed by altering the DNA sequence in P_luxC. In an analogous experiment, tetr operator sequence variants also suppress defective repression phenotypes of TetR proteins with substitutions at T40 (23). Thus, LuxR likely functions similarly to TetR at repressed promoters by binding to a single palindromic operator site via specific interactions with residues in the DNA binding domain. LuxR is not like TetR when one considers activated promoters. At activated promoters, LuxR recognizes a different consensus motif and binds three sites in the activated promoter P_luxC. Multiple LuxR binding sites commonly exist in each promoter (59% of the LuxR-bound regions contain 2 or more sites; see Fig. S4 in the supplemental material; for example, P_luxC). LuxR may facilitate DNA looping by binding to multiple sites, a mechanism that has been proposed for the LuxR homolog SmR (24).

Our biochemical analyses suggest that the DNA sequence of the LuxR binding site is not the only factor that specifies activation or repression, because LuxR mutants that activated P_luxC, for example, could not activate all LuxR-activated promoters. Likewise, LuxR mutants that repressed P_luxC could not repress all LuxR-repressed promoters. In addition, DNA binding affinity alone cannot account for all LuxR transcription activity because LuxR had the weakest affinity for P_luxC site 2, which is the critical binding site in that promoter in terms of regulation. It is also peculiar that LuxR exhibits the strongest affinity for P_luxC site 3 but this site is not required for activation in vivo. Thus, features such as the DNA sequence, number and relative locations of LuxR binding sites, and productive LuxR interactions with RNA polymerase and other transcription factors likely combine to dictate whether LuxR activates or represses a given promoter and to what extent. For example, we know that the cAMP receptor protein (CRP) and MetR regulate P_luxC (25), and LuxR may interact with these proteins to activate transcription (Fig. 7). We are currently exploring LuxR interactions with RNA polymerase and other transcription factors at P_luxC as a model to understand the mechanism of LuxR activation.

Our studies of LuxR gene regulation support a role for LuxR as a dual-function global transcriptional regulator. In many ways, our analysis suggests that LuxR is more similar to CRP than to TetR. CRP is an activator and repressor of transcription of >200 genes (26–31). CRP binds to a conserved 22-bp operator sequence (32–35), and the positioning of the CRP site dictates its mode of action (36–38). CRP DNA binding affinity varies between promoters, with the highest affinities corresponding to those sites that are most similar to the consensus site (32, 35, 39). Finally, activation-defective mutants of CRP have been identified, and they contain substitutions in amino acids that interact with RNA polymerase (40, 41). While these general parallels suggest that LuxR functions similarly to CRP, there are two striking contrasts: first, there is no known ligand that controls LuxR activity, and second, LuxR repression requires a symmetrical palindrome, whereas LuxR activation requires only a half-site. We are currently determining how the position of LuxR binding sites with respect to RNA polymerase at promoters correlates with activation or repression.

As the master regulator of quorum-sensing gene expression, LuxR controls the timing of expression of hundreds of genes in response to changes in cell density. The concentration of LuxR increases as autoinducers accumulate (7). This LuxR protein concentration gradient enables LuxR to control promoters via different binding affinities at various cell densities, producing a temporal pattern of gene expression (7). Absent other modulatory features, promoters containing the highest-affinity binding sites will be regulated first during the transition from LCD to HCD. For example, VIBHAR_05222 is repressed 2-fold by LuxR at LCD and 7-fold at HCD. Thus, because LuxR has a high binding affinity for the binding site at P_luxC, it is repressed even by the low concentrations of LuxR present at LCD. In contrast, luxC is one of the final genes to be activated in response to quorum sensing (data not shown), which fits with our observation that LuxR has a weak affinity for P_luxC site 2. Thus, DNA binding affinity, coupled to other features, results in a finely choreographed pattern of gene expression. We propose that LuxR, although structurally similar to TetR, has evolved unique characteristics that enable it to differentially control the genes in the quorum-sensing regulon in response to quorum-sensing cues.

MATERIALS AND METHODS

Bacterial strains and media. Escherichia coli strains S17-1 Apir, DH10B (Electromax; Invitrogen), BL21(DE3) (Invitrogen), and derivatives were grown with aeration at 37°C in Luria-Bertani (LB) medium with 40 μg/ml kanamycin and 10 μg/ml chloramphenicol. V. harveyi strain BB120 (BAA-1116) and derivatives were grown with aeration at 30°C in Luria marine (LM) medium with 100 μg/ml kanamycin (Sigma) and 10 μg/ml chloramphenicol (Sigma). Plasmids were transferred from E. coli to V. harveyi by conjugation (42).

Molecular methods. E. coli strains S17-1 Apir and DH10B were used for cloning. PCR reactions used iProof DNA polymerase (Bio-Rad). Restriction enzymes, T4 polynucleotide kinase, calf intestinal phosphatase (CIP), and T4 DNA ligase were purchased from New England Biolabs (NEB). Oligonucleotides were purchased from Integrated DNA Technologies. The Genomener II EZClone Domain mutagenesis kit (Stratagene) was used for random mutagenesis. The QuikChange mutagenesis kit (Stratagene) was used to introduce mutations into plasmids. Cloning procedures and sequences of PCR primers are available upon request. All plasmid constructs were confirmed by sequencing by Genewiz, Inc. RNA isolation, cDNA synthesis, and quantitative real-time PCR (qRT-PCR) reactions were carried out as described elsewhere (42). Samples were nor-
an input DNA and IP DNA from an empty vector control (pJV139). See following ChIP samples were sequenced: the input DNA and IP DNA from representative ChIP samples was prepared for sequencing using the Illumina ChIP-seq sample prep kit and verified by qRT-PCR. The DNA from four independent experiments.

**Inducible expression of luxR.** luxR was expressed under control of the P_m promoter from a plasmid (pV239) constitutively expressing lacP. Overnight E. coli cultures containing this plasmid and derivatives were diluted 1:1,000 and grown at 30°C for 16 h, and samples were analyzed by FACS or qRT-PCR. Overnight V. harveyi cultures containing the plasmid and derivatives were diluted 1:1,000 and grown to an approximate OD_{600} of 0.2, 10 μM IPTG was added, and the cultures were grown for 3 additional hours. FLAG-luxR was expressed under the control of the P_{luxR} promoter (pV057) as previously described (7). To induce FLAG-luxR expression, overnight cultures of E. coli strains containing this construct were diluted 1:1,000 in the presence of 1 mM IPTG and 10 μM theophylline, and samples were measured by FACS or harvested for RNA isolation after 16 h of growth at 30°C.

**ChIP-seq.** Plasmids expressing either FLAG-luxR (pAP116) or FLAG-luxR R17C (pST012); a DNA-binding-defective luxR mutant and empty vector controls (pSLS3 or pV139) were conjugated into a V. harveyi ΔluxR strain (KM669). The ChIP protocol is based on previously published methods (44) and the Affymetrix ChIP assay protocol with several modifications. Overnight cultures were diluted 1:50,000 and grown for 16 h at 30°C. Fifty OD_{600} units of cells were cross-linked and washed as previously described (44), and cells were lysed in 1 ml of lysis buffer (1× protease inhibitors [Sigma], 50 μg ml⁻¹ lysozyme, 1 mM Bugbuster, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride [PMSF]) for 20 min at room temperature on a rotator. Following lysis, the DNA was sheared by sonication to an average size of 100 to 1,000 bp. The supernatant was clarified at 13,000 rpm for 10 min at 4°C. Immunoprecipitation reactions contained a 200-μl aliquot of input sample, 800 μl of IP buffer (50 mM HEPES-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1 mM PMSF), and 40 μl EZ-view anti-FLAG agarose beads (Sigma) (equilibrated in Tris-buffered saline [TBS]) and were carried out for 2 h at room temperature on a rotator. Following immunoprecipitation, beads were collected and washed (Affymetrix ChIP assay protocol). Immunoprecipitated complexes were eluted, and cross-links were reversed as described elsewhere (Affymetrix ChIP assay protocol). Samples (input DNA and IP DNA) were analyzed by qRT-PCR to assess the quality of the immunoprecipitation from four independent experiments. DNA from representative ChIP samples was prepared for sequencing using the Illumina ChIP-seq sample prep kit and verified by qRT-PCR. The following ChIP samples were sequenced: the input DNA and IP DNA from FLAG-luxR (pAP116), IP DNA from FLAG-luxR R17C (pST012), and input DNA and IP DNA from an empty vector control (pV139). See the supplemental material for a description of ChIP-seq data analysis procedures.

**Fluorescent reporter assays.** LuxR transcriptional regulation of P_{5222} and P_{luxC} was measured in E. coli strains carrying pJV064, containing a transcriptional fusion of mCherry to P_{5222} and a transcriptional fusion of GFP to P_{luxC}. Overnight E. coli DH10B cultures containing pJV239 (or derivatives) and pJV064 were diluted 1:1,000 and grown for 16 h at 30°C. GFP and mCherry fluorescence was measured using a 1420 Victor2 multilabel counter (Wallac) or on a Becton Dickinson FACSaria cell sorter using FACS Diva software.

**Genetic screens.** Mutant luxR libraries were generated by random mutagenesis of the luxR or FLAG-luxR ORF. For the wild-type luxR screen, 796,100 luxR mutants (average, 1.8 mutations per clone) were pooled, and DNA was extracted and transformed into E. coli DH10B containing pJV064. Colonies (338,500) were pooled, and the cultures were diluted 1:1,000 and grown at 30°C for 16 h. The FLAG-luxR screen was performed similarly except the mutant library culture was grown in medium containing 1 mM IPTG and 10 μM theophylline. Mutant clones (86,850) were obtained (average, 3.5 mutations per clone), DNA was extracted and transformed into DH10B carrying pJV064, and 580,000 colonies were pooled for screening. In all experiments, mutants were sorted into two groups using FACS: (i) high mCherry fluorescence (no repression) and high GFP fluorescence (wild-type activation), and (ii) low mCherry fluorescence (wild-type repression) and low GFP fluorescence (no activation). Positive clones were retested and sequenced. All mutants showed identical phenotypes in the presence or absence of the FLAG tag.

To screen for mutations in P_{5222}, an oligonucleotide containing the binding site (GTACTGACAAAAAAGTTAAT) was purchased from IDT with 3% randomization and inserted in place of the wild-type site in pJV064. Twenty-two thousand four hundred ten mutant clones (average, 2.1 mutations per clone) were pooled, and DNA was extracted and transformed into E. coli DH10B containing the plasmid expressing luxR T52M/124V (pV241). Forty-six thousand colonies were pooled, and the culture was diluted 1:100 and grown for 3 h. Populations of cells were sorted using FACS to obtain cells exhibiting high GFP fluorescence (wild-type activation) and low mCherry fluorescence (wild-type repression). Clones were sequenced and analyzed in the presence of wild-type luxR, luxR T52M/124V, or an empty vector (pJV036).

**EMSAs.** LuxR proteins were purified and EMSA substrates (see Table S3 in the supplemental material) were annealed as previously described (7). Labeling reactions (100 fmol of double-stranded DNA [dsDNA] substrate, 10 μCi of [γ-32P]ATP, T4 polynucleotide kinase [NEB], and kinase buffer) were incubated for 30 min at 37°C, and the labeled substrates were purified on ProbeQuant G-50 Micro columns (GE Healthcare). EMSA reactions (12 μl) were performed as previously described (7) with two modifications. Reaction mixtures contained 0.1 nM dsDNA substrate and the LuxR protein in dilution buffer (20 mM imidazole, pH 7.5, 300 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol [DTT], and 5% glycerol). DNA binding measurements were determined using ImageQuant software (GE Healthcare) and GraphPad Prism software.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at [http://mbio.asm.org/llookup/suppl/doi:10.1128/mBio.00378-13/-/DCSupplemental](http://mbio.asm.org/llookup/suppl/doi:10.1128/mBio.00378-13/-/DCSupplemental).

**Text S1, DOX file, 0.1 MB.**

Figure S1, TIF file, 0.7 MB.

Figure S2, TIF file, 0.5 MB.

Figure S3, TIF file, 0.8 MB.

Figure S4, TIF file, 0.2 MB.

Table S1, DOX file, 0.1 MB.

Table S2, DOX file, 0.2 MB.

Table S3, DOX file, 0.2 MB.

Table S4, DOX file, 0.1 MB.

Table S5, DOCX file, 0.1 MB.

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