

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

Julia C. van Kessel,^a Luke E. Ulrich,^{b,c} Igor B. Zhulin,^{c,d} Bonnie L. Bassler^{a,e}

Department of Molecular Biology, Princeton University, Princeton, New Jersey, USA^a; Agile Genomics LLC, Mount Pleasant, South Carolina, USA^b; Department of Microbiology, University of Tennessee, Knoxville, Tennessee, USA^c; Computer Science and Mathematics Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee, USA^d; Howard Hughes Medical Institute, Princeton University, Princeton, New Jersey, USA^e

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 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.

Received 28 May 2013 Accepted 5 June 2013 Published 9 July 2013

Citation van Kessel JC, Ulrich LE, Zhulin IB, Bassler BL. 2013. Analysis of activator and repressor functions reveals the requirements for transcriptional control by LuxR, the master regulator of quorum sensing in *Vibrio harveyi*. *mBio* 4(4):e00378-13. doi:10.1128/mBio.00378-13.

Editor Ronald Taylor, Dartmouth Medical School

Copyright © 2013 van Kessel et al. This is an open-access article distributed under the terms of the [Creative Commons Attribution-Noncommercial-ShareAlike 3.0 Unported license](https://creativecommons.org/licenses/by-nc-sa/3.0/), which permits unrestricted noncommercial use, distribution, and reproduction in any medium, provided the original author and source are credited.

Address correspondence to Bonnie L. Bassler, bbassler@princeton.edu.

Bacteria use the cell-cell communication process called quorum sensing to measure and respond to changes in the number and species relatedness of bacteria in the environment. Quorum sensing involves the production and detection of extracellular signaling molecules called autoinducers. At low cell density (LCD), autoinducer levels are below the concentration required for detection, and so bacteria behave as individuals. As bacteria grow to high cell density (HCD), autoinducers accumulate. When a critical-threshold concentration of autoinducers is achieved, bacteria detect them and express genes required for group behaviors, such as biofilm formation, virulence factor production, and bioluminescence (1). In the model quorum-sensing bacterium *Vibrio harveyi*, information encoded in autoinducers is funneled into the control of the production of LuxR, the master regulator of quorum-sensing gene expression.

LuxR is the founding member of a group of homologous pro-

teins that control quorum-sensing responses in all vibrios, including HapR (*Vibrio cholerae*), SmcR (*Vibrio vulnificus*), LitR (*Vibrio fischeri*), and OpaR (*Vibrio parahaemolyticus*) (2). LuxR-type proteins are members of the TetR family of transcription factors, which are ubiquitous in bacteria and share a conserved helix-turn-helix (HTH) DNA binding motif in their N-terminal domains (3). TetR proteins function as dimers to recognize and bind DNA sequences possessing dyad symmetry. Many TetR proteins bind small molecules; for example, the canonical TetR protein binds tetracycline. In the unbound state, TetR binds to two DNA binding sites: one to autorepress its own expression and one to repress expression of *tetA*, which confers tetracycline resistance. Upon binding to tetracycline, TetR releases the DNA, allowing transcription. The crystal structures of two LuxR homologs, HapR and SmcR, have been solved, and they have identical domain folds and the same relative domain arrangements as the TetR protein QacR

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*, *luxR*, *luxC*, *qrgB*, and *qrr4* 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 consen-

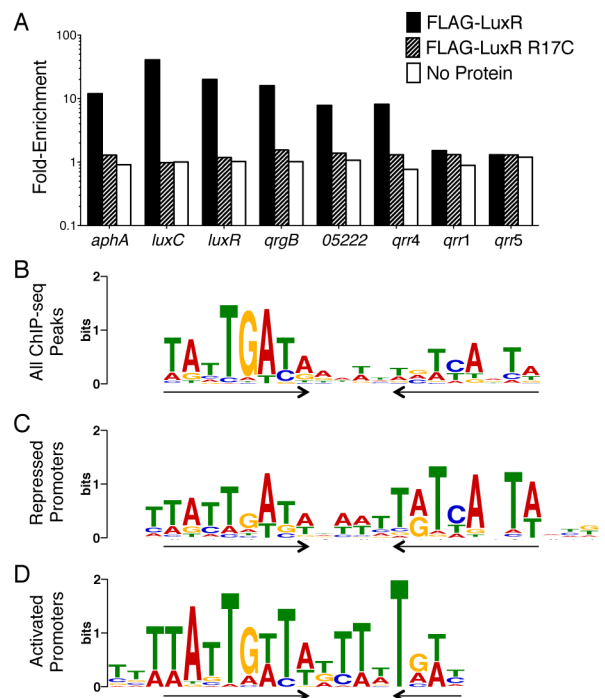


FIG 1 LuxR directly regulates 115 genes. (A) qRT-PCR analysis of LuxR occupancy at promoters in a *V. harveyi* $\Delta luxR$ strain (KM669) expressing FLAG-LuxR (pAPI116), FLAG-*luxR* R17C (pST012, a DNA binding mutant), and an empty vector (pSLS3, denoted as “no protein”). Fold enrichment at each promoter represents the ratio of immunoprecipitated DNA to input DNA. These data represent four independent experiments. (B to D) LuxR DNA binding motifs from MEME analyses of groups of ChIP-seq data peaks: all peaks (B), peaks present upstream of repressed promoters (C), or peaks present upstream of activated promoters (D). Inverted arrows denote the dyad symmetry.

sus 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

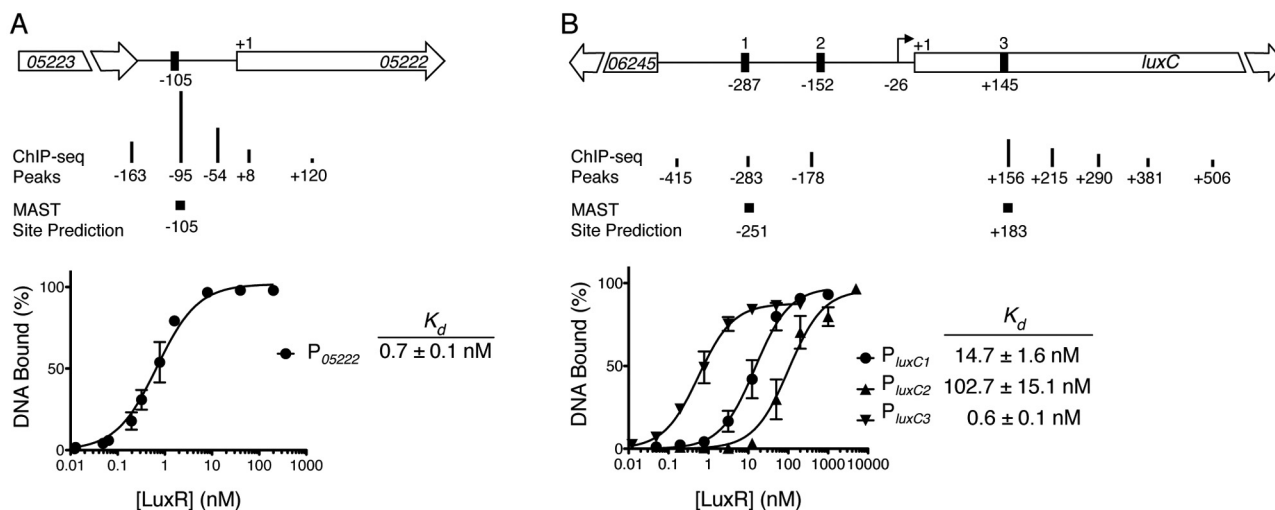


FIG 2 LuxR binding to activated and repressed promoters. Diagrams illustrating the locations and relative strengths of LuxR binding (ChIP-seq peaks) at the *VIBHAR_05222* promoter region (A) or the *luxC* promoter region (B). Sites determined *in vitro* (black boxes) or by MAST are shown relative to 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.

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 *VIBHAR_05222* locus using the MAST (Motif Alignment and Search Tool) software tool (15, 17, 18). We identified only one binding site (Fig. 2A), and LuxR bound this site with high affinity ($K_d = 0.7$ nM) (Fig. 2A). We performed EMSAs with DNA fragments that tile across the entire promoter and verified that this is the only site bound by LuxR *in vitro* (see Fig. S1E in the supplemental material). The sizes of the DNA fragments analyzed during standard ChIP-seq analysis (~200 bp) are larger than the LuxR binding sites (28 bp). Because of this, at some promoters, such as P_{05222} , a single LuxR binding site appears as a wide peak and is interpreted to be multiple peaks by the bioinformatics analysis.

To study a typical case of a gene activated by LuxR, we chose the promoter driving expression of the luciferase operon (*luxCDABE*, *VIBHAR_06244-VIBHAR_06240*), the most highly activated locus controlled by LuxR. Bioinformatic analysis of the ChIP-seq data identified eight LuxR binding peaks near *luxC* (P_{luxC}). Scanning this region with the LuxR PWM by using MAST revealed only two sites, one of which is located within the *luxC* open reading frame (ORF) (denoted as sites 1 and 3 in Fig. 2B). Previous studies showed LuxR bound to an additional site that we do not

identify by MAST (denoted as site 2 in Fig. 2B) (8, 12). LuxR bound all three sites *in vitro*, with the highest binding affinity for site 3 ($K_d = 0.6$ nM) and the weakest affinity for site 2 ($K_d = 102.7$ nM) (Fig. 2B). Others have shown that removing the high-affinity site 3 does not affect the level of LuxR activation *in vivo*, and thus site 3 is not required for P_{luxC} activation (12). Similarly, we observed that a $P_{luxC-gfp}$ reporter construct lacking site 3 is fully activated by LuxR *in vivo* (see Fig. S1F in the supplemental material). Therefore, we focused on sites 1 and 2 to determine the site(s) required for activation *in vivo*. A randomized sequence that is not bound by LuxR (see Fig. S1G) was inserted in place of the LuxR binding site at position 1 or position 2. Randomizing site 1 did not alter activation, whereas randomizing site 2 eliminated activation (see Fig. S1F). Collectively, these results demonstrate that LuxR binds to multiple binding sites *in vitro* and *in vivo*, but only site 2 is necessary for *luxC* activation *in vivo*.

Mutations in *luxR* separate activation and repression at representative promoters. Our ChIP-seq data indicated that LuxR binds distinct sequences in the promoters of activated and repressed genes (Fig. 1), suggesting that there could be different requirements for LuxR for activation and repression. To investigate the mechanisms underpinning LuxR activation and repression, we performed a screen to identify LuxR mutants that differentiate between activated and repressed promoters. We screened a library of random *luxR* mutants for two phenotypes: (i) LuxR variants that cannot activate but can repress (activation-defective mutants) and (ii) LuxR variants that can activate but cannot repress (repression-defective mutants). We used our two representative promoters, P_{luxC} and P_{05222} , to assay activation and repression, respectively. A *gfp* reporter was fused to P_{luxC} and an *mCherry* reporter was fused to P_{05222} to facilitate simultaneous screening by fluorescence-assisted cell sorting (FACS).

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

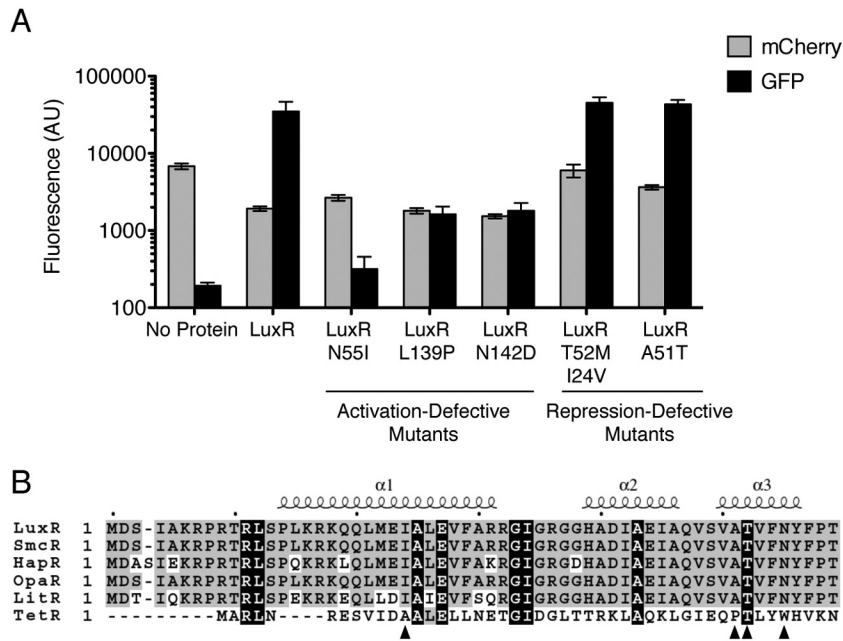


FIG 3 LuxR mutants defective for activation or repression. (A) LuxR mutants with decreased activation of P_{luxC} (activation-defective mutants) and decreased repression of P_{05222} (repression-defective mutants). *E. coli* strains containing an empty vector (pJV036, denoted as “No Protein”), expressing *luxR* (pJV239), or expressing *luxR* mutants (N55I [pJV240], L139P [pJV242], N142D [pJV261], T52M/124V [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. fischeri* (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.

these, LuxR N55I and LuxR N55K exhibited the most severe phenotypes (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/124V is completely defective for repression, while LuxR A51T is partially defective (Fig. 3A). We separated the LuxR T52M/124V mutations by constructing LuxR T52M and LuxR I24V. The LuxR T52M mutant was inactive at both the activated and repressed 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 substitutions are shown in an alignment of the DNA binding domains of LuxR homologs and *E. coli* TetR (Fig. 3B). Several of the mutations lie in the conserved HTH (N55I and other N55 substitutions, T52M/124V and A51T), including a substitution of the T52 residue that is conserved between LuxR and TetR. We return to this point below.

Mutations in *luxR* affect regulation of specific promoters. To test the generality of the activation and repression defects for the LuxR mutant proteins, we measured their activities at other LuxR-regulated promoters in *V. harveyi*. First, we tested whether the activation-defective mutants were also defective at other activated promoters. In addition to eliminating activation of *luxC*, the LuxR N55I variant showed decreased activation of P_{p08175} (Fig. 4). In contrast, the activation-defective mutants LuxR L139P and LuxR N142D activated P_{05020} but could not activate P_{p08175} (Fig. 4; see also Fig. S2 in the supplemental material). All three activation-defective mutants fully repressed P_{05222} but did show defects in repression at other promoters (for example, P_{aphA}) (Fig. 4; see also Fig. S2).

We next tested the generality of the repression-defective mu-

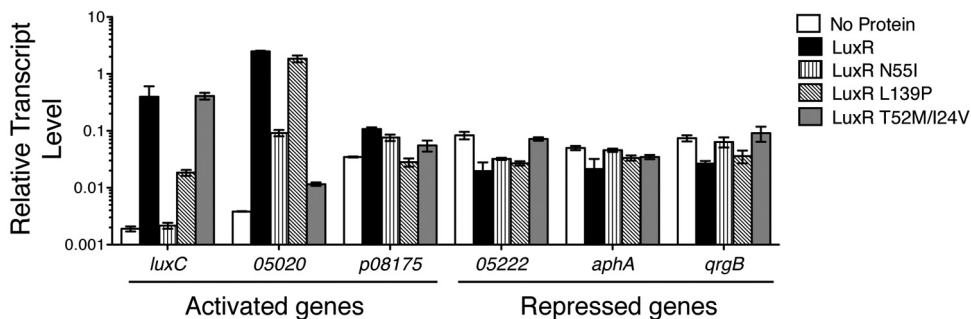


FIG 4 LuxR mutants are defective for regulating specific promoters in *V. harveyi*. Transcript levels of genes regulated by LuxR were assayed by qRT-PCR from a *V. harveyi* $\Delta luxR$ strain (KM669) containing an empty vector (pJV036, denoted as “No Protein”) or expressing *luxR* (pJV239), *luxR* N55I (pJV240), *luxR* L139P (pJV242), or *luxR* T52M/124V (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_{05222} , 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_{05222} using EMSAs with purified proteins. The activation-defective mutants LuxR N55I and LuxR L139P both bound the P_{05222} 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_{05222} . 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_{05222} because it is impaired for DNA binding at this particular promoter.

We next tested DNA binding at sites 1 and 2 in P_{luxC} , 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

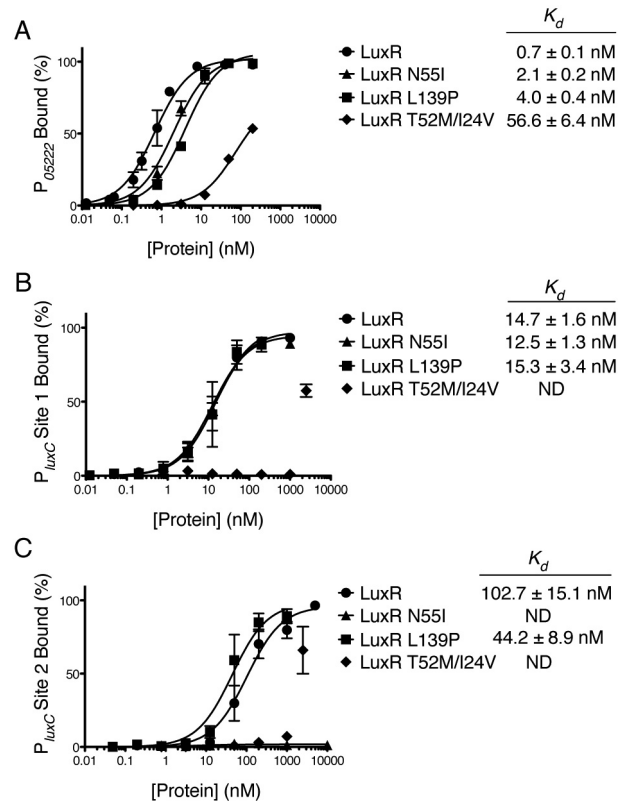


FIG 5 LuxR mutant proteins are defective for DNA binding. DNA binding curves from quantitative EMSAs are shown for LuxR, LuxR N55I, LuxR L139P, and LuxR T52M/I24V at the P_{05222} binding site (A), P_{luxC} binding site 1 (B), and P_{luxC} binding site 2 (C) (see Fig. S3 in the supplemental material for representative gels showing data used to calculate binding curves). Dissociation constants (K_d) are shown for each protein at each binding site at which a curve could be fit to the data. Error bars represent the standard deviations of three independent measurements.

(P_{05222} G \rightarrow T and P_{05222} T \rightarrow 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

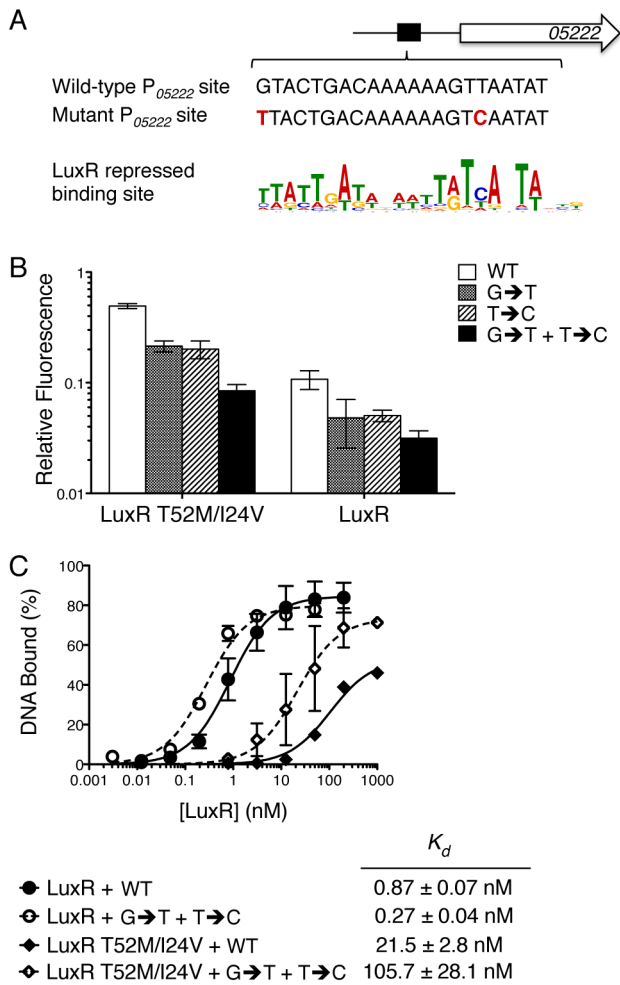


FIG 6 The LuxR T52M/I24V defect is suppressed by altering the corresponding DNA binding site. (A) The P_{05222} binding site and the two suppressor mutations (G \rightarrow T and T \rightarrow C) aligned with the LuxR binding site consensus motif from repressed promoters. (B) *E. coli* strains expressing *luxR* (pJV239), *luxR* T52M/I24V (pJV241), or containing an empty vector (pJV036) were assayed for transcriptional regulation of P_{05222} . Fluorescence was measured from P_{05222} -*mCherry* with either the wild-type P_{05222} site (WT, pJV141), mutation G \rightarrow T (pJV230), mutation T \rightarrow C (pJV227), or mutations G \rightarrow T and T \rightarrow C (pJV233). Fluorescence was normalized to the empty vector control. Error bars represent the standard deviations of measurements of three biological samples. (C) DNA binding curves from quantitative EMSAs for LuxR and LuxR T52M/I24V bound to either the P_{05222} binding site (WT) or the P_{05222} mutant binding site containing two mutations (G \rightarrow T + T \rightarrow C). Dissociation constants (K_d) are shown for each protein at each site. Error bars represent the standard deviations of three independent measurements.

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

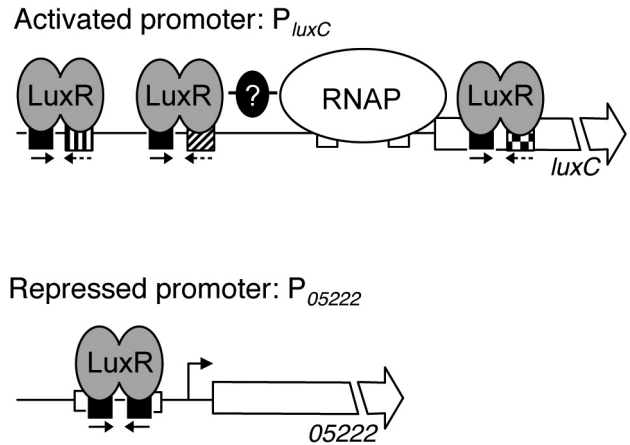


FIG 7 Model for LuxR transcriptional control. At activated promoters (e.g., P_{luxC}), LuxR interacts with RNA polymerase (denoted RNAP) and/or other transcription factors to promote transcription. At repressed promoters (e.g., P_{05222}), LuxR binding occludes RNA polymerase, which prevents transcription. LuxR binding site sequences at repressed promoters are symmetrical palindromes (denoted by black boxes with inverted arrows), whereas binding site sequences at activated promoters contain only one-half of the palindrome. Furthermore, LuxR binding site sequences at each activated promoter vary, resulting in distinct LuxR binding affinities (denoted by the different fill patterns for the binding site boxes in the P_{luxC} promoter).

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 palindrome. Second, LuxR bound a single site at the example repressed promoter P_{05222} , 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_{05222} . In an analogous experiment, *tet* 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 example 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 SmcR (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_{05222} 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_{05222} , 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 λ pir, DH10B (Electromax; Invitrogen), BL21(DE3) (Invitrogen), and derivatives were grown with aeration at 37°C in Luria-Bertani (LB) medium with 40 μ g ml⁻¹ kanamycin and 10 μ g ml⁻¹ chloramphenicol. *V. harveyi* strain BB120 (BAA-1116) and derivatives were grown with aeration at 30°C in Luria marine (LM) medium with 100 μ g ml⁻¹ kanamycin (Sigma) and 10 μ g ml⁻¹ chloramphenicol (Sigma). Plasmids were transferred from *E. coli* to *V. harveyi* by conjugation (42).

Molecular methods. *E. coli* strains S17-1 λ pir 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 Genemorph 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-

malized to the internal standard *hfg*, and the standard curve method or the $\Delta\Delta C_T$ method was used for data analysis.

For quantitative Western blot analyses of LuxR production in wild-type cells, *V. harveyi* BB120 cells were collected at an optical density at 600 nm (OD_{600}) of 1.0 (HCD), and cell counts were determined by viability plating (see Fig. S1B in the supplemental material). For quantitative Western blots of mutant LuxR proteins, cells were collected from *V. harveyi* $\Delta luxR$ mutant strains expressing wild-type or mutant *luxR* following induction (see Fig. S2 and see below). Pellets were resuspended at 0.01 OD_{600} units per μ l in lysis buffer (Bugbuster [Novagen], 50 μ g ml^{-1} lysozyme, and 1 U ml^{-1} Benzonase [Novagen]). Lysates were serially diluted to determine the linear range for quantification of LuxR bands, and proteins were analyzed by SDS-PAGE on 12% polyacrylamide gels. Protein transfer, membrane blocking, and incubations with anti-LuxR primary antibodies, anti-mouse secondary antibodies, and anti-rabbit secondary antibodies were performed as described elsewhere (43). Anti-beta' antibodies raised against *E. coli* beta' (Rpoc) were purchased from Neo-clone and used at a 1:10,000 dilution. Chemiluminescence was carried out using a SuperSignal West Pico kit (Pierce). Protein bands were quantified using ImageQuant software.

Inducible expression of *luxR*. *luxR* was expressed under control of the P_{tac} promoter from a plasmid (pJV239) constitutively expressing *lacI^q*. 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 μ M IPTG was added, and the cultures were grown for 3 additional hours. FLAG-*luxR* was expressed under the control of the $P_{tactheo}$ promoter (pJV057) 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 μ M 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 pJV139) were conjugated into a *V. harveyi* $\Delta luxR$ 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 \times protease inhibitors [Sigma], 50 μ g ml^{-1} lysozyme, 1 \times 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 reaction mixtures contained a 200- μ l aliquot of input sample, 800 μ l 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 μ l 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 (pJV139). See the supplemental material for a description of ChIP-seq data analysis procedures.

Fluorescent reporter assays. LuxR transcriptional regulation of P_{05222} and P_{luxC} was measured in *E. coli* strains carrying pJV064, containing a

transcriptional fusion of *mCherry* to P_{05222} 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 either on 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 μ M 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_{05222} , an oligonucleotide containing the binding site (GTACTGACAAAAAGTTAATAT) 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/I24V (pJV241). 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/I24V, or an empty vector (pJV036).

EMSA. 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 [ds-DNA] substrate, 10 μ Ci 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 μ l) 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/lookup/suppl/doi:10.1128/mBio.00378-13/-/DCSupplemental>.

Text S1, DOCX 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, DOCX file, 0.1 MB.

Table S2, DOCX file, 0.2 MB.

Table S3, DOCX file, 0.2 MB.

Table S4, DOCX file, 0.1 MB.

Table S5, DOCX file, 0.1 MB.

ACKNOWLEDGMENTS

We thank Colleen O'Loughlin, Zhuo Li, Amanda Izzo, and Anne McCabe for assistance with experiments. We thank Matthew Bochman and Steven Rutherford for insightful comments. We appreciate Donna Storton, Jessica Buckles, and Lance Parsons for assistance with Illumina sequencing.

This work was supported by the Howard Hughes Medical Institute, National Institutes of Health (NIH) grant 5R01GM065859 and National Science Foundation (NSF) grant MCB-0343821 to B.L.B., NIH grant GM07225 to I.B.Z., and NIH fellowship F32GM089019 to J.C.V.K.

REFERENCES

- Rutherford ST, Bassler BL. 2012. Bacterial quorum sensing: its role in virulence and possibilities for its control. *Cold. Spring Harb. Perspect. Med.* 2:11. doi: [10.1101/cshperspect.a012427](https://doi.org/10.1101/cshperspect.a012427).
- Kim Y, Kim BS, Park YJ, Choi WC, Hwang J, Kang BS, Oh TK, Choi SH, Kim MH. 2010. Crystal structure of SmcR, a quorum-sensing master regulator of *Vibrio vulnificus*, provides insight into its regulation of transcription. *J. Biol. Chem.* 285:14020–14030.
- Ramos JL, Martínez-Bueno M, Molina-Henares AJ, Terán W, Watanabe K, Zhang X, Gallegos MT, Brennan R, Tobes R. 2005. The TetR family of transcriptional repressors. *Microbiol. Mol. Biol. Rev.* 69:326–356.
- De Silva RS, Kovacicova G, Lin W, Taylor RK, Skorupski K, Kull FJ. 2007. Crystal structure of the *Vibrio cholerae* quorum-sensing regulatory protein HapR. *J. Bacteriol.* 189:5683–5691.
- Schumacher MA, Miller MC, Grkovic S, Brown MH, Skurray RA, Brennan RG. 2002. Structural basis for cooperative DNA binding by two dimers of the multidrug-binding protein QacR. *EMBO J.* 21:1210–1218.
- Waters CM, Bassler BL. 2006. The *Vibrio harveyi* quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. *Genes Dev.* 20:2754–2767.
- van Kessel JC, Rutherford ST, Shao Y, Utria AF, Bassler BL. 2013. Individual and combined roles of the master regulators AphA and LuxR in control of the *Vibrio harveyi* quorum-sensing regulon. *J. Bacteriol.* 195:436–443.
- Swartzman E, Meighen EA. 1993. Purification and characterization of a poly(dA-dT) lux-specific DNA-binding protein from *Vibrio harveyi* and identification as LuxR. *J. Biol. Chem.* 268:16706–16716.
- Lee DH, Jeong HS, Jeong HG, Kim KM, Kim H, Choi SH. 2008. A consensus sequence for binding of SmcR, a *Vibrio vulnificus* LuxR homologue, and genome-wide identification of the SmcR regulon. *J. Biol. Chem.* 283:23610–23618.
- Tsou AM, Cai T, Liu Z, Zhu J, Kulkarni RV. 2009. Regulatory targets of quorum sensing in *Vibrio cholerae*: evidence for two distinct HapR-binding motifs. *Nucleic Acids Res.* 37:2747–2756.
- Pompeani AJ, Irgon JJ, Berger MF, Bulyk ML, Wingreen NS, Bassler BL. 2008. The *Vibrio harveyi* master quorum-sensing regulator, LuxR, a TetR-type protein is both an activator and a repressor: DNA recognition and binding specificity at target promoters. *Mol. Microbiol.* 70:76–88.
- Miyamoto CM, Smith EE, Swartzman E, Cao JG, Graham AF, Meighen EA. 1994. Proximal and distal sites bind LuxR independently and activate expression of the *Vibrio harveyi* lux operon. *Mol. Microbiol.* 14:255–262.
- Hammer BK, Bassler BL. 2003. Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Mol. Microbiol.* 50:101–104.
- Svenningsen SL, Waters CM, Bassler BL. 2008. A negative feedback loop involving small RNAs accelerates *Vibrio cholerae*'s transition out of quorum-sensing mode. *Genes Dev.* 22:226–238.
- Bailey TL, Elkan C. 1994. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. *Proc. Int. Conf. Intell. Syst. Mol. Biol.* 2:28–36.
- Zhang Y, Qiu Y, Tan Y, Guo Z, Yang R, Zhou D. 2012. Transcriptional regulation of opaR, qrr2-4 and aphA by the master quorum-sensing regulator OpaR in *Vibrio parahaemolyticus*. *PLoS One* 7:e34622. doi: [10.1371/journal.pone.0034622](https://doi.org/10.1371/journal.pone.0034622).
- Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren J, Li WW, Noble WS. 2009. MEME SUITE: tools for motif discovery and searching. *Nucleic Acids Res.* 37:W202–W208. doi: [10.1093/nar/gkp335](https://doi.org/10.1093/nar/gkp335).
- Bailey TL, Gribskov M. 1998. Combining evidence using p-values: application to sequence homology searches. *Bioinformatics* 14:48–54.
- Tuch BB, Mitrovich QM, Homann OR, Hernday AD, Monighetti CK, De La Vega FM, Johnson AD. 2010. The transcriptomes of two heritable cell types illuminate the circuit governing their differentiation. *PLoS Genet.* 6:e1001070. doi: [10.1371/journal.pgen.1001070](https://doi.org/10.1371/journal.pgen.1001070).
- Zordan RE, Miller MG, Galgoczy DJ, Tuch BB, Johnson AD. 2007. Interlocking transcriptional feedback loops control white-opaque switching in *Candida albicans*. *PLoS Biol.* 5:e256. doi: [10.1371/journal.pbio.0050256](https://doi.org/10.1371/journal.pbio.0050256).
- Georlette D, Ahn S, MacAlpine DM, Cheung E, Lewis PW, Beall EL, Bell SP, Speed T, Manak JR, Botchan MR. 2007. Genomic profiling and expression studies reveal both positive and negative activities for the *Drosophila* Myb MuvB/dREAM complex in proliferating cells. *Genes Dev.* 21:2880–2896.
- Li XY, MacArthur S, Bourgon R, Nix D, Pollard DA, Iyer VN, Hechmer A, Simirenko L, Stapleton M, Luengo Hendriks CL, Chu HC, Ogawa N, Inwood W, Sementchenko V, Beaton A, Weiszmann R, Celniker SE, Knowles DW, Gingeras T, Speed TP, Eisen MB, Biggin MD. 2008. Transcription factors bind thousands of active and inactive regions in the *Drosophila* blastoderm. *PLoS Biol.* 6:e27. doi: [10.1371/journal.pbio.0060027](https://doi.org/10.1371/journal.pbio.0060027).
- Baumeister R, Helbl V, Hillen W. 1992. Contacts between Tet repressor and tet operator revealed by new recognition specificities of single amino acid replacement mutants. *J. Mol. Biol.* 226:1257–1270.
- Jeong HS, Kim SM, Lim MS, Kim KS, Choi SH. 2010. Direct interaction between quorum-sensing regulator SmcR and RNA polymerase is mediated by integration host factor to activate vvpE encoding elastase in *Vibrio vulnificus*. *J. Biol. Chem.* 285:9357–9366.
- Chatterjee J, Miyamoto CM, Zouzoulas A, Lang BF, Skouris N, Meighen EA. 2002. MetR and CRP bind to the *Vibrio harveyi* lux promoters and regulate luminescence. *Mol. Microbiol.* 46:101–111.
- Aiba H. 1983. Autoregulation of the *Escherichia coli* crp gene: CRP is a transcriptional repressor for its own gene. *Cell* 32:141–149.
- Musso RE, Di Lauro R, Adhya S, de Crombrughe B. 1977. Dual control for transcription of the galactose operon by cyclic AMP and its receptor protein at two interspersed promoters. *Cell* 12:847–854.
- Grainger DC, Hurd D, Harrison M, Holdstock J, Busby SJ. 2005. Studies of the distribution of *Escherichia coli* cAMP-receptor protein and RNA polymerase along the *E. coli* chromosome. *Proc. Natl. Acad. Sci. U. S. A.* 102:17693–17698.
- Zheng D, Constantinidou C, Hobman JL, Minchin SD. 2004. Identification of the CRP regulon using in vitro and in vivo transcriptional profiling. *Nucleic Acids Res.* 32:5874–5893.
- Gama-Castro S, Jiménez-Jacinto V, Peralta-Gil M, Santos-Zavaleta A, Peñaloza-Spinola MI, Contreras-Moreira B, Segura-Salazar J, Muñoz-Rascado L, Martínez-Flores I, Salgado H, Bonavides-Martínez C, Abreu-Goodger C, Rodríguez-Penagos C, Miranda-Ríos J, Morett E, Merino E, Huerta AM, Treviño-Quintanilla L, Collado-Vides J. 2008. RegulonDB (version 6.0): gene regulation model of *Escherichia coli* K-12 beyond transcription, active (experimental) annotated promoters and Textpresso navigation. *Nucleic Acids Res.* 36:D120–D124. doi: [10.1093/nar/gkn491](https://doi.org/10.1093/nar/gkn491).
- Shimada T, Fujita N, Yamamoto K, Ishihama A. 2011. Novel roles of cAMP receptor protein (CRP) in regulation of transport and metabolism of carbon sources. *PLoS One* 6:e20081. doi: [10.1371/journal.pone.0020081](https://doi.org/10.1371/journal.pone.0020081).
- Berg OG, von Hippel PH. 1988. Selection of DNA binding sites by regulatory proteins. II. The binding specificity of cyclic AMP receptor protein to recognition sites. *J. Mol. Biol.* 200:709–723.
- Ebright RH, Cossart P, Gicquel-Sanzey B, Beckwith J. 1984. Molecular basis of DNA sequence recognition by the catabolite gene activator protein: detailed inferences from three mutations that alter DNA sequence specificity. *Proc. Natl. Acad. Sci. U. S. A.* 81:7274–7278.
- Ebright RH, Cossart P, Gicquel-Sanzey B, Beckwith J. 1984. Mutations that alter the DNA sequence specificity of the catabolite gene activator protein of *E. coli*. *Nature* 311:232–235.
- Busby S, Ebright RH. 1999. Transcription activation by catabolite activator protein (CAP). *J. Mol. Biol.* 293:199–213.
- Kolb A, Busby S, Buc H, Garges S, Adhya S. 1993. Transcriptional regulation by cAMP and its receptor protein. *Annu. Rev. Biochem.* 62:749–795.
- Xiong XF, de la Cruz N, Reznikoff WS. 1991. Downstream deletion analysis of the lac promoter. *J. Bacteriol.* 173:4570–4577.
- Aiba H. 1985. Transcription of the *Escherichia coli* adenylate cyclase gene is negatively regulated by cAMP-cAMP receptor protein. *J. Biol. Chem.* 260:3063–3070.
- Kolb A, Busby S, Herbert M, Kotlarz D, Buc H. 1983. Comparison of the binding sites for the *Escherichia coli* cAMP receptor protein at the lactose and galactose promoters. *EMBO J.* 2:217–222.
- Irwin N, Ptashne M. 1987. Mutants of the catabolite activator protein of *Escherichia coli* that are specifically deficient in the gene-activation function. *Proc. Natl. Acad. Sci. U. S. A.* 84:8315–8319.
- Eschenlauer AC, Reznikoff WS. 1991. *Escherichia coli* catabolite gene

- activator protein mutants defective in positive control of lac operon transcription. *J. Bacteriol.* 173:5024–5029.
42. Rutherford ST, van Kessel JC, Shao Y, Bassler BL. 2011. AphA and LuxR/HapR reciprocally control quorum sensing in vibrios. *Genes Dev.* 25:397–408.
 43. Shao Y, Bassler BL. 2012. Quorum-sensing non-coding small RNAs use unique pairing regions to differentially control mRNA targets. *Mol. Microbiol.* 83:599–611.
 44. Grainger DC, Overton TW, Reppas N, Wade JT, Tamai E, Hobman JL, Constantinidou C, Struhl K, Church G, Busby SJ. 2004. Genomic studies with Escherichia coli MelR protein: applications of chromatin immunoprecipitation and microarrays. *J. Bacteriol.* 186:6938–6943.
 45. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. *Bioinformatics* 23:2947–2948.
 46. Gouet P, Courcelle E, Stuart DI, Métoz F. 1999. ESPript: analysis of multiple sequence alignments in PostScript. *Bioinformatics* 15:305–308.