AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

MINIREVIEW

Quorum Sensing Gene Regulation by LuxR/HapR Master Regulators in Vibrios

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ABSTRACT The coordination of group behaviors in bacteria is accomplished via the cell-cell signaling process called quorum sensing. Vibrios have historically been models for studying bacterial communication due to the diverse and remarkable behaviors controlled by quorum sensing in these bacteria, including bioluminescence, type III and type VI secretion, biofilm formation, and motility. Here, we discuss the *Vibrio* LuxR/HapR family of proteins, the master global transcription factors that direct downstream gene expression in response to changes in cell density. These proteins are structurally similar to TetR transcription factors but exhibit distinct biochemical and genetic features from TetR that determine their regulatory influence on the quorum sensing gene network. We review here the gene groups regulated by LuxR/HapR and quorum sensing and explore the targets that are common and unique among *Vibrio* species.

KEYWORDS quorum sensing, LuxR, HapR, SmcR, gene regulation, *Vibrio*, *Vibrio cholerae*, *Vibrio harveyi*

Accepted manuscript posted online 8 May 2017

Citation Ball AS, Chaparian RR, van Kessel JC. 2017. Quorum sensing gene regulation by LuxR/HapR master regulators in vibrios. J Bacteriol 199:e00105-17. https://doi.org/10.1128/JB.00105-17.

Editor William Margolin, McGovern Medical School

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Quorum sensing is a type of cell-cell communication used by a wide variety of bacteria. Through the detection of small signaling peptides or molecules termed autoinducers, cells monitor and respond to changes in the surrounding bacterial population and coordinate group behaviors. The first evidence of quorum sensing was the observation that a bacterial phenotype correlated with changes in population density (1–3). We now know that a plethora of bacterial activities are regulated by quorum sensing, ranging from bioluminescence and motility to protease production and toxin secretion (4, 5).

Owing to the easily monitored bioluminescent quorum sensing phenotype of several *Vibrio* species, vibrios have become major model organisms for studies of bacterial communication. In Gram-negative bacteria, most known quorum sensing gene regulatory systems are controlled through direct binding of an autoinducer to a cytosolic transcription factor. These quorum sensing systems are named LuxI/LuxR systems after the *Vibrio fischeri* system that was originally discovered (6, 7). The LuxI protein is the autoinducer synthase, and the LuxR protein is the transcriptional regulator that binds autoinducer as a ligand, allowing it to dimerize and bind DNA to control quorum sensing-regulated genes (7–9). LuxI/LuxR proteins are found in a wide variety of Gram-negative bacteria, and many are involved in quorum sensing signaling (1). However, *V. fischeri* seems to be the exception rather than the rule in the *Vibrio* genus. Most other vibrios for which quorum sensing has been characterized have systems with membrane-bound autoinducer receptors (Fig. 1) (10–19). These histidine kinase receptors initiate a signaling cascade that culminates in the expression of a TetR-type transcription factor, which regulates quorum sensing genes (20, 21). The members of the *Vibrio* TetR family of master quorum sensing regulators are highly conserved and include HapR in *Vibrio cholerae*, SmcR in *Vibrio vulnificus*, LitR in *V. fischeri*, OpaR in *Vibrio parahaemolyticus*, VanT in *Vibrio anguillarum*, and VtpR in *Vibrio tubiashii* (22–27). The TetR-type master regulator of quorum sensing genes in *Vibrio harveyi* was also named

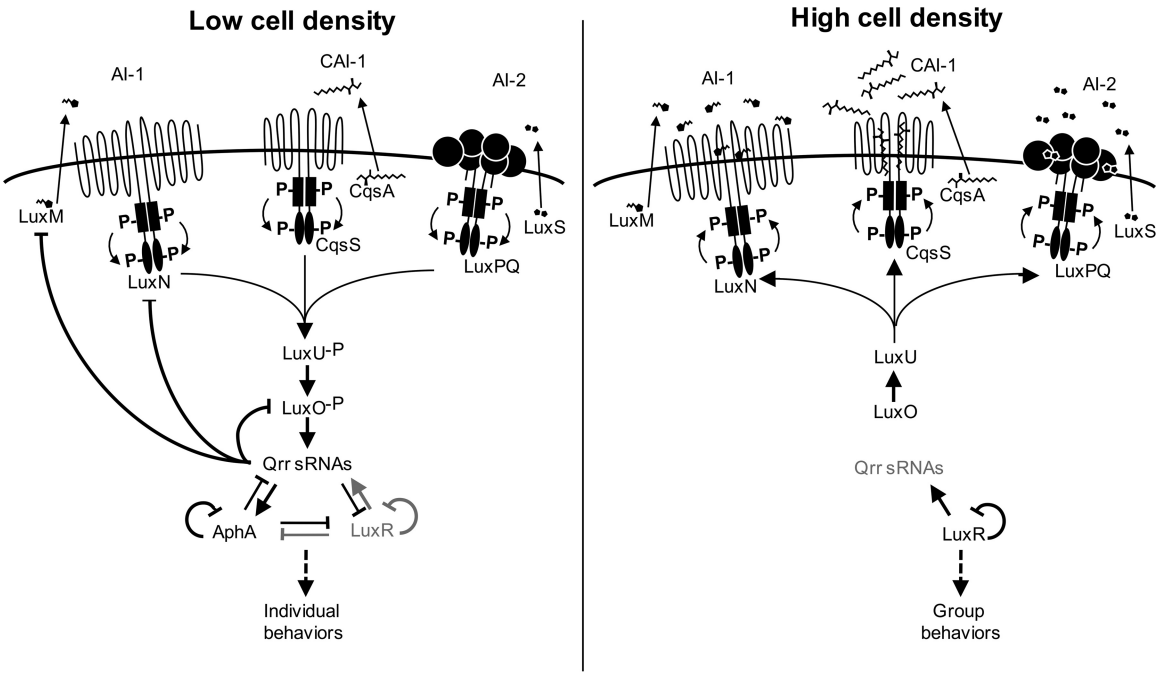

FIG 1 Model of the *V. harveyi* quorum sensing system. The autoinducer synthases LuxM, CqsS, and LuxS produce AI-1, CAI-1, and AI-2, respectively. At LCD, autoinducers are at low concentrations in the external environment. LuxO~P activates *qrr* gene expression. The Qrr sRNAs activate the expression of AphA and repress the expression of LuxR, LuxM, and LuxO. High levels of AphA and low levels of LuxR together regulate individual behavior genes. AphA autorepresses its expression and feeds back to repress the expression of the *qrr* genes and *luxR*. LuxR autorepresses its expression, represses *aphA*, and activates the *qrr* genes. At HCD, autoinducer concentrations are high, and AI-1, CAI-1, and AI-2 bind to the LuxN, CqsS, and LuxPQ receptors, respectively. The receptors dephosphorylate LuxU. Thus, LuxU does not phosphorylate LuxO, and the Qrrs are not expressed. LuxR is expressed at high levels, and AphA is not expressed. LuxR regulates group behavior genes. LuxR autorepresses its own expression and feeds back to repress *aphA* transcription and to activate *qrr* transcription.

LuxR. However, it is structurally, biochemically, and genetically distinct from the *V. fischeri* LuxR protein that binds an autoinducer as part of the LuxI/LuxR regulatory system (20, 28–31). *V. harveyi* LuxR and its *Vibrio* homologs do not require a ligand to dimerize or bind DNA (28, 32, 33). In fact, *V. fischeri* encodes a LuxR/HapR homolog called LitR in its central quorum sensing system in addition to the LuxI/LuxR system (21, 34). Further, LuxR, HapR, and SmcR can cross-complement activity in the respective strains, thus supporting the grouping of these proteins as functional homologs (22, 35).

THE *VIBRIO* QUORUM SENSING PATHWAY CULMINATES IN EXPRESSION OF AphA AND LuxR/HapR

V. harveyi was the first of the vibrios shown to have membrane-bound receptors that bind autoinducers (16). Thus, *V. harveyi* has become an ideal model organism for studying quorum sensing pathways in other vibrios where similar quorum sensing system architectures have been identified involving membrane-bound autoinducer receptors, small regulatory RNAs (sRNAs), and LuxR homologs acting as master regulators. These include *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*, and *Vibrio alginolyticus* (15, 22–24, 34, 36–41). In *V. harveyi*, the autoinducers are produced and diffuse through the cell membrane (Fig. 1). When *Vibrio* cells are growing at low cell density (LCD), there is an insufficient concentration of autoinducers in the extracellular environment to bind the membrane receptors (10, 11, 16, 40, 42), and they function as kinases to phosphorylate the phosphotransfer protein LuxU (43). LuxU transfers phosphate to the response regulator LuxO, and phosphorylated LuxO (LuxO~P) activates the transcription of the sRNAs termed quorum regulatory RNAs (Qrrs) (44, 45). The Qrrs both positively and negatively posttranscriptionally regulate the expression of quorum sensing genes (46). At LCD, translation of the transcription factor AphA is activated by the Qrrs (47), while production of the transcription factor LuxR is negatively regulated

by the Qrrs (45). Thus, at LCD, AphA is at its highest level and LuxR is at its lowest level (48). As autoinducers accumulate in the surrounding environment at high cell density (HCD), the membrane-bound receptors bind the autoinducers and switch to acting as phosphatases, removing the phosphates from the regulatory circuit. LuxO is dephosphorylated and the Qrrs are no longer expressed (44). Thus, AphA is no longer produced, and LuxR expression is high (48, 49). LuxR and AphA are the two master transcription factors that control 99% of the quorum sensing regulons: AphA is the LCD regulator, and LuxR is the HCD regulator (47, 48). The remainder of genes are post-transcriptionally controlled by the Qrrs (50). Even at LCD when LuxR levels are lowest, LuxR controls >80 genes and AphA controls >100 genes (48). At HCD, LuxR regulates hundreds of genes.

The roles of LuxR and AphA as the master regulators of quorum sensing gene expression are conserved across the *Vibrio* genus, even when other factors within the signaling cascade differ (12, 15, 47). For example, *V. harveyi* has three cognate membrane-bound receptors (Fig. 1), LuxN, LuxPQ, and CqsS, and each binds a specific autoinducer (autoinducer 1 [AI-1], AI-2, and cholera autoinducer 1 [CAI-1], respectively) (41). *V. cholerae* has four autoinducer receptors that feed into the same circuit to regulate HapR, one of which is unlike canonical *Vibrio* receptors in that it is not membrane bound (11–13). A new autoinducer-receptor pair was identified in *V. cholerae*; binding of the autoinducer 3,5-dimethylpyrazin-2-ol (DPO) is required for dimerization and function of the cytosolic transcription factor VqmA, which indirectly inhibits biofilm formation (51). The autoinducer-receptor systems in *V. parahaemolyticus* are likely similar to those in *V. harveyi*, based on phylogenetic analyses and signaling assays (10, 14, 16, 42), whereas only the LuxPQ system that detects AI-2 has been identified in *V. vulnificus* (40). The number of Qrrs in different *Vibrio* species varies from one to five among vibrios, and they act either additively or redundantly (15, 45, 52–56). Further, the Qrr regulon in *V. harveyi* (~20 genes) does not appear to include the type VI secretion genes, which are regulated by the Qrrs in *V. cholerae* (50, 57).

There are several feedback loops in the quorum sensing circuit (Fig. 1). The Qrrs positively regulate AphA and negatively regulate LuxO, LuxM, and LuxR (46). LuxR represses *aphA* expression, and AphA represses *luxR* expression (47, 58). Further, because AphA and LuxR are autorepressors and regulate the transcription of the Qrrs, the concentrations of LuxR and AphA in the cell are highly controlled (29, 45, 59). Collectively, these feedback loops ensure precise expression levels of LuxR and AphA, drive transitions between LCD and HCD, determine the effective range of autoinducer concentrations, and prevent small fluctuations in autoinducer concentration from having a large impact on gene expression (59–62).

AphA, THE LCD MASTER REGULATOR

AphA is the LCD master quorum sensing regulator. It belongs to the MarR family of transcriptional regulators, and like many members of this family, it has a conserved winged helix DNA binding motif at its N terminus. AphA has a unique antiparallel coiled-coil dimerization domain at the C terminus (63, 64). The *V. harveyi* AphA protein shares 86% identity with *V. cholerae* and 96% with *V. parahaemolyticus* and has a consensus binding sequence of ATATGCAN$_6$TGCATAT (65). In addition to positive regulation by the Qrrs, *V. cholerae* AphA expression is activated by binding of two transcriptional regulators, Lrp and VpsR, to the *aphA* promoter (47, 66). AphA is induced by high cyclic di-GMP levels in *V. cholerae* through binding of cyclic di-GMP to VpsR, which impacts the expression of downstream genes (67). Importantly, AphA gene regulation is critical for pathogenesis (64, 68). At the *V. cholerae tcpPH* operon, AphA directly interacts with coactivator AphB to positively regulate the expression of cholera toxin and the toxin-coregulated pilus to initiate the virulence cascade (69). In *V. harveyi*, AphA directly represses the expression of *aphA*, *luxR*, and the *qrr* genes (47). Although it is not known whether an AphA interaction with AphB is required for regulation of all these genes, at least for *qrr*4 repression, AphB is not required (47). Further, quorum sensing regulatory genes have not been found to be part of the AphB regulon under

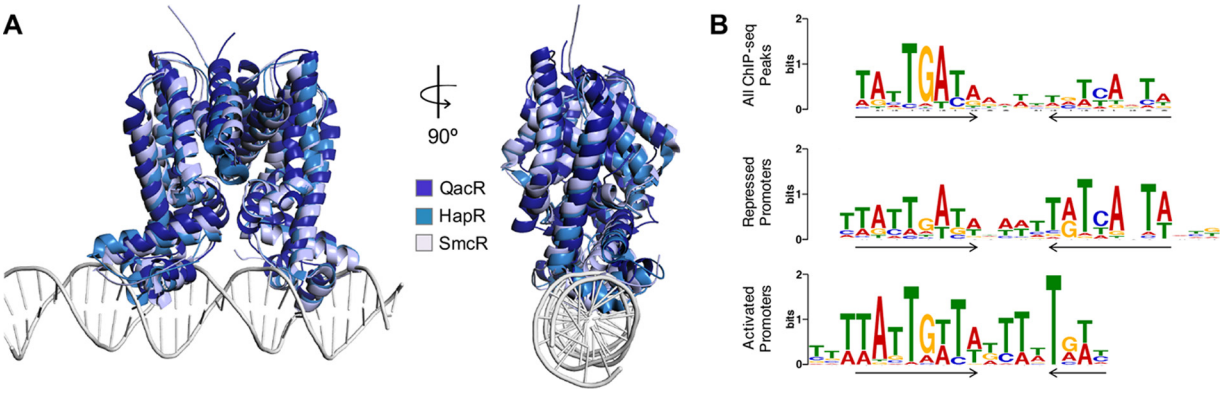

FIG 2 LuxR/HapR homolog structural and DNA binding properties. (A) Superimposed crystal structures of the TetR proteins QacR, HapR, and SmcR (32, 33, 84). Structures were superimposed with DaliLite (142) and the figures created with PyMOL. The DNA-bound QacR dimer superimposed with HapR or SmcR both resulted in a root mean square deviation (RMSD) of 3.1. Superimposition of HapR to SmcR results in an RMSD of 1.5. HapR is shown in cyan (PDB: 2PBX), SmcR is shown in light blue (PDB: 3KZ9), and for the QacR-DNA structure, QacR is shown in dark blue and the DNA in gray (PDB: 1JT0) (32, 33, 84). (B) LuxR DNA binding motifs from ChIP-seq data grouped by the location of the binding site peak. Top, all LuxR DNA binding peaks from ChIP-seq; middle, peaks in promoters of repressed genes; bottom, peaks in promoters of activated genes. The arrows indicate dyad symmetry in the binding site. Image reproduced with permission (74).

conditions that have been tested (47, 70). In *V. harveyi*, AphA controls 170 genes at LCD, and among these are >40 type III secretion system (T3SS) genes that are repressed by AphA (47, 48). Repression of T3SS genes at LCD by AphA and at HCD by LuxR results in the expression of T3SS genes at mid-cell density, and these genes are upregulated >1,000-fold during infection (48, 71). Thus, AphA plays a critical role in virulence gene regulation at LCD in vibrios.

THE *VIBRIO* LuxR/HapR FAMILY: DISTINCT TetR PROTEINS

LuxR shares high amino acid identity with other LuxR-type proteins: 71% with HapR (*V. cholerae*), 96% with OpaR (*V. parahaemolyticus*), 93% with SmcR (*V. vulnificus*), and 60% with LitR (*V. fischeri*). Structural determination of the LuxR homologs HapR and SmcR revealed that these proteins are TetR family transcription factors with a characteristic helix-turn-helix DNA binding motif in the N-terminal domain (Fig. 2A) (32, 33). Although the shared amino acid identity of the *Vibrio* LuxR homologs and other TetR family proteins is low, a high level of conservation exists at the secondary and tertiary levels. For example, SmcR shares just 27% similarity with the TetR-type regulator from *Staphylococcus aureus*, QacR, yet it is clear from examining the root mean square deviation (RMSD) values that these proteins are structural orthologs (Fig. 2A).

Although LuxR proteins function as dimers and exhibit TetR structural features (Fig. 2A) (72), they are distinct from most other TetR proteins in numerous ways. First, LuxR family proteins act as both activators and repressors, whereas TetR-type proteins typically act only as repressors (22, 47, 49, 72–76). Second, LuxR proteins regulate hundreds of genes (48, 77–79), while TetR-type proteins generally regulate 1 to 2 genes (72). For example, the *V. harveyi* LuxR regulon is 625 genes, and the *V. cholerae* HapR regulon is 100 genes (48, 80). Third, LuxR proteins bind to multiple binding sites within the promoter regions of some genes, unlike most TetR-type proteins that have a single binding site per promoter (49, 73, 74, 78, 81). The presence of multiple LuxR binding sites in a single promoter tends to correlate with genes that are activated, but this is not strictly the case. Regarding activated promoters, there are eight LuxR binding sites in the *luxCDABE* locus, which drives the expression of the bioluminescence genes, and two LuxR binding sites in the *betIBA-proXWV* promoter, which controls the expression of the osmotic stress genes (82, 83). However, there are two LuxR binding sites in the *luxR* promoter that are autorepressed by LuxR (29, 31, 82). Based on chromatin immunoprecipitation sequencing (ChIP-seq) data, there are 227 LuxR binding sites in the promoters of 115 genes, yielding an average of ~2 sites per promoter (74). Of note,

some TetR-type proteins, such as QacR, bind cooperatively as dimers of dimers on opposite sides of the DNA helix (84–86). While the mechanism of DNA binding by LuxR proteins is unclear in the absence of a DNA-bound structure, it is possible that LuxR proteins bind DNA in this manner.

A fourth difference between LuxR proteins and typical TetR proteins is in regard to the conservation of the binding site sequence. LuxR proteins bind to a 20- to 22-bp consensus binding motif with dyad symmetry, which is a typical binding site organization and length for TetR proteins (Fig. 2B) (72). However, for each of the *Vibrio* LuxR-type proteins that has been examined, the palindrome is asymmetric, with a preference for one side of the palindrome (Fig. 2B) (73, 74, 78, 81). The asymmetry of the consensus palindrome is produced from the combination of sites from activated and repressed promoters: repressed binding sites generally retain symmetrical inverted repeats, while activated binding sites contain only half of the palindrome (Fig. 2B) (74). The asymmetric nature of the LuxR binding motif may be an artifact of the locations of the binding sites in activated and repressed promoters. Alternatively, LuxR recognition of various DNA sequences may be connected to the structure of LuxR and possibly to bending of the DNA helix. Some TetR proteins alter DNA structure: TetR induces a 17° bend, whereas QacR bends DNA 3° and widens the DNA major groove (84, 87). Indeed, some of the alignment differences between SmcR/HapR and QacR occur in the helix-turn-helix region (Fig. 2A). This may be due to superimposition of the apo structures of HapR/SmcR onto the DNA-bound structure of QacR. Thus, even structural homologs may interact with DNA in different ways, which remains to be explored for the *Vibrio* LuxR family of regulators.

Finally, LuxR proteins exhibit various binding affinities for their binding sites, ranging from 0.5 nM to >100 nM (74). The strength of LuxR binding to its consensus binding site is postulated to have a strong impact on the timing of LuxR gene expression (49, 82). Because LuxR expression increases 10-fold in a gradient between LCD and HCD (49, 88), the range of LuxR concentrations at different quorum sensing phases likely influences gene expression at different times during population growth based on the affinity of LuxR for the binding site(s) in promoters. LuxR-type proteins also interact with other proteins to activate transcription via synergistic DNA binding and possibly direct protein-protein interactions (76, 82, 89). LuxR binds DNA synergistically with integration host factor (IHF) in *V. harveyi*, which is necessary for the precise timing of bioluminescence gene expression during quorum sensing (82). IHF also plays a role in bending DNA at the *vvpE* promoter in *V. vulnificus* to facilitate an interaction between SmcR and RNA polymerase for transcription activation (76). These many features of LuxR generate a complex transcription profile: genes are activated and repressed both at LCD and HCD, and the changes range from 2- to 200-fold (48, 77–80, 82).

LuxR/HapR REGULATION OF GROUP BEHAVIORS IN VIBRIOS

LuxR and its homologs were named according to the genes they were initially discovered to regulate. For example, HapR is the regulator of the hemagglutinin/protease A (*hapA*) in *V. cholerae* (22), *V. parahaemolyticus* OpaR is the regulator of opacity (e.g., capsule production), and LuxR is the regulator of bioluminescence (e.g., lux) (24, 28, 89). However, it has become clear that these proteins control transcription on a global scale, influencing the expression of hundreds of genes (4, 5). The advent of global transcriptomic techniques, like microarrays and RNA sequencing (RNA-seq), uncovered the regulons of LuxR, HapR, SmcR, and OpaR, as well as the entire quorum sensing regulons (including the AphA and sRNA-regulated genes) of *V. cholerae* and *V. harveyi* (50, 74, 77–80, 82, 90). These experiments supply a comprehensive view of genes controlled by LuxR homologs, including directly and indirectly regulated genes. As a global transcription factor, LuxR directly regulates 115 promoters out of a total of 625 genes (74). The SmcR regulon experiment identified only direct regulatory targets, of which there were 121 genes (78). Conversely, for HapR and OpaR, only the global regulons (direct and indirect combined) were identified and contain 100 genes and 267 genes, respectively (77, 80). The regulon data for HapR, OpaR, SmcR, and LuxR were

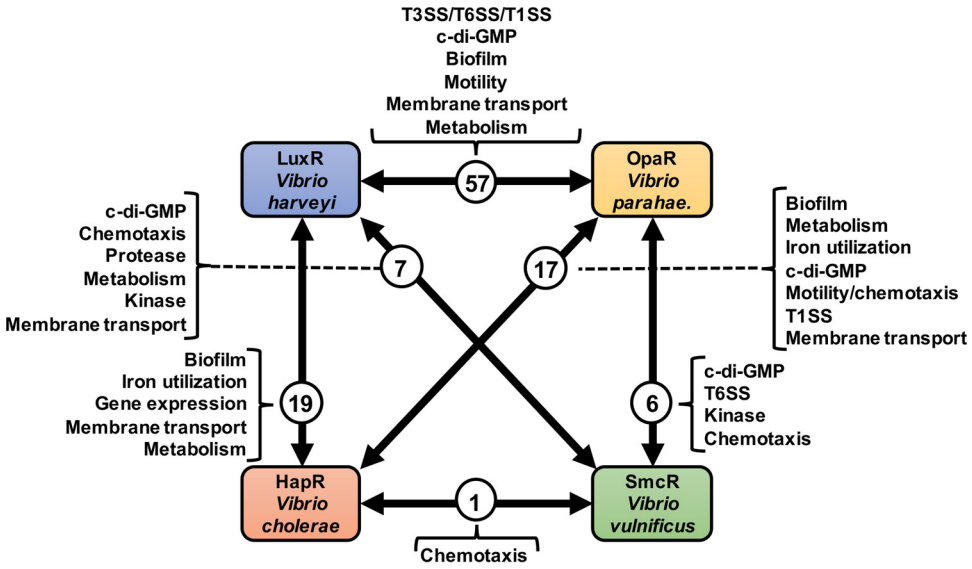

FIG 3 Overlapping regulons of *Vibrio* LuxR-type regulators. The LuxR, HapR, SmcR, and OpaR regulons, which were previously published (77, 78, 80, 82), were analyzed to identify mutual gene constituents via pairwise reciprocal BLAST searches (143). The numbers of homologous genes identified between pairs of regulons are shown in circles and connected by arrows. Genes associated with major cellular processes are listed in the brackets extending from each circled number. Lists are not comprehensive; the paired lists contain genes encoding hypothetical proteins with no annotated function. *Vibrio parahae.*, *Vibrio parahaemolyticus*.

collected using different approaches, including microarrays (HapR and LuxR), RNA-seq (LuxR and OpaR), and DNA pulldowns combined with bioinformatics (SmcR). The samples were collected at various cell densities and on different media. However, even though methods to gather these data are quite different, there are striking similarities among the LuxR, HapR, SmcR, and OpaR regulons. We examined these existing regulon data sets to identify genes with similar functions that are regulated by quorum sensing in multiple *Vibrio* species. The global gene regulons were analyzed for LuxR, HapR, and OpaR, while the SmcR direct regulon was used. Genes with similar function between pairs of *Vibrio* species were revealed by performing pairwise reciprocal BLAST searches (Fig. 3). Genes were considered "shared" between species if reciprocal protein BLAST searches yield the same best hit. Shared genes can be listed as associated with one pair or with multiple paired comparisons. Clearly, many gene classes are common among quorum sensing regulons, yet some gene groups and modes of regulation are extraordinarily different. Of particular interest are the pathways found in multiple *Vibrio* LuxR regulons, which include the group behaviors of motility, metabolism, production of public goods, cyclic di-GMP signaling, biofilm production, and secretion.

Motility. Although regulation of motility and/or chemotaxis is common to *Vibrio* regulons (Fig. 3), the regulation of these genes is remarkably different among *Vibrio* species (77, 78, 80, 82). In *V. harveyi* and *V. cholerae*, LuxR and HapR activate the expression of swimming motility genes, producing cells that display the highest level of motility at HCD (80, 91). This phenotype is also apparent in *V. vulnificus* and *V. tubiashii*, as *smcR* and *vtpR* mutants show reduced swimming motility (26, 38). Conversely, in *V. parahaemolyticus*, quorum sensing appears to modulate motility in the opposite fashion. OpaR inhibits swarming motility by repressing lateral flagellar genes, and AphA activates swimming and swarming behaviors at LCD. Thus, *V. parahaemolyticus* displays the highest levels of motility at LCD (68, 92). Similarly, motility is repressed by quorum sensing in the more distantly related species *V. fischeri* (18). In addition, multiple chemotaxis genes are found in the LuxR regulons, and several of them are common between vibrios (77, 78, 80, 82). In *V. harveyi*, LuxR positively regulates chemotaxis genes, whereas in *V. parahaemolyticus* and *V. cholerae*, OpaR and HapR negatively regulate homologous genes. These regulatory differences may be

explained by the different niches inhabited by these bacteria. In *V. cholerae*, evidence suggests that activation of motility genes at HCD via quorum sensing promotes the detachment of *V. cholerae* from host epithelial cells and propels the bacteria into the intestinal lumen for shedding back into the environment (93). Conversely, in *V. fischeri*, swimming motility is essential to initiate the symbiosis with *Euprymna scolopes* (94–96). Following colonization, *V. fischeri* downregulates motility genes at HCD in a quorum sensing-dependent manner, suggesting that motility is not important during maintenance of the symbiosis (18).

Metabolism. The expression of several metabolic enzymes is regulated by LuxR homologs (Fig. 3) (77, 80, 82), suggesting a key role for quorum sensing control of metabolism. Indeed, the Qrrs in *V. cholerae* repress the expression of the AlsSDO pathway, which is important for pyruvate metabolism (97). As the *V. cholerae* population grows, more pyruvate is fluxed through the AlsSDO pathway, which produces neutral metabolites instead of toxic organic acids, thereby allowing the population to remain stable in stationary phase. Similarly, in *V. fischeri*, as the population grows, acetate accumulates in the medium, which effectively lowers the local pH and can toxify the environment (98). Quorum sensing via LitR activates the production of acetyl coenzyme A (acetyl-CoA) synthase (Acs), which converts acetate into acetyl-CoA and counteracts the acidification (98). In addition, the quorum sensing network in *V. parahaemolyticus* represses 64 genes associated with the transport and metabolism of amino acids, carbohydrates, and lipids (99). A Δ*luxO* mutant exhibits significant fitness defects, while an Δ*opaR* mutant shows fitness advantages when grown on a variety of different carbon sources (e.g., glucose, gluconate, mannose, ribose, and arabinose). These results suggest that OpaR balances metabolic flux in *V. parahaemolyticus* in a manner similar to HapR in *V. cholerae* (99).

Public goods. Quorum sensing within the *Vibrio* clade appears to regulate a number of genes associated with importing/exporting shared molecules that benefit the both the producers and nonproducers in the local community, which are termed "public goods" (Fig. 3) (77, 78, 80, 82). In *V. harveyi*, light production and metalloprotease production are considered public goods and are directly regulated by LuxR (31, 100, 101). Additionally, the metalloproteases VtpA/VtpB and EmpA are activated by VtpR in *V. tubiashii* and VanT in *V. anguillarum*, and the alkaline serine protease ProA is activated by LuxR*val* in *V. alginolyticus* (25–27). Quorum sensing also represses siderophore production in both *V. harveyi* and *V. vulnificus* (102, 103). The production of extracellular polysaccharide (EPS) for biofilms is modulated by quorum sensing and LuxR proteins (see section below) (104), and chitin metabolism genes are controlled at various levels by quorum sensing in *V. cholerae* (105–107).

Because public goods usually comprise biomolecules that can be utilized by both producers and nonproducers, social "cheaters" can arise within bacterial populations that are at quorum. Cheaters often originate from mutations that yield defective quorum sensing systems, such as in *V. cholerae*, in which ~50% of natural isolates contain mutations in *hapR* (108). These mutants can outcompete wild-type cells *in vitro*, suggesting that such mutations could easily spread in the environment. A similar effect is observed for *V. fischeri litR* and *luxO* mutants (34, 109). However, bacterial populations can police themselves against cheaters via a number of mechanisms, including toxin production and metabolite exclusion (110, 111). In *V. harveyi*, constitutive cooperators (Δ*luxO* mutant) and cheaters (Δ*luxR* mutant) have reduced growth yields or are outcompeted by the wild type under various conditions (112). Thus, a functional quorum sensing system can act as a controlling mechanism for the cooperative expression of global behaviors that are fine-tuned for growth performance.

Cyclic di-GMP signaling. Quorum sensing in vibrios regulates the production of cyclic di-GMP (c-di-GMP), which is an important intracellular signaling molecule in prokaryotes (54, 113). Levels of c-di-GMP are modulated by two classes of cellular enzymes, diguanylate cyclases (which contain GGDEF motifs) and cyclic diguanylate phosphodiesterases (which contain EAL/HD-GYP motifs) (114, 115). In *V. cholerae*, *V.*

vulnificus, *V. parahaemolyticus*, and likely other vibrios, c-di-GMP is critical for coordinating biofilm formation and motility (116–120). LuxR homologs regulate the expression of a number of genes encoding diguanylate cyclases and cyclic diguanylate phosphodiesterases (Fig. 3). However, in *V. harveyi* and *V. parahaemolyticus*, some of the genes encoding these enzymes are upregulated, while others are downregulated by quorum sensing. *V. cholerae* is known to possess 62 genes that encode proteins capable of modulating the concentration of c-di-GMP inside the cell (121), and some of these control motility in opposite manners (122). Intracellular c-di-GMP signaling functions via a high-specificity model, in which individual diguanylate cyclase c-di-GMP production rates and levels drive distinct changes in transcription and downstream phenotypes (e.g., biofilm formation) (123). This is in contrast to a low-specificity model, in which total cellular c-di-GMP concentrations control transcriptional responses. High-specificity c-di-GMP signaling may enable cells to independently control pathways through these segregated c-di-GMP microdomains. Quorum sensing controls the expression of some diguanylate cyclases in vibrios, but not all, which in turn regulate downstream transcription via high-specificity c-di-GMP signaling. This type of network design likely enables fine-tuning of the population-wide quorum sensing response toward specific pathway control via c-di-GMP specificity for phenotypic outputs.

Biofilm production. A plethora of research has shown that quorum sensing and LuxR-type proteins control the expression of biofilm genes (24, 67, 104, 124–130). Accordingly, multiple genes involved in EPS biosynthesis are common among the LuxR regulons examined (Fig. 3). In *V. cholerae*, HapR represses the VpsR and VpsT activators of biofilm formation, which results in biofilm formation at LCD and repression of biofilm genes at HCD (113, 121, 124). Conversely, SmcR and OpaR activate biofilm gene expression at HCD (24, 127). As discussed above, quorum sensing also indirectly affects the expression of biofilm genes through the modulation of c-di-GMP levels (128). The opposite biofilm lifestyles of these vibrios likely are connected to niche adaptation and/or environmental signals. *V. cholerae* biofilm production at LCD is thought to promote persistence in natural aqueous environments by providing protection from stresses (93). Biofilms also appear to protect cells from acid stresses during infection and colonization of the host (129). Following attachment, there are "biofilm-like" microcolonies that form (131), although it is not clear if these are similar to biofilms formed on abiotic surfaces (132).

Secretion systems. The type III and type VI secretion systems (T3SS and T6SS, respectively) are intimately linked to quorum sensing in several *Vibrio* species (Fig. 3) (41, 49, 57, 71, 90, 133). T3SS and T6SS are complex syringe-like structures that are capable of penetrating proximal cellular membranes to deliver effector proteins that interfere with various cellular processes to cause cell death (134). T3SS are generally used to breach eukaryotic membranes, whereas T6SS can target both eukaryotic and prokaryotic membranes. In *V. harveyi* and *V. parahaemolyticus*, LuxR and OpaR repress expression of the T3SS operons, including those encoding the structural proteins, effector proteins, and the transcription factors in the system (41, 49, 135). Not only is the expression of T3SS vastly different between LCD and HCD in culture, but these operons are also highly upregulated during infection in a quorum sensing-dependent manner (71). While other vibrios, such as *V. cholerae*, contain T3SS, the regulatory connection to quorum sensing remains to be explored (136). In addition, the expression of T6SS is activated by quorum sensing in vibrios. LuxR/HapR and the Qrrs activate and repress the expression of T6SS operons in *V. cholerae* and *V. harveyi*, respectively (57, 90, 133). In the case of *V. anguillarum*, the LuxR homolog VanT represses the expression of *hcp*, which encodes a T6SS structural protein that is necessary for delivering effector proteins into the target cell (137, 138). HapR also activates *qstR*, which encodes the transcription factor that controls the expression of T6SS and competence genes (139, 140). Although the mechanism of T6SS regulation has not yet been examined in *V. vulnificus*, the SmcR regulon includes *hcp*, and thus, T6SS is controlled by quorum

sensing (78). Interestingly, a type II secretion system has been recently revealed in *V. cholerae*, and this gene cluster is regulated by quorum sensing (141).

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

For decades, *Vibrio* species have served as excellent model systems for studying quorum sensing, cell-cell interactions, and pathogenesis. Many of the intricacies of quorum sensing networks have been uncovered and yielded a fundamental understanding of the role of LuxR/HapR-type proteins. It is clear that LuxR protein expression is regulated through a phosphocascade circuit that responds to different autoinducers produced by various members of the *Vibrio* clade. The balance between AphA and LuxR protein concentrations is at the center of most *Vibrio* quorum sensing networks and directs changes in downstream gene expression. Furthermore, the Qrrs are critical for managing these concentrations, allowing rapid transitions from LCD to HCD or *vice versa*. Ultimately, the concentration of LuxR determines the regulation of hundreds of genes downstream. The mechanism by which this is accomplished is unclear, and many questions remain. For example, what are the multiple binding sites required for activation? How does LuxR affect transcription initiation? Do LuxR protein-protein interactions play roles in activation and/or repression? Does LuxR binding alter the DNA structure? These questions can be answered with biochemical, biophysical, and structural assays with LuxR proteins to gain a better understanding of the regulatory mechanisms of these transcription factors.

As global regulators, LuxR homologs regulate the expression of hundreds of genes to produce group behaviors. The elucidation of the LuxR regulons by transcriptomics in multiple *Vibrio* species revealed that these master regulators likely control a variety of cellular processes in addition to those discussed above. These findings have prompted the question: what other group behaviors are controlled by LuxR? LuxR homologs control biofilm formation, secretion, and c-di-GMP signaling, but there are likely others. It was only recently revealed that quorum sensing is connected to metabolism and the osmotic stress response. Indeed, hundreds of genes regulated by LuxR proteins in vibrios are annotated as hypothetical proteins with unknown function. Future research should focus on careful examination of these genes and patterns of expression that may reveal their function. Further experimental and bioinformatics examination of *Vibrio* quorum sensing regulons may guide the field toward important functional genes. Our simple search for orthologs in pairs of *Vibrio* quorum sensing transcriptomes revealed interesting classes of genes, such as chemotaxis and membrane transport genes. Because these four transcriptomes were determined under various assay conditions and methods, a side-by-side comparison of transcriptomes from vibrios under the same assay conditions would likely yield more comprehensive results. These types of transcriptomic and bioinformatics studies in *Vibrio* species reveal gene functions that benefit different habitats or lifestyles and may be particularly beneficial in identifying genes that are critical for pathogenesis.

Following the field's deep focus on bacterial cell-cell signaling molecules, a breadth of knowledge now exists on autoinducer synthesis, structures, receptors, and signal transduction components, as well as the regulatory mechanisms of the sRNAs, LuxR, and AphA. The downstream genes regulated by quorum sensing signaling (e.g., motility and biofilm formation) are also fairly well studied, although, as discussed above, there are likely many gene classes regulated by quorum sensing that have yet to be discovered. Thus, on the surface, it appears that the general signaling scheme in vibrios is elucidated: high concentrations of autoinducer signals drive changes in LuxR and AphA concentrations that up- or downregulate gene groups. However, there are major gaps in connecting signaling to behavior. What gene expression changes occur during transitions from LCD to HCD or from HCD to LCD? Certainly, these transitions occur under various conditions as cells grow in communities, move around, infect hosts, and become dispersed in the environment. What happens at midlevel concentrations of autoinducers or in the presence of one dominant autoinducer? A few research studies have hinted that there are discrete groups of genes controlled by various levels of

autoinducers, suggesting that there is a specific series of events that occur over the growth of a bacterial culture as autoinducers accumulate. Because many quorum sensing studies are performed in planktonic monoculture, it is difficult to assess the effects of signal concentrations on the transcriptome or to determine how to separate these effects from those resulting from concurrent changes in nutrient availability or the accumulation of toxic by-products. As more autoinducer-receptor pairs are uncovered in vibrios, the web of signaling circuits becomes complex. With strong depth in the field and ever-improving technologies, we are now poised to ask questions such as: how does autoinducer signaling affect the timing of gene expression? How is this timing affected by nutrient availability during planktonic or biofilm growth? Do different autoinducers confer distinct levels of gene expression? How does the growth of mixed species cultures affect quorum sensing gene expression, and does this impact the physiology and/or ecology of these bacteria? It will be illuminating to explore the complex connections between autoinducer signaling and the control of group behavior genes in vibrios.

ACKNOWLEDGMENTS

We thank Douglas Rusch and Ram Podicheti from the Indiana University Center for Genomics and Bioinformatics for analyses of the quorum sensing regulons. We thank Susanne Ressl for analyses of LuxR protein structures. We also thank Matthew Bochman and Ankur Dalia for comments on the manuscript.

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