Iron- and Quorum-sensing Signals Converge on Small Quorum-regulatory RNAs for Coordinated Regulation of Virulence Factors in Vibrio vulnificus*

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Vibrio vulnificus is a marine bacterium that causes human infections resulting in high mortality. This pathogen harbors five quorum-regulatory RNAs (Qrr1–5) that affect the expression of pathogenicity genes by modulating the expression of the master regulator SmcR. The qrr genes are activated by phosphorylated LuxO to different degrees; qrr2 is strongly activated; qrr3 and qrr5 are moderately activated, and qrr1 and qrr4 are marginally activated and are the only two that do not respond to cell density-dependent regulation. Qrrs function redundantly to inhibit SmcR at low cell density and fully repress when all five are activated. In this study, we found that iron inhibits qrr expression in three distinct ways. First, the iron-ferric uptake regulator (Fur) complex directly binds to qrr promoter regions, inhibiting LuxO activation by competing with LuxO for cis-acting DNA elements. Second, qrr transcription is repressed by iron independently of Fur. Third, LuxO expression is repressed by iron independently of Fur. We also found that, under iron-limiting conditions, the five Qrrs functioned additively, not redundantly, to repress SmcR, suggesting that cells lacking iron enter a high cell density mode earlier and could thereby modulate expression of virulence factors sooner. This study suggests that iron and quorum sensing, along with their cognate regulatory circuits, are linked together in the coordinated expression of virulence factors.

Vibrio vulnificus is a marine bacterium that causes septicemia and wound infection and is acquired either through a wound or through the gastrointestinal tract upon consumption of contaminated raw fish or water (1). Numerous virulence factors have been identified for this pathogen, including those for siderophore synthesis and iron acquisition (2–9). Repeats toxin (RTX), encoded by rtxA1, is important both in vitro and in vivo for survival during infection (10). Vulnibactin, a catechol siderophore, is essential for scavenging iron from human transferrin and therefore important for virulence (11, 12).

Bacterial pathogens experience a variety of stresses from natural or host environments during host infection, such as nutrient limitation, temperature changes, osmotic stress, and oxidative stress (13). Pathogenic bacteria have evolved sophisticated mechanisms through which to control gene expression under these differing environments by sensing relevant environmental factors and swiftly adapting to improve survival and pathogenicity.

It is well known that iron plays an important role in regulating virulence factors in pathogenic bacteria (14). In V. vulnificus, iron is necessary for growth and increased host mortality in vivo (15), and scavenging host iron is vitally important for its pathogenicity (16). Bacteria produce small molecules called siderophores that specifically bind Fe(III), ensuring iron acquisition from iron-scarce environments such as that in a host (14). V. vulnificus produces both hydroxamate- and phenolate-type siderophores (17). Mutants with impaired catechol (phenolate) siderophore production are less virulent when compared with wild type V. vulnificus (11), and vulnibactin is essential for utilization of transferrin- and lactoferrin-bound iron in vivo (18). Ferric uptake regulator (Fur)2 is the major iron-responsive transcriptional regulator in Gram-negative bacteria (19). In the presence of iron, Fur acts as a dimer to bind the consensus 19-bp palindromic Fur box (5′-GATAAATGATAATCATCAT-3′) present in the promoter regions of target genes, and it represses transcription by inhibiting the binding of RNA polymerase (20). The Fur-iron complex regulates a series of genes, including those for sidereophore synthesis and iron acquisition (21). In V. vulnificus, Fur is a 149-amino acid protein known to repress sidereophore biosynthesis and utilization as well as heme utilization (11, 22, 23). Fur also directly regulates the expression of virulence factors such as VvhA in V. vulnificus (24).

Bacterial cell density is another factor that affects a broad range of cellular activities, including virulence. Regulation in response to cell density is accomplished through the quorum-sensing pathway, which monitors diffusible signal molecules that accumulate at high cell density, and subsequently modulates genes associated with survival and virulence (25). The quorum-sensing pathway in V. vulnificus is similar to that of Vibrio harveyi and Vibrio cholerae. V. vulnificus harbors a homolog of V. harveyi LuxS, which is an enzyme that synthesizes the autoinducer-2 signaling molecule (26, 27). However, in a well studied V. vulnificus strain, MO6-24/O, whose genome has...

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‡ The abbreviations used are: Fur, ferric uptake regulator; Qrr, quorum regulatory RNA.
been completely sequenced (GenBank\textsuperscript{TM} accession number CP002469.1 for chromosome I and CP002470.1 for chromosome II) (28), there are no genes for the biosynthesis of either autoinducer-1 or cholaera autoinducer-1. Homologs of LuxPQ, the cognate receptor for autoinducer-2 in \textit{V. harveyi}, and LuxU and LuxO, which are involved in a phospho-relay, were identified in \textit{V. vulnificus} (29–33). The autoinducer signal converges on LuxO, a nitrogen regulatory protein (NtrC) homolog, which in turn regulates the master regulator SmcR (34–36).

Involvement of small RNA molecules called quorum regulatory RNAs (Qrrs) in quorum sensing has been well documented (37). In \textit{V. harveyi} and \textit{V. cholerae}, Qrrs are transcribed at low cell density in a \(a^2\)-dependent manner and repress expression of the master regulators LuxR and HapR by pairing with untranslated regions of the coding genes (37–40). Bioinformatics analysis suggests the existence of five Qrrs in \textit{V. vulnificus} (37). Qrrs are highly conserved at the nucleotide level among \textit{Vibrio} species but vary in number and mechanism. \textit{V. harveyi} has five Qrrs that function additively on LuxR expression (38). \textit{V. cholerae} has four Qrrs that function redundantly on HapR (37). \textit{V. fischeri} has only one Qrr that fully represses LitR (41). Considering the conservation of quorum-sensing pathways among \textit{Vibrio} species (33), it is hypothesized that Qrrs in \textit{V. vulnificus} repress the expression of SmcR, a homolog of \textit{V. harveyi} LuxR. In \textit{V. vulnificus}, the quorum-sensing master regulator SmcR is responsible for regulating expression of various virulence factors, and mutations in SmcR significantly attenuate the cytotoxicity of \textit{V. vulnificus} (42). At high cell density, SmcR inhibits the expression of \textit{vvhA}, which encodes hemolysin (27, 36), but it up-regulates the expression \textit{vvpE}, which encodes elastase (43). SmcR also inhibits the transcription of \textit{rtxA}A, a major virulence factor in \textit{V. vulnificus} (44), and it inhibits vulnibactin synthesis by binding to the promoter region of \textit{vvsAB} (23).

In this work, we show that Qrrs in \textit{V. vulnificus} are also regulated by quorum-sensing signaling via LuxO and modulate the expression of virulence factors via SmcR. Furthermore, we observed that Qrrs are responsive to iron concentration and that both quorum sensing and iron sensing converge at Qrrs to coordinately control virulence factors.

**Experimental Procedures**

**Bacterial Strains, Plasmids, and Culture Conditions**—Strains and plasmids used in this study are listed in Table 1. For \textit{Escherichia coli} strains, Luria-Bertani (LB) medium was used for...
TABLE 2—Continued

| Primer | Sequence (from 5’ to 3’) |
|--------|--------------------------|
| qrr4-R | GCTACATTTTTAACCAAAAATGC |
| qrr4-F | GAGTGAGATCACGCGCATGATAG |
| qrr5-R | GCTACATTTTTAACCAAAAATGC |
| qrr5-F | GAGTGAGATCACGCGCATGATAG |
| qrr6-R | GCTACATTTTTAACCAAAAATGC |
| qrr6-F | GAGTGAGATCACGCGCATGATAG |
| qrr7-R | GCTACATTTTTAACCAAAAATGC |
| qrr7-F | GAGTGAGATCACGCGCATGATAG |
| qrr8-R | GCTACATTTTTAACCAAAAATGC |
| qrr8-F | GAGTGAGATCACGCGCATGATAG |

TABLE 2

| Primers and sequences used in the study |

| For construction of ΔluxOΔfur
| fur-K1 | TCTAGACGTTAAAGAGAAAATACTGC |
| fur-K2 | GAGTTTATTGTTATACATATGAG |
| fur-K3 | GCCAGTGATATTCGATGATGATG |
| fur-K4 | GCTGACATTTTTAACCAAAAATGC |
| fur-K5 | GCTACATTTTTAACCAAAAATGC |
| fur-K6 | GCTACATTTTTAACCAAAAATGC |
| fur-K7 | GCTACATTTTTAACCAAAAATGC |

| For construction of Δqrr14
| qrr1-K1 | TCTAGACGTTAAAGAGAAAATACTGC |
| qrr1-K2 | GAGTTTATTGTTATACATATGAG |
| qrr1-K3 | GCCAGTGATATTCGATGATGATG |
| qrr1-K4 | GCTGACATTTTTAACCAAAAATGC |
| qrr1-K5 | GCTACATTTTTAACCAAAAATGC |
| qrr1-K6 | GCTACATTTTTAACCAAAAATGC |
| qrr1-K7 | GCTACATTTTTAACCAAAAATGC |

| For primer extension
| qrr1-PE | AACTATGATCTCTCTACACCTCATCAT |
| qrr2-PE | TTTATTGTTATGAACTGAGAA |
| qrr3-PE | GCTATTTTCTGTTGCAAGATC |
| qrr4-PE | GCTATTTTCTGTTGCAAGATC |
| qrr5-PE | GCTATTTTCTGTTGCAAGATC |

| For construction of ΔluxOΔfur
| ΔluxO-R | TCTAGACGTTAAAGAGAAAATACTGC |
| ΔluxO-F | GAGTTTATTGTTATACATATGAG |
| ΔluxO-K1 | GCCAGTGATATTCGATGATGATG |
| ΔluxO-K2 | GCTGACATTTTTAACCAAAAATGC |
| ΔluxO-K3 | GCTACATTTTTAACCAAAAATGC |
| ΔluxO-K4 | GCTACATTTTTAACCAAAAATGC |
| ΔluxO-K5 | GCTACATTTTTAACCAAAAATGC |
| ΔluxO-K6 | GCTACATTTTTAACCAAAAATGC |
| ΔluxO-K7 | GCTACATTTTTAACCAAAAATGC |

| For construction of Δqrr14
| qrr1-PE | AACTATGATCTCTCTACACCTCATCAT |
| qrr2-PE | TTTATTGTTATGAACTGAGAA |
| qrr3-PE | GCTATTTTCTGTTGCAAGATC |
| qrr4-PE | GCTATTTTCTGTTGCAAGATC |
| qrr5-PE | GCTATTTTCTGTTGCAAGATC |

| For Qrr complementation and ΔluxOΔfur construction
| C9150-F | TTTATTGTTATGAACTGAGAA |
| C9150-R | GCTATTTTCTGTTGCAAGATC |

| For qPCR
| ΔluxO-F | TCTAGACGTTAAAGAGAAAATACTGC |
| ΔluxO-R | GAGTTTATTGTTATACATATGAG |

| For qPCR
| ΔluxO-F | TCTAGACGTTAAAGAGAAAATACTGC |
| ΔluxO-R | GAGTTTATTGTTATACATATGAG |

| For ΔluxOΔfur
| ΔluxO-F | TCTAGACGTTAAAGAGAAAATACTGC |
| ΔluxO-R | GAGTTTATTGTTATACATATGAG |

| For Δqrr14
| Δqrr14-F | TTTATTGTTATGAACTGAGAA |
| Δqrr14-R | GCTATTTTCTGTTGCAAGATC |

| For ΔluxOΔfur
| ΔluxO-F | TCTAGACGTTAAAGAGAAAATACTGC |
| ΔluxO-R | GAGTTTATTGTTATACATATGAG |

| For Δqrr14
| Δqrr14-F | TTTATTGTTATGAACTGAGAA |
| Δqrr14-R | GCTATTTTCTGTTGCAAGATC |

| For ΔluxOΔfur
| ΔluxO-F | TCTAGACGTTAAAGAGAAAATACTGC |
| ΔluxO-R | GAGTTTATTGTTATACATATGAG |

| For Δqrr14
| Δqrr14-F | TTTATTGTTATGAACTGAGAA |
| Δqrr14-R | GCTATTTTCTGTTGCAAGATC |

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Continued

culture at 37 °C. For V. vulnificus, LBS medium (LB medium supplemented with 1.5% NaCl) was used for culture at 30 °C. All media components were purchased from Difco (Sparks, MD), and antibiotics were purchased from Sigma. For iron-limiting conditions, the iron chelator 2,2’-dipyridyl was used at a concentration of 200 μM.

Construction of ΔluxOΔfur and LuxOD47E Mutants, Deletions in qrrs, and Δqrr1–ΔΔsmcR—To construct ΔluxOΔfur, the primers fur-K1 and fur-KR1 (Table 2) were used for amplification of the upstream region of fur, and the primers fur-KF2 and fur-KR2 were used for the downstream region of fur. S17-1 Δpir harboring pDM4-fur, containing the fur upstream and downstream sequences, was constructed for conjugation with V. vulnificus ΔluxO. For construction of the V. vulnificus mutant luxOΔfurD47E, megaprimed PCR (45) was performed using the primers LuxO-F, LuxO-D47E, and LuxO-R (Table 2). The resulting 1424-bp PCR product was ligated into the pGEM-T Easy vector, and the generation of the mutation was confirmed by nucleotide sequencing. After digestion with Apal and Xba1, the resulting DNA fragment was cloned into pDM4 to construct pDM4-luxOD47E, followed by mobilization from S17-1Δpir to MO6-24/O via conjugation. A double crossover was performed as described above, and the subsequent mutation was confirmed by DNA sequencing. For construction of Δqrr1, a deletion in the qrr1 gene and the qrr1 upstream region was amplified by PCR using the primers qrr1-KF1 and qrr1-KR1, and the qrr1 downstream region was amplified by PCR with the primers qrr1-KF2 and qrr1-KR2 (Table 2). The PCR products were ligated and cloned into the pre-digested suicide vector pDM4. The resulting plasmid was mobilized from S17-1 Δpir to V. vulnificus by conjugation. A double crossover was selected on LB containing 10% sucrose. Colonies that grew in sucrose but were sensitive to chloramphenicol were selected, and the mutation was confirmed by PCR and sequencing. Construction of Δqrr2, Δqrr3, Δqrr4, Δqrr5, Δqrr14 (double mutant), Δqrr134 (triple mutant), Δqrr1345 (quadruple mutant), and Δqrr1–5 (quintuple mutant) was performed in a similar manner. For qrr2 mutants, the primers qrr2-KF1, qrr2-KR1, qrr2-KF2, and qrr2-KR2 were used. For qrr3 mutants, the primers qrr3-KF1, qrr3-KR1, qrr3-KF2, and qrr3-KR2 were used. For qrr4 mutants, the primers qrr4-KF1, qrr4-KR1, qrr4-KF2, and qrr4-KR2 were used. For qrr5 mutants, the primers qrr5-KF1, qrr5-KR1, qrr5-KF2, and qrr5-KR2 were used. For construction of Δqrr1–ΔΔsmcR, plasmid pDM4–SMCRKO was employed (23).
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Primer Extension to Determine qrr Transcriptional Start Sites—The transcriptional start sites of the five qrr genes were determined using the PrimeScript™ first strand cDNA synthesis kit (Takara, Ohtsu, Japan) and primers complementary to each qrr (Table 2). RNA was purified as described previously (46). Total RNA was extracted from wild type MO6-24/O cultured in LBS broth and harvested at log phase using the RNaseasy mini kit (Qiagen, Valencia, CA). Reverse transcription reactions were performed at 42 °C for 1 h and then inactivated at 70 °C. The same primers were used to generate the sequencing ladder using the Top™ DNA sequencing kit (Bioneer, Seoul, Korea). These constructs were subsequently mobilized from S17-1 into V. vulnificus [H9004 fur, harboring pHK-pfur] and V. vulnificus [H9004 luxO]. The resulting PCR products were digested using KpnI and XbaI and ligated into the transcription primers listed in Table 2. The resulting PCR products were separated on a 6% denaturing polyacrylamide gel alongside the corresponding sequencing ladders and analyzed with a Fuji BAS 1500 Image Analyzer (Fujifilm, Tokyo, Japan).

Construction of the Transcriptional Reporter Fusions luxO-luxAB and qrr-luxAB and Measurement of Luciferase Activity—DNA fragments containing the promoter regions of luxO and each of the five qrr genes were amplified using the primers listed in Table 2. The resulting PCR products were digested using KpnI and XbaI and ligated into the transcription reporter plasmid pHK0011 (47), generating pHK-pluxO, pHK-pqrr1, pHK-pqrr2, pHK-pqrr3, and pHK-pqrr5. These constructs were subsequently mobilized from S17-1 to V. vulnificus wild type MO6-24/O, LuxO, LuxOD46E, Δfur, and ΔluxOΔfur. n-Decyl-aldehyde was added to 10 μl of each culture diluted in 500 μl of phosphate-buffered saline (PBS) (final concentration, 0.06% (v/v)). Luminescence was measured using a luminometer (Lumat LB 9507, Berthold Technologies, Bad Wildbad, Germany). Relative light units (light units/cell) were calculated using a transfectable plasmid containing luxO coding region as an internal standard.

Quantitative Real Time PCR—To analyze Qrr-regulated wpvE and vvhA expression in V. vulnificus, overnight cultures of V. vulnificus were subcultured into fresh LBS broth. Samples were harvested at log phase (A600 of ~0.6) and stationary phase (A600 >2.0), and RNA was purified using the RNaseasy mini kit (Qiagen). RNA concentration was determined using a Biophotometer (Eppendorf, Hamburg, Germany). Reverse transcription was performed using the PrimeScript™ RT (Takara, Ohtsu, Japan) reagent kit. Quantitative PCR was performed in a 96-well PCR plate using SYBR® Premix Ex Taq™ and the ABI PRISM 7500 real time PCR system (Applied Biosystems, Carlsbad, CA). Primers are listed in Table 2. rpsL was used as an endogenous control. Relative RNA expression was analyzed by 7500 SDS software (Applied Biosystems, Carlsbad, CA).

Purification of Strep-LuxO and Strep-Fur—A DNA fragment containing the luxO-coding region was amplified by PCR using the primers LuxO-Strep-F and LuxO-Strep-R (Table 2) and subcloned into pASK-IBA™-7 (IBA, Göttingen, Germany), which results in a Strep-tag II at the N terminus of LuxO. The resulting plasmid, pASK-IBA-LuxO, was transformed into E. coli BL21 (DE3) (Novagen, Madison, WI). The Strep-LuxO fusion protein was induced using 200 ng/ml anhydrotetracycline and purified using Strep-Tactin-Septarose (IBA) according to the manufacturer’s instructions. Fur was cloned into the expression vector pASK-IBA7 (22) to construct pASK-IBA-Fur and expressed and purified in the same way as LuxO. LuxO and Fur protein purity were assessed by performing SDS-PAGE, and protein concentration was assessed using a Lowry assay (48).

Electrophoresis Mobility Shift Assay—To prepare probes for the electrophoresis mobility shift assay (EMSA), DNA fragments containing the promoter regions of each qrr gene or rpsL were amplified by PCR using the primers ErpsL-F and ErpsL-R, Eqr1-F and -R for qrr1, Eqr2-F and -R for qrr2, Eqr3-F and -R for qrr3, Eqr4-F and -R for qrr4, and Eqr5-F and -R for qrr5 (Table 2). The products were subsequently labeled with [γ-32P]ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Fur binding to the upstream region of each qrr gene was performed in a 20-μl reaction containing Fur binding buffer (10 mM HEPES, 100 mM KCl, 10 μg/ml dl-dC and 10% glycerol, pH 7.5, with the supplementation of 25 μM MnSO4 or 1 mM EDTA). Ten ng of each DNA probe was incubated with increasing amounts of purified Fur protein (23).

After incubation at 30 °C for 30 min, 4 μl of sucrose dye (0.25% bromphenol blue, 0.25% xylene cyanol, and 40% sucrose) was added to the reaction. Samples were separated by 5% neutral PAGE. DNA was visualized using the BAS 1500 imaging system (Fujifilm, Tokyo, Japan). Binding between LuxO and the upstream region of each qrr gene was performed in a 20-μl volume reaction containing LuxO binding buffer (10 mM Tris, pH 7.5, 300 mM NaCl, 1 mM MgSO4, 1 mM DTT, 10% glycerol, and 10 μg/ml dl-dC). Ten ng of each probe was incubated with increasing amounts of LuxO (0, 25, 50, 100 nM, 200, 400, and 800 nM). For the EMSA competition study between LuxO and Fur, LuxO (800 nM) and Fur (1 μM or 2 μM) were added separately or together to the binding mix containing 10 ng of each LuxO...
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**qrr** probe. Binding reactions were performed in LuxO binding buffer with a supplementation of 100 μM MnSO₄.

**DNase I Footprinting Assay**—To identify the Fur-binding sequences of the **qrr** genes, DNA fragments containing the promoter region of each **qrr** gene were amplified by PCR using primers listed in Table 2 (Eqr1-F and Eqr1-PE for **qrr1**, Eqr2-F and Eqr2-PE for **qrr2**, Eqr3-F and Eqr3-PE for **qrr3**, Eqr4-F and Eqr4-PE for **qrr4**, and Eqr5-F and Eqr5-PE for **qrr5**). Eqr1-F, Eqr2-F, Eqr3-F, Eqr4-F, and Eqr5-F were pre-labeled with [γ⁻³²P]ATP using T4 PNK (New England Biolabs). Each probe (200 ng) was incubated with increasing concentrations of Fur in a 50-μl reaction (10 mM Tris, pH 7.5, 300 mM NaCl, 1 mM MgSO₄, 1 mM DTT, 100 μM MnSO₄, 10% glycerol) at 30 °C for 30 min. After the addition of 50 μl MgCl₂-CaCl₂ solution (10 mM MgCl₂, 5 mM CaCl₂), samples were treated with 0.12 units of RQ1 RNase-free DNase I (Promega, Madison, WI) for 2 min. The DNase reaction was terminated with 90 μl of stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS). The digested DNA was harvested by ethanol precipitation and dissolved in 10 μl of formamide loading dye (98% formamide, 0.025% bromphenol blue, 0.025% xylene cyanol FF) and separated on a 6% polyacrylamide-urea gel alongside the sequencing ladder generated by the same labeled primer.

**Results**

**Identification of Five Quorum-regulatory RNAs in V. vulnificus**—**Qrrs** were first identified in *V. cholerae* and *V. harveyi* (37), and prior to our study, these small RNAs had not been examined in *V. vulnificus*. Through a homologous sequence search using bioinformatic tools, the following five Qrrs were found in *V. vulnificus*: **qrr1**, located between VVMO6_RS10095 (encoding LuxO) and VVMO6_RS10100 (encoding exonuclease ABC subunit B) on chromosome I; **qrr2**, located between VVMO6_RS10105 (encoding a membrane protein) and

![Figure 1](image-url)
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A

B

C

| LuxO (nM) | LuxO (nM) | LuxO (nM) | LuxO (nM) | LuxO (nM) | LuxO (nM) |
|-----------|-----------|-----------|-----------|-----------|-----------|
| 0         | 100       | 500       | 1000      | 1000      | 1000      |

Cold probe LuxO

LuxO-probe complex

LuxO

rpsL

qrr1

qrr2

qrr3

qrr4

qrr5

Free probe
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VVMO6_RS15235 (encoding a hypothetical protein) on chromosome II; qrr3, located between VVMO6_RS20600 (the magnesium transporter gene mgtE) and VVMO6_RS20605 (encoding an AraC family transcriptional regulator) on chromosome II; qrr4, located between VVMO6_RS17015 (encoding a methyl-accepting chemotaxis protein) and VVMO6_RS17020 (encoding a transcriptional regulator) on chromosome II; and qrr5, located between VVMO6_RS17415 (encoding a hypothetical protein) and VVMO6_RS17420 (encoding a membrane-associated phospholipid phosphatase) on chromosome II.

To confirm that these five Qrrs are indeed expressed in V. vulnificus and to identify transcriptional start sites, we performed primer extension experiments using total RNA extracted from wild type V. vulnificus cells grown to exponential phase (Fig. 1A).

Phosphorylated LuxO Directly Activates Qrr Genes—The luxO gene in V. vulnificus (NCBI accession number ABG81424) shows 90 and 85% identity to that of V. harveyi (NCBI accession number P0C5SSS) and V. cholerae (NCBI accession number NP_230666), respectively. In V. harveyi and V. cholerae, Qrrs are expressed in a cell density-dependent manner. At low cell density, phosphorylated LuxO acts as an enhancer to activate the σ54-initiated transcription of qrr genes. Consequently, master regulators such as LuxR in V. harveyi are repressed (37, 38). However, at high cell density, LuxO is dephosphorylated and no longer activates transcription of qrr genes, which also relieves master regulator repression.

Sequences in the promoter region of Qrr-encoding genes were analyzed using ClustalW2 to locate regulatory protein-binding sites (50). The consensus LuxO-binding sequence (5′-TTGCACW3TGCAAA-3′) found in V. cholerae (37) is present in the promoter region of all five V. vulnificus qrr genes (Fig. 1B), suggesting that LuxO regulates qrr expression as it does in V. cholerae. In V. harveyi, LuxO with an Asp to Glu mutation at residue 47 (luxOD47E) mimics phosphorylated LuxO and constitutively activates Qrr expression (31). To investigate whether Qrrs in V. vulnificus are expressed in a cell density-dependent manner through LuxO phosphorylation, we constructed a luxOD47E mutant and compared qrr expression in wild type V. vulnificus, a luxO mutant (ΔluxO), and luxOD47E using transcriptional fusions to each of the five qrr genes (qrr1–5–luxAB) (Fig. 2A). We observed a common expression pattern for each of the five qrr genes in all three phenotypic backgrounds. At low cell densities, expression levels in the luxOD47E strain were similar to wild type. However, at high cell densities, expression levels were higher in the luxOD47E strain than in wild type. Expression of all qrr genes was low at all growth stages in ΔluxO, suggesting that LuxO is required for the activation of Qrr expression. Differences in the pattern and magnitude of expression between each of the qrr genes in wild type cells were observed. The qrr1 and qrr4 genes showed a relatively low level of expression regardless of growth stage, although LuxO did appear to be required for expression. The expression level of qrr2 was highest, and yet it still decreased at high cell density. The expression patterns of qrr3 and qrr5 were similar to those of qrr2 but with approximately half the magnitude.

In general, in the luxOD47E mutant, expression levels increased with increasing cell density. Measurements of LuxO levels at various growth stages revealed that LuxO expression was low at low cell density and increased at higher cell density (Fig. 2B).

Next, we used electrophoresis mobility shift assays to analyze direct interactions between LuxO and the promoter regions of each of the qrr genes, which were predicted to have LuxO-binding sites (Fig. 2C). We found that DNA fragments for all five qrr promoters were bound by LuxO. The strongest binding appeared to be between LuxO and qrr2, where as little as 200 nM LuxO was enough to shift a significant amount of the qrr2 fragment into a bound complex (see qrr2, 5th lane in Fig. 2C). By comparison, binding of LuxO to qrr3 and qrr5 was not as strong (see 6th lane for each in Fig. 2C), and binding to qrr1 and qrr4 was the weakest.

Qrrs Repress Expression of SmcR—The quorum-sensing master regulator SmcR in V. vulnificus is homologous to LuxR in V. harveyi and HapR in V. cholerae (42, 51, 52). Qrrs in V. cholerae and V. harveyi inhibit the translation of their respective master regulator by binding to the 5′-untranslated region (UTRs) of each (37, 38). Alignment of the smcR 5′-UTR with the Qrr sequences of V. vulnificus suggested that Qrrs can form a hybrid structure with the region overlapping the ribosomal binding site (RBS) (Fig. 3A) suggesting that Qrrs may also inhibit SmcR translation in this species. To test this, we examined SmcR expression in wild type V. vulnificus and in Δqrr1–5 strains through Western blotting of protein extracts from cells grown to both log phase and stationary phase (Fig. 3B). In wild type, SmcR expression was barely detectable at low cell density but was significantly higher in cells that had reached stationary phase. In Δqrr1–5, SmcR was expressed abundantly in both log and stationary phases. These results suggest that Qrrs control SmcR expression through repression at low cell density but not at high cell density. We also examined SmcR expression in an hfq deletion mutant. The hfq gene encodes an sRNA chaperone required for Qrr function in V. harveyi and V. cholerae (37). Results for the Δhfq strain were similar to those for Δqrr1–5 suggesting that SmcR inhibition by Qrrs requires Hfq. SmcR expression in ΔluxO cells was similar to that of Δqrr1–5, suggesting low qrr expression when LuxO is absent (Fig. 2A). SmcR expression was not detected in luxOD47E cells. Qrr2, Qrr3, and Qrr5 were abundantly expressed in luxOD47E cells at both low and high cell density (Fig. 2A), again suggesting that LuxO is required for qrr expression.

Figure 2. LuxO activates qrr transcription. A, expression of qrr genes in wild type, luxOD47E, and ΔluxO cells; B, expression of luxO in wild type cells. Expression levels of the five qrr genes and luxO were quantitatively measured using the luxAB reporter gene fusion at various growth stages as described under “Experimental Procedures.” Data shown are averages of experiments done in technical triplicate, and error bars denote the standard deviations. C, binding of purified LuxO to DNA upstream of each of the qrr genes as demonstrated by electrophoresis mobility shift assay. A 32P-labeled DNA fragment (10 ng), including the promoter region of each qrr gene, was incubated with purified LuxO at the following concentrations: 0, 25, 50, 100, 200, 400, and 800 nM. Unlabeled probe (300 ng) was used in a competition experiment and is shown at the far right of each gel image. The promoter region of rpsl (30S ribosomal protein S12) was employed as a negative control. The position of the free probe and the LuxO-probe complex are indicated by arrows. These results are representative of three independent experiments. RLU, relative light units.
FIGURE 3. Qrrs repress expression of SmcR redundantly in *V. vulnificus*. A, nucleotide sequences of five Qrrs that potentially base pair with the 5′-untranslated region (UTR) of SmcR. Putative pairing sequences are boxed in the Qrr sequences and are marked with asterisks in the 5′-UTR of SmcR. The initiation codon and ribosome-binding site of SmcR are noted. B, repression of SmcR by Qrrs at log phase in *V. vulnificus*. Wild type, ΔsmcR, Δqrr1–5, ΔluxO, Δhfr, luxOD47E, and Δqrr1–5 cells were harvested at both log phase (A600 0.6 – 0.7) and stationary phase (A600 2.0), and 10 µg of lysate was subjected to Western blotting using an antibody against SmcR. The upper panel represents the relative densities of the bands shown in the lower panel. Band intensities were quantified using MultiGauge version 3.0 software (Fujifilm, Tokyo, Japan). Values are averages normalized to the intensity of the Δqrr1–5 (stationary phase) sample from biological experiments done in triplicate. **, p < 0.005; NS, not significant in Student’s t test with p > 0.05. C, regulation of SmcR by individual Qrrs. Wild type *V. vulnificus* cells harboring pRK415, Δqrr1–5 harboring pRK415, and Δqrr1–5 harboring pRK415-qr4 through pRK415-qr5 were harvested at log phase (A600 ~ 0.6). Ten µg of lysate was subjected to Western blotting using an antibody against SmcR. This result is representative of three independent experiments. D, SmcR expression in cell extracts from qrr deletion strains was measured by Western blot hybridization. Wild type *V. vulnificus* MO6-24/O, Δqrr1, Δqrr2, Δqrr3, Δqrr4, Δqrr5, Δqrr14, Δqrr134, Δqrr1345, and Δqrr1–5 were harvested at log phase (A600 ~ 0.6). Ten µg of lysate was subjected to Western blotting using an antibody against SmcR. This result is representative of three independent experiments.
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To test whether each of the Qrrs could inhibit SmcR expression, we performed complementation experiments by returning each qrr gene back into the Δqrr1–5 mutant individually on pRK415 vector constructs (53). SmcR expression was determined by Western blotting of cell extracts obtained at log phase (Fig. 3C). Introduction of any one of the five qrr genes significantly restored inhibition of SmcR levels, similar to what was observed in wild type cell extracts. To determine whether the regulation of SmcR by the Qrrs is redundant or additive, we then constructed strains containing individual qrr mutations and measured SmcR expression levels in cells grown to log phase (Fig. 3D). None of the five single mutants significantly affected SmcR expression. We then measured SmcR expression in mutant strains containing combinations of qrr genes: Δqrr14, Δqrr134, Δqrr1345, and Δqrr1–5. Expression of SmcR in the double or triple mutants was no different from wild type and was only slightly increased in the quadruple mutant Δqrr1345. Only the quadruple mutant Δqrr1–5 showed full expression of SmcR. These results suggest that Qrrs in V. vulnificus act redundantly in SmcR regulation and that full derepression of SmcR requires depletion of all five Qrrs.

Qrrs Regulate Expression of the Virulence Factors VvpE and VvhA via SmcR—SmcR directly activates vvpE, a gene encoding the virulence factor metalloprotease, in V. vulnificus (43). Because Qrrs affect SmcR levels, we predicted that they would also affect vvpE expression. Consistent with a previous report in which vvpE was shown to have an RpoS-dependent promoter, vvpE expression was higher at stationary phase than at log phase (Fig. 4A). When compared with wild type cells, vvpE expression in cells at stationary phase was ~15-fold lower in ΔsmcR and Δqrr1–5ΔsmcR. In ΔluxO and Δqrr1–5 strains, where SmcR expression is promoted, there was ~8-fold higher vvpE expression than in wild type. These results suggest that Qrrs inhibits vvpE expression in V. vulnificus, very likely by affecting the levels of SmcR.

Hemolysin, encoded by vvhA, is another important virulence factor in V. vulnificus. This gene was shown to be repressed by SmcR through the action of the transcription factor HlyU (44). To confirm that Qrrs affect vvhA expression via SmcR, we assessed vvhA expression in qrr mutants (Fig. 4B). At log phase, ΔluxO and Δqrr1–5, in which SmcR is derepressed, respectively, showed 5- and 50-fold lower expression of VvhA compared with wild type, whereas luxOD47E, ΔluxOΔsmcR, ΔsmcR, and Δqrr1–5ΔsmcR showed higher vvhA expression compared with wild type. At stationary phase, wild type, ΔluxO, and Δqrr1–5, which had similar levels of SmcR expression (Fig. 3B), also had similar levels of VvhA expression, whereas luxOD47E, ΔsmcR, ΔluxOΔsmcR, and Δqrr1–5ΔsmcR showed ~10-fold higher expression of vvhA as compared with wild type. These results suggest that Qrrs activate vvhA through SmcR. It is noteworthy that vvhA expression in stationary phase is higher than that in log phase, independent of SmcR, which suggests that an additional unknown factor is involved in vvhA regulation. Taken together, our data suggest that quorum-sensing signals are transduced to Qrrs, which regulate SmcR to modulate the expression of virulence factors in V. vulnificus, similar to what has been observed for V. harveyi and V. cholerae.

Iron Represses Qrr Expression in V. vulnificus through Both Fur-dependent and Fur-independent Ways—Iron is scarce in the natural environment and in the host. The LuxU-LuxO-
SmC signal transduction pathway may be regulated not only by the availability of autoinducer molecules as an indication of cell density, but also by other environmental factors, among which iron is particularly important. We showed that the iron-Fur complex represses the expression of SmC by directly binding to the promoter region of this gene (49). From this, we hypothesized that qrr expression might also be affected by iron.

To test this, we examined qrr expression under both iron-rich and iron-limiting conditions. *V. vulnificus* strains containing each individual qrr gene transcriptionally fused to the luxAB reporter were grown in rich medium with or without the iron chelator 2,2’-dipyridyl and quantitatively assessed for qrr expression. Depletion of iron led to a significant up-regulation of all five qrr genes in wild type cells (Fig. 5A). When this experiment was performed using cells grown in AB minimal medium with or without the supplementation of FeSO₄, iron repression was also observed (data not shown). We therefore concluded that the presence of iron represses qrr expression.

Fur is a global transcriptional regulator involved in the iron response, and it directly binds to the promoter regions of target genes when iron is present (54). We hypothesized that iron-dependent Qrr repression is elicited by Fur. To test this, Qrr expression was compared in wild type and Δfur cells under iron-rich and iron-limiting conditions (Fig. 5A). Under iron-rich conditions, expression levels of qrr2, qrr3, qrr4, and qrr5 were −2.0, 2.2, 3.2, and 1.5 times higher, respectively, in Δfur as compared with wild type. No significant difference was observed for qrr1 (Fig. 5A). Introduction of a wild type copy of *fur* into the Δfur strain restored qrr repression (data not shown). Expression of each of the qrr genes in the Δfur strain was further increased when the iron chelator was added. Notably, qrr expression levels were not significantly different between wild type and Δfur in the presence of the chelator. Taken together, these results suggest that Fur represses qrr2-5 in the presence of iron but that there is also an iron-regulatory mechanism that represses the five qrr genes independently of Fur.

As Qrr expression is dependent on LuxO, we explored the possibility that luxO plays a role in iron-dependent regulation of qrr genes by assessing qrr expression in a ΔluxO mutant and in a ΔluxODΔfur double mutant. As expected, qrr expression in these two mutants was lower than in wild type. However, there was no significant difference in qrr expression between the ΔluxO and ΔluxODΔfur strains (Fig. 5A). Under iron-limiting conditions, qrr expression in these mutants was significantly increased, suggesting that, even without LuxO, qrr expression was further decreased by iron, independent of Fur.

Fur binds directly to the promoter regions of all five qrr genes—To test our assumption that Fur affects qrr expression by binding the promoter region and inhibiting transcription of these genes, we performed EMSA using ³²P-labeled DNA fragments of the qrr promoter regions and purified Fur protein (Fig. 5B). Purified Fur and the qrr probes were incubated in the presence of either 25 μM Mn²⁺ (instead of Fe²⁺) or 1 mM EDTA. We observed that Fur could bind to all five qrr promoters in the presence of Mn²⁺ in a density-dependent manner, suggesting that Fur acts as a repressor of the qrr genes. However, Fur affinity was lost when Mn²⁺ was not present.

Although Fur binds to the upstream region of qrr1, expression of this gene was not significantly different in wild type versus Δfur strains (Fig. 5A). This discrepancy led us to hypothesize that qrr1 expression is not high enough (see Fig. 2A) to clearly show Fur-mediated repression. To test this possibility, we employed pLuxOD47E, which constitutively expresses active LuxO (Table 1), in a ΔluxO background. When LuxOD47E was supplied in trans, qrr1 expression levels were two times the levels in ΔluxO alone (Fig. 5C). These results suggest that, under our experimental conditions, qrr1 expression is low due to a low affinity for LuxO. Comparing qrr1 expression in ΔluxOΔfur + LuxOD47E with that in ΔluxO + LuxOD47E showed that expression was higher in the absence of Fur. We concluded that qrr1 is indeed repressed by iron in a Fur-dependent manner, but this repression was not detectable in wild type cells due to a low level of qrr1 expression.

Fur competes with LuxO in binding to qrr promoters—The above results suggested that both Fur and LuxO bind to regions upstream of the qrr genes and exert opposite effects on qrr expression. Furthermore, repression of qrr genes by Fur in the presence of iron was only seen upon LuxO activation (Fig. 5, A and C). We therefore hypothesized that Fur-binding sites overlap with LuxO-binding sites in qrr promoters, leading to competition for binding by the two regulatory proteins. To test this hypothesis, we performed an EMSA competition experiment between LuxO and Fur in the presence of divalent ions (Fig. 6A). Increasing Fur concentrations led to the formation of more Fur-qrr complexes and fewer LuxO-qrr complexes. These results suggest that the Fur-iron complex effectively competes for binding with LuxO, thereby inhibiting qrr expression.

We identified the specific qrr nucleotide sequences bound by Fur using a DNAse I footprinting assay (Fig. 6B). With respect to the transcription start site, the Fur-binding regions are located at −185 to −41 for qrr1, at −119 to −51 for qrr2, at −203 to −152 and −145 to −110 for qrr3, at −155 to −94 for qrr4, and at −153 to −121 and −82 to −16 for qrr5. All regions bound by
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Fur contain sequences homologous to the known Fur consensus binding box (5’-GATAATGATAATCATTATC-3’) (data not shown). Comparing the Fur binding regions to the LuxO consensus binding sequence, we observed that the binding sites for these two proteins overlap in all five of the qrr promoters. This result is consistent with the competition binding results.
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shown in Fig. 6A. We conclude that Fur represses qrr transcription by physically interfering with the binding of LuxO.

LuxO Is Repressed by Iron in a Fur-independent Manner—Because LuxO activates qrr transcription, we hypothesized that iron might regulate luxO expression. To test this, we used a luxO-luxAB transcriptional fusion construct in both wild type and Δfur strains. We found that luxO expression was significantly induced in the presence of an iron chelator in both wild type and Δfur. Expression levels were not significantly different between wild type and Δfur, indicating that Fur itself exerts no effect on luxO expression (Fig. 7) and suggesting that iron inhibits luxO expression in a Fur-independent manner. Low expression of LuxO under iron-rich conditions might also lead to even lower levels of qrr transcription.

Qrrs Function Additively to Repress SmcR under Iron-limiting Conditions—We assessed levels of SmcR expression under iron-limiting conditions in wild type, luxOD47E, ΔluxO, and Δqrr1–5 cells by Western blotting (Fig. 8A). In wild type cells grown under iron-rich conditions and harvested in log phase, SmcR was not detected. However, when iron was limiting, low levels of SmcR expression were observed. In luxOD47E mutants, SmcR was not detected under either condition. However, SmcR expression was observed in ΔluxO and Δqrr1–5 cells regardless of iron conditions. In a previous study, we showed that the Fur-iron complex directly inhibits smcR transcription (49). In this study, we confirmed that the transcriptional levels of smcR were lower under iron-rich conditions in all four strains (data not shown). Nevertheless, SmcR was not decreased by iron in ΔluxO and Δqrr1–5. It is possible that the iron-mediated transcriptional repression of smcR is not strong enough to affect protein levels, especially at high cell density when SmcR translation is no longer inhibited by Qrrs. Consequently, the prediction is that SmcR levels would be repressed by iron at low cell density but not affected at high cell density. To test this model, we measured SmcR levels at various growth stages in wild type cells. At low cell density, SmcR was not detectable regardless of iron levels (Fig. 8B). However, SmcR expression was induced at a much earlier growth phase under iron-limiting conditions than under iron-rich conditions, and it reached a similar level under both conditions when cells were at high density. This pattern was also observed for SmcR-directed VvpE expression in our previous study (49). This result suggests that the direct repressive effect of iron on smcR is not strong and that at stationary phase, when no Qrrs are expressed, smcR expression is fully derepressed making repression by Fur-iron negligible. When iron is limiting, SmcR is expressed at an earlier growth stage compared than under iron-rich conditions, making cells more sensitive to the effects of cell density. To investigate this further, we measured the expression of smcR under iron-limiting conditions using qrr deletion strains (Fig. 8C). SmcR expression in Δqrr2, Δqrr3, and Δqrr5 was significantly derepressed as compared with wild type but was not significantly different in Δqrr1 and Δqrr4. SmcR expression in the double mutant Δqrr14 was barely different from wild type. This is in agreement with our observation that qrr1 and qrr4 are expressed at low levels and therefore cannot effectively inhibit expression of SmcR regardless of iron levels. In contrast, Δqrr2, Δqrr3, and Δqrr5 had much higher levels of SmcR expression, consistent with higher expression of qrr2, qrr3, and qrr5 in wild type cells (Fig. 2A). The other multiple mutant strains, Δqrr134, Δqrr1345, and Δqrr1–5, showed gradually increasing levels of smcR expression, suggesting that Qrrs function additively to repress smcR under iron-limiting conditions.

Discussion

In this study, we characterized five quorum-regulatory RNAs in V. vulnificus that control the expression of the master regulator SmcR. Based on the magnitude and pattern of expression, the five Qrrs could be separated into three groups, with differ-

FIGURE 6. Fur competes with LuxO for binding to regions upstream of qrr genes. A, electrophoresis mobility shift assay of binding competition between LuxO and Fur for qrr promoter regions. Ten ng of each qrr promoter was incubated with either LuxO, Fur, or both. Lanes 1–6 include each probe incubated with the following: lane 1, no protein; lane 2, 1 μM Fur; lane 3, 2 μM Fur; lane 4, 4,800 nM LuxO; lane 5, 8,800 nM LuxO with 1 μM Fur; lane 6, 8,000 nM LuxO with 2 μM Fur. Positions of probes bound and shifted by LuxO or Fur are indicated by arrows. This result is representative of two independent experiments. B, DNase I footprinting of Fur protein binding to each qrr. A 32P-labeled probe (200 ng) was incubated with increasing concentrations of Fur (0, 250 and 500 nM, and 1 μM). The nucleotide sequences protected by Fur (shaded boxes), the p2-binding site (unshaded boxes), and the consensus LuxO-binding sites (hatched boxes) are indicated.

FIGURE 7. Effects of iron on luxO transcriptional levels. Luciferase activity representing transcriptional levels of luxO was measured in wild type V. vulnificus, and Δfur. Cells were cultured with or without 200 μM iron chelator 2,2′-dipyridyl, which was supplemented when cells were at an A600 of ~0.2. Luminescence and cell density were measured at an A600 ~0.6. Relative light units (RLU) represent light units normalized to cell density (luminescence/A600). Results are averages from three independent samples, and error bars denote standard deviations (**, p < 0.005; NS, not significant in Student's t test with p > 0.05).
ences that may be attributed to the varying affinity of LuxO for cis-acting elements in the upstream promoter region of each. qrr2 has three LuxO-binding sites, one of which is a perfect match to the canonical LuxO consensus binding sequence (Fig. 1B) (37). qrr3, qrr4, and qrr5 each have two binding sites, one of which is a perfect match to the consensus. qrr1 has only one
binding site that differs from the consensus sequence by two nucleotides (Fig. 1B). These differences likely affect the binding affinity of LuxO for each qrr, which we observed in preliminary experiments using EMSA (Fig. 2B). Differences in expression between qrr genes have also been observed in _V. cholerae_ and _V. harveyi_ (37, 38).

It is not clear why _V. vulnificus_ employs multiple Qrrs for the purpose of repressing SmcR. _V. cholerae_, _V. harveyi_, and _Vibrio parahemolyticus_ harbor four, five, and five Qrrs, respectively, to repress the LuxR-type regulator, so our observations are consistent with what has been found in these other _Vibrio_ species (37, 38). A collection of Qrrs with redundant activities might help guarantee strong repression of SmcR when cells are at a low density. It is also possible that some or all of the Qrrs regulate targets other than SmcR. In _V. harveyi_ and _V. cholerae_, _aphA_ is activated by Qrrs at low cell density, and this gene product then regulates the expression of ~300 additional genes, including numerous virulence factors (55). An _aphA_ homolog was identified in _V. vulnificus_ (56), and although expression of this gene was not directly affected by Qrrs, it was repressed by SmcR.3 We speculate that there are other as yet unidentified regulatory proteins that are activated at low cell density in _V. vulnificus_. Qrrs are expressed at low cell densities and derepressed under iron-limiting conditions, suggesting that they may play a role in the regulation of genes required under these conditions. Recent studies have shown that Qrrs also directly regulate target genes not involved in quorum sensing, such as the type VI secretion system, genes associated with biofilm formation, and numerous other recently discovered genes in _V. cholerae_ (57–59). A recent study showed that even a single Qrr can transduce quorum-sensing signals through multiple mechanisms. In _V. harveyi_, Qrrs act through different mechanisms for different targets and employ unique base pairing regions to discriminate between targets (40, 60). The presence of a variety of non-conserved regions among the five _V. vulnificus_ Qrrs suggests that each may also regulate specific target genes independently. If there are conditions under which repression of a particular _qrr_ gene is necessary to properly manipulate regulation of one of these unique targets, the presence of the remaining Qrrs might suffice to transduce the quorum-sensing signal and regulate SmcR.

It is possible that some or all Qrrs are involved in transduction of non-quorum-sensing signals. The iron-dependent regulation of Qrrs and _luxO_ shown in this study might represent the first example of such multiple roles for Qrrs. These results suggest that the presence of multiple Qrrs make it possible to simultaneously monitor multiple environmental signals and coordinately modulate various target genes to fine-tune gene expression and elicit efficient and effective responses under a given condition. Identifying the effects of other environmental conditions on Qrr expression may reveal more mechanisms by which these quorum-sensing signals are affected.

This study showed that iron affects Qrr expression in three different ways (Fig. 9). First, iron directly represses _qrr_ transcription by antagonizing LuxO-mediated activation. Second, iron represses transcription of all five _qrr_ genes independently of Fur through the action of an unknown factor (labeled _X_ in Fig. 9). Third, transcription of LuxO is repressed by iron and an unknown factor independently of Fur thereby repressing _qrr_ transcription. We determined that several factors involved in cell density, iron, and growth stage, including SmcR, IscR (61), and RpoS (62), are irrelevant to the observed iron-associated regulation (data not shown) and are therefore unlikely candidates for the unknown factor (_X_ in Fig. 9). This unknown factor remains to be identified.

Iron affects _smcR_ expression both positively and negatively. In the presence of iron, _luxO_ and _qrr_ expression are repressed, resulting in up-regulation of _smcR_. Conversely, the iron-Fur complex represses _smcR_ expression by directly binding to the promotor region (49). However, it appears that iron-Fur-mediated repression is not strong enough to effectively repress _smcR_ expression at high cell density when the _qrr_ genes are strongly inhibited by iron (Fig. 8). Therefore, the overall effect of iron is the up-regulation of _smcR_ expression, leading to a greater cell response to quorum sensing, _i.e._ an earlier transition to high cell density mode in the absence of iron leads to activation of the quorum-sensing response. The biological role of the direct repression of _smcR_ by the Fur-iron complex is likely modula-
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