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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 Gram-negative marine bacteria 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 cytolysin detected in serum and skin lesions of infected mice, hemolysin, phospholipase, capsular polysaccharide, insulin-degrading enzyme, and metalloproteases (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′-GATAATGATAATCATTATC-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 siderophore synthesis and iron acquisition (21). In *V. vulnificus*, Fur is a 149-amino acid protein known to repress siderophore 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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[2] The abbreviations used are: Fur, ferric uptake regulator; Qrr, quorum regulatory RNA.

TABLE 1
Strains and plasmids used in this study

Bacterial strains and plasmids	Derivation/relevant characteristics	Ref. or source
E. coli		
DH5α	λ^- ϕ80d*lacZ*ΔM15Δ (*lacZYA-argF*)*U169 recA1 endA1 hsdR17* ($r_K^- m_K^-$) *supE44 thi-1 gyrA relA1*	Our collection
BL21(DE3)	F^-*ompT hsdSB* (r_B^- m_B^-) *gal dcm* (DE3)	Novagen
S17-1	C600::RP4 2-(Tc::Mu) (*Km*::Tn7) *thi pro hsdR hsdM*$^+$ *recA*	70
S17-1λ*pir*	λ*pir* lysogen of S17-1	70
V. vulnificus		
MO6-24/O	Clinical isolate; virulent	Our collection
HS031 (Δ*smcR*)	Derivative of MO6-24/O, with a deletion in *smcR*	43
HLM101 (Δ*fur*)	Derivative of MO6-24/O, with a deletion in *fur*	71
*luxO*D47E	Derivative of MO6-24/O, with LuxO Asp-47 to Glu	This study
KPM201 (Δ*luxO*)	Derivative of MO6-24/O, *luxO*::*nptI*	36
MIBE301	Derivative of MO6-24/O *luxO*::*nptI*, with a deletion in *smcR* (This strain is also called as Δ*luxO*Δ*smcR*)	23
Δ*luxO*Δ*fur*	Derivative of MO6-24/O *luxO*::*nptI*, with a deletion in *fur*	This study
Δ*qrr1*	Derivative of MO6-24/O, with a deletion in *qrr1*	This study
Δ*qrr2*	Derivative of MO6-24/O, with a deletion in *qrr2*	This study
Δ*qrr3*	Derivative of MO6-24/O, with a deletion in *qrr3*	This study
Δ*qrr4*	Derivative of MO6-24/O, with a deletion in *qrr4*	This study
Δ*qrr5*	Derivative of MO6-24/O, with a deletion in *qrr5*	This study
Δ*qrr14*	Derivative of MO6-24/O, with deletion in *qrr1* and *qrr4*	This study
Δ*qrr134*	Derivative of MO6-24/O, with deletion in *qrr1*, *qrr2*, and *qrr4*	This study
Δ*qrr1345*	Derivative of MO6-24/O, with deletion in *qrr1*, *qrr2*, *qrr3*, and *qrr4*	This study
Δ*qrr1–5*	Derivative of MO6-24/O, with deletion in *qrr1*, *qrr2*, *qrr3*, *qrr4*, and *qrr5*	This study
Δ*qrr1–5*Δ*smcR*	Derivative of Δ*qrr1–5* with deletion in *smcR*	This study
Plasmids		
pASK-IBA-7	Expression vector, Ap^r	IBA
pASK-IBA-Fur	pASK-IBA7 with *V. vulnificus fur*	23
pASK-IBA-LuxO	pASK-IBA7 with *V. vulnificus luxO*	This study
pHK0011	pRK415, a promoterless *luxAB*, Tc^r	47
pH*K-qrr1*	pHK0011 with *qrr1* promoter fused to *luxAB*	This study
pHK-*qrr2*	pHK0011 with *qrr2* promoter fused to *luxAB*	This study
pHK-*qrr3*	pHK0011 with *qrr3* promoter fused to *luxAB*	This study
pHK-*qrr4*	pHK0011 with *qrr4* promoter fused to *luxAB*	This study
pHK-*qrr5*	pHK0011 with *qrr5* promoter fused to *luxAB*	This study
pHK-*luxO*	pHK0011 with *luxO* promoter fused to *luxAB*	This study
pRK415	IncP *ori*, broad host range vector; *oriT* of RP4, Tc^r	73
pRK415-*qrr1*	pRK415 with *V. vulnificus qrr1*	This study
pRK415-*qrr2*	pRK415 with *V. vulnificus qrr2*	This study
pRK415-*qrr3*	pRK415 with *V. vulnificus qrr3*	This study
pRK415-*qrr4*	pRK415 with *V. vulnificus qrr4*	This study
pRK415-*qrr5*	pRK415 with *V. vulnificus qrr5*	This study
pDM4	Suicide vector, *ori*R6K, Cm^r	74
pDM4-SMCRKO	pDM4 with upstream and downstream sequence of *smcR*	23
pGEM®-T Easy	Cloning vector, Ap^r	Promega
pBBR1-MCS4	Broad host range expression vector; Ap^r	72
pLuxO47E	pBBR1-MCS4 with *luxO*D47E	This study

been completely sequenced (GenBank™ 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 cholera autoinducer-1. Homologs of LuxPQ, the cognate receptor for autoinducer-2 in *V. harveyi*, and LuxU and LuxO, which are involved in a phospho-relay, were identified in *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 *V. harveyi* and *V. cholerae*, Qrrs are transcribed at low cell density in a σ^{54}-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 *V. vulnificus* (37). Qrrs are highly conserved at the nucleotide level among *Vibrio* species but vary in number and mechanism. *V. harveyi* has five Qrrs that function additively on LuxR expression (38). *V. cholerae* has four Qrrs that function redundantly on HapR (37). *V. fischeri* has only one Qrr that fully represses LitR (41). Considering the conservation of quorum-sensing pathways among *Vibrio* species (33), it is hypothesized that Qrrs in *V. vulnificus* repress the expression of SmcR, a homolog of *V. harveyi* LuxR. In *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 *V. vulnificus* (42). At high cell density, SmcR inhibits the expression of *vvhA,* which encodes hemolysin (27, 36), but it upregulates the expression *vvpE*, which encodes elastase (43). SmcR also inhibits the transcription of *rtxA*1, a major virulence factor in *V. vulnificus* (44), and it inhibits vulnibactin synthesis by binding to the promoter region of *vvsAB* (23).

In this work, we show that Qrrs in *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 *Escherichia coli* strains, Luria-Bertani (LB) medium was used for

TABLE 2

Primers and sequences used in the study

Primer	Sequence (from 5′ to 3′)
For construction of Δ*luxO*Δ*fur*	
fur-KF1	TCTAGACGTTAAAGAGAAAATACTGC
fur-KR1	GGATCCAAGACCAGCATCCTTTAGCGC
fur-KF2	GGATCCAATCCAGACGCACATAAACG
fur-KR2	CTCGAGTCAGAGACTTTGGGTGTTAAC
For construction of *luxO*D47E	
LuxO-F	GGGCCCTTGAAGCGTAATATCAAAGATATT
LuxO-D47E	CATTCCAGATCTTATTCTGCTGGAATTACGTCTAC
LuxO-R	TCTAGATCTTGTACCTCTTTCCAGG
For construction of *qrr* deletions	
*qrr*1-KF1	CTCGAGATAAATGGCTTGTCTCCAC
*qrr*1-KR1	GAATTCACTTTCGTTTTTGCATTGTT
*qrr*1-KF2	GAATTCAGCCAATAGTGAAATGACTG
*qrr*1-KR2	AGATCTAACTGAATACTTGCATG
*qrr*2-KF1	AGATCTGAGCAGCAGAATAAAGCTGCC
*qrr*2-KR1	CCGAATTCAAGGGTCGAGAAGTATTATGCA
*qrr*2-KF2	GGAATTCGATTTGGGAGATGGCGCTCAA
*qrr*2-KR2	AGATCTCATGAAATCTCACGAAAAACA
*qrr*3-KF1	AGATCTGTCACCATTGGTCTGTTTGAA
*qrr*3-KR1	CCTCTAGAAAATTCCGCGTTTTTACCATG
*qrr*3-KF2	CCTCTAGACCGATCTAACTTCCCTACGAA
*qrr*3-KR2	AGATCTCAGGGTTATCGTGATAAATGA
*qrr*4-KF1	AGATCTATCGATCGCCAGTTGATTGAG
*qrr*4-KR1	GGAATTCCGTATCAAATCGACGTATTTA
*qrr*4-KF2	GGAATTCAGAACATTTGGCATAACAGCT
*qrr*4-KR2	AGATCTGCCATCTGTGTGCTCACGATG
*qrr*5-KF1	AGATCTGCCATCTGTGTGCTCACGATA
*qrr*5-KR1	CGAATTCCCAACCCTATTATTTGCTACA
*qrr*5-KF2	GGAATTCTCAATGTCACCCAAATGGTT
*qrr*5-KR2	AGATCTAAAGCAGCACCTGCGATCACA
For primer extension	
qrr1-PE	AACAGTACTTCACTATTGGCATC
qrr2-PE	TTATGTTGAGTGAACAATGGTA
qrr3-PE	GCTTTTACATGTGACAAATCA
*qrr*4-PE	GTATATATGTGTGAACAAGTCATA
*qrr*5-PE	GCTGTATATATTTGTGAACAATCAG
For construction of transcriptional reporter fusions to *luxO* and *qrr* genes	
P*luxO*-F	GGGGTACCGAAATCCCGCAAGCAGAAA
P*luxO*-R	TTTCTAGAACGCCCAGTACCCACGATA
P*qrr1*-F	GGGGTACCTTCTACCATCAGCAAGTAGCG
P*qrr1*-R	GCTCTAGAAACAACGTCAGTTGGCTAGGT
P*qrr2*-F	GGGGTACCCCATCGCTAAACCTTTTAAG
P*qrr2*-R	GCTCTAGAGTATTCACTAACAACGTCAG
P*qrr3*-F	GGGGTACCGGTTTCTTTGCCTTTCTTGGC
P*qrr3*-R	GCTCTAGACTAGGTGACCCTCGGCTTAA
P*qrr4*-F	GGGGTACCCCGGCTAAGAAAATGGAAATC
P*qrr4*-R	GCTCTAGACCTCGGCTTAATAAGGGTCAC
P*qrr5*-F	GGGGTACCTCAACACTAGAGGAAGGGCG
P*qrr5*-R	GCTCTAGATCACTAACAACGTCAGTTGGCT
For Qrr complementation and p*luxO* D47E construction	
C*qrr*1-F	TTTCTAGATGGCATGAGA TAGGAGCGATAGA
C*qrr*1-R	GGGGTACCAGAAGCGTGATGAAATTGAAAAG
C*qrr*2-F	TTTCTAGAGACATAACGCTCCCTGCCTTC
C*qrr*2-R	GGGGTACCTCGGATTTTTCTTTCCACACC
C*qrr*3-F	TTTCTAGAAACCACACTGACATCACACTCC
C*qrr*3-R	GGGGTACCTTTTTGAATAATGAATCTCTCG
C*qrr*4-F	TTTCTAGAAATCGGATTTATATCAAGCGTTT
C*qrr*4-R	GGGGTACCTCGTCTTATTTGTTCTCGTGGCG
C*qrr*5-F	TTTCTAGAAATTTACCCTGGGATAGAGCAGT
C*qrr*5-R	GGGGTACCTGAAATACCTCATCACAAACAAG
CLuxO-F	GGGGTACCGGCTAGATTATGCAACAAATAACG
CLuxO-R	TTTCTAGAATTTCCTCAGTCAACGAGGC
For qPCR	
vvpE-F	TTTACGCTACTTCGACCAACCCTC
vvpE-R	ATCTCAAAACCCTTACGCACATTC
vvhA-F	CGCAGAATGAGAACAAAAACTACCA
vvhA-R	ATCAAACACCAAGGTCTTCGAGTAG
rpsL-F	AGGAGCACTCGGTTGTTCTTATC
rpsL-R	GACCTTGTTTACGGTTGTTCACG
For strep-LuxO purification	
LuxO-Strep-F	CCGGAATTCATGCAACAAATAACGACAACG
LuxO-Strep-R	CCCTCGAGGATTTAGTAATTCCATTATGC

TABLE 2—*Continued*

Primer	Sequence (from 5′ to 3′)
For gel shift assay	
E*qrr*1-F	GTACCCACGATATTGATATCG
E*qrr*1-R	GCCATTGTTATTTGCAAAATGC
E*qrr*2-F	CGAGTATACCCAGATTCATGTC
E*qrr*2-R	CTGAATATCCCATATATTAACTC
E*qrr*3-F	TGACTTTATCACCCCAAACCAC
E*qrr*3-R	CACCATGGTTTCTGTGATATTG
E*qrr*4-F	GAGTGAGATCACGCGCATGATAG
E*qrr*4-R	CACGAATTCCGTATCAAATCGAC
E*qrr*5-F	GCTGGAAACGTTGAAAGAAGTC
E*qrr*5-R	GCTACATTTTTAACCAAAATGC
ErpsL-F	TGAATCGCGACTAAGCACCAATAT
ErpsL-R	GTGAAAAATCTAATCCCCAACCAC

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 Δqrr1–5ΔsmcR—To construct Δ*luxO*Δ*fur*, the primers *fur*-KF1 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 λ*pir* harboring pDM4-*fur,* containing the *fur* upstream and downstream sequences, was constructed for conjugation with *V. vulnificus* Δ*luxO*. For construction of the *V. vulnificus* mutant *luxO*D47E, megaprimer 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 ApaI and XbaI, the resulting DNA fragment was cloned into pDM4 to construct pDM4-*luxO*D47E, followed by mobilization from S17-1λ*pir* 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 Δ*qrr1*, 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 λ*pir* 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 Δ*qrr2*, Δ*qrr3*, Δ*qrr4*, Δ*qrr5*, Δ*qrr14* (double mutant), Δ*qrr134* (triple mutant), Δ*qrr1345* (quadruple mutant), and Δ*qrr1–5* (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 Δ*qrr1–5*Δ*smcR*, plasmid pDM4-SMCRKO was employed (23).

Primer Extension to Determine qrr Transcriptional Start Sites—The transcriptional start sites of the five *qrr*s 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 RNeasy 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). The generated cDNAs 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-p*luxO*, pHK-p*qrr1*, pHK-p*qrr2*, pHK-p*qrr3*, pHK-p*qrr4*, and pHK-p*qrr5*. These constructs were subsequently mobilized from S17-1 into *V. vulnificus* wild type MO6-24/O, Δ*luxO*, LuxOD46E, Δ*fur*, and Δ*luxO*Δ*fur*. *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/A_{600}), which are light units normalized to cell densities, represent the transcription levels of corresponding genes.

Complementation of Δqrr1–5 and Construction of pLuxO-D47E—DNA fragments containing each *qrr* were amplified using the following primers: C*qrr1*-F and C*qrr1*-R for *qrr1*; C*qrr2*-F and C*qrr2*-R for *qrr2*; C*qrr3*-F and C*qrr3*-R for *qrr3*; C*qrr*4-F and C*qrr*4-R for *qrr4*; and C*qrr*5-F and C*qrr*5-R for *qrr5* (Table 2). The PCR products were digested with XbaI and KpnI and ligated into pRK415 to obtain pRK415-*qrr1*, pRK415-*qrr2*, pRK415-*qrr3*, pRK415-*qrr4*, and pRK415-*qrr5*. These constructs were mobilized to *V. vulnificus* Δ*qrr1–5* from S17-1 by conjugation. Using *V. vulnificus* mutant *luxO*D47E genomic DNA as a template, the *luxO*-coding region was amplified using the primers CLuxO-F and CLuxO-R (Table 2) and ligated into pBBR1-MCS4, generating pLuxOD47E. The resulting plasmid was mobilized from S17-1 to *V. vulnificus* Δ*luxO* and Δ*luxO*Δ*fur*, harboring pHK-*qrr1*.

Western Blot Analysis of SmcR—For analysis of SmcR expression, overnight cultures of *V. vulnificus* were subcultured into fresh LBS broth. Cells were harvested at log phase (A_{600} of ~0.6) and stationary phase (A_{600} >2.0) and washed with PBS. Cells were lysed by ultrasonication, and the total protein concentration was assessed using the Lowry method (48). Next, 10 μg of protein was resolved by SDS-PAGE on a 12% polyacrylamide gel. After transfer to a Whatman Protran BA 83 nitrocellulose membrane (GE Healthcare UK Ltd., Buckinghamshire, UK), proteins were treated with anti-SmcR antibody (49) for 1 h at room temperature. Goat anti-rat IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA) was used as a secondary antibody. SmcR expression was visualized using Western blotting Luminol Reagent (Santa Cruz Biotechnology).

Quantitative Real Time PCR—To analyze Qrr-regulated *vvpE* and *vvhA* expression in *V. vulnificus*, overnight cultures of *V. vulnificus* were subcultured into fresh LBS broth. Samples were harvested at log phase (A_{600} of ~0.6) and stationary phase (A_{600} >2.0), and RNA was purified using the RNeasy 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® Primex 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-Sepharose (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 E*rpsL*-F and E*rpsL*-R, E*qrr*1-F and -R for *qrr1*, E*qrr*2-F and -R for *qrr2*, E*qrr*3-F and -R for *qrr3*, E*qrr*4-F and -R for *qrr4*, and E*qrr*5-F and -R for *qrr5* (Table 2). The products were subsequently labeled with [γ-^{32}P]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 dI-dC and 10% glycerol, pH 7.5, with the supplementation of 25 μM $MnSO_4$ 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 $MgSO_4$, 1 mM DTT, 10% glycerol, and 10 μg/ml dI-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

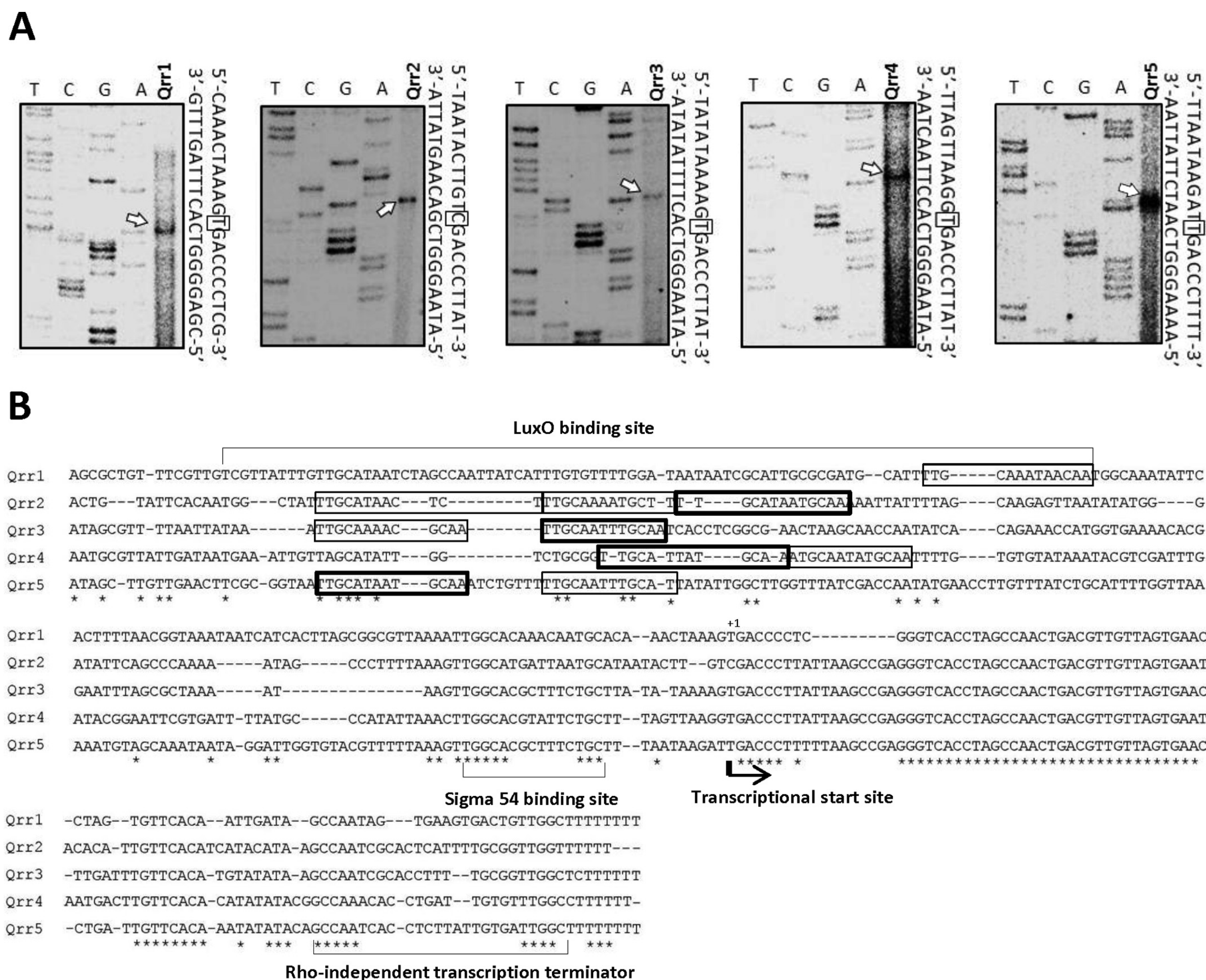


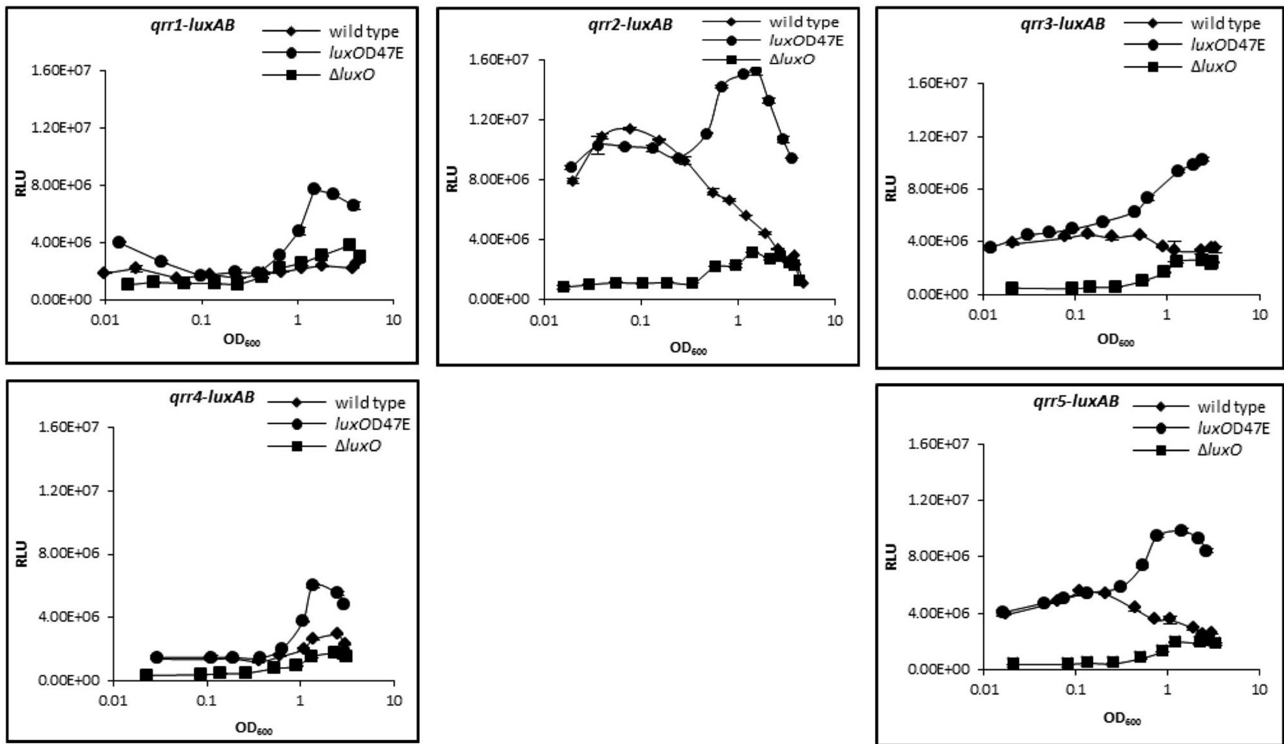
FIGURE 1. **Identification of *qrr* transcriptional start sites in *V. vulnificus*.** *A, qrr* transcriptional start sites were identified through primer extension experiments. T, C, G, and A represent the sequencing ladders. Transcriptional start sites are indicated with *arrows* on the gel images, and are *boxed* in the sequence to the *right* of each image. *B,* alignment of the *qrr* coding regions and upstream regions was performed using ClustalW. The transcriptional start sites are denoted with *arrows*, and the putative σ^{54}-binding sites and Rho-independent terminator sequences are *underlined*. Putative LuxO-binding sites perfectly matched to the consensus sequences are denoted with *thick-line boxes*, and those partially matched with the consensus sequence are denoted with *thin-line boxes*.

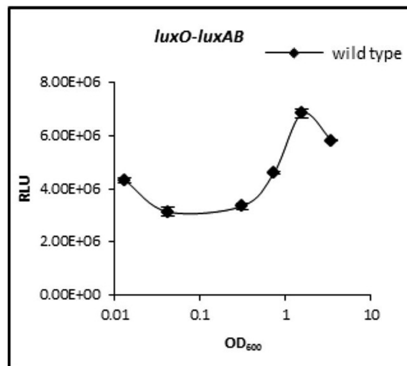
qrr probe. Binding reactions were performed in LuxO binding buffer with a supplementation of 100 μM $MnSO_4$.

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 (E*qrr1*-F and *qrr1*-PE for *qrr1*, E*qrr2*-F and *qrr2*-PE for *qrr2*, E*qrr3*-F and *qrr3*-PE for *qrr3*, E*qrr4*-F and *qrr4*-PE for *qrr4*, and E*qrr5*-F and *qrr5*-PE for *qrr5*). E*qrr1*-F, E*qrr2*-F, E*qrr3*-F, E*qrr4*-F, and E*qrr5*-F were pre-labeled with [γ-^{32}P]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 $MgSO_4$, 1 mM DTT, 100 μM $MnSO_4$, 10% glycerol) at 30 °C for 30 min. After the addition of 50 μl $MgCl_2$·$CaCl_2$ solution (10 mM $MgCl_2$, 5 mM $CaCl_2$), 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_RS15230 (encoding a membrane protein) and


A
qrr1-luxAB
wild type
luxOD47E
ΔluxO
RLU
OD600
qrr2-luxAB
qrr3-luxAB
qrr4-luxAB
qrr5-luxAB


B
luxO-luxAB
wild type
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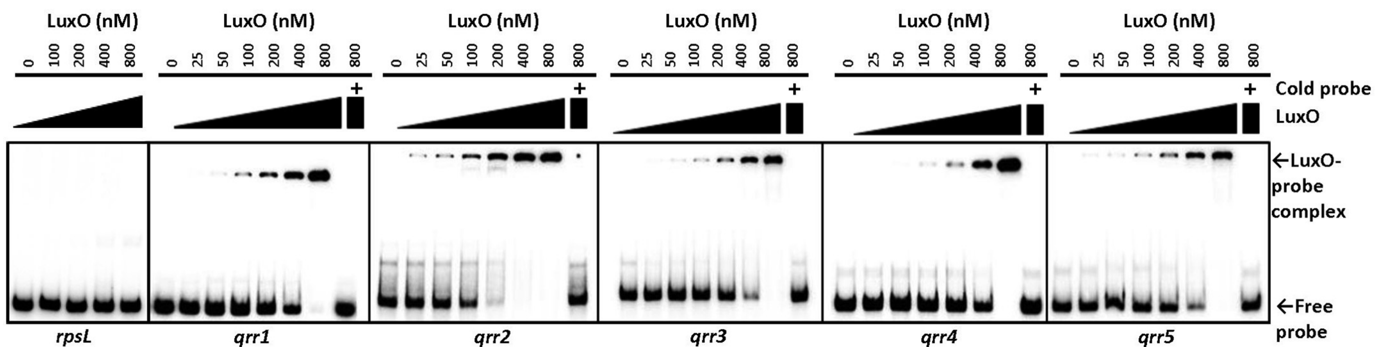
OD600


C
LuxO (nM)
Cold probe
LuxO
←LuxO-probe complex
←Free probe
rpsL
qrr1
qrr2
qrr3
qrr4
qrr5

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. 1*A*).

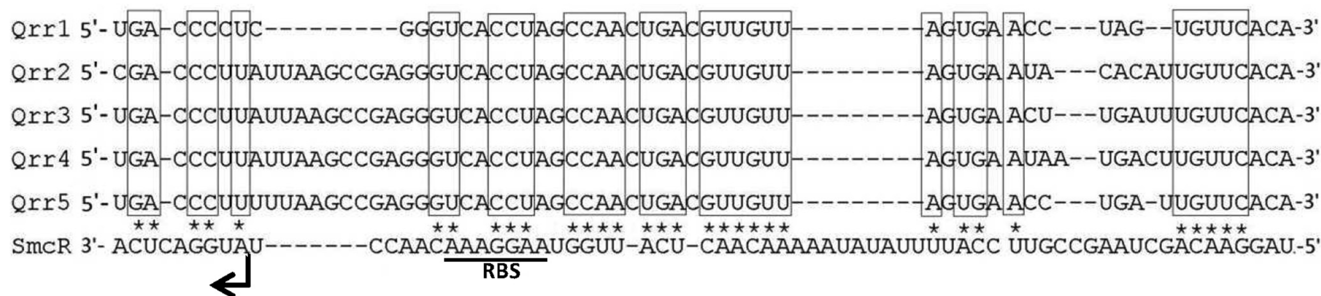
Phosphorylated LuxO Directly Activates qrr Genes—The *luxO* gene in *V. vulnificus* (NCBI accession number ABG81424) shows 90 and 85% identity to that of *V. harveyi* (NCBI accession number P0C5S5) and *V. cholerae* (NCBI accession number NP_230666), respectively. In *V. harveyi* and *V. cholerae*, Qrrs are expressed in a cell density-dependent manner. At low cell density, phosphorylated LuxO acts as an enhancer to activate the σ^{54}-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.

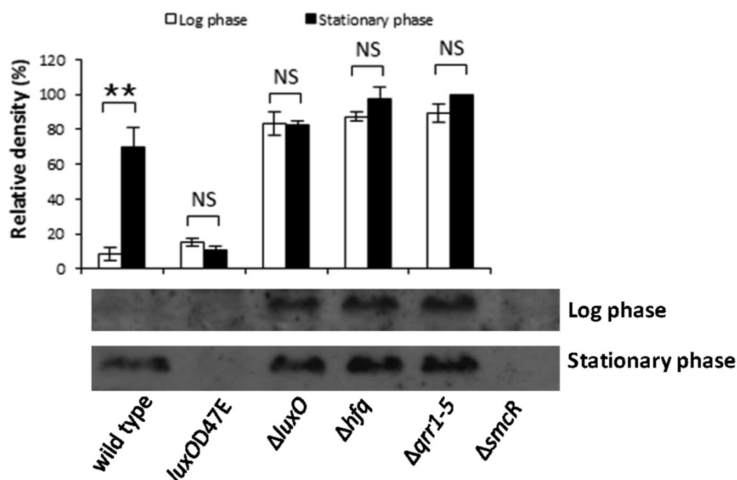
Sequences in the promoter region of Qrr-encoding genes were analyzed using ClustalW2 to locate regulatory protein-binding sites (50). The consensus LuxO-binding sequence (5′-TTGCAW$_3$TGCAA-3′) found in *V. cholerae* (37) is present in the promoter region of all five *V. vulnificus qrr* genes (Fig. 1*B*), 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 (*luxO*D47E) 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 *luxO*D47E mutant and compared *qrr* expression in wild type *V. vulnificus*, a *luxO* mutant (Δ*luxO*), and *luxO*D47E using transcriptional fusions to each of the five *qrr* genes (*qrr*(1–5)-*luxAB*) (Fig. 2*A*). 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 *luxO*D47E strain were similar to wild type. However, at high cell densities, expression levels were higher in the *luxO*D47E strain than in wild type. Expression of all *qrr* genes was low at all growth stages in Δ*luxO*, 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 *luxO*D47E 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. 2*B*).

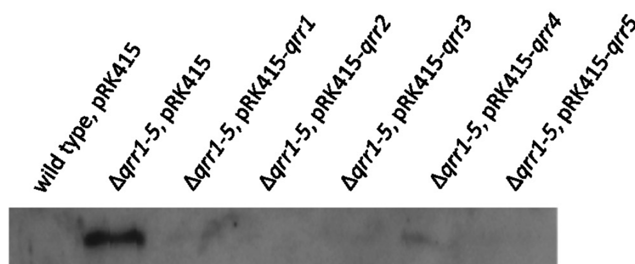
Next, we used electrophoresis mobility shift assays to analyze direct interactions between LuxO and the promoter regions of each of the *qrr* genes, which were predicted to have LuxO-binding sites (Fig. 2*C*). We found that DNA fragments for all five *qrr* promoters were bound by LuxO. The strongest 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. 2*C*). By comparison, binding of LuxO to *qrr3* and *qrr5* was not as strong (see *6th lane* for each in Fig. 2*C*), 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. 3*A*) 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 Δ*qrr1–5* strains through Western blotting of protein extracts from cells grown to both log phase and stationary phase (Fig. 3*B*). In wild type, SmcR expression was barely detectable at low cell density but was significantly higher in cells that had reached stationary phase. In Δ*qrr1–5*, 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 Δ*hfq* strain were similar to those for Δ*qrr1–5* suggesting that SmcR inhibition by Qrrs requires Hfq. SmcR expression in Δ*luxO* cells was similar to that of Δ*qrr1–5*, suggesting low *qrr* expression when LuxO is absent (Fig. 2*A*). SmcR expression was not detected in *luxO*D47E cells. Qrr2, Qrr3, and Qrr5 were abundantly expressed in *luxO*D47E cells at both low and high cell density (Fig. 2*A*), again suggesting that LuxO is required for *qrr* expression.

FIGURE 2. **LuxO activates *qrr* transcription.** *A,* expression of *qrr* genes in wild type, *luxO*D47E, and Δ*luxO* 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 ^{32}P-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.







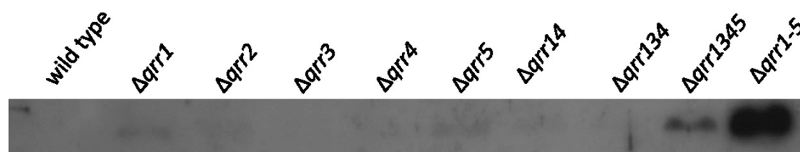


FIGURE 3. **Qrrs repress expression of SmcR redundantly in *V. vulnificus*.** *A,* nucleotide sequences of five Qrrs that potentially base pair with the 5′-untranslated region (*UTR*) of SmcR. Putative pairing sequences are *boxed* in the Qrr sequences and are marked with *asterisks* in the 5′-UTR of 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, Δ*smcR*, Δ*qrr1–5*, Δ*luxO*, Δ*hfq*, *luxO*D47E, and Δ*qrr1–5* cells were harvested at both log phase (A_{600} 0.6 – 0.7) and stationary phase (A_{600} >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 Δ*qrr1–5* (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, Δ*qrr1–5* harboring pRK415, and Δ*qrr1–5* harboring pRK415-*qrr1* through pRK415-*qrr5* were harvested at log phase (A_{600} ~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, Δ*qrr1*, Δ*qrr2*, Δ*qrr3*, Δ*qrr4*, Δ*qrr5*, Δ*qrr14*, Δ*qrr134*, Δ*qrr1345*, and Δ*qrr1–5* were harvested at log phase (A_{600} ~0.6). Ten μg of lysate was subjected to Western blotting using an antibody against SmcR. This result is representative of three independent experiments.

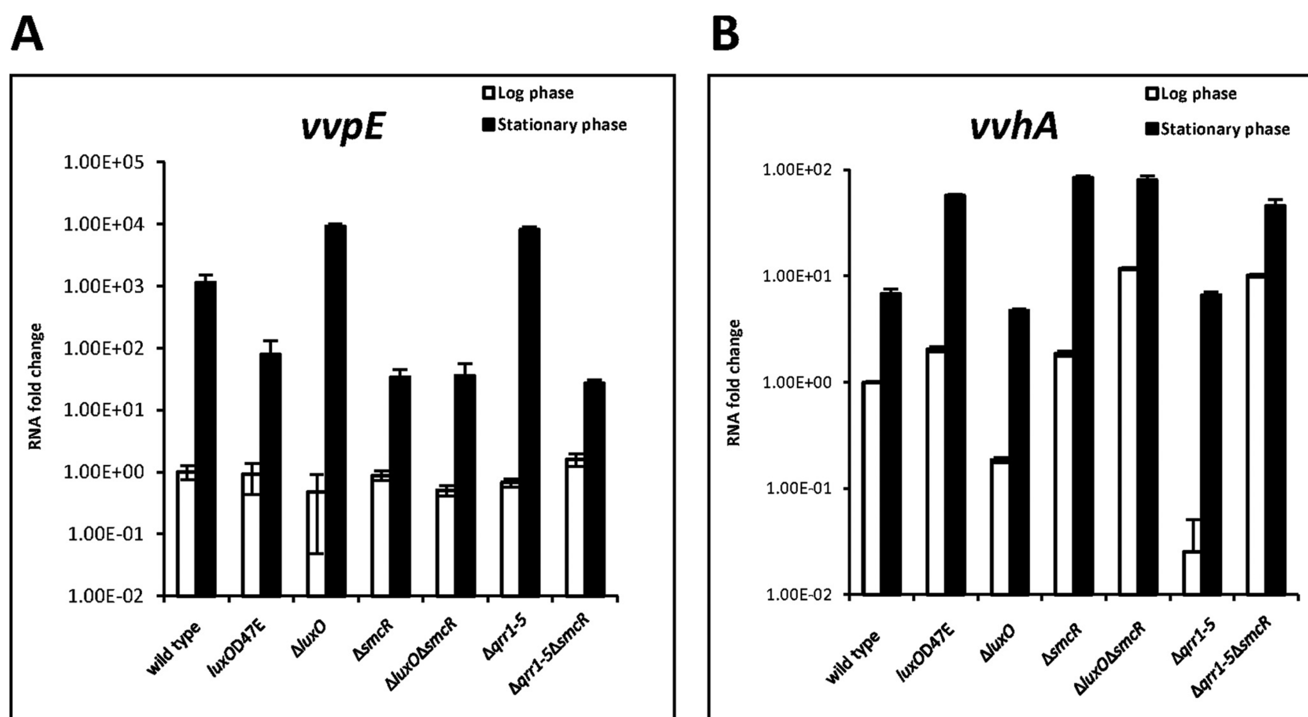


FIGURE 4. **Qrr-dependent regulation of virulence factor expression.** *A,* expression levels of *vvpE* in various strains as measured by quantitative RT-PCR. RNA from wild type *V. vulnificus*, *lux*OD47E, Δ*luxO*, Δ*smcR*, Δ*luxO*Δ*smcR*, Δ*qrr1–5*, and Δ*qrr*Δ*smcR* was purified from cells at log phase (A_{600} ~0.6) and stationary phase (A_{600} >2.0). RNA fold changes represent the *vvpE* expression level normalized to wild type. *B,* expression levels of *vvhA* in various strains as measured by quantitative RT-PCR. Values are averages from biological experiments done in triplicate. *Error bars* indicate the standard deviations.

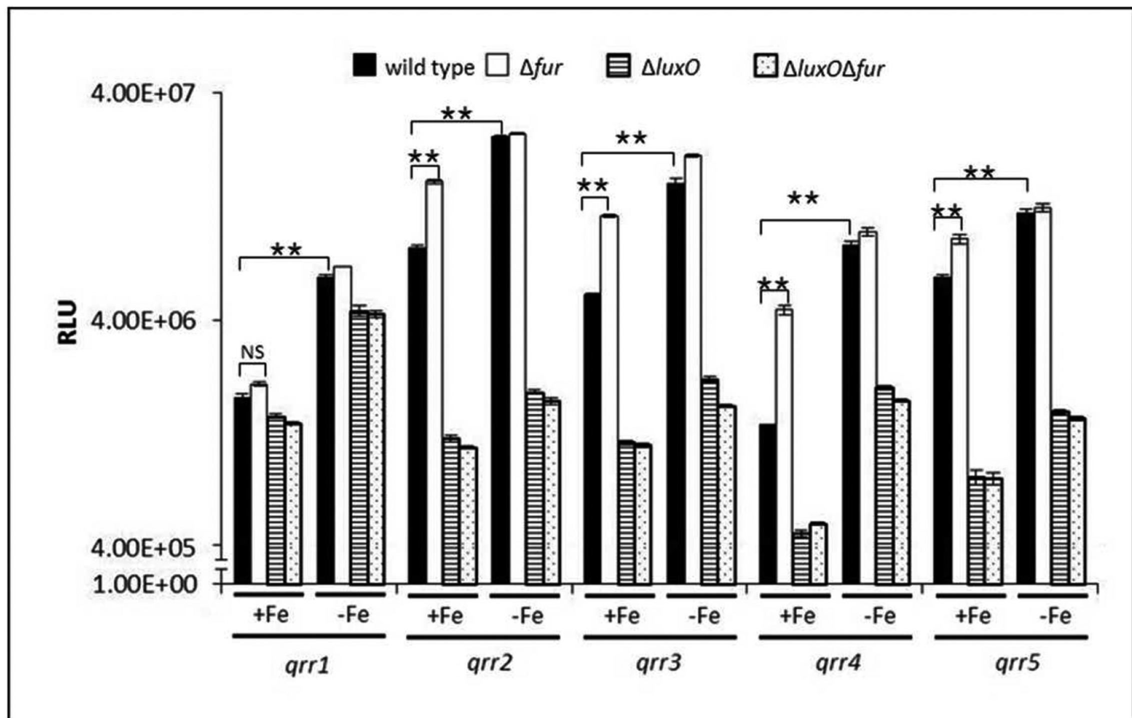
To test whether each of the Qrrs could inhibit SmcR expression, we performed complementation experiments by returning each *qrr* gene back into the Δ*qrr1–5* mutant individually on pRK415 vector constructs (53). SmcR expression was determined by Western blotting of cell extracts obtained at log phase (Fig. 3*C*). 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. 3*D*). None of the five single mutants significantly affected SmcR expression. We then measured SmcR expression in mutant strains containing combinations of *qrr* genes: Δ*qrr14*, Δ*qrr134*, Δ*qrr1345*, and Δ*qrr1–5*. Expression of SmcR in the double or triple mutants was no different from wild type and was only slightly increased in the quadruple mutant Δ*qrr1345*. Only the quadruple mutant Δ*qrr1–5* 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. 4*A*). When compared with wild type cells, *vvpE* expression in cells at stationary phase was ~15-fold lower in *lux*OD47E, where SmcR expression is repressed, and ~30-fold lower in Δ*smcR* and Δ*qrr1–5*Δ*smcR*. In Δ*luxO* and Δ*qrr1–5* 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. 4*B*). At log phase, Δ*luxO* and Δ*qrr1–5*, in which SmcR is derepressed, respectively, showed 5- and 50-fold lower expression of VvhA compared with wild type, whereas *lux*OD47E, Δ*luxO*Δ*smcR*, Δ*smcR,* and Δ*qrr1–5*Δ*smcR* showed higher *vvhA* expression compared with wild type. At stationary phase, wild type, Δ*luxO*, and Δ*qrr1–5*, which had similar levels of SmcR expression (Fig. 3*B*), also had similar levels of VvhA expression, whereas *lux*OD47E, Δ*smcR*, Δ*luxO*Δ*smcR*, and Δ*qrr1–5*Δ*smcR* 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-

A



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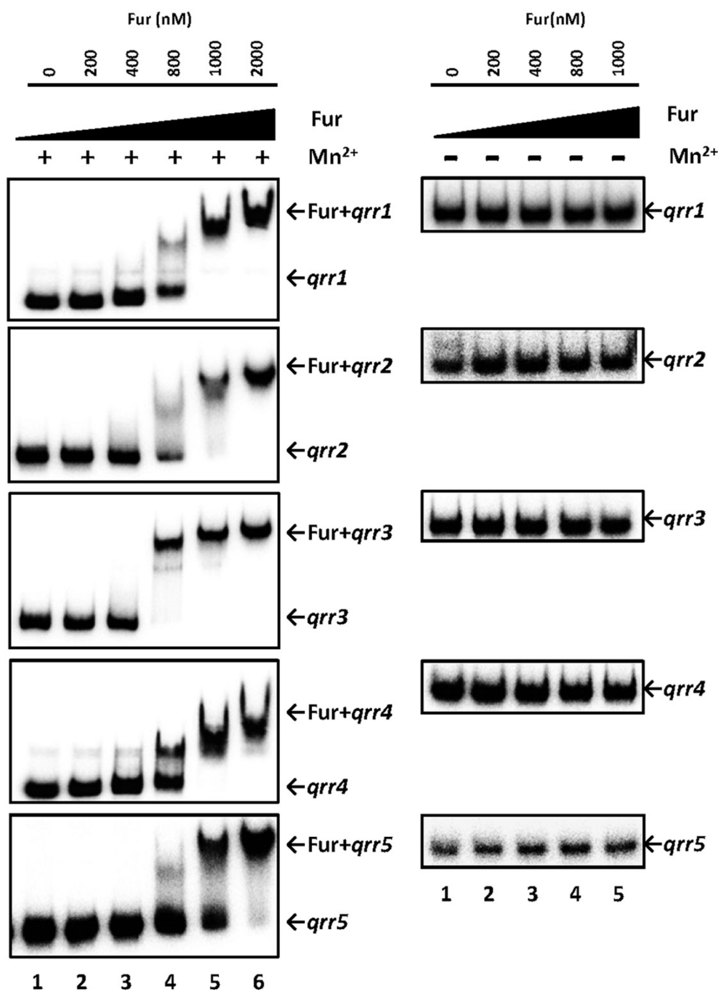

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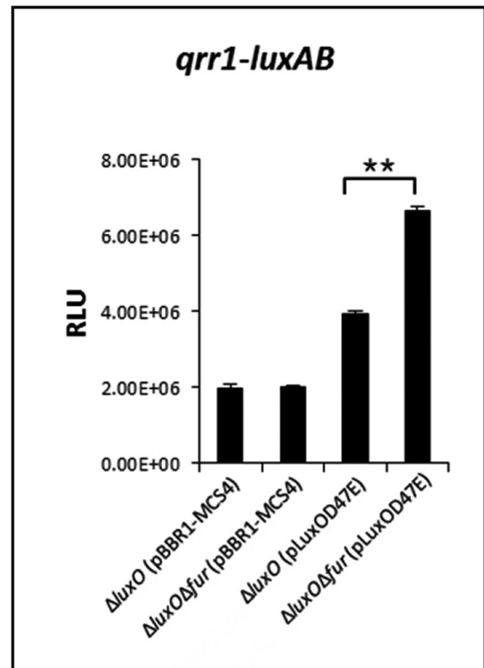


SmcR signal transduction pathway may be regulated not only by the availability of autoinducer molecules as an indication of cell density, but also by other environmental factors, among which iron is particularly important. We showed that the iron-Fur complex represses the expression of SmcR by directly 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. 5*A*). When this experiment was performed using cells grown in AB minimal medium with or without the supplementation of $FeSO_4$, 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 Δ*fur* cells under iron-rich and iron-limiting conditions (Fig. 5*A*). 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 Δ*fur* as compared with wild type. No significant difference was observed for *qrr1* (Fig. 5*A*). Introduction of a wild type copy of *fur* into the Δ*fur* strain restored *qrr* repression (data not shown). Expression of each of the *qrr* genes in the Δ*fur* strain was further increased when the iron chelator was added. Notably, *qrr* expression levels were not significantly different between wild type and Δ*fur* 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 Δ*luxO* mutant and in a Δ*luxO*Δ*fur* 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 Δ*luxO* and Δ*luxO*Δ*fur* strains (Fig. 5*A*). 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 ^{32}P-labeled DNA fragments of the *qrr* promoter regions and purified Fur protein (Fig. 5*B*). Purified Fur and the *qrr* probes were incubated in the presence of either 25 μM Mn^{2+} (instead of Fe^{2+}) or 1 mM EDTA. We observed that Fur could bind to all five *qrr* promoters in the presence of Mn^{2+} in a density-dependent manner, suggesting that Fur acts as a repressor of the *qrr* genes. However, Fur affinity was lost when Mn^{2+} was not present.

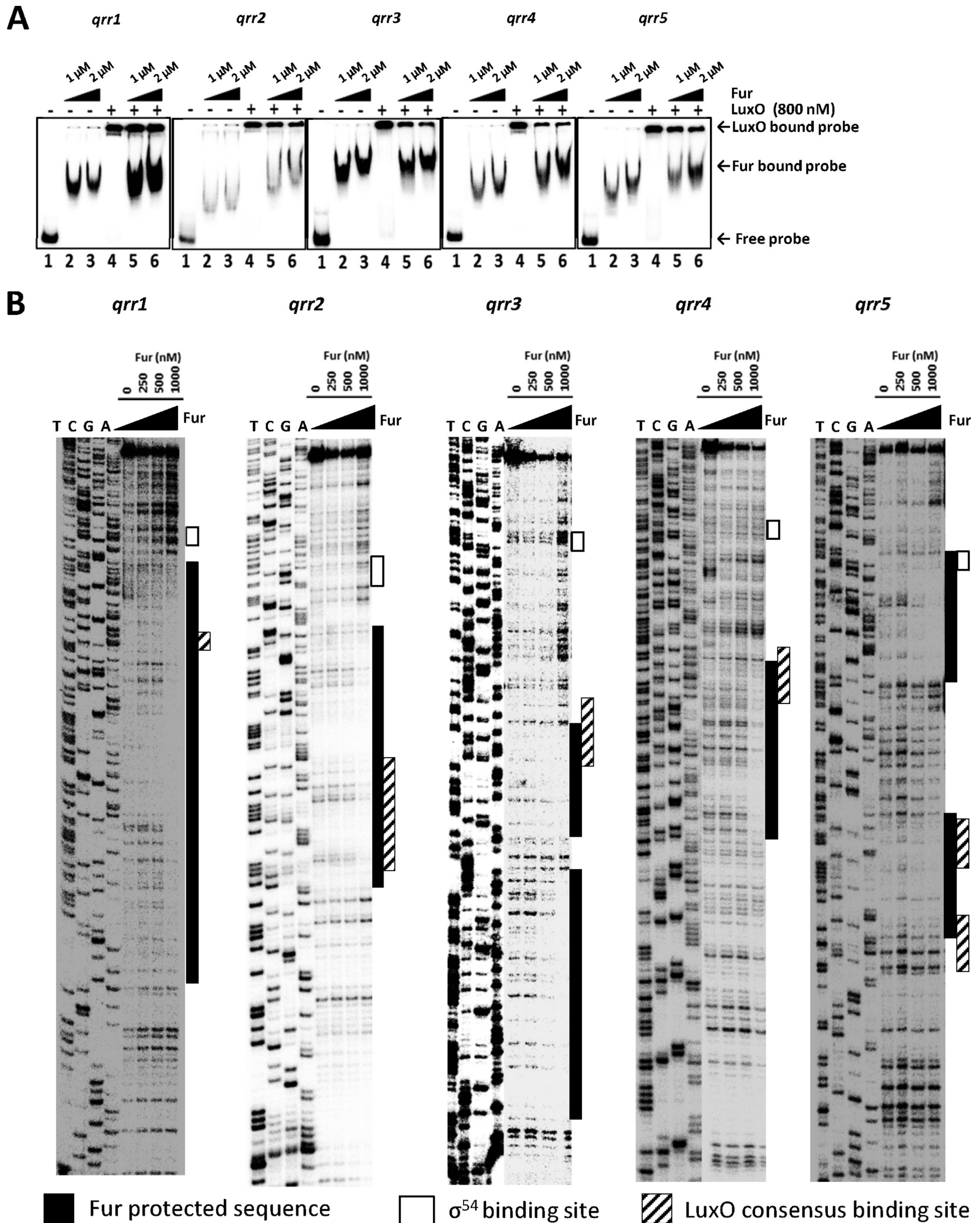
Although Fur binds to the upstream region of *qrr1*, expression of this gene was not significantly different in wild type *versus* Δ*fur* strains (Fig. 5*A*). This discrepancy led us to hypothesize that *qrr1* expression is not high enough (see Fig. 2*A*) to clearly show Fur-mediated repression. To test this possibility, we employed pLuxOD47E, which constitutively expresses active LuxO (Table 1), in a Δ*luxO* background. When LuxOD47E was supplied in *trans*, *qrr1* expression levels were two times the levels in Δ*luxO* alone (Fig. 5*C*). These results suggest that, under our experimental conditions, *qrr1* expression is low due to a low affinity for LuxO. Comparing *qrr1* expression in Δ*luxO*Δ*fur* + LuxOD47E with that in Δ*luxO* + 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. 6*A*). 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. 6*B*). 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

FIGURE 5. ***qrr* transcription regulated by iron in *V. vulnificus*.** *A,* regulation of *qrr* transcription by iron and Fur. Luciferase activity represents levels of *luxAB*-transcriptional reporter fusions to *qrr1*, *qrr2*, *qrr3*, *qrr4*, and *qrr5* in wild type *V. vulnificus*, Δ*fur*, Δ*luxO*, and Δ*fur*Δ*luxO* harboring each of the plasmids pHK-*qrr1*, pHK-*qrr2*, pHK-*qrr3*, pHK-*qrr4*, and pHK-*qrr5*. Bacteria were cultured with or without 200 μM of the iron chelator 2,2′-dipyridyl, which was supplemented when cells were at an A_{600} of ~0.2. Cell density and luminescence were measured at log phase (A_{600} of ~0.6), as described under "Experimental Procedures." Relative light units (*RLU*) represent light units normalized to cell density (luminescence/A_{600}). Values are averages from three biological experiments, and *error bars* denote standard deviations. *B,* binding of Fur to the promoter regions of *qrr* genes as determined by electrophoresis mobility shift assay. Ten ng of DNA probes, including *qrr* promoter regions, were incubated with increasing concentrations of Fur in the presence (*left panel*) or absence (*right panel*) of 100 μM $MnSO_4$. *Lanes 1–6* represent Fur concentrations of 0, 200, 400, and 800 nM and 1 and 2 μM, respectively. *C,* Fur significantly represses transcription of *qrr1* in the presence of overexpressed *luxO*D47E. Δ*luxO* and Δ*fur*Δ*luxO* harboring pHK-*qrr1* and pLuxOD47E or pBBR1-MCS4 were cultured until log phase. Values are averages from three independent experiments, and *error bars* denote standard deviations (A_{600} of ~0.6). Expression of *qrr1* was measured as described above (**, $p < 0.005$; *NS,* not significant in Student's *t* test with $p > 0.05$).

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



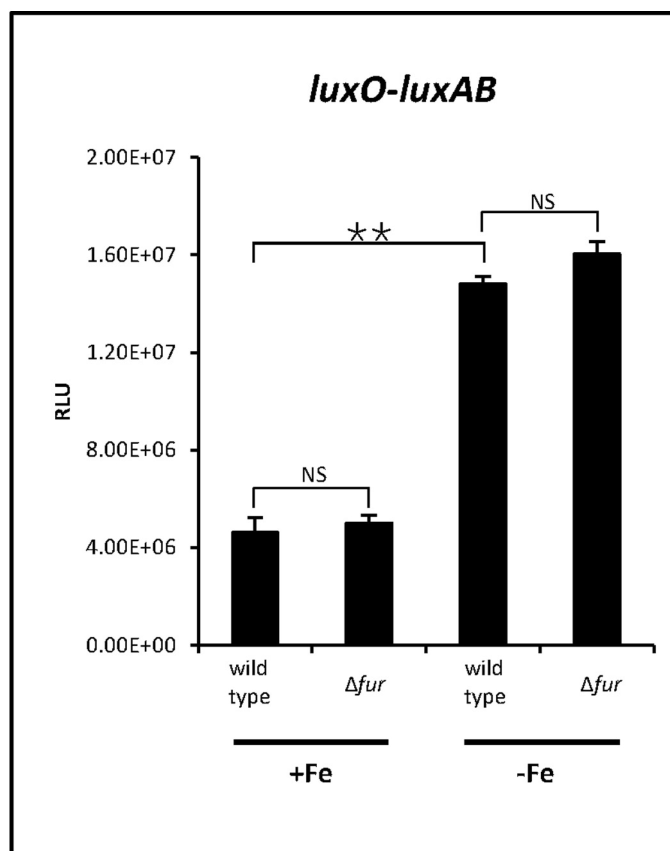


FIGURE 7. **Effects of iron on *luxO* transcriptional levels.** Luciferase activity representing transcriptional levels of *luxO* was measured in wild type *V. vulnificus,* and Δ*fur*. Cells were cultured with or without 200 μM iron chelator 2,2′-dipyridyl, which was supplemented when cells were at an A_{600} of ~0.2. Luminescence and cell density were measured at an A_{600} ~0.6. Relative light units (*RLU*) represent light units normalized to cell density (luminescence/A_{600}). 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 $p > 0.05$).

shown in Fig. 6*A*. 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 Δ*fur* strains. We found that *luxO* expression was significantly induced in the presence of an iron chelator in both wild type and Δ*fur*. Expression levels were not significantly different between wild type and Δ*fur*, indicating that Fur itself exerts no effect on *luxO* expression (Fig. 7) and suggesting that iron inhibits *lux*O 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, *lux*OD47E, Δ*luxO*, and Δ*qrr1–5* cells by Western blotting (Fig. 8*A*). 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 *lux*OD47E mutants, SmcR was not detected under either condition. However, SmcR expression was observed in Δ*luxO* and Δ*qrr1–5* 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 Δ*luxO* and Δ*qrr1–5*. 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. 8*B*). 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 assessed the expression of *smcR* under iron-limiting conditions using *qrr* deletion strains (Fig. 8*C*). SmcR expression in Δ*qrr2*, Δ*qrr3*, and Δ*qrr5* was significantly derepressed as compared with wild type but was not significantly different in Δ*qrr1* and Δ*qrr4*. SmcR expression in the double mutant Δ*qrr14* 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, Δ*qrr2*, Δ*qrr3*, and Δ*qrr5* had much higher levels of SmcR expression, consistent with higher expression of *qrr2, qrr3,* and *qrr5* in wild type cells (Fig. 2*A*). The other multiple mutant strains, Δ*qrr134*, Δ*qrr1345*, and Δ*qrr1–5*, 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*, 800 nM LuxO; *lane 5*, 800 nM LuxO with 1 μM Fur; *lane 6*, 800 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 ^{32}P-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 σ^{54}-binding site (*unshaded boxes*), and the consensus LuxO-binding sites (*hatched boxes*) are indicated.

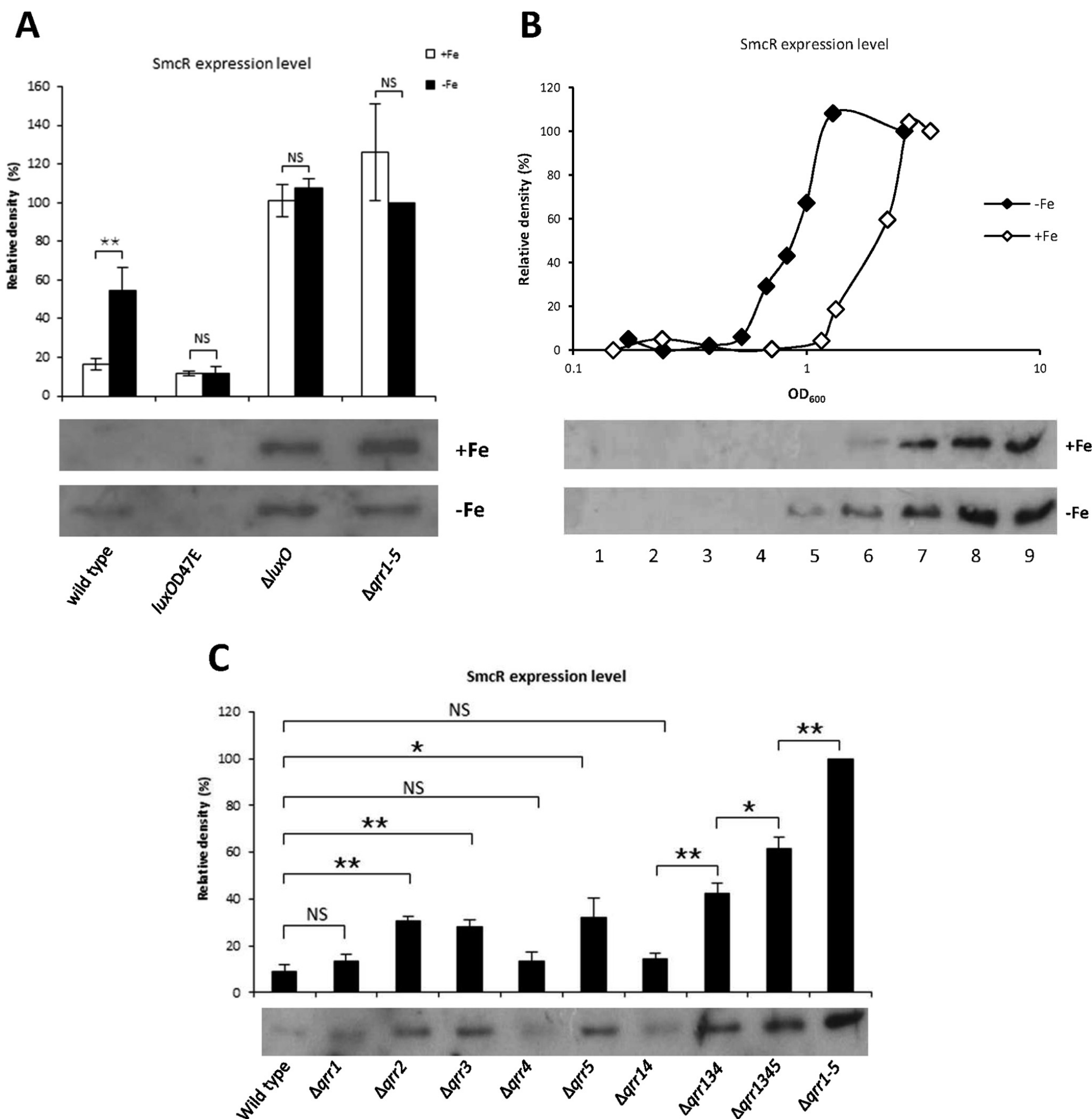


FIGURE 8. **Effect of iron on SmcR regulation.** *A,* Western blot hybridization of SmcR in wild type, *luxO*D47E, Δ*qrr1–5,* and Δ*luxO* under iron-rich and iron-limiting conditions. The iron chelator 2,2′-dipyridyl (200 μM), was supplemented when cells were at an A_{600} of ~0.2, and cells were harvested at an A_{600} of ~0.6. Ten μg of each lysate sample was loaded onto the gel. The *upper panel* represents the relative densities of bands in the hybridization shown in the *lower panel*. Values are averages normalized to the intensity of the Δ*qrr1–5* (without iron) sample from three biological experiments. *Error bars* denote standard deviations (**, $p < 0.005$; *NS,* not significant in Student's *t* test with $p > 0.05$). *B,* SmcR expression under iron-rich and iron-limiting conditions. Wild type *V. vulnificus* was cultured with or without 100 μM of 2,2′-dipyridyl supplemented at an A_{600} of ~0.1. Cells were harvested for Western blotting at different growth stages as indicated, and 10 μg of each lysate were loaded onto the gel. Wild type cells without iron chelator were harvested at A_{600} readings of 0.148, 0.240, 0.502, 0.709, 1.156, 1.335, 2.22, 2.75, and 3.395 (*lanes 1–9*, respectively). Wild type cells supplemented with the iron chelator were harvested at A_{600} readings of 0.172, 0.242, 0.382, 0.527, 0.671, 0.822, 0.998, 1.296, and 2.62 (*lanes 1–9*, respectively). The *upper panel* represents the relative densities of bands shown in the *lower panel*. Values are averages normalized to the intensity of the sample in *lane 9*. *C,* expression of SmcR under iron-limiting conditions. *V. vulnificus* mutants, including Δ*qrr1*, Δ*qrr2*, Δ*qrr3*, Δ*qrr4*, Δ*qrr5*, Δ*qrr14*, Δ*qrr134*, Δ*qrr1345*, and Δ*qrr1–5*, were cultured with 200 μM 2,2′-dipyridyl added at an A_{600} of ~0.2. Cells were harvested for Western blotting at an A_{600} of ~0.4, and 10 μg of each lysate was loaded onto the gel. The *upper panel* represents the relative densities of bands shown in the *lower panel*. Values are averages normalized to the intensity of the Δ*qrr1–5* sample from three biological experiments (**, $p < 0.005$; *, $p < 0.01$; *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. 1*B*) (37). *qrr3*, *qrr4*, and *qrr5* each have two binding sites, one of which is a perfect match to the consensus. *qrr1* has only one

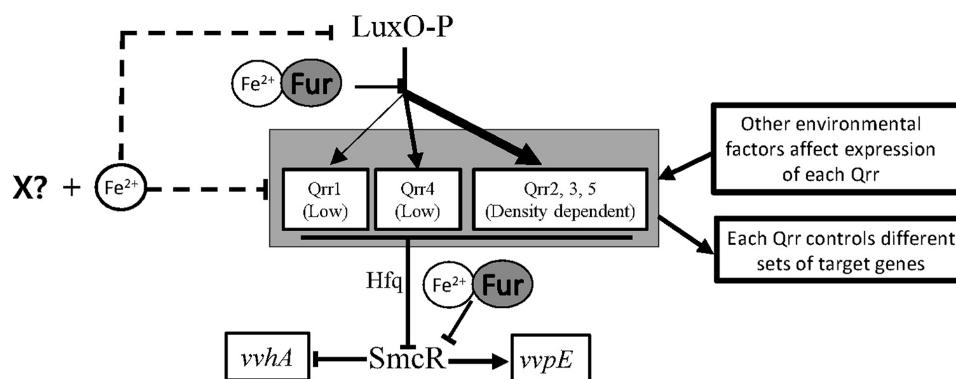


FIGURE 9. **Working model for the regulation of the quorum-sensing signaling pathway by cell density and iron in *V. vulnificus*.** At low cell density, phosphorylated LuxO activates σ^{54}-dependent transcription of the five *qrr* genes to different degrees and with different patterns. It is possible that the expression of each *qrr* gene is also affected by environmental factors other than cell density and that each Qrr has its own target genes to regulate. Qrr redundantly represses expression of SmcR with assistance from Hfq. SmcR is a global transcriptional regulator involved in regulation of various virulence factors, including activation of *vvpE* and repression of *vvhA*. The Fur-iron complex directly represses the expression of both Qrr and SmcR. Iron also inhibits the transcription of *luxO* and the five *qrr* genes, possibly through an unidentified factor labeled *X*.

binding site that differs from the consensus sequence by two nucleotides (Fig. 1*B*). These differences likely affect the binding affinity of LuxO for each *qrr*, which we observed in preliminary experiments using EMSA (Fig. 2*B*). 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 parahemeolyticus* 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 promoter 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-

[3] Y. Wen, I. H. Kim, and K.-S. Kim, unpublished results.

tion at high cell density, where Qrrs are not expressed and cannot be regulated by iron. In summary, by employing this dual regulatory system, cells can effectively turn on the quorum-sensing response in the presence of iron at low cell density and attenuate the response in the presence of iron at high cell density.

We further showed that Qrrs repress SmcR redundantly under iron-rich conditions (Fig. 5) but additively under iron-limiting conditions (Fig. 8*D*). It is possible that under iron-limiting conditions, *smcR* is no longer repressed by the Fur iron complex (49), meaning that stronger Qrr control is needed to keep SmcR repressed. Regardless of the molecular basis underlying the additive action of Qrrs under iron-limiting conditions, such a regulatory mode results in a faster transition from low cell density mode to the high cell density mode (Fig. 8, *B* and *C*). Iron availability is an important limitation for pathogenic microorganisms to overcome to survive and thrive in a host environment. Rapid transition to high cell density mode under these conditions may allow pathogens to more quickly express virulence factors and thereby acquire iron.

The transition from single-cell mode to quorum-sensing mode involves more than LuxO-P, Qrrs, and SmcR. Complicated feedback loops, dose compensation, and self-regulation are also involved in the precise timing of the switch between high and low cell density modes. In *V. cholerae*, four Qrrs compensate for each other to calibrate total Qrr activity through the Qrr-LuxO and HapR-Qrr feedback loops and ensure the timing of quorum sensing (39, 63). HapR self-repression and LuxR-Qrr feedback ensure the fast elimination of LuxR during the transition from high cell density to low cell density (63, 64). HapR and LuxO also constrain their expression through self-repression (64, 65). As all of these factors are affected by iron, these regulatory mechanisms need to be carefully studied under iron-limiting conditions.

Cell density is important for the survival and pathogenesis of bacteria as they encounter the differing conditions of a host environment, and iron and cell density influence one another by affecting the growth rate of cells (66–68). Alternatively, cell density might also directly affect the availability of iron. Therefore, cognate signal transduction systems for the quorum-sensing and iron-regulation pathways regulate both pathways and coordinate the regulation of virulence genes. In *Pseudomonas aeruginosa*, PrrF1 and PrrF2, small RNAs involved in iron-dependent regulation, activate quorum sensing by promoting the production of an autoinducer (69). In *V. vulnificus*, both SmcR and Fur repress the transcription of *vvhA* and the vulnibactin synthesis gene *vvsAB* (23). SmcR expression is directly repressed through binding of the Fur-iron complex, leading to an attenuated quorum-sensing response under iron-rich conditions (49). In summary, this study showed that cognate signal transduction pathways for iron and quorum sensing converge on Qrrs to control the expression of virulence factors and ensure optimal growth conditions for the pathogen while in the host.

Author Contributions—Y. W. and K. S. K. designed the study and wrote the paper. Y. W. and I. H. K. performed the experiments.

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