



VqsA, a Novel LysR-Type Transcriptional Regulator, Coordinates Quorum Sensing (QS) and Is Controlled by QS To Regulate Virulence in the Pathogen *Vibrio alginolyticus*

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ABSTRACT The quorum sensing (QS) system controls bacterial group behaviors in response to cell density. In vibrios, LuxR and AphA are two master QS regulators (MQSRs) controlling gene expression in response to high or low cell density. Other regulators involved in the regulation of these two MQSRs and QS pathways remain to be determined. Here, we performed bacterial one-hybrid (B1H)-assay-based screens of transcriptional factors (TFs) to identify TFs that can directly regulate the expression of *luxR* and *aphA* from a library of 285 TFs encoded by the fish pathogen *Vibrio alginolyticus*. A total of 7 TFs were identified to bind to the promoters of both *luxR* and *aphA*. Among these TFs, the novel LysR-type transcriptional regulator (LTTR) VqsA could activate LuxR and repress AphA transcription. Meanwhile, LuxR and AphA exerted feedback inhibition and activation of *vqsA* expression, respectively, indicating that VqsA coordinates QS and is also regulated by QS. In addition, VqsA inhibited its own expression by directly binding to its own promoter region. The VqsA-binding sites in the promoter regions of *luxR* and *aphA* as well as the binding sites of LuxR, AphA, and VqsA in the *vqsA* gene were uncovered by electrophoretic mobility shift assays (EMSAs) and DNase I footprinting analysis. Finally, VqsA was verified to play essential roles in QS-regulated phenotypes, i.e., type VI secretion system 2 (T6SS2)-dependent interbacterial competition, biofilm formation, exotoxin production, and *in vivo* virulence of *V. alginolyticus*. Collectively, our data showed that VqsA is an important QS regulator in *V. alginolyticus*.

IMPORTANCE Investigation of the mechanism of regulation of quorum sensing (QS) systems will facilitate an understanding of bacterial pathogenesis and the identification of effective QS interference (QSI) targets. Here, we systematically screened transcriptional factors (TFs) that modulate the expression of the master QS regulators (MQSRs) LuxR and AphA, and a novel LysR-type transcriptional regulator, VqsA, was identified. Our data illuminated the mechanisms mediating the interaction among LuxR, AphA, and VqsA as well as the effects of these regulators on the expression and output of QS. The impaired expression of virulence genes as a result of *vqsA* disruption demonstrated that VqsA is an important player in QS regulation and pathogenesis and may be the third MQSR involved in sensing environmental signals by vibrios to coordinate QS responses. This study will facilitate the development of strategies to interfere with QS and effectively control this pathogen that plagues the aquaculture industry.

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Quorum sensing (QS) enables bacteria to collectively coordinate gene expression in response to cell density. LuxR and AphA are two master QS regulators (MQSRs) in the QS circuits of vibrios (1–3). AphA is involved in the regulation of gene expression in the low-cell-density (LCD) stage, while LuxR is involved in the high-cell-density (HCD) stage. The separate or combined activities of these two MQSRs regulate the expression of ~600 genes in *Vibrio harveyi*, leading to a variety of cooperative behaviors, including bioluminescence, biofilm formation, motility, and virulence (4, 5). Reciprocal repression of these two MQSRs enables these regulators to be individually expressed at the low- or high-cell-density stage (3, 4). The mechanism of QS-based signaling in response to cell densities and autoinducer (AI) concentrations has been well established in *V. harveyi* and other vibrios (2).

MQSR production is strictly regulated at multiple levels to obtain concentration gradients for the temporal regulation of gene expression at different cell densities while avoiding the potentially high fitness cost of runaway expression and protein bursts under nonideal conditions (4, 6). Indeed, various HapR (a LuxR homolog) regulators are deployed in *Vibrio cholerae* to optimize the QS output at the transcriptional level (e.g., Fis, VqmA, HapR, AphA, VarS/VarA, and FliA/FlgM) (3, 7–11), the posttranscriptional level (e.g., small RNAs [sRNAs] associated with *csrA* and *grr* genes) (12, 13), and the post-translational level (e.g., VarS/VarA) (10, 14), constituting a sophisticated regulation network. These regulators and other uncharacterized factors play essential roles in the interactions of QS pathways with environmental signals and thus are important targets for the development of nonantibiotic therapeutics and drugs, known as QS interference (QSI) therapies (15).

Vibrio alginolyticus is a Gram-negative bacterium and an opportunistic pathogen that causes intra- and extraintestinal diseases or wound infections in humans and infects a large number of sea animals (16). Known virulence factors of *V. alginolyticus* include extracellular proteases, e.g., Asp (16), MviN (17), ProA (18), and Pep (19). These virulence factors and other virulence-related genes are closely regulated by the LuxR-LuxO-centered QS regulatory system in *V. alginolyticus* (20). The QS signaling network of *V. alginolyticus* is quite similar to that of *V. harveyi*, including the three types of AIs that are produced and sensed by LuxS/PQ, LuxM/N, and CqsA/S (21–23). At low cell density, AphA binds to the promoter of *luxR* and 48 other genes and regulates the expression of these genes (24). At high cell density, LuxR can also act a global regulator, binding directly upstream of and regulating the expression of more than 100 genes in response to QS signals, as also seen in *V. harveyi* (reference 4 and our unpublished data). Autorepression of LuxR occurs via the binding of LuxR to the promoter at two sites, known as LuxR-binding site I (RBSI) and RBSII (6). In addition to LuxR and AphA, the promoter of *luxR* is also indirectly regulated by LuxT and directly regulated by AphB and the alternative sigma factor RpoE, which works in conjunction with core RNA polymerase in response to changes in temperature (6, 23, 25). However, whether there are other factors that can regulate QS in the bacterium remains largely unknown.

In this study, we employed a modified bacterial one-hybrid (B1H) system to systematically screen a library of 285 transcriptional factors (TFs) in *V. alginolyticus* to identify *luxR* and *aphA* regulators. A total of 7 TFs were identified to bind to and regulate the promoters of both *luxR* and *aphA*. Among these factors, the hypothetical protein V12G01_18672 (referred to here as VqsA [*V*ibrio *q*uorum *s*ensing *a*ctivator]), which encodes a novel LysR-type transcriptional regulator (LTTR) and the deletion of which leads to the greatest reduction in exotoxin production, was chosen for further analysis. Electrophoretic mobility shift assays (EMSAs) and DNase I footprinting analysis confirmed that VqsA regulates *luxR* and *aphA* expression by binding directly to the promoter DNA of these genes. VqsA is important for the virulence of the bacterium and may be the third MQSR coordinating QS and virulence gene expression.

RESULTS

Systematic screening revealed multiple regulators binding to the promoters of *luxR* and *aphA* and regulating QS output. We used a previously described strategy (26) to adapt the BacterioMatch II two-hybrid system (Stratagene, La Jolla, CA, USA) for identifying protein-protein interactions to a bacterial one-hybrid system (B1H) for the detection of specific protein-DNA interactions *in vivo* (Fig. 1A; see also Fig. S1 in the supplemental material). The B1H system consists of a selected bait promoter DNA cloned into the reporter plasmid pBXcmT carrying the selectable genes HIS3 and *aadA*. A specific transcription factor (TF) is expressed as a fusion with the alpha-subunit of RNA polymerase (RNAP) from the pTRG plasmid. This pair of recombinant vectors is cotransformed into the host strain (Fig. S1). If the TF protein interacts with the target DNA, the protein recruits and stabilizes the binding of RNAP at the promoter and thus activates the transcription of the HIS3 reporter gene in the presence of the compound 3-amino-1,2,4-triazole (3-AT). A second reporter gene, *aadA*, encoding a protein that confers streptomycin resistance (Str^r), provides an additional form of selection to validate the DNA-protein interaction (Fig. 1A) (26). To search for potential TFs involved in the regulation of *luxR* and *aphA* expression in *V. alginolyticus*, the promoter regions of these genes (P_{luxR} and P_{aphA}) were individually cloned into the T/A cloning site upstream of HIS3-*aadA* in pBXcmT. The B1H system was first validated by the growth of the cotransformants harboring pTRG::LuxR and pBXcmT:: P_{luxR} plasmids with a known LuxR- P_{luxR} interaction pair (6) on the plate with selective medium containing 3-AT and Str (positive control) (Fig. 1B). No growth of the cotransformants encoding LuxR and P_{luxR} Δ RBSI/II with mutated LuxR-binding sites I and II (6) was observed on the selection plates (negative control). All these cotransformants grew on the LB plate in the absence of 3-AT and Str (Fig. 1B). To further validate the B1H system, AphA was observed to bind to the promoter regions of both *aphA* and *luxR* (Table S1), as previously documented (24).

The *V. alginolyticus* genome encodes ~285 predicted TFs with putative DNA-binding domains. The TF genes were individually cloned into the pTRG plasmid and then screened using P_{luxR} and P_{aphA} as bait sequences in pBXcmT in the B1H system. The comprehensive P_{luxR} and P_{aphA} screens yielded 16 and 19 putative TFs, respectively, by interacting with the *luxR* and *aphA* promoters, respectively (Table S1). Many of the P_{luxR} - and P_{aphA} -associated TFs belong to the LysR-type transcriptional regulator (LTTR) family, the most common type of prokaryotic DNA-binding protein, suggesting the importance of LTTRs in regulating QS. Moreover, 7 TFs, i.e., AsnC, SoxR, MetR, 12G01_02325, 01665, 06711, and 18672, exhibited binding affinities toward both P_{luxR} and P_{aphA} and supported the growth of the cotransformants harboring the TF and the P_{luxR} or P_{aphA} pairs (Fig. 1B) (Table 1). The electrophoretic mobility shift assays (EMSAs) showed that all TFs bound efficiently to both P_{luxR} and P_{aphA} in a concentration-dependent manner, whereas the negative control (*gyrB*) remained unbound (Fig. 1C and 2C). These data confirmed the interaction between the promoters and the selected TFs.

We further generated the in-frame deletion mutants of the 7 selected TFs, and none of these mutants exhibited growth defects compared to the wild-type (WT) strain (Fig. S2). Hide powder azure (HPA) digest assays and Western blot analyses were performed to investigate the roles of these TFs in the production of alkaline serine protease (Asp), a QS-controlled exotoxin (16). Compared to the WT strain, the Asp yield was seen to be drastically reduced in the $\Delta luxR$ mutant and significantly increased in the $\Delta aphA$ mutant (Fig. 1D and E) (6, 24). The $\Delta soxR$, $\Delta metR$, and $\Delta 06711$ mutant strains showed markedly increased Asp production, while the $\Delta asnC$, $\Delta 02325$, $\Delta 01665$, and $\Delta 18672$ mutants were significantly impaired in Asp production (Fig. 1D and E). Taken together, these data demonstrate that various novel positive and negative regulators bind directly to the promoters of MQSR-encoding genes and are involved in QS pathways.

VqsA regulates the expression of *luxR* by directly binding to a specific binding site. Among the above-mentioned 7 selected TFs, we were intrigued by 12G01_18672 (here referred to as VqsA), as the disruption of this gene drastically impaired the ability of *V. alginolyticus* EPGs to produce the exotoxin (Fig. 1E). The *vqsA* gene encodes a

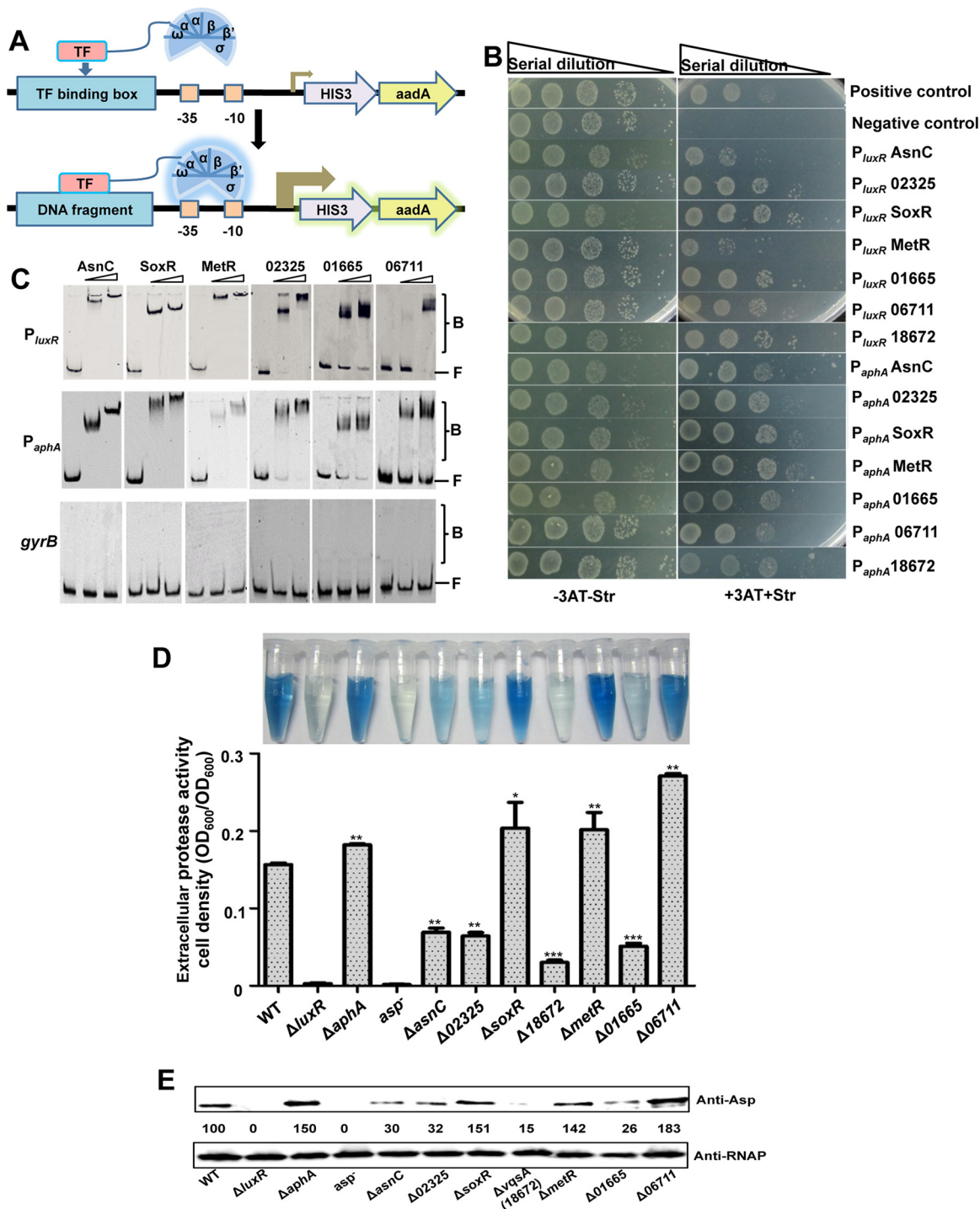


FIG 1 Screening of TFs bound to the promoter DNA of *luxR* and *aphA* *in vitro*. (A) Diagrams for the modified bacterial one-hybrid (B1H) assay. If the TF interacts with the target DNA, the TF recruits and stabilizes the binding of RNA polymerase at the weak promoter (shown as a turned arrow), thus activating the transcription of the HIS3 reporter gene, conferring 3-AT resistance, and the gene *aadA*, conferring Str resistance; this confirms the DNA-protein interaction. (B) B1H identification of TFs interacting with promoter DNA of *aphA* and *luxR*. Positive growth of cotransformants with pBXcmT harboring *P_{luxR}* or *P_{aphA}* and the pTRG derivatives expressing AsnC, 02325, SoxR, MetR, 01665, 06711, or 18672 on selective plates are shown. Cotransformants harboring pTRG::LuxR with pBXcmT::*P_{luxR}* and pTRG::LuxR with pBXcmT::*P_{luxR}* ΔRBSI/II were used as positive and negative controls (6), respectively. (C) EMSA analysis of protein-DNA interactions. Cy5-labeled *P_{luxR}* or *P_{aphA}* DNA was added to reaction mixtures with different concentrations of the AsnC, SoxR, MetR, 02323, 01665, and 06711 proteins and a 10-fold excess of nonspecific competitor [poly(dI:dC)]. The unrelated *gyrB* DNA was used as a negative control. B, bound DNA; F, unbound DNA. (D) Hide powder azure (HPA) digestion assays were performed to analyze extracellular Asp activity of the supernatants of 9-h cultures. Asp activity was normalized by dividing the total protease activity by the cell density of each strain. The results are presented as the mean ± SD (*n* = 3). *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 based on analysis of variance (ANOVA) comparisons. (E) Western blot analysis was performed to analyze Asp yields in the WT and in the indicated deletion mutant strains using concentrated

(Continued on next page)

TABLE 1 Seven transcriptional regulators identified to bind to the promoters of both *aphA* and *luxR*

Gene ID ^a	Protein ^b	Molecular mass (kDa)	pI	Function	Location of HTH ^c	Binding with ^d :	
						P _{luxR}	P _{aphA}
V12G01_10813	AsnC	17.32	4.67	Transcriptional regulator AsnC	N, C	+++	++++
V12G01_02325	HP	42.19	4.66	Transcriptional activator	N	++++	++++
V12G01_02620	SoxR	16.20	7.59	SoxR protein	N	+++++	++++
V12G01_16177	MetR	34.09	7.35	LysR family MetR	N	+++	++++
V12G01_01665	HP	15.47	7.57	Hypothetical regulator	N	++++	+++
V12G01_06711	HP	33.52	4.87	LysR family protein	N	++++	+++
V12G01_18672	VqsA	32.84	7.25	LysR family protein	N	++++	++++

^aID, identification.

^bHP, hypothetical protein.

^cHTH, helix-turn-helix domain and the location of this domain in the protein (N- or C-terminal end).

^dThe observed growth of the serial dilutions of cotransformants harboring both the indicated promoter DNA and the pTRG plasmid expressing the selected protein on LB agar plates containing 3-AT and Str. Plus signs indicate the level of growth of the indicator strains used for B1H assays.

292-amino-acid (aa) protein annotated as an LTTR that is highly conserved in vibrios. We first confirmed that complementation with a plasmid carrying the *vqsA* gene restored LuxR expression in the $\Delta vqsA$ mutant to the WT level (Fig. 2A). In the QS cascade of vibrios, LuxO represses the expression of LuxR (2, 6). Compared to the $\Delta luxO$ (6) and $\Delta vqsA$ mutants, the $\Delta vqsA \Delta luxO$ double-mutant strain showed significantly lower LuxR production than the $\Delta luxO$ mutant (Fig. 2A). These results contradict the idea that VqsA regulates LuxR expression via LuxO and suggest that VqsA might act on the LuxR regulatory pathway independent of LuxO and upstream QS cascades (Fig. 2A). The regulation by VqsA of the expression of *luxR* was further confirmed by reverse transcription-quantitative PCR (qRT-PCR) analysis, showing that the transcript levels of *luxR* in $\Delta vqsA$ mutant cells are significantly lower than those in *vqsA*⁺ mutant cells after 9 h of growth (Fig. 2B).

EMSAs showed that VqsA bound specifically to a DNA probe that included the promoter region of the *luxR* gene in a concentration-dependent manner in the presence of an excess amount (10-fold) of the nonspecific poly(dI:dC) competitor (Fig. 2C, lanes 1 to 8) and could be titrated out by the addition of the unlabeled *luxR* probe (Fig. 2C, lanes 9 and 10). The value of the dissociation constant (K_d) for the binding of VqsA to *luxR* was calculated to be 0.55 μ M (Fig. 2D). Next, a dye-primer-based DNase I footprinting assay was performed on both strands of a DNA fragment encompassing the promoter of *luxR*. We compared the electropherograms with and without addition of the VqsA protein to determine the specific VqsA-binding site (VBS), 5'-TGAGTGTAATGGTGCATATGCACAAGGTCAA-3' (88 bp upstream of ATG), which mapped to the -35 box and -10 box in the promoter region of *luxR* (Fig. 2E). The absence of the specific binding site abolished the binding of VqsA to the *luxR* promoter (Fig. 2F). Taken together, these data indicate that VqsA binds to the *luxR* promoter, positively regulating transcription and expression of the *luxR* gene.

VqsA directly represses *aphA* expression. We next asked whether VqsA is a transcriptional regulator of *aphA*. The MQSR AphA governs the LCD QS gene expression and is highly expressed in the first 2 h of growth (24). Western blotting was performed with an AphA-specific antibody to test AphA expression in the WT, $\Delta aphA$ mutant, and $\Delta vqsA$ mutant strains and in the complement *vqsA*⁺ strain at 2 h in Luria-Bertani medium supplemented with 3% NaCl (LBS). A notable increase in *aphA* expression was observed in the $\Delta vqsA$ mutant strain, whereas *aphA* expression in the *vqsA*⁺ complement strain decreased to the WT level (Fig. 3A). qRT-PCR analysis also confirmed that the *aphA* transcript level was higher in the $\Delta vqsA$ mutant strain than in the WT strain and was significantly lower in the *vqsA*⁺ complement strain (Fig. 3B), indicating that VqsA represses the transcription of *aphA*.

FIG 1 Legend (Continued)

supernatant with Asp-specific antibodies. RNAP was used as the loading control for the supernatants that were obtained from the same number of cells. The numbers indicate the densitometric measurements.

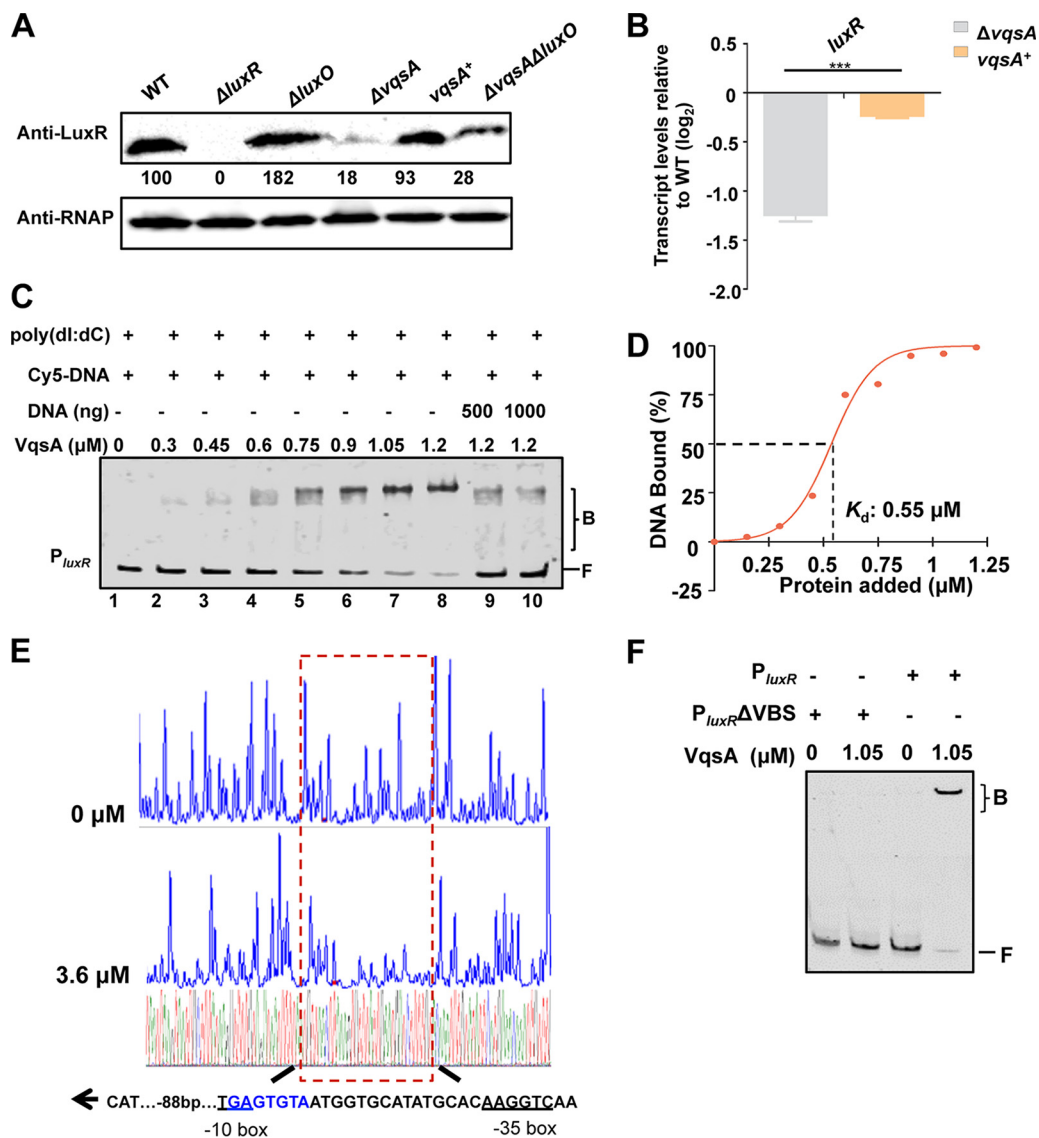


FIG 2 VqsA regulates *luxR* expression by directly binding to the *luxR* promoter. (A) Western blot analysis showing LuxR expression in WT and $\Delta luxR$, $\Delta luxO$, $\Delta vqsA$, *vqsA*⁺, and $\Delta vqsA \Delta luxO$ mutant cells grown in LBS for 9 h. RNAP was used as a loading control. The numbers indicate the densitometric measurements. (B) qRT-PCR analysis of *luxR* transcripts in the WT, $\Delta vqsA$ mutant, and *vqsA*⁺ mutant cells grown in LBS for 9 h; 16S rRNA was selected as a control. The results are displayed as the mean \pm SD ($n = 3$). ***, $P < 0.001$ based on Student's *t* test. (C) EMSA examination of the binding of VqsA to P_{luxR} . The amount of VqsA protein used was as indicated, and 20 ng of each Cy5-labeled probe was added to the EMSA reactions. The shifts were verified to be specific in experiments in which 25- to 50-fold excess of unlabeled specific DNA and nonspecific competitor DNA [poly(dI:dC)] were used. (D) Plot showing the binding affinity of VqsA for the *luxR* promoter. The densitometric intensities of bound DNA fragments were plotted against VqsA concentrations. The indicated dashed line represents the concentration of VqsA that caused half-maximal binding (K_d). (E) DNase I footprinting analysis of VqsA binding to a binding site in the *luxR* promoter (shown in the dashed box). Electropherograms show a DNase I digestion of the P_{luxR} promoter probe after incubation with 0 or 3.6 μ M VqsA. The corresponding nucleotide sequences that were protected by VqsA are indicated below, with the binding motif highlighted in blue. The direction of transcription (as depicted with an arrow), the location of the partial -10 box and -35 box (underlined), and the VqsA-binding site relative to the start codon ATG are shown. (F) EMSA investigation of the binding of VqsA to the specific VqsA-binding site (VBS) in the *luxR* promoter. The P_{luxR} variant lacking the VBS sequence ($P_{luxR} \Delta VBS$) was also investigated.

EMSAs were then performed to investigate the binding of VqsA to the *aphA* promoter probe. The result showed that P_{aphA} had affinity for the purified VqsA protein in a concentration-dependent manner in the presence of excess (10-fold) poly(dI:dC) competitor and could be titrated out by a higher concentration of unlabeled P_{aphA} probe (Fig. 3C). The K_d value for the binding of VqsA to *aphA* was calculated to be 0.35 μ M (Fig. 3D). In addition, the DNase I footprinting assay with both strands of the P_{aphA}

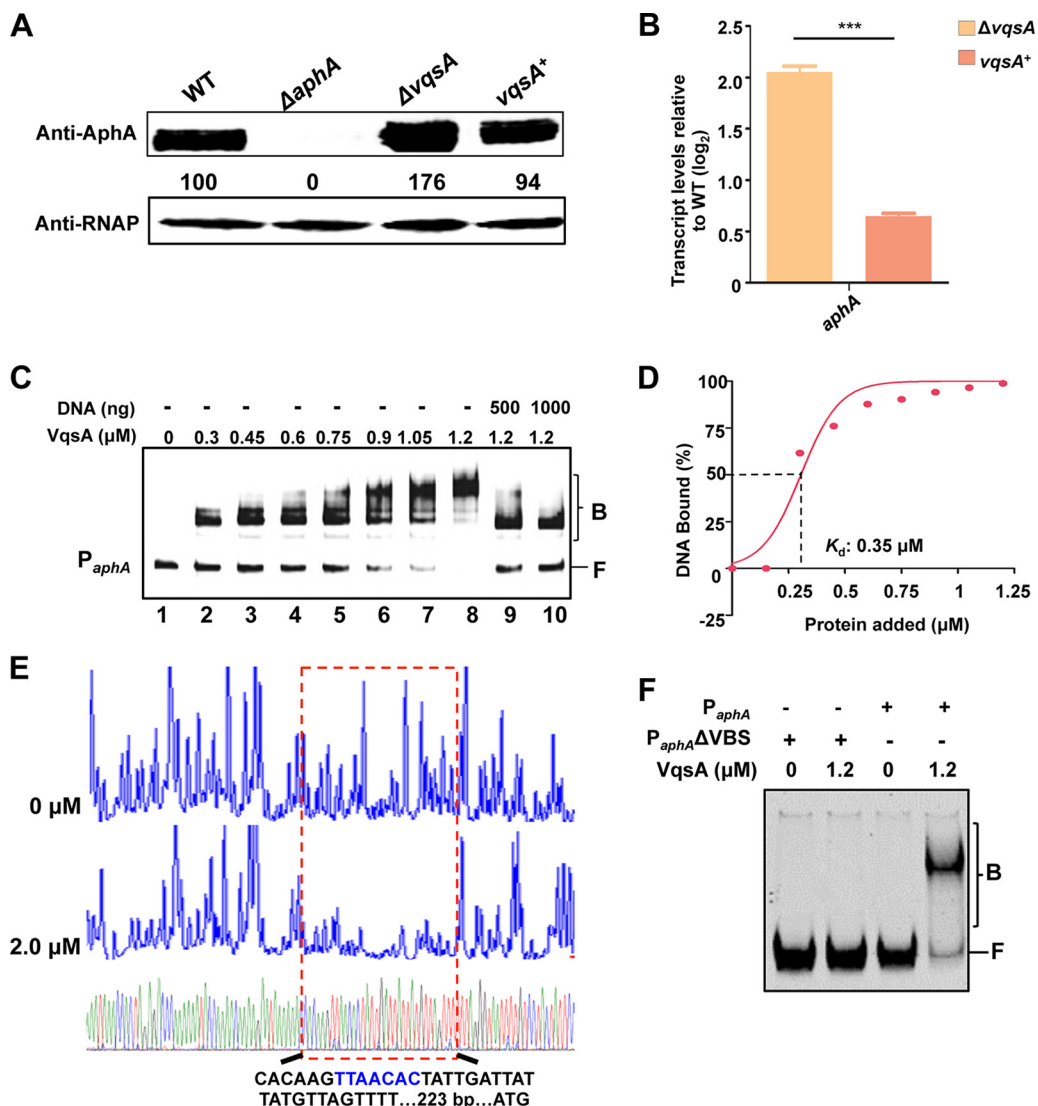


FIG 3 VqsA is involved in *aphA* expression by binding directly to the promoter of *aphA*. (A) Western blot analysis of AphA expression in WT, Δ vqsA mutant, and vqsA⁺ mutant cells cultured in LBS medium for 2 h. RNAP was used as a loading control. The numbers indicate the densitometric measurements. (B) qRT-PCR analysis of the *aphA* transcripts. The WT, Δ vqsA mutant, and vqsA⁺ mutant strains were cultured in LBS for 2 h, and mRNA transcripts were detected by qRT-PCR; 16S rRNA was selected as a control. The results are displayed as the mean \pm standard deviation (SD) ($n = 3$), ***, $P < 0.001$ based on Student's *t* test. (C) EMSA analysis of the binding of VqsA to P_{aphA} . The amount of VqsA protein used is indicated, and 20 ng of each Cy5-labeled probe was added to the EMSA reactions. The shifts were verified to be specific in experiments in which 25- to 50-fold excess of unlabeled specific DNA and nonspecific competitor DNA [poly(dI:dC)] were used. (D) Plot showing the affinity of the binding of VqsA to the *aphA* promoter. The densitometric intensities of bound DNA fragments were plotted against VqsA concentrations. (E) DNase I footprinting analysis of the binding of VqsA to a binding site in P_{aphA} . Electropherograms show the P_{aphA} promoter probe treated with DNase I after incubation with 0 or 2.0 μ M VqsA. (F) EMSA performed with purified VqsA and the *aphA* promoter DNA (P_{aphA}) or its variant lacking the specific VqsA-binding site ($P_{aphA} \Delta$ VBS). Dashed boxes indicate the binding site revealed by DNase I footprinting assays.

probe uncovered a specific VqsA-protected region, 5'-CACAAGTTAACTATTGATTAT TATGTTAGTTTT-3' (223 bp upstream of ATG), in the *aphA* promoter region (Fig. 3E). The absence of the specific binding site abolished the capacity of VqsA to bind to the *aphA* promoter (Fig. 3F). Taken together, these data along with the results of B1H assays (Fig. 1B) demonstrated that VqsA binds to the *aphA* promoter, negatively regulating the transcription of the *aphA* gene.

LuxR, AphA, and VqsA bind to the promoter region of vqsA in vitro. EMSAs and DNase I footprinting assays were carried out to test the binding of LuxR, AphA, and VqsA to the *vqsA* promoter region *in vitro* (Fig. 4). Both LuxR and AphA bound efficiently

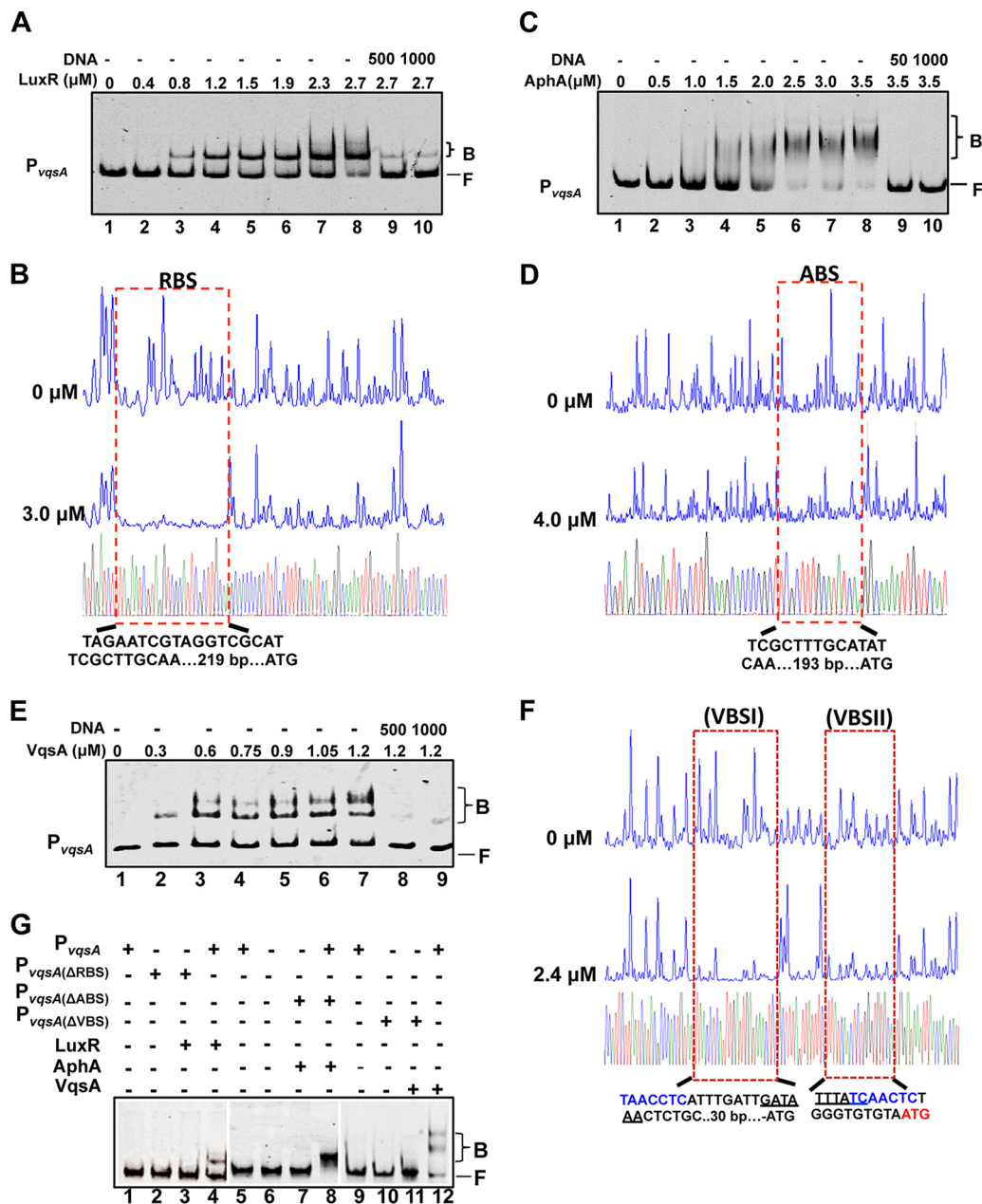


FIG 4 AphA, LuxR, and VqsA bind to P_{vqsA} . (A, C, and E) EMSAs were performed with purified LuxR (A), AphA (C), and VqsA (E). The amounts of the proteins used are indicated, and 20 ng of each Cy5-labeled P_{vqsA} probe was added to the EMSA reactions. The shifts were verified to be specific in experiments in which 25- to 50-fold excess of unlabeled specific DNA and nonspecific competitor DNA [poly(dI:dC)] were added. (B, D, and F) DNase I footprinting analysis of LuxR (B), AphA (D), or VqsA (F) binding to their distinct binding sites in P_{vqsA} . Electropherograms of a DNase I digestion of 400 ng of the P_{vqsA} promoter probe after incubation with 0 or 3.0 μ M LuxR, 0 or 4.0 μ M AphA, and 0 or 2.4 μ M VqsA are shown. The nucleotide sequences that were protected by LuxR, AphA, or VqsA are as indicated below. The specific palindromic sequences are underlined. Dashed boxes indicate the binding site revealed by DNase I footprinting assays. (G) EMSA investigation of the binding of LuxR, AphA, or VqsA to their specific binding sites, RBS, ABS, and VBS, in the *luxR* promoter region. The WT and the variant P_{vqsA} lacking the related binding sites were also investigated.

and specifically to a probe encompassing the entire P_{vqsA} in a concentration-dependent manner in the presence of high concentrations (10-fold excess of the probe DNA) of a nonspecific poly(dI:dC) competitor and could be titrated out by the unlabeled DNA probe (Fig. 4A and C). The K_d values for the binding of LuxR and AphA to the *vqsA* promoter were calculated to be 1.5 μ M and 1.74 μ M, respectively. The specific binding sites of LuxR (RBS) and AphA (ABS) on P_{vqsA} were further mapped with DNase I

footprinting assays (Fig. 4B and D). These data suggested that *vqsA* could also be regulated via feedback by QS through the combined activities of LuxR and AphA.

LTTRs usually exhibit autorepression (27). To investigate the autorepression of VqsA, an EMSA was first performed, and the results indicated that VqsA bound directly to the *vqsA* promoter at two sites (Fig. 4E) with a calculated K_d value of $0.73 \mu\text{M}$. These results indicated that VqsA has a much higher binding affinity toward the *vqsA* promoter than LuxR and AphA and thus might exhibit strong autorepression. A DNase I footprinting assay on both strands of P_{vqsA} confirmed that VqsA bound to the promoter at the 5'-TAACCTCATTGATTGATAAACTCTGC-3' (VBS1) site, -30 bp relative to the start codon (ATG), and the 5'-TTTATCAACTCTGGGTGTGCA-3' (VBSII) site, immediately upstream of the start codon (Fig. 4F). Moreover, the perfect palindromic sequence GA TAAA was identified in these two binding sites (Fig. 4F). When the respective LuxR, AphA, and VqsA-binding sites were deleted on P_{vqsA} , the ability of these regulators to bind to the promoter was abolished (Fig. 4G, lanes 3, 7, and 11). A VqsA-specific-binding motif (TAAACTC) was generated with the MEME suite algorithms (<http://meme-suite.org>) ($P < 10^{-12}$) by providing the sequences of the four binding sites in the promoter regions of *luxR*, *aphA*, and *vqsA* as input (Fig. 2E, 3E, and 4F). Taken together, these results showed that the *vqsA* promoter was bound to and could be regulated by LuxR, AphA, and VqsA.

Regulation of the activity of the *vqsA* promoter by LuxR, AphA, and VqsA. We then tested the roles of VqsA, LuxR, and AphA in the expression of *vqsA*. As an investigation of the overall effect of these proteins on *vqsA* expression, cells cultured for 9 h and approaching the stationary-growth phase (24) were used for P_{vqsA} -*luxAB* or qRT-PCR transcriptional assays. P_{vqsA} activity was significantly enhanced in the cells with the deletion of the *vqsA* gene and was restored to the wild-type (WT) levels in the *vqsA*⁺ complement strain after 9 h of incubation (Fig. 5A), indicating negative regulation of *vqsA* expression by VqsA, a typical autoregulatory feature of LTTRs (27). In qRT-PCR assays, upon measuring the transcript levels of *vqsA* in the WT, $\Delta luxR$ mutant, and $\Delta aphA$ mutant strains and in the complemented *luxR*⁺ and *aphA*⁺ strains, the transcript level of *vqsA* was significantly higher, by 5-fold, in the $\Delta luxR$ mutant than that in the WT, but lower by ~ 2 -fold in the $\Delta aphA$ mutant; the levels of the *vqsA* transcripts in the *luxR*⁺ and *aphA*⁺ complemented strains were restored to WT levels (Fig. 5B). We also performed an epistatic test of the functions of LuxR and AphA in the transcriptional control of *vqsA*. The $\Delta luxR \Delta aphA$ double mutant and the $\Delta luxR$ mutant strain overexpressing *aphA* showed levels of *vqsA* transcripts similar to those of the $\Delta luxR$ mutant strain, while the $\Delta aphA$ mutant strain overexpressing *luxR* exhibited a *vqsA* transcript level comparable to that of the *luxR*⁺ mutant strain in 9-h cultures (Fig. 5B), demonstrating that LuxR exerts dominant control over *vqsA* expression at the HCD stage. In addition, a 5'-RACE experiment was performed as previously described (28) using RNA extracted from the WT, $\Delta vqsA$ mutant, and $\Delta luxR$ mutant strains grown for 9 h in LBS. The deletion of *vqsA* or *luxR* resulted in a stronger sequencing signal than that observed for the WT after reverse transcriptional analysis (Fig. S3), which facilitated the identification of the transcriptional start site (TSS), a "G" at position -516 relative to the start codon ATG (Fig. 5C).

We further characterized the roles of the binding sites of AphA (ABS), LuxR (RBS), and VqsA (VBSI and VBSII) in the P_{vqsA} region on the expression of *vqsA* (Fig. 5C and D). As expected, P_{vqsA} activity was significantly attenuated in the mutant of ABS, P_{vqsA} MABS, indicating that this *cis*-acting element and AphA were essential for activation of the *vqsA* promoter. Moreover, the single nucleotide substitutions in the RBS P_{vqsA} MRBS resulted in increasing the activity of the *vqsA* promoter to a level higher than that observed for WT P_{vqsA} . Furthermore, the activity of the *vqsA* promoter with a mutated VBSI, P_{vqsA} MVBSI, and/or VBSII (P_{vqsA} MVBSII or P_{vqsA} MVBSI/II) was higher than that of the WT promoter. Collectively, these data confirmed that *vqsA* expression was repressed by LuxR and VqsA but activated by AphA, supporting the idea that *vqsA* expression is

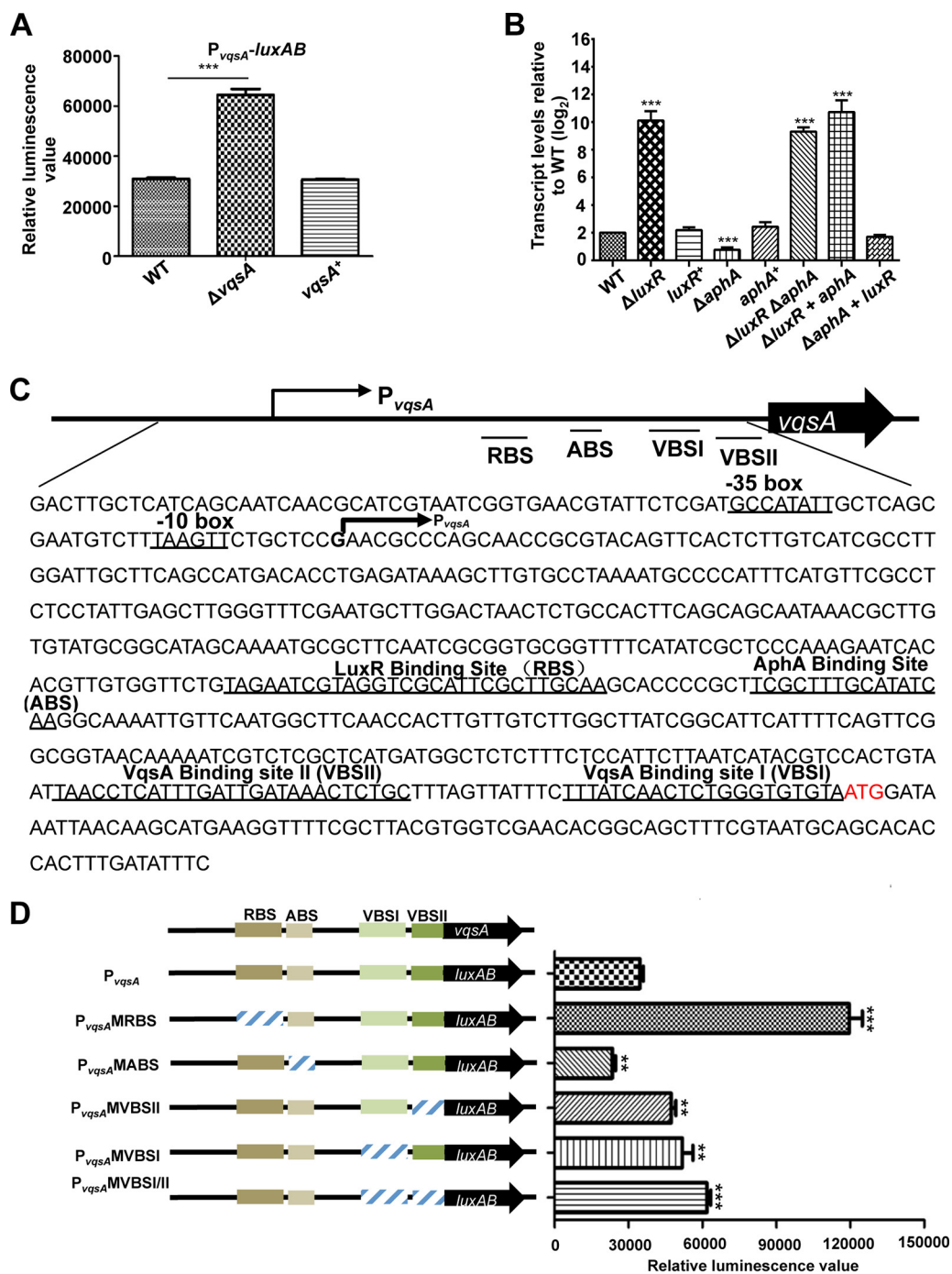


FIG 5 The expression of *vqsA* is regulated by AphA, LuxR, and VqsA at the transcriptional level. (A) Transcriptional analysis of P_{vqsA} -*luxAB* in the WT and $\Delta vqsA$ mutant strains cultured in LBS for 9 h. The results are shown as the mean \pm SD ($n = 3$). ***, $P < 0.001$ based on Student's t test. (B) qRT-PCR analysis of the *vqsA* transcripts in $\Delta luxR$, $\Delta aphA$, $luxR^+$, and $aphA^+$ mutants and in the $\Delta luxR \Delta aphA$ double mutant, as well as the related strains exhibiting epistatic expression ($\Delta luxR aphA$ mutant and $\Delta aphA luxR$ mutant) cultured in LBS for 9 h, and mRNA transcripts were detected by qRT-PCR; 16S rRNA was selected as a control. The results are displayed as the mean \pm SD ($n = 3$); **, $P < 0.01$; ***, $P < 0.001$, based on Student's t test relative to the WT results. (C) The nucleotide sequence of P_{vqsA} . The LuxR-binding site (RBS), AphA-binding site (ABS), VqsA-binding site I (VBSI), and VqsA-binding site II (VBSII) and the -10 box and -35 box are underlined. The P_{vqsA} transcription start site as determined by 5'-RACE is labeled with an arrow, and the translational start codon (ATG) is in red. (D) The promoter activities of P_{vqsA} and its variants fused to *luxAB*. Fluorescence levels were assayed for each of the strains. The data are shown as the mean \pm standard error of the mean (SEM) ($n = 3$). **, $P < 0.01$; ***, $P < 0.001$, based on Student's t test relative to the WT P_{vqsA} promoter.

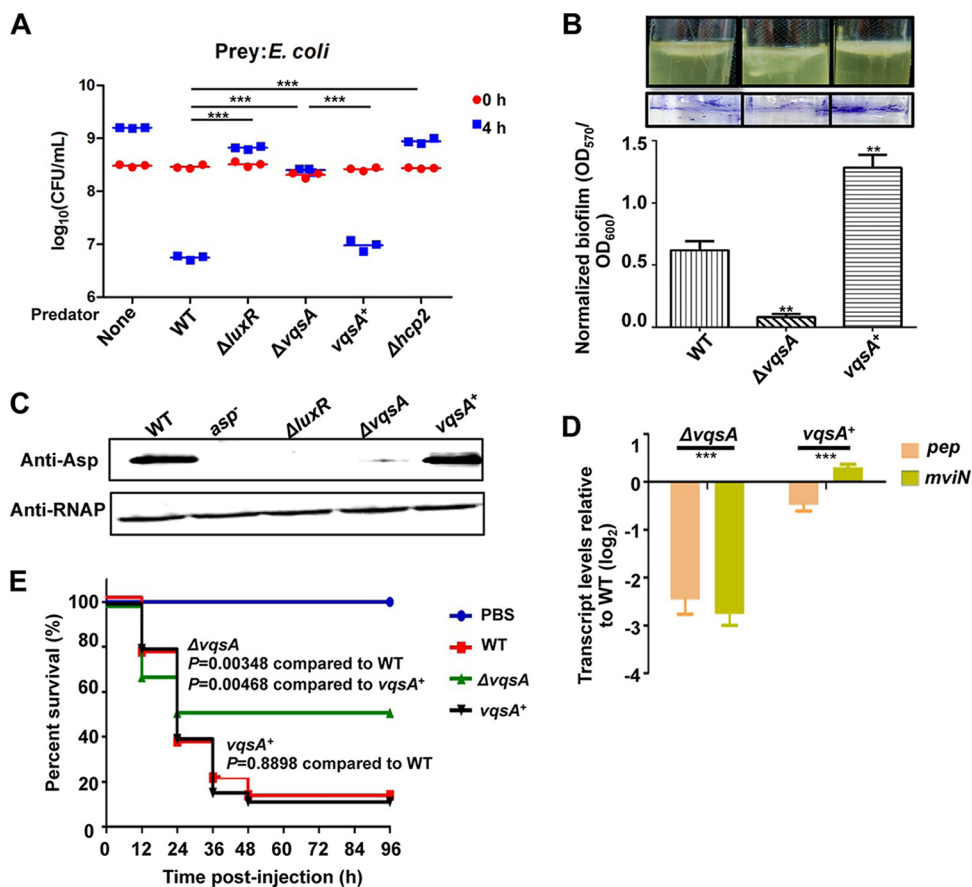


FIG 6 Essential roles of VqsA in interbacterial competition activity, biofilm formation, exotoxin production, and virulence. (A) CFU of *E. coli* before ($t = 0$) and after ($t = 4$) a 4-h coculture with the indicated *V. alginolyticus* predator strains or *E. coli* alone. The $\Delta hcp2$ mutant strain deficient in interbacterial competition capacity was used as a control. The data represent the results from three independent experiments. ***, $P < 0.001$ by an unpaired two-tailed t test. (B) Biofilm formation in glass tubes after 48 h was quantitatively assayed with crystal violet staining of the biofilm cells. For the colorimetric assay of the released crystal violet from the biofilm cells, the OD_{570} was measured and normalized with the OD_{600} of the cultures. **, $P < 0.01$ based on Student's t test relative to the WT strain. (C) Western blot analysis was performed to assay Asp yields in the $\Delta vqsA$ and $vqsA^+$ mutant strains. RNAP was used as the loading control. (D) qRT-PCR analysis of the transcriptional levels of the *pep* and *mviN* genes. The WT, $\Delta vqsA$ mutant, and $vqsA^+$ mutant strains were cultured in LBS for 9 h, and the mRNA transcripts were detected by qRT-PCR; 16S rRNA was selected as a control. ***, $P < 0.001$, Student's t test relative to the $vqsA^+$ mutant strain. (E) Groups of 30 fish were infected with the indicated strains. The number of fish that survived was recorded for 4 days. The P values were calculated using a Kaplan-Meier survival analysis with a log rank test with Prism software (version 6.01).

subject to a sophisticated regulation network in which AphA, LuxR, and VqsA are participants.

Essential roles of VqsA in bacterial killing capacity, biofilm formation, exotoxin production, and *in vivo* virulence toward fish. Previous investigations had demonstrated that QS regulates the bacterial killing via type VI secretion system 2 (T6SS2) in *V. alginolyticus* (29, 30). We tested whether VqsA is involved in bacterial killing capacity, a QS-controlled phenotype. We found that the WT strain killed *Escherichia coli* MC4100 in coculture and reduced the starting CFU of *E. coli* $\sim 100\times$ when the strains were cocultured at 30°C for 4 h on LBS agar plates. Hcp2 is a structural component of T6SS2 that is essential for bacterial killing activity, and the ability to kill bacteria was abolished in the $\Delta hcp2$ mutant strain (30). Compared to the $\Delta luxR$ and $\Delta hcp2$ mutant strains, which are deficient in interbacterial competition activity (30), the $\Delta vqsA$ mutant also exhibited markedly decreased killing of *E. coli*, and the complement $vqsA^+$ strain restored the bacterial killing capacities (Fig. 6A). Furthermore, biofilm formation was significantly decreased in the $\Delta vqsA$ mutant strain in comparison to the WT strain and

the complement strain (Fig. 6B). Western blotting confirmed the essential role of *vqsA* in the positive regulation of exotoxin Asp production in *V. alginolyticus* (Fig. 1D, 1E, and 6C). Similarly, the transcription of MviN and Pep, two additional exotoxins that are essential for pathogenesis in *V. alginolyticus* (17, 18), was also observed to be positively regulated by VqsA (Fig. 6D). These data demonstrate that VqsA is involved in QS pathways to regulate a plethora of group behaviors.

To further evaluate the influence of VqsA on the virulence of *V. alginolyticus*, adult zebrafish ($n = 30$) were challenged via intramuscular (i.m.) injection with 5×10^6 CFU/fish of the WT, $\Delta vqsA$ mutant, or *vqsA*⁺ mutant strain, as previously described (23). The fish mortalities caused by the $\Delta vqsA$ mutant challenge were much lower than those observed with the WT and complementation strains. The survival rates of the fish challenged with the $\Delta vqsA$ mutant (~45%) were significantly higher than those of fish challenged with the WT (~17%) or *vqsA*⁺ mutant (~16%) strains (Fig. 6E). The significant virulence attenuation resulting from *vqsA* abrogation suggested that VqsA is involved in the *in vivo* virulence of *V. alginolyticus*. Taken together, these data indicate that VqsA plays essential roles in coordinating QS and virulence expression in *V. alginolyticus*.

DISCUSSION

In vibrios, QS is essential for the regulation of virulence gene expression, and the identification of QS regulators will facilitate the development of QSI strategies. With this goal in mind, here, we performed a comprehensive screen of QS regulators and identified multiple novel QS regulators involved in the regulation of the MQSRs LuxR and/or AphA in the marine pathogen *V. alginolyticus*, uncovering the complicated regulation network of QS and pathogenesis in the bacterium. In addition, a large portion of the QS regulators, including the LTTR family proteins (Tables 1 and S1), contain ligand-binding domains, which suggest their roles in the sensing of various physiological or biochemical signals to modulate QS output. The LTTR protein MetR has been shown to be involved in QS regulation in response to methionine metabolism in *V. harveyi* (31). AphB, a low-pH- and oxygen-responsive LTTR protein (32, 33), regulates QS by directly binding to the *luxR* promoter and controlling LuxR expression at the transcriptional level in *V. alginolyticus* (25). Moreover, identification of the oxidative-stress-responsive SoxR (34) and the feast/famine regulatory protein AsnC (35) as being involved in QS regulation also suggested that various physiological signals might affect QS regulation.

As QS directly or indirectly regulates ~600 (approximately 15%) genes in vibrios (4, 36, 37), it is reasonable to suggest that other unrelated regulatory pathways could be integrated into the preexisting QS regulatory circuits to modify gene expression in response to environmental cues. Our EMSA, DNase I footprinting assay, and transcriptional assay indicated that the LTTR family protein VqsA is involved in QS control (Fig. 1 and 6) via regulation of the expression of the MQSRs AphA and LuxR. We are greatly interested in the signals to which VqsA might respond to regulate QS. We modeled the structure of VqsA and found that the ligand-binding domain of VqsA is structurally related to AphB and CrgA. AphB has been reported to form a tetramer (38), while CrgA family proteins form octameric rings for DNA binding in response to specific stimuli to regulate gene expression (39). The molecular model indicated that the ligand-binding domain of VqsA also contains Cys residues Cys189 and Cys166 (Fig. S4), which might mediate intra-/intermolecular interactions in a manner similar to the thiol-switch Cys235 in AphB to sense oxidative stresses and ultimately regulate QS gene expression (38, 40).

Our transcriptional assays indicated that VqsA, similar to other LTTRs, acts as both an autorepressor and activator/repressor of target promoters (Fig. 2, 3, and 5) (27). Our analysis uncovered two specific binding sites (VBS1 and VBSII) upstream of the translational start site of P_{vqsA} . In addition, the palindromic repeat GATAAA identified in these two flanking VqsA-binding sites of P_{vqsA} might facilitate the formation of a hairpin structure to repress autotranscription (Fig. 4F). Although the expression of AphA was

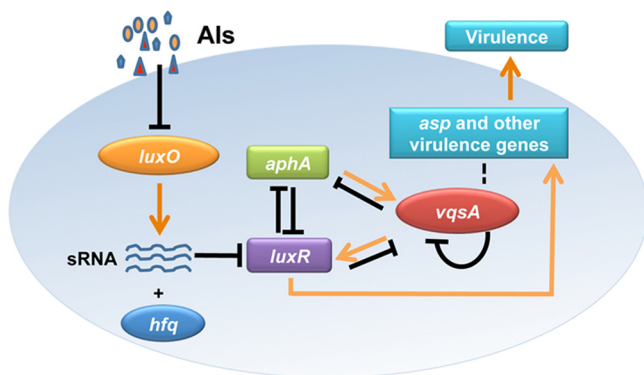


FIG 7 Schematic summarization of the regulatory roles of VqsA in QS circuits and virulence gene expression in *V. alginolyticus*. The pathways are illustrated with arrows (activation) or with bar-ended lines (repression) and are discussed in the text. The putative pathway indicating that VqsA might directly regulate the expression of some virulence-associated genes is depicted as dashed lines. sRNA, small RNA.

repressed by VqsA (Fig. 3A and B), our data did not disclose an additional VqsA-binding site in proximity to the binding site identified in the promoter region of *aphA* (Fig. 3E). We speculate that VqsA might interact with other proteins to collectively repress the expression of *aphA*. Unlike CrgA or other LTTRs that bind to a 50- to 60-bp stretch of DNA (27), our DNase I footprinting assays revealed a region of 21 to 34 bp protected by VqsA, including a previously unreported conserved 7-bp consensus sequence, TAAACTC, in both the activated and repressed promoter regions (Fig. 2E, 3E, and 4F). Moreover, VqsA appears to facilitate *luxR* transcription by binding to the template strand of the *luxR* promoter at nucleotides (nt) -40 to -9 relative to the transcription start site (Fig. 2) (6), a region usually essential for RNA polymerase binding and for the formation of an open promoter complex to initiate transcription. It is unusual to observe a factor binding to the -10 element to facilitate gene transcription in Gram-negative bacteria, although in mycobacteria, the transcription factors CarD and RbpA are essential for formation of the transcriptionally competent open complex by directly interacting with RNAP and the -10 region to activate transcription (41). Taking these analyses together, our data indicate that VqsA might adopt special DNA-binding mechanisms to regulate gene expression. It is important to note that all these binding analyses were performed *in vitro*. Chromatin immunoprecipitation sequencing (ChIP-seq) and structural analysis could further dissect the mechanisms via which VqsA regulates QS.

We were intrigued by the role of VqsA in the regulation of QS output. Our data indicated that similar to the MQSRs LuxR and Apha (6, 24), VqsA regulates the expression of LuxR and Apha (Fig. 2 and 3), and *vqsA* expression is also subject to tight regulation by LuxR, Apha, and VqsA (Fig. 4 and 5). Specifically, LuxR and VqsA repress *vqsA* expression, while Apha promotes the transcription of *vqsA*. Epistasis analysis of LuxR and Apha further revealed a dominant role for LuxR in the regulation of *vqsA* at HCD stages (Fig. 5B). All these analyses support a model in which VqsA is the third MQSR in *V. alginolyticus* (Fig. 7). As *V. harveyi* Apha and LuxR regulate the expression of a large number of genes (37), VqsA might also regulate the expression of various genes directly or indirectly via Apha or LuxR and may be associated with the QS regulatory network to control various virulence-associated phenotypes (Fig. 6 and 7). Future work should focus on (i) the individual and combined roles of these three MQSRs in controlling QS regulons in different vibrios, and (ii) the mutual and reciprocal control of the expression of these MQSRs to control QS in growing cells and in different growth stages, as well as under various environmental conditions. These studies shall enrich our knowledge of the architecture of QS systems and their regulation.

In summary, here, we identified that the LysR family regulator VqsA regulates QS by binding to the promoters of *aphA* and *luxR* to negatively and positively regulate the

expression of these genes, respectively. The expression of *vqsA* is autorepressed and feedback controlled by AphA and LuxR. VqsA also regulates the expression of exotoxins and other QS-controlled virulence factors and plays essential roles in survival in zebrafish. The modified B1H system proved to be a powerful tool to uncover new regulators in QS signaling cascades and led to the discovery of VqsA as an important player in QS regulation and pathogenesis. VqsA may be the third master QS regulator (MQSR) in vibrios to coordinate QS-mediated responses (Fig. 7). The discovery of VqsA along with various as-yet-uncharacterized regulators involved in QS circuits provided a genetic framework to dissect the complex regulatory network of QS and thereby facilitate the development of QSI strategies to effectively control pathogens that plague the aquaculture industry.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids that were used in this study are listed in Table 2. The *V. alginolyticus* wild-type (WT) and derivative strains were grown at 30°C in Luria-Bertani (LB) medium supplemented with 3% NaCl (LBS). *Escherichia coli* strains were grown at 37°C in LB medium. When appropriate, the medium was supplemented with carbenicillin (Carb; 100 µg/ml), chloramphenicol (Cm; 25 µg/ml), kanamycin (Km; 100 µg/ml), or L-arabinose (0.04% [wt/vol]).

Construction of the deletion mutants, complement strains, and the protein expression strains. In-frame deletion mutant strains and complement strains were constructed as previously described using specific primers (Table 3) and isothermal assembly (6, 42). Briefly, overlap PCR was used to generate DNA fragments for the in-frame deletion. These fragments were then cloned into the XbaI-digested R6K-based suicide vector pDM4 by isothermal assembly and transformed into *E. coli* DH5α *λpir* cells, as previously described (6). After being sequenced, the resulting plasmids were transformed into *E. coli* SM10 *λpir* and then mated into EPGS by conjugation. Double-crossover transformants were selected sequentially on LBS medium containing Carb and Cm and then on LBS with 12% (wt/vol) sucrose. Generation of the targeted mutants was confirmed by PCR using a primer pair targeting the upstream and downstream regions of the designed deletion region (Table 3) to sequence the region. To construct the complement strains, the genes spanning the predicted promoter region and open reading frame (ORF) were cloned into the modified pBAD33 plasmid harboring the *mob* gene (pBAD33-*mob*) and a FLAG tag downstream of the cloned gene (24). The derivative plasmid was conjugated into the related mutant strains.

The pET28a derivative plasmids used for VqsA, AsnC, SoxR, MetR, 02325, 01665, 06711, AphA, and LuxR expression were generated as previously described (6). Briefly, the ORF sequences were amplified by PCR using the primers shown in Table 3. Each ORF sequence was inserted into pET28a with a His₆ tag at the carboxyl terminal. Then, the recombinant plasmid was transformed into *E. coli* BL21(DE3). Successful generation of the plasmids was confirmed by PCR using primer pairs (Table 3) to sequence the recombinant region. The pDM8-P_{*luxR*}-*luxAB* constructs used for the promoter activity assays of *vqsA* and *aphA* with pDM8 and *luxAB* were generated as previously described (24).

Cloning of *V. alginolyticus* transcription factors and regulatory sequences of the target genes for bacterial one-hybrid assays. The genes of 285 probable transcription factors were amplified from *V. alginolyticus* EPGS by using gene-specific primers (a subset of the primers are shown in Table 3) and cloned into the pTRG plasmid. The promoters of the *V. alginolyticus* *aphA* and *luxR* genes were amplified using primers specific to these promoters (Table 3) and inserted into the reporter vector pBXcmT, as previously described (25, 26). The *E. coli* XL1-Blue MRF9 kanamycin-resistant (Km^r) strain was used for propagating all pBXcmT and pTRG recombinant plasmids. The cotransformants with pairs of pBXcmT/pTRG plasmids in the reporter strain were tested on LB medium containing 34 mg/ml Cm, 50 mg/ml Km, and 15 mg/ml tetracycline (Tet). Positive cotransformants were screened on plates containing 16 mg/ml streptomycin (Str) and 20 mM 3-amino-1,2,4-triazole (3-AT). The plates were incubated at 30°C for ~3 to 4 days.

ECP activity assay and immunoblots of Asp, LuxR, and AphA. Extracellular protease (ECP) activity was assayed by using hide powder azure (HPA) digestion, as previously described (43). In brief, the strains were grown in LBS at 30°C for 9 h. The cell density at 600 nm (OD₆₀₀) was measured. The cultures were centrifuged at 8,000 × *g*, and the supernatants were filtered through 0.22-µm-pore-size filters. Then, 10 mg of HPA and 1 ml of phosphate-buffered saline (PBS [pH 7.2]) were added to 1 ml of the filtered supernatants. The mixture was incubated with shaking at 37°C for 2 h, followed by the addition of 10% trichloroacetic acid into the mixture to stop the reaction. Finally, the total protease activity was measured at 600 nm. ECP activity was normalized for each strain by dividing the total activity by the OD₆₀₀.

For the Asp, AphA, and LuxR immunoblotting assays, bacterial cell pellets at low cell density (2 h) or high cell density (9 h) were suspended in 1 volume of PBS to normalize the culture densities based on OD₆₀₀ values. The ECP from equal cell numbers was mixed with phenylmethylsulfonyl fluoride (PMSF) at a ratio of 1:100 on ice for 30 min, and the mixtures were ultrafiltered through a Millipore tube with a 5-kDa molecular weight cutoff. The concentrated ECP was mixed with the sample-loading buffer and processed as described previously (43). In addition, the samples were heated with 20% SDS-PAGE loading buffer. Then, 15 µl of each normalized sample was loaded onto a 12% denaturing polyacrylamide gel. The proteins were resolved using electrophoresis and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in a 12% solution of skim milk powder, incubated with a 1:3,000 dilution of Asp-, LuxR- or AphA-specific rabbit antibodies, and then incubated with a 1:2,000

TABLE 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Reference or source
Strains		
<i>E. coli</i>		
DH5α <i>λpir</i>	Host for π-requiring plasmids	Lab collection
SM10 <i>λpir</i>	Host for π-requiring plasmids, conjugal donor	Lab collection
BL21(DE3)	Host strain for protein expression	Novagen
XL1-Blue MRF9	B1H reporter; host strain for propagating pBXcmT and pTRG recombinants, Km ^r	Agilent
MC4100	Prey bacterium used for interbacterial competition assay	Lab collection
<i>V. alginolyticus</i>		
EPGS	Wild type, isolated from aquiculture farm of the South China Sea (CCTCC AB 209306), Amp ^r	Lab collection
<i>asp</i> mutant	EPGS Δ <i>asp</i> , Cm ^r	16
Δ <i>luxR</i> mutant	EPGS Δ <i>luxR</i>	16
Δ <i>aphA</i> mutant	EPGS Δ <i>aphA</i>	24
Δ <i>luxO</i> mutant	EPGS Δ <i>luxO</i>	20
<i>luxR</i> ⁺ strain	Δ <i>luxR</i> mutant/pMMB- <i>luxR</i> , Cm ^r	18
<i>aphA</i> ⁺ strain	Δ <i>aphA</i> mutant/pBAD33- <i>aphA-flag-1</i> , Cm ^r	24
Δ <i>asnC</i> mutant	EPGS Δ <i>asnC</i>	This study
Δ02325 mutant	EPGS Δ02325	This study
Δ <i>soxR</i> mutant	EPGS Δ <i>soxR</i>	This study
Δ <i>metR</i> mutant	EPGS Δ <i>metR</i>	This study
Δ01665 mutant	EPGS Δ01665	This study
Δ06711 mutant	EPGS Δ06711	This study
Δ <i>vqsA</i> mutant	EPGS Δ18672 (<i>vqsA</i>)	This study
Δ <i>vqsA</i> Δ <i>luxO</i> mutant	EPGS Δ <i>luxO</i> Δ18672	This study
Δ <i>luxR</i> Δ <i>aphA</i> mutant	EPGS Δ <i>luxR</i> Δ <i>aphA</i>	This study
BL21/pET28a- <i>aphA</i>	BL21/pET28a- <i>aphA</i> , Km ^r	6
BL21/pET28a- <i>luxR</i>	BL21/pET28a- <i>luxR</i> , Km ^r	6
XL1-Blue MRF9/pTRG::LuxR+pBXcmT::P _{luxR}	XL1-Blue MRF9 carrying pTRG-LuxR+pBXcmT-P _{luxR}	This study
XL1-Blue MRF9/pTRG::LuxR+pBXcmT::P _{luxR} ΔRBSI/II	XL1-Blue MRF9 carrying pTRG-LuxR+pBXcmT-P _{luxR} ΔRBSI/II	This study
XL1-Blue MRF9/pTRG::AsnC+pBXcmT::P _{luxR}	XL1-Blue MRF9 carrying pTRG-AsnC+pBXcmT-P _{luxR}	This study
XL1-Blue MRF9/pTRG::02325+pBXcmT::P _{luxR}	XL1-Blue MRF9 carrying pTRG-02325+pBXcmT-P _{luxR}	This study
XL1-Blue MRF9/pTRG::SoxR+pBXcmT::P _{luxR}	XL1-Blue MRF9 carrying pTRG-SoxR+pBXcmT-P _{luxR}	This study
XL1-Blue MRF9/pTRG::MetR+pBXcmT::P _{luxR}	XL1-Blue MRF9 carrying pTRG-MetR+pBXcmT-P _{luxR}	This study
XL1-Blue MRF9/pTRG::01665+pBXcmT::P _{luxR}	XL1-Blue MRF9 carrying pTRG-01665+pBXcmT-P _{luxR}	This study
XL1-Blue MRF9/pTRG::06711+pBXcmT::P _{luxR}	XL1-Blue MRF9 carrying pTRG-06711+pBXcmT-P _{luxR}	This study
XL1-Blue MRF9/pTRG::18672+pBXcmT::P _{luxR}	XL1-Blue MRF9 carrying pTRG-18672+pBXcmT-P _{luxR}	This study
XL1-Blue MRF9/pTRG::AsnC+pBXcmT::P _{aphA}	XL1-Blue MRF9 carrying pTRG-AsnC+pBXcmT-P _{aphA}	This study
XL1-Blue MRF9/pTRG::02325+pBXcmT::P _{aphA}	XL1-Blue MRF9 carrying pTRG-02325+pBXcmT-P _{aphA}	This study
XL1-Blue MRF9/pTRG::SoxR+pBXcmT::P _{aphA}	XL1-Blue MRF9 carrying pTRG-SoxR+pBXcmT-P _{aphA}	This study
XL1-Blue MRF9/pTRG::MetR+pBXcmT::P _{aphA}	XL1-Blue MRF9 carrying pTRG-MetR+pBXcmT-P _{aphA}	This study
XL1-Blue MRF9/pTRG::01665+pBXcmT::P _{aphA}	XL1-Blue MRF9 carrying pTRG-01665+pBXcmT-P _{aphA}	This study
XL1-Blue MRF9/pTRG::18672+pBXcmT::P _{aphA}	XL1-Blue MRF9 carrying pTRG-18672+pBXcmT-P _{aphA}	This study
<i>vqsA</i> ⁺ strain	Δ18672 mutant carrying pBAD33-18672, Cm ^r	This study
Δ <i>aphA luxR</i> mutant	Δ <i>aphA</i> mutant carrying pMMB- <i>luxR</i>	This study
Δ <i>luxR aphA</i> mutant	Δ <i>luxR</i> mutant carrying pBAD33:: <i>aphA-flag-1</i>	This study
EPGS/pDM8-P _{luxR} - <i>luxAB</i>	EPGS/pDM8-P _{luxR} - <i>luxAB</i> , Cm ^r	This study
EPGS Δ18672/pDM8-P _{luxR} - <i>luxAB</i>	Δ18672 mutant/pDM8-P _{luxR} - <i>luxAB</i> , Cm ^r	This study
EPGS/pDM8-P _{aphA} - <i>luxAB</i>	EPGS/pDM8-P _{aphA} - <i>luxAB</i> , Cm ^r	This study
EPGS Δ18672/pDM8-P _{aphA} - <i>luxAB</i>	Δ18672 mutant/pDM8-P _{aphA} - <i>luxAB</i> , Cm ^r	This study
EPGS/pDM8-P _{asp} - <i>luxAB</i>	EPGS/pDM8-P _{asp} - <i>luxAB</i> , Cm ^r	This study
EPGS Δ18672/pDM8-P _{asp} - <i>luxAB</i>	Δ18672 mutant/pDM8-P _{asp} - <i>luxAB</i> , Cm ^r	This study
EPGS/pDM8-P _{vqsA} - <i>luxAB</i>	EPGS/pDM8-P _{vqsA} - <i>luxAB</i> , Cm ^r	This study
EPGS Δ18672/pDM8-P _{vqsA} - <i>luxAB</i>	Δ18672 mutant/pDM8-P _{vqsA} - <i>luxAB</i> , Cm ^r	This study
EPGS Δ <i>luxR</i> /pDM8-P _{vqsA} - <i>luxAB</i>	Δ <i>luxR</i> mutant/pDM8-P _{vqsA} - <i>luxAB</i> , Cm ^r	This study
EPGS Δ <i>aphA</i> /pDM8-P _{vqsA} - <i>luxAB</i>	Δ <i>aphA</i> mutant/pDM8-P _{vqsA} - <i>luxAB</i> , Cm ^r	This study
EPGS/pDM8-P _{vqsA} MRBS- <i>luxAB</i>	EPGS/pDM8-P _{vqsA} MRBS- <i>luxAB</i> , Cm ^r	This study
EPGS/pDM8-P _{vqsA} MABS- <i>luxAB</i>	EPGS/pDM8-P _{vqsA} MABS- <i>luxAB</i> , Cm ^r	This study
EPGS/pDM8-P _{vqsA} MVBSI- <i>luxAB</i>	EPGS/pDM8-P _{vqsA} MVBSI- <i>luxAB</i> , Cm ^r	This study
EPGS/pDM8-P _{vqsA} MVBSII- <i>luxAB</i>	EPGS/pDM8-P _{vqsA} MVBSII- <i>luxAB</i> , Cm ^r	This study
EPGS/pDM8-P _{vqsA} MVBSI/II- <i>luxAB</i>	EPGS/pDM8-P _{vqsA} MVBSI/II- <i>luxAB</i> , Cm ^r	This study
BL21/pET28a- <i>asnC</i>	BL21/pET28a- <i>asnC</i> , Km ^r	This study
BL21/pET28a- <i>metR</i>	BL21/pET28a- <i>metR</i> , Km ^r	This study
BL21/pET28a- <i>soxR</i>	BL21/pET28a- <i>soxR</i> , Km ^r	This study
BL21/pET28a- <i>vqsA</i>	BL21/pET28a- <i>vqsA</i> , Km ^r	This study

(Continued on next page)

TABLE 2 (Continued)

Strain or plasmid	Relevant characteristics ^a	Reference or source
Plasmids		
pDM4	Suicide vector, <i>pir</i> dependent, R6K, SacBR, Cm ^r	47
pBAD33-mob	pBAD33, <i>mob</i> , Cm ^r	24
pDM8	pSup202 derivative containing promoterless <i>lacZ</i> , Cm ^r	47
pET28a- <i>aphA</i>	pET28a carrying <i>aphA</i> ORF, Km ^r	6
pET28a- <i>luxR</i>	pET28a carrying <i>luxR</i> ORF, Km ^r	6
pDM8-P _{<i>luxR</i>} - <i>luxAB</i>	pDM8 with promoter region of <i>luxR</i> and <i>luxAB</i> , Cm ^r	6
pDM8-P _{<i>aphA</i>} - <i>luxAB</i>	pDM8 with promoter region of <i>aphA</i> and <i>luxAB</i> , Cm ^r	24
pMMB- <i>luxR</i>	pMMB206 derivative containing <i>luxR</i> _{val} from bp -782 to 1408, Cm ^r	18
pBAD33:: <i>aphA-flag-1</i>	pBAD33 derivative with P _{<i>aphA</i>} -driven expression of AphA-Flag fusion protein, Cm ^r	24
pBXcmT-P _{<i>aphA</i>}	pBXcmT with promoter region of <i>aphA</i> , Cm ^r	This study
pBXcmT-P _{<i>luxR</i>}	pBXcmT with promoter region of <i>luxR</i> , Cm ^r	This study
pBXcmT-P _{<i>luxR</i>} ΔRBSI/II	pBXcmT with <i>luxR</i> promoter deleted LuxR binding box I/II, Cm ^r	This study
pTRG-LuxR	pTRG with <i>luxR</i> ORF, Tet ^r	This study
pTRG-AsnC	pTRG with <i>asnC</i> ORF, Tet ^r	This study
pTRG-MetR	pTRG with <i>metR</i> ORF, Tet ^r	This study
pTRG-SoxR	pTRG with <i>soxR</i> ORF, Tet ^r	This study
pTRG-02325	pTRG with 02325 ORF, Tet ^r	This study
pTRG-01665	pTRG with 01665 ORF, Tet ^r	This study
pTRG-06711	pTRG with 06711 ORF, Tet ^r	This study
pTRG-18672	pTRG with 18672 ORF, Tet ^r	This study
pDM4-18672	pDM4 with 18672 fragment deleted from nt 4 to 876, Cm ^r	This study
pDM4-02325	pDM4 with 02325 fragment deleted from nt 4 to 1146, Cm ^r	This study
pDM4- <i>asnC</i>	pDM4 with <i>asnC</i> fragment deleted from nt 4 to 462, Cm ^r	This study
pDM4- <i>metR</i>	pDM4 with <i>metR</i> fragment deleted from nt 4 to 876, Cm ^r	This study
pDM4- <i>soxR</i>	pDM4 with <i>soxR</i> fragment deleted from nt 4 to 909, Cm ^r	This study
pDM4-06711	pDM4 with 06711 fragment deleted from nt 4 to 876, Cm ^r	This study
pDM4-01665	pDM4 with 18672 fragment deleted from nt 4 to 411, Cm ^r	This study
pBAD33-18672	18672-expressing plasmid driven by native promoter	This study
pET28a-18672	pET28a carrying 18672 ORF, Km ^r	This study
pET28a- <i>asnC</i>	pET28a carrying <i>asnC</i> ORF, Km ^r	This study
pET28a- <i>metR</i>	pET28a carrying <i>metR</i> ORF, Km ^r	This study
pET28a- <i>soxR</i>	pET28a carrying <i>soxR</i> ORF, Km ^r	This study
pET28a-02325	pET28a carrying 02325 ORF, Km ^r	This study
pET28a-01665	pET28a carrying 01665 ORF, Km ^r	This study
pET28a-06711	pET28a carrying 06711 ORF, Km ^r	This study
pDM8-P _{<i>asp</i>} - <i>luxAB</i>	pDM8 with promoter region of <i>asp</i> and <i>luxAB</i> , Cm ^r	This study
pDM8-P _{<i>vqsA</i>} - <i>luxAB</i>	pDM8 with promoter region of <i>vqsA</i> and <i>luxAB</i> , Cm ^r	This study
pDM8-P _{<i>vqsA</i>} MRBS- <i>luxAB</i>	pDM8 with mutated LuxR box of <i>vqsA</i> promoter and <i>luxAB</i> , Cm ^r	This study
pDM8-P _{<i>vqsA</i>} MABS- <i>luxAB</i>	pDM8 with mutated AphA box of <i>vqsA</i> promoter and <i>luxAB</i> , Cm ^r	This study
pDM8-P _{<i>vqsA</i>} MVBSI- <i>luxAB</i>	pDM8 with mutated VqsA box I of <i>vqsA</i> promoter and <i>luxAB</i> , Cm ^r	This study
pDM8-P _{<i>vqsA</i>} MVBSII- <i>luxAB</i>	pDM8 with mutated VqsA box II of <i>vqsA</i> promoter and <i>luxAB</i> , Cm ^r	This study
pDM8-P _{<i>vqsA</i>} MVBSI/II- <i>luxAB</i>	pDM8 with mutated VqsA box I/II of <i>vqsA</i> promoter and <i>luxAB</i> , Cm ^r	This study

^aKm^r, kanamycin resistance; Amp^r, ampicillin resistance; Cm^r, chloramphenicol resistance.

dilution of the horseradish peroxidase (HRP)-conjugated secondary antibody. RNAP was used for the loading controls, as previously described. Then, the blots were visualized with the enhanced chemiluminescence (ECL) reagent in a Western blotting kit provided by Thermo Fisher Scientific, Shanghai, China.

qRT-PCR assays. Bacteria were cultured overnight and then diluted 1:100 in LBS. The cells were then grown at 30°C and harvested at the indicated time points. An RNA extraction kit (Tiangen, Beijing, China) was used to isolate the total RNA. In addition, the RNA samples were digested with DNase I to degrade DNA contaminants. One microgram of RNA was used to generate cDNA using random primers (TaKaRa, Dalian, China). In addition, the cDNA was amplified with specific primer pairs with FastStart universal SYBR green master (Tiangen) on a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The specific primers used are shown in Table 3. Three independent qRT-PCR experiments were performed (each in triplicate), and the transcript levels were normalized to 16S rRNA in each sample by the $\Delta\Delta C_T$ method (44).

Bioluminescence assays. Bioluminescence assays were performed at least three times, as previously described (24). Briefly, the strains were cultured overnight, diluted to 6×10^6 CFU/ml in LBS, and then incubated with shaking at 30°C. Every hour for 9 h, a 200- μ l aliquot was sampled. After adding 1% decanal to the samples, the bioluminescence was measured on a Berthold Orion II bioluminescence reader (Berthold Detection Systems, Pforzheim, Germany), as previously described (45). Meanwhile, the bacterial growth was also monitored by measuring the optical density at 600 nm (OD₆₀₀) (BioTek, Winooski, VT, USA).

Purification of VqsA and other proteins. The VqsA open reading frame (ORF) sequence was amplified by PCR using the primers shown in Table 3. The ORF sequence was inserted into pET28a with

TABLE 3 Primers used in this study

Primer name	Primer sequence (5' to 3')	Target	
18672-P1	AGTACGCGTCACTAGTGGGGCCCTTCTAGAGAAGGTGATTTGGCAGTGCGATGTA	18672 deletion mutant	
18672-P2	CTTTATGGGGTGACGTACACACCCAGAGTTGATAAAG		
18672-P3	AACTCTGGGTGTGTACGTACACCCATAAAGCGGCGACAATT		
18672-P4	TAACAATTTGTGGAATTCCTGGGAGAGCTTAAATGCAGTCAAGGGGTAGCGTCC		
18672-OUT-F	AGGATCGCTTCCCTGCTCTGCCG	02325 deletion mutant	
18672-OUT-R	AGAACC GCCGATGGAATGT		
18672-IN-F	ATGGATAAATTAACAAGCATGAAGG		
18672-IN-R	CGACCAAGGTACGGTTTTTAAGGGA		
02325-P1	AGTACGCGTCACTAGTGGGGCCCTTCTAGATCAGCCACGCATTGCGAATAGAACG		
02325-P2	TACTACTCGCTTCAAGTGTCTGTGCTTCTCGTAATTGTCTT		
02325-P3	CGAGAAGCACAGCACTTGAACGCGAGTGTAAAGAACCTGCCT		
02325-P4	TAACAATTTGTGGAATTCCTGGGAGAGCTGTATTATCCTGATAACCCCTCTGT		
02325-OUT-F	ATGCAAACCGTATTGAACCGAAAAG		
02325-OUT-R	CGGCGAATGCGGTAAAAAGTAAAAG		
02325-IN-F	ATGGAAAATCGCACAGTCATTACAAC	soxR deletion mutant	
02325-IN-R	TTACAACGTGTTGAGGACATCCGCG		
soxR-P1	AGTACGCGTCACTAGTGGGGCCCTTCTAGATTCTTGGGTGAAGGCATTGTTGGTC		
soxR-P2	GAATAGAGATAATCGCCTTGACTCCCTGTGTATCAA		
soxR-P3	CACAGGGAGTCAAGGCGATTATCTCTATTCTTTGTCGAA		
soxR-P4	TAACAATTTGTGGAATTCCTGGGAGAGCTTAGCAAGCGAATAATGGAATAAACA		
soxR-OUT-F	AAGCAAACCTTTTCATACCCACAT		
soxR-OUT-R	GATTTACTTGCCGAAGAACGAGACC		
soxR-IN-F	TTCTGGAGTGAAAGTATCTGCCTA		
soxR-IN-R	CCGAGTGGTCTTTCCCTAATACATC		
asnC-P1	AGTACGCGTCACTAGTGGGGCCCTTCTAGAGAGTGGTCACTGACGATGAGAACTT	asnC deletion mutant	
asnC-P2	ATTTACGTTTCGGTTACTATGATTTCCGTTAGGGATAGAG		
asnC-P3	AACGGAAATCATAGTAACCGAAACGTAATCCGT		
asnC-P4	TAACAATTTGTGGAATTCCTGGGAGAGCTAAGAGAAAGTCGCGGTAGTT		
asnC-OUT-F	GTTGTTTTAGATCGATGATGCTGC		
asnC-OUT-R	TATTTTGCCTCAGCTAGAATTCGGC		
asnC-IN-F	GCGCAATATTA AAAA ACTGATGGC		
asnC-IN-R	GTTCTGCAACGATATCAATGTCTCC		
06711-P1	AGTACGCGTCACTAGTGGGGCCCTTCTAGATCTGCAATCCAATCAAATGAACAA		06711 deletion mutant
06711-P2	TACTGATAAAAATGTGATTTGACACAAACCAATAAATGAATTC		
06711-P3	TGGTTGTGTCAAATCACATTTTATCAGTGAGCGTC		
06711-P4	TAACAATTTGTGGAATTCCTGGGAGAGCTTCTAAGACCGCTTTTCCAATGGG		
06711-OUT-F	ACGCTCAGACTTCGCAAGGGTAA		
06711-OUT-R	ACAACCGATAACATTACGAAAGCCG		
06711-IN-F	GTATGTCGATTAAGGCATTAGAGAA		
06711-IN-R	GCTCAATTTATCTGCTAACCCAGTC		
01665-P1	AGTACGCGTCACTAGTGGGGCCCTTCTAGAACCTAAGCGAGATTGCAGCAGTTCA	06115 deletion mutant	
01665-P2	GCGAAATTATCGAGGATTTCTGACTCCAGTTCCGAATTGCG		
01665-P3	ACTGGAGTACGAAATCCTCGATAATTTCCGCATCACTGCG		
01665-P4	TAACAATTTGTGGAATTCCTGGGAGAGCTAAGTGGAAAGCTGGTATGGTTGCGAAG		
01665-OUT-F	ATTTGTTCAAGCGTTCCATCGTGAG		
01665-OUT-R	TACTTTCTTTATGTTGTTGTTGCGT		
01665-IN-F	TGTCTTTGCCAACGCCTA		
01665-IN-R	TCTTTATGGAGAGATAGGAAGGTCC		
metR-P1	AGTACGCGTCACTAGTGGGGCCCTTCTAGAACCTATTGGTCATATCCGATGC		metR deletion mutant
metR-P2	CATAACCCAAATCTGAATTCCTTTCATCCCTTTGTTGCTC		
metR-P3	GGATGAAAGGAATTCAGATTTGGGTTATGAAAGGCTTAGCG		
metR-P4	TAACAATTTGTGGAATTCCTGGGAGAGCTGTGATGACGAGCTGCAAGGTTTA		
metR-OUT-F	GCGGCCAGCTCACTTCAAACGTATC		
metR-OUT-R	GCCGATCTTGCTGTCTTTAGCTATG		
metR-IN-F	AGCTAAAACATTTGCGGACACTCAC		
metR-IN-R	CGCGGTTGCGAAAAAGCTTGTAAAG		
18672-Flag-F	CCATACCCGTTTTTTTGGGTAGCGAATTCACAGGAGGAATTAACCATGAAATTAGATGACTTAAACCTG	18672 expressed in pBAD33-mob	
18672-Flag-R1	TCGTCTGCTTTGTAGTCTGAGCCACCGCCACCGTTGTTCACTTTTGTGTTGGAAAG		
18672-Flag-R2	GGTCAGCATGGGTACCTTTCTCCTCTTAAATTAATCTGCTGCTGCTCCTTGTAGTCTGA		
18672-Flag2-F	AGAGGACACATGGATAAAATTAACAAGCATGAAGG		
pBAD33mob-C'Flag-R	CTTGATGCCTGCGAGTTCGATTACTTGTGCTGCTGCTCCTTGTAGTCTGA	Fluorescence assay	
P ₁₈₆₇₂ -luxAB-F	ATTGCTGACGGTGCAGCGATCCGGGGAATTCAGCAACCGCTACAGTTCACTCTT		
P ₁₈₆₇₂ -luxAB-R	GTCGACCTGCAGCCCAAGCTTATCGATTTCGTACACACCCAGAGTTGATAAAG		
P _{aphA} -luxAB-F	ATTGCTGACGGTGCAGCGATCCGGGGAATTCAGCAGAGCTGCTATACCCGACGCGT		
P _{aphA} -luxAB-R	GTCGACCTGCAGCCCAAGCTTATCGATTTCGGCTACTTATACTTTCACTGCGCTC		
luxAB-pDM8-P4	GTCGACCTGCAGCCCAAGCTTATCGATTTCGTTACGAGTGGTATTTGACGATGTTG		

(Continued on next page)

TABLE 3 (Continued)

Primer name	Primer sequence (5' to 3')	Target
P ₁₈₆₇₂ -F	TGCCTGCAGGTCGACGATTCGGCATTCAATTTTCAGTTCGGCGG	EMSA or DNase I footprinting
P ₁₈₆₇₂ -R	CATGGTCGGTGAAATATCAAAGTGG	
P _{luxR} -F	AGTCGCACGCCAAACGATCACC	P _{luxR} ΔCBS
P _{luxR} -R	ATCCATTTTCCTTGCCATTTG	
P _{aphA} -F	ACTCCCGGGAGCAGAGCTGCTATACCGCACGCGT	P _{aphA} ΔCBS
P _{aphA} -R	ACTCCCGGGGCTACTTATACTTTTCAGTGCCTC	
P _{asp} -F	TGCCTGCAGGTCGACGATAATCCATTCAGCATAGCAATCG	P _{asp} ΔCBS
P _{asp} -R	TTGCCCTATTCCTTATTTGAGC	
P _{luxR} ΔCBS-1	CATCTGTAGAACAATTTATATACAG	P ₁₈₆₇₂ ΔCBS/II
P _{luxR} ΔCBS-2	TGATTATTTTAATAGTTGCTTAAAGCAGTGACATCTGTAGAACAATTTA	
P _{luxR} ΔCBS-3	ATCCATTTTCCTTGCCATTTGAGTTGATATTGGGGTAACCCCTAATTGATTATTTAATAGTTGCTT	P ₁₈₆₇₂ MRBS
P _{aphA} ΔCBS-1	AATTACCCTAAGTTTTTTTATTCTTTTATTTTGGTAT	
P _{aphA} ΔCBS-2	ATAAAAACTTAGGGTAATTTTGTCTACTATTCT	P ₁₈₆₇₂ MABS
P _{asp} ΔCBS-1	GATCATTCAATTAATAAATTATCTACAAAACCTTATAAAGC	
P _{asp} ΔCBS-2	GATAATTTTTAATTGAATGATCTTTTGTACAAATTTG	P ₁₈₆₇₂ MCBSI
P18672ΔCBSI/II-1	GAAATAACTAAAATTACAGTGGACGTATGATTAAGAA	
P18672ΔCBSI/II-2	CGTCCACTGTAATTTTAGTTATTTTCATGGATAAATTAACAAGCATGAAGG	P ₁₈₆₇₂ MCBSII
P18672MRBS-1	TCCGTATCAAGCATCGGAGCCAGAACCACAACGTGTGATT	
P18672MRBS-2	GCTTGATACGGATAGGTACCGCACCCCGCTTCGCTTTG	qRT-PCR
P18672MABS-1	GGTCGCGTACCCTATCAGCGGGGTGCTTGCAGCGAAT	
P18672MABS-2	GATAGGGTACGCGACCGGCAAAATTTGTTCAATGGCTTCAACCA	P ₁₈₆₇₂ MCBSI
P18672MCBSI-1	TACTCTGGGCGACCGACCGTCTTGGCATTACAGTGGACGTATGATT	
P18672MCBSI-2	CGGTGCCCCAGAGTATTTAGTTATTTCTTTATCAAC	P ₁₈₆₇₂ MCBSII
P18672MCBSII-1	GTACACAACTCTGGTCGCCCCGAAATAACTAAAGCAGAGTT	
P18672MCBSII-2	ACCAGAGTTTGTGTACATGGATAAATTAACAAGCATGA	5'-RACE
aphA-RT-F	GAAAGCAAGCCACCAACAGG	
aphA-RT-R	CGACCAGCGTCTGTAATGGA	BIH
luxR-RT-F	CCTACACGCGAAGACTTGGT	
luxR-RT-R	TCTTGGCTGACAAGCTCGAT	P ₁₈₆₇₂ MCBSI
18672-RT-F	CCTTGAGCAACATGCCCCCTA	
18672-RT-R	ATCGGGTGTGCTGTTTCCA	P ₁₈₆₇₂ MCBSII
asp-RT-F	TGCTCAGCGAAACGACTTCA	
asp-RT-R	TAGTACCAGCAACGCGGTTT	5'-RACE
16sRNA-F	AAAGCACTTTCAGTCGTGAGGAA	
16sRNA-R	TGCCCTTACGCCCAAGTAAT	P ₁₈₆₇₂ MCBSI
RACE-adapter	GCGCGAATTCCTGTAGAACGAAC	
18672-RACE-nested-1	ACGGTTGATCAGCGTCTGG	5'-RACE
18672-RACE-nested-2	GATCAGCGTCTGGATCAGGTT	
18672-RACE	TTACAGTTCCGGTTGGACGG	BIH
RNA-Linker	AUAUGCGCGAAUUCUGUAGAACGAACACUAGAAGAAA	
pBXcmT-F	CCTCGGACTGAACACGTTGGCT	P ₁₈₆₇₂ MCBSI
pBXcmT-R	GTGTAAGCCCGGGGTGCCTAA	
pTRG-LuxR-F	CGGGATCCATGGCAAGGAAATGGATATGGACT	P ₁₈₆₇₂ MCBSII
pTRG-LuxR-R	CGGAATTCACAGCAGACAATAAAAAGCCCCA	
pTRG-18672-F	CGGGATCCATGGATAAATTAACAAGCATGA	P ₁₈₆₇₂ MCBSI
pTRG-18672-R	CGGAATTCCTTATGGATTCACATGAGAAAGA	
pTRG-02325-F	CGGGATCCATGGAAATCGCACAGTCATTAC	P ₁₈₆₇₂ MCBSII
pTRG-02325-R	CGGAATTCCTTACAACGTGTTGAGGACATCC	
pTRG-06711-F	CGGGATCCATGAACTTAACTCAAGTAGAAG	P ₁₈₆₇₂ MCBSI
pTRG-06711-R	CGGAATTCCTTAGAAGTCAAATAAATACTCG	
pTRG-AsnC-F	CGGGATCCATGAAATTAGATGACTTAAACCTG	P ₁₈₆₇₂ MCBSII
pTRG-AsnC-R	GGAATTCGTGAATGTTATAAGCGATCA	
pTRG-01665-F	CGGGATCCATGTCTTTTGCCCAACGCCTAGCCA	P ₁₈₆₇₂ MCBSI
pTRG-01665-R	CGGAATTCCTCAAAGTACTCGTTTTTAGATTCT	
pTRG-MetR-F	CGGGATCCATGATAGAGCTAAAACATTTGC	P ₁₈₆₇₂ MCBSII
pTRG-MetR-R	CGGAATTCCTTAAAGCCACCTTGATCCGTCA	
pTRG-SoxR-F	CGGGATCCATGGAAATCAGCGTAGGAGAAG	P ₁₈₆₇₂ MCBSI
pTRG-SoxR-R	CGGAATTCCTACTCTTCTTAAACGTTAAG	
pTRG-F	TGGCTGAACAACTGGAAGCT	P ₁₈₆₇₂ MCBSII
pTRG-R	ATTCGTGCCCCGCCATAA	

a His₆ tag at the carboxyl terminal. Then, the recombinant plasmid was transformed into *E. coli* BL21(DE3). The strain was grown to an OD₆₀₀ of ~0.8 to 1.0 at 25°C, and expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were grown for ~14 to 15 h to allow VqsA expression. Finally, the protein was purified using nickel affinity chromatography. The purification of overexpressed AsnC, SoxR, MetR, 02325, 01665, 06711, AphA, and LuxR was also conducted as described above.

Electrophoretic mobility shift and DNase I footprinting assays. For the electrophoretic mobility shift assays (EMSAs), purified His₆-tagged proteins were incubated with different Cy5-labeled DNA probes (Table 3) in 20 μ l of loading buffer (10 mM NaCl, 0.1 mM dithiothreitol [DTT], 0.1 mM EDTA, 10 mM Tris [pH 7.4]). After the mixture was incubated at 25°C for 30 min, the samples were resolved by 6% polyacrylamide gel electrophoresis in 0.5 \times Tris-boric acid-EDTA (TBE) buffer on ice at 100 V for 120 min. Then, the gels were scanned using a Typhoon FLA 9500 imager (GE Healthcare, Uppsala, Sweden). Dye-primer-based DNase I footprinting assays were performed as previously described (6). In brief, the promoter regions of *aphA*, *luxR*, or *vqsA* were amplified by PCR with *PFU* DNA polymerase and specific primers labeled with 6-carboxyfluorescein (6-FAM) at the 5' ends (Table 3). The probes with the FAM label were purified using a Wizard SV gel and PCR clean-up system (Promega, Beijing, China) and then quantified using a NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific). In each test, 400 ng of the probe was incubated with different amounts of VqsA, AphA, or LuxR in a total volume of 40 μ l. Subsequently, the mixtures were incubated for 30 min at 25°C. After incubation, 10 μ l of a solution containing DNase I (0.015 units) and CaCl₂ (100 nM) was added to the mixtures. The mixtures were then incubated for 60 s at 25°C. The reactions were stopped with 140 μ l of DNase I stop solution (0.15% SDS 200 mM, 30 mM EDTA, and unbuffered sodium acetate). Next, the samples were extracted by the phenol-chloroform extraction method and precipitated with ethanol. After evaporation of the ethanol, the pellets were dissolved in 10 μ l of Milli-Q water. Then, 0.1 μ l of GeneScan-500 LIZ size standards (Applied Biosystems) and 7.9 μ l of HiDi formamide (Applied Biosystems) were added to the 2 μ l of the digested DNA. Finally, the mixtures were analyzed using a 3730 DNA Analyzer with a G5 dye set that was run on an altered default genotyping module that increased the injection time to 30 s and the injection voltage to 3 kV. GeneMapper 4.0 (Applied Biosystems) was used to analyze the results.

5'-RACE. The 5'-rapid amplification of cDNA ends (RACE) experiments were performed as previously described (28). Six micrograms of total RNA was extracted using the TRIzol reagent (28) from WT, Δ *vqsA* mutant, and Δ *luxR* mutant cultures and subjected to dephosphorylation using tobacco acid pyrophosphatase (TAP) (Epicentre) for 60 min at 37°C. The total RNA was ligated with an RNA oligonucleotide linker by using T4 RNA ligase (New England BioLabs, Beverly, MA, USA), according to the manufacturer's instructions. cDNA was synthesized using avian myeloblastosis virus (AMV) reverse transcriptase (RT) (TaKaRa), according to the manufacturer's instructions with a specific primer, 18672-RACE. PCR amplification was performed using the nested primers RACE-adaptor and either 18672-RACE-nested-1 or 18672-RACE-nested-2 (Table 3). Finally, the single band was extracted, subcloned, and sequenced.

Bacterial killing assay. The interbacterial killing assays were performed three times, as previously described (30). Overnight cultures were used for the interbacterial killing assays. Cultures were first normalized to an OD₆₀₀ of 0.5, and the cells were mixed in a predator-to-prey ratio of 4:1. Twenty-five microliters of these mixtures was spotted on LBS agar plates and then cocultured at 30°C for 4 h. The CFU counts of the strains in the mixtures spotted on LBS agar plates at $t = 0$ were determined by plating 10-fold serial dilutions on appropriate plates. After 4 h, the bacterial spots were collected from the LBS agar plates, and the CFU counts of the surviving predator and prey were determined. An empty pBAD33 or pRK415 plasmid that could replicate stably in the corresponding host was used to determine *E. coli* and *V. alginolyticus* resistance to chloramphenicol or tetracycline, respectively (30).

Biofilm formation assays. The biofilm assay was performed as previously described (43). Overnight cultures of WT, Δ *vqsA* mutant, *vqsA*⁺ mutant, and other strains were diluted to an OD₆₀₀ of 1.0. Fifty microliters of the cultures was inoculated into 5 ml of LBS in glass tubes and grown at 30°C without shaking for 48 h. After staining with 2% crystal violet for 20 min and washing, the biofilms were dissolved in 33% (vol/vol) acetic acid, and the absorbance of the solutions at 570 nm was measured. The experiments were performed at least three times (Fig. 6B).

Infection of fish. WT and Δ *vqsA* mutant bacteria were incubated at 30°C overnight and then inoculated into fresh medium and grown for 9 h. All of the strains were harvested and then serially diluted with PBS. Zebrafish weighing approximately 0.25 g were infected with the strains at doses ranging from 10⁴ to 10⁷ CFU/fish via intramuscular injection, according to a previously described method (46). Thirty fish were infected with each dilution, and three parallel experiments were performed. Then, the fish were cultured for 72 h, and vibriosis-induced death was observed and confirmed by isolating *V. alginolyticus* strains from the dead fish. LD₅₀ values were calculated according to a previously described method (46).

Ethics statement. All animal experiments presented in this study were approved by the Animal Care Committee of the East China University of Science and Technology (grant 2006272). The Experimental Animal Care and Use Guidelines from the Ministry of Science and Technology of China (MOST-2011-02) were strictly followed.

Accession number(s). The draft genome sequence of EPGS has been deposited in GenBank under the accession number [GCA_001273715.1](https://doi.org/10.1128/AEM.00444-18).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00444-18>.

SUPPLEMENTAL FILE 1, PDF file, 1.4 MB.

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