The Transcriptional Regulator VqmA Increases Expression of the Quorum-Sensing Activator HapR in *Vibrio cholerae*

Zhi Liu, Ansel Hsiao, Adam Joelsson, and Jun Zhu*

Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

Vibrio cholerae is the causative agent of the severe diarrheal disease cholera. A number of environmental stimuli regulate virulence gene expression in *V. cholerae*, including quorum-sensing signals. At high cell densities, quorum sensing in *V. cholerae* invokes a series of signal transduction pathways in order to activate the expression of the master regulator HapR, which then represses the virulence regulon and biofilm-related genes and activates protease production. In this study, we identified a transcriptional regulator, VqmA (VCA1078), that activates *hapR* expression at low cell densities. Under in vitro inducing conditions, constitutive expression of VqmA represses the virulence regulon in a HapR-dependent manner. VqmA increases *hapR* transcription as measured by the activity of the *hapR-lacZ* reporter, and it increases HapR production as measured by Western blotting. Using a heterogenous *luxCDABE* cosmid, we found that VqmA stimulates quorum-sensing regulation at lower cell densities and that this stimulation bypasses the known LuxO–small-RNA regulatory circuits. Furthermore, we showed that VqmA regulates *hapR* transcription directly by binding to its promoter region and that expression of *vqmA* is cell density dependent and autoregulated. The physiological role of VqmA is also discussed.

The gram-negative bacterium *Vibrio cholerae* is the causative agent of cholera, an acute dehydrating diarrhea still endemic in many parts of the developing world (6). The ability of *V. cholerae* strains to cause severe enteric infection in humans is dependent on the expression of cholera toxin (CT) and a pilus colonization factor known as the toxin-coregulated pilus (TCP). The genes involved in CT and TCP production are controlled by a series of signal transduction cascades. Two membrane-localized complexes thought to respond to environmental signals, ToxRS and TcpPH, activate the transcription of *toxT*. ToxT, in turn, activates the transcription of the virulence regulon (ToxR regulon), including the cholera toxin genes *ctxA* and *ctxB* and the *tcp* genes responsible for TCP biosynthesis. In addition, Skorupski and coworkers have identified AphA as an activator of *tcpPH* expression (18, 29). The environmental cues influencing the expression of virulence genes in vivo are poorly characterized, but extensive studies have indicated the importance of a variety of stimuli, including temperature, pH, bile salts, and quorum-sensing signals (15, 19).

Single-celled bacteria are able to produce and respond to small diffusible molecules called autoinducers. These molecules accumulate as cell density increases and regulate the expression of a range of genes to control a variety of physiological functions, in a process known as quorum sensing (10, 33). Many gram-negative bacteria use a set of diffusible *N*-acyl homoserine lactones (AHLs) that generally serve as cell-to-cell communication signals. The key regulatory components of these signaling systems are LuxI-type proteins, which act as AHL synthases, and LuxR-type proteins, which serve as AHL receptors and AHL-dependent transcription factors (9). The

quorum-sensing system in *V. cholerae*, which does not possess AHL-dependent signal transduction pathways, has been shown to respond to at least two autoinducer molecules: CAI-1, whose structure has yet to be solved, and AI-2, a furanosyl borate diester also produced by *Vibrio harveyi* and many other bacteria.

In *V. cholerae*, quorum sensing has been shown to negatively regulate virulence gene expression (26, 38). Accumulation of these autoinducers modulates the activity of a central regulator, LuxO, via membrane receptors CqsS and LuxPQ (26). At low cell densities, LuxO actively represses the expression of another key quorum-sensing regulator, HapR, by activating the expression of a set of small RNAs (21). At high cell densities, LuxO is inactivated, and thus *hapR* expression is activated. In *V. cholerae*, quorum sensing has been shown to negatively regulate virulence gene expression (26, 38). HapR decreases *tcpPH* transcription indirectly by repressing transcription of *aphA* (18). HapR also represses the *vps* (*Vibrio* polysaccharide synthesis) operon, thus negatively regulating biofilm formation (13, 35, 37). In addition, HapR directly up-regulates the expression of *hapA*, which produces secreted hemagglutinin (HA)/protease, responsible for detachment of the vibrios from the intestinal epithelium (7, 17, 23).

Recent studies have suggested that quorum-sensing regulation in *V. cholerae* is far more complicated than previously thought. The VarS/VarA two-component sensory system comprises an additional quorum-sensing-dependent regulatory input, which uses an additional set of small RNAs together with the global regulatory protein CsrA to modulate gene expression in *V. cholerae* (20). Furthermore, *hapR* expression is repressed at high cell densities by HapR itself, although the significance of this autorepression is unknown (22). In this study, we identified an additional regulator, VCA1078, that modulates the *V. cholerae* quorum-sensing regulon by increasing the expression of *hapR*. Genetic and biochemical studies suggest that

* Corresponding author. Mailing address: Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, PA 19104. Phone: (215) 573-4104. Fax: (215) 898-9557. E-mail: junzhu@mail.med.upenn.edu.

TABLE 1. Strains and plasmids used in this study

Strains or plasmids	Relevant characteristic(s)	Source or reference
V. cholerae strains		
C6706	El Tor, wild type	31
C6706lacZ	*lacZ* deletion	38
C6706mpc	*luxO* constitutive allele (L104Q)	32
MM194	*hapR* deletion	38
ZLV101	*vqmA* deletion in C6706lacZ	This study
ZLV102	pMal-c2x in ZLV101	This study
ZLV103	pMal-c2x in C6706lacZ	This study
ZLV104	pZL3 in ZLV101	This study
ZLV105	pZL3 in C6706lacZ	This study
ZLV106	*hapR-lacZ* in ZLV102	This study
ZLV107	*hapR-lacZ* in ZLV103	This study
ZLV108	*hapR-lacZ* in ZLV104	This study
AHV301	*vqmA-lacZ* in C6706lacZ, *vqmA* disrupted	This study
AHV302	*vqmA-lacZ* in C6706lacZ, with intact *vqmA*	This study
Plasmids		
pMal-c2x	Cloning vector to make MBP fusions	New England Biolabs
pKP302	*lacZ* transcriptional fusion vector	28
pGP704	R6K origin vector, *mob* RP4	27
pWM91	R6K origin vector with *sacB*	24
pJZ142	*hapR-lacZ* on pVIK112 for integrating into the *V. cholerae* genome	38
pBB1	*luxCDABE* of *V. harveyi* on a cosmid	1
pJZ244	Promoterless *lacZ* of *V. cholerae* in pGP704	This study
pJZ177	*vqmA* flanking region cloned into pWM91	This study
pJZ179	*vqmA* coding sequence in pMal-c2x, in frame with *malE*	This study
pZL3	P*tac*-*vqmA*, *vqmA* coding sequence in pMal-c2x	This study
pZL4	Same as pJZ179 with *vqmA* DNA binding sequence deleted	This study
pZL5	*hapR* promoter region in pKP302	This study
pAH301	Internal fragment of *vqmA* cloned into pJZ244	This study
pAH302	Intact 5′-end fragment of *vqmA* cloned into pJZ244	This study

this regulator binds directly to the *hapR* promoter. We thus name VCA1078 VqmA (*Vibrio q*uorum *m*odulator A).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *Escherichia coli* and *V. cholerae* strains used in this study are listed in Table 1 and were propagated in LB containing appropriate antibiotics at 37°C, unless otherwise noted. The plasmid harboring P*tac*-controlled *vqmA* was constructed by PCR amplifying the *vqmA* (VCA1078) coding sequence and cloning it into the pMal-c2x vector (New England Biolabs), resulting in pZL3 (no *malE*) or pJZ179 (in frame with *malE*). A *vqmA* in-frame deletion was constructed by overlapping PCR of the *vqmA* flanking region and cloning into pWM91 (24). The resulting plasmid was then introduced into *V. cholerae* strains and selected for double homologous recombination events. Plasmids containing *vqmA* lacking its helix-turn-helix DNA binding domain sequences (ΔHTH) were constructed by digesting pJZ179 with NruI and XbaI, filling in with Klenow fragment, and religating. The *hapR-lacZ* reporter plasmid was constructed by cloning the PCR-amplified *hapR* promoter

region into the transcriptional LacZ reporter vector pKP302 (28). The *vqmA-lacZ* transcriptional reporter fusions were constructed by cloning of either an internal fragment of *vqmA* or intact 5′ *vqmA* sequences into a suicide plasmid containing promoterless *lacZ* (pJZ244) on pGP704 (27). The resulting plasmids were then introduced into *V. cholerae* strains lacking *lacZ* and screened for homologous recombination events.

Microarray analysis. The primers for PCR amplification of all *V. cholerae* full-length open reading frames (ORFs) were synthesized as previously published (5). All PCR products were spotted onto GAPSII slides (Corning) using OminiGrid 100 (Gene Machines, Inc) (performed by Penn Microarray Core Facilities). Strains ZLV102 and ZLV105 were grown to mid-log phase in AKI medium, which is optimized for virulence gene expression (16), containing appropriate antibiotics and 0.1 μM isopropyl-β-D-thiogalactopyranoside (IPTG). The RNA was isolated using TRIzol reagent (Invitrogen) and cleaned with an RNeasy kit (QIAGEN). Fluorescently labeled cDNA was prepared by direct incorporation of fluorescent nucleotide analogues (Cy3-dCTP and Cy5-dCTP) during a first-strand randomly primed reverse transcription reaction. The differentially labeled cDNAs were combined and subsequently applied to the array surface under conditions that favor hybridization. Microarray slides were scanned using a GenePix 4000B (Axon, Inc). For every ORF-specific spot, the resulting fluorescence intensity of each of the labels was measured, and intensities were compared using the GenePix Pro 6.0 software system (Axon, Inc).

Luminescence assay. Cosmid pBB1, carrying the *V. harveyi lux* operon (1), was introduced into *V. cholerae* strains by electroporation. The resulting strains were grown in LB with appropriate antibiotics at 30°C overnight, diluted to a concentration of 1:1,000 in fresh LB, and incubated at 30°C. Luminescence was read at 30-min intervals for 7 h using a Bio-Tek Synergy HT spectrophotometer. Cultures were diluted, and cell density was monitored by counting CFU. Relative light units are defined as 10^3 light units/CFU ml^{-1}.

Western blotting (immunoblotting) of HapR. Strains were grown in LB overnight and inoculated at 1:100 into LB broth. Samples were withdrawn at the time points indicated. Cell pellets were lysed, and total protein was measured by a Bio-Rad protein assay kit. Portions (0.1 mg) of total protein were size fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and immunoblotted with affinity-purified polyclonal anti-HapR rabbit antiserum.

Detection of cholera toxin. GM1 ganglioside enzyme-linked immunosorbent CT assays (12) were carried out following overnight incubations of *V. cholerae* strains with shaking at 37°C in AKI medium with appropriate antibiotics and IPTG (0.1 μM).

β-Galactosidase activity assays. *E. coli* or *V. cholerae* strains containing LacZ reporter fusions were grown at 37°C in LB with IPTG (0.1 μM). Samples were withdrawn at the time points indicated and then assayed for β-galactosidase activity as previously described (25).

Gel retardation assays. Maltose-binding protein (MBP)-VqmA fusions were purified through amylose columns according to the manufacturer's instructions (New England Biolabs). PCR products containing different lengths of the *hapR* promoter region were digested with XbaI and end labeled using [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I. Binding reaction mixtures contained 0.1 ng DNA and 0.5 μg wild-type or ΔHTH VqmA proteins in a buffer consisting of 10 mM Tris-HCl (pH 7.9), 1 mM EDTA, 1 mM dithiothreitol, 60 mM KCl, 30 mg/ml calf thymus DNA, 20 mg/ml bovine serum albumin, and 10% glycerol. After 20 min of incubation at room temperature, samples were size fractionated using 5% polyacrylamide gels in 0.5× TAE buffer (20 mM Tris-acetate, 1 mM EDTA [pH 8.5]). The radioactivity of free DNA and VqmA-DNA complexes was visualized by using a StormB840 PhosphorImager (Molecular Dynamics).

Infant mouse colonization assay. The infant mouse colonization assay has been described previously (11). Briefly, *V. cholerae* mutant strains were mixed with the wild-type strain, and approximately 10^5 cells were inoculated into 6-day-old CD-1 suckling mice. After a 20-h period of colonization, intestinal homogenates were collected, and the ratio of mutant to wild-type bacteria was determined.

RESULTS

Overexpression of the LuxR family protein VqmA represses virulence gene expression in vitro. In bacteria, gene expression is often highly regulated by various transcription factors. This is supported by the large numbers of conserved transcriptional regulators revealed by microbial genome sequencing. For ex-

TABLE 2. Differential gene expression in *vqmA* mutant and *vqmA*(Con) strains in AKI medium

ORF	ID	*vqmA*(Con)/*vqmA* mutant expression ratio[a]	SD[b]
Pathogenesis			
TagD protein	VC0824	−2.7	0.0
Toxin-coregulated pilus biosynthesis protein P	VC0826	−3.2	0.0
Toxin-coregulated pilus biosynthesis protein H	VC0827	−2.6	0.0
Toxin-coregulated pilin TcpA	VC0828	−3.3	0.1
Toxin-coregulated pilus biosynthesis protein B	VC0829	−2.6	0.0
Toxin-coregulated pilus biosynthesis protein Q	VC0830	−3.2	0.1
Toxin-coregulated pilus biosynthesis protein C	VC0831	−2.3	0.1
Toxin-coregulated pilus biosynthesis protein R	VC0832	−2.3	0.0
Toxin-coregulated pilus biosynthesis protein D	VC0833	−3.6	0.1
Toxin-coregulated pilus biosynthesis protein S	VC0834	−2.7	0.0
Toxin-coregulated pilus biosynthesis protein T	VC0835	−4.0	0.1
Toxin-coregulated pilus biosynthesis protein E	VC0836	−2.2	0.0
Toxin-coregulated pilus biosynthesis protein F	VC0837	−2.7	0.2
TCP virulence regulatory protein ToxT	VC0838	−2.9	0.1
Leader peptidase TcpJ	VC0839	−3.6	0.0
Accessory colonization factor AcfB	VC0840	−2.3	0.0
Accessory colonization factor AcfC	VC0841	−5.2	0.0
Cholera enterotoxin beta subunit	VC1456	−3.4	0.1
Cholera enterotoxin A subunit	VC1457	−2.6	0.1
Hemolysin	VCA0219	−8.8	0.0
Regulation			
LuxR family transcriptional regulator VqmA	VCA1078	40.0	8.0
Hemagglutinin/protease regulatory protein HapR	VC0583	4.3	0.3
LuxR family transcriptional regulator	VCA0888	20.8	5.5
Metabolism			
Malate dehydrogenase	VC0432	−3.7	0.0
Aconitate hydratase 2	VC0604	−2.9	0.0
Carbon starvation protein A (putative)	VC0687	−4.5	0.0
Glutaredoxin	VC1146	5.2	0.5
Alpha-acetolactate decarboxylase	VC1589	4.4	0.4
Acetolactate synthase	VC1590	2.5	0.1
Glyceraldehyde 3-phosphate dehydrogenase	VC2000	3.3	0.4
2-Oxoglutarate dehydrogenase E1 component	VC2087	−3.3	0.1
GTP-binding protein TypA	VC2744	2.4	0.2
Glutamate-ammonia ligase	VC2746	2.9	0.4
Glycerophosphoryl diester phosphodiesterase	VCA0136	−30.3	0.0
GMP reductase	VCA0197	4.5	1.8
Acrobic glycerol-3-phosphate dehydrogenase	VCA0657	−24.9	0.0
Glycerol kinase	VCA0744	−47.3	0.0
Glycerol uptake facilitator protein	VCA0745	−18.3	0.0
3-Oxoacyl-(acyl carrier protein) synthase III	VCA0751	−3.1	0.1
Proline dehydrogenase	VCA1073	−4.6	0.0
Protein processing/transport			
16-kDa heat shock protein A	VC0018	4.3	0.3
Peptide ABC transporter ATP-binding protein	VC0170	−2.7	0.0
Peptide ABC transporter periplasmic peptide-binding protein	VC0171	−2.7	0.0
Na^+/H^+ antiporter (putative)	VC0389	2.8	0.2
PTS system glucose-specific IIBC component[c]	VC2013	3.6	0.1
Glycerol-3-phosphate transporter	VCA0137	−63.9	0.0
Maltose ABC transporter permease protein	VCA0943	2.7	0.5
Maltose ABC transporter permease protein	VCA0944	3.7	0.8
Unknown function			
	VC0134, VC0491, VC0669, VC0770, VC1492, VC2155, VCA0324, VCA0367, VCA0483, VCA0587, VCA0887	>2	
	VC0669, VC1492, VC2155, VCA1097	<−2	

[a] ZLV102 and ZLV104 were grown in AKI medium in the presence of IPTG (0.1 μM). Positive values represent activation in ZLV104 [*vqmA*(Con)]; negative values represent repression. Average ratios from two independent experiments are shown.
[b] From two independent experiments.
[c] PTS, phosphotransferase.

ample, in the *V. cholerae* genome, there are eight open reading frames annotated as LuxR family proteins (14). LuxR family proteins are thought to share an AHL interaction region and DNA binding motif (9). However, while all annotated LuxR family proteins in *V. cholerae* share a conserved helix-turn-helix DNA binding motif (3), they show very weak homology to the conserved AHL binding domains (data not shown). Furthermore, no AHL synthase genes are present in the *V. cholerae* genome, and no AHL signals can be detected under laboratory culture conditions using a sensitive AHL bioassay strain (36) (data not shown). Thus, these LuxR-type proteins in *V. cholerae* may not be involved in the process of interaction with self-produced AHL signals. To investigate the genes possibly regulated by these LuxR family proteins, we performed microarray analysis to compare the transcriptional profiles of strains with chromosomal in-frame deletions of these genes to those of strains with plasmids overexpressing the corresponding genes. Artificially activating these regulatory genes enabled us to detect target genes that otherwise might not be affected under the experimental conditions we normally use. Interestingly, overexpression of one of the annotated LuxR family genes, VCA1078, which is predicted to encode a 319-amino-acid protein sharing a conserved carboxyl-terminal DNA binding motif with only the LuxR protein of *Vibrio fischeri*, leads to the repression of the entire ToxR virulence regulon (Table 2). For example, genes in the TCP island are repressed 2.3- to 5.2-fold in the VCA1078-overexpressing strain. Similarly, the *ctxA* and *ctxB* genes, which encode the two subunits of cholera toxin, are also inhibited (threefold) in such a strain. The inhibition of virulence gene expression is not due to a general inhibitory effect of protein overproduction, since no virulence gene repression was observed when other LuxR family proteins were overexpressed (data not shown) or with expression of the control protein MalE. We renamed VCA1078 *vqmA* (*Vibrio quorum-sensing modulator A*), because its gene product regulates the quorum-sensing system of *V. cholerae* (see below).

Repression of virulence genes by constitutively expressed VqmA is HapR dependent. To verify the microarray data, we measured the production of CT, a major virulence determinant, after growth in AKI medium, which is known to induce virulence factor production (16). The strains with constitutively expressed VqmA produced 93% less cholera toxin than a wild-type strain (Fig. 1). Deletion of *vqmA* did not affect CT production.

Microarray analysis indicated that the expression of two regulators, the LuxR family protein VCA0888 and HapR, was up-regulated when VqmA was constitutively expressed (Table 2). Overexpression of VCA0888 did not affect CT production (data not shown), indicating that VCA0888 is not involved in virulence gene regulation. However, previous studies have demonstrated that HapR represses virulence gene expression by repressing *aphA* transcription (18, 38). Remarkably, *hapR* expression is increased more than fourfold in the strain constitutively expressing VqmA [VqmA(Con)]. This suggests that VqmA may act on virulence gene expression through HapR. To test this possibility, we examined CT production in a *hapR* mutant. In contrast to *hapR*⁺ strains harboring the P*tac*-*vqmA* plasmid (producing only 7% of the CT made in wild-type strains), overexpression of VqmA in the *hapR* mutant had only
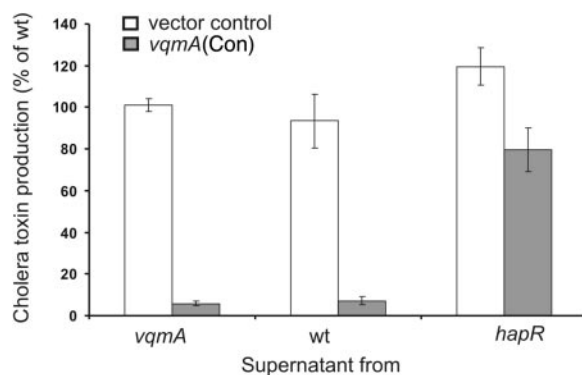

FIG. 1. Constitutive expression of *vqmA* inhibits cholera toxin production in a HapR-dependent manner. *V. cholerae* strains (*vqmA*, wild type [wt], or *hapR*) with pMal-c2x (vector control) (white bars) or pZL3 (constitutively expressed *vqmA*) (gray bars) were grown in AKI medium in the presence of 0.1 μM IPTG. CT in supernatants was measured using a CT enzyme-linked immunosorbent assay. Data are expressed as percentages of the level of CT produced by wild-type C6706 grown under the same conditions. Results are representative of three experiments ± standard deviations.

a minimal effect on CT production (80% of the CT produced by the wild type) (Fig. 1), while a plasmid constitutively expressing *hapR* in such a strain restored the repression of CT production (data not shown). These results indicated that the inhibitory effect of VqmA on the virulence regulon required functional HapR.

VqmA activates *hapR* transcription at low cell densities. Our microarray experiment revealed that VqmA regulates *hapR* transcription. To further confirm the microarray data and to investigate how VqmA affects *hapR* transcription, we monitored the expression of a chromosomal *hapR-lacZ* transcriptional fusion in a *vqmA* deletion mutant, a wild-type *vqmA* strain, and a strain with multiple copies of constitutively expressed *vqmA* as a function of cell density. Figure 2A shows that *hapR* expression is higher at low cell densities (optical density at 600 nm [OD$_{600}$], <0.6) in strains with a single copy of *vqmA* (wild type) and strains constitutively expressing *vqmA* than in *vqmA* deletion mutants. However, at high cell densities (OD$_{600}$, ≥1.5), the expression of *hapR* is similar in all three strains tested.

To further confirm that VqmA regulates *hapR*, we used a Western blot assay to measure HapR production in strains with plasmids with different forms of *vqmA*. Equal amounts of total protein were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. In the wild-type strain, HapR was detectable when mid-log phase was reached, although little HapR was found in *vqmA* mutants at these cell densities (Fig. 2B). In contrast, when VqmA was constitutively expressed, HapR was detected at lower cell densities (OD$_{600}$, ≈0.2). At high cell densities, all strains produced similar amounts of HapR protein. We interpret these data as indicating that VqmA contributes to *hapR* regulation primarily at low cell densities.

VqmA increases HapR-activated gene expression. In addition to repressing virulence gene expression, the quorum-sensing regulator HapR also activates the heterologous promoter of the *V. harveyi luxCDABE* operon (26). To study the rela-
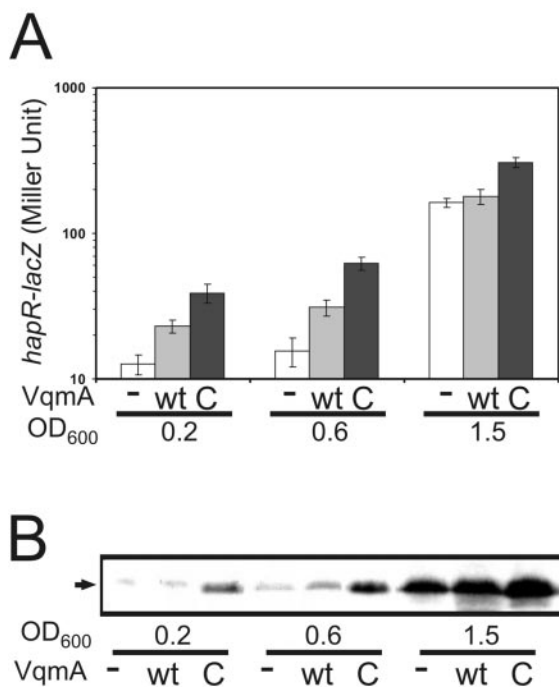
FIG. 2. VqmA increases *hapR* transcription at low cell densities. (A) Chromosomal *hapR-lacZ* expression in the *vqmA* deletion strain (−) (white bars), wild-type *vqmA* (wt) (gray bars), and the strain with constitutively expressed *vqmA* (C) (black bars) at the cell densities indicated. Results are representative of three experiments ± standard deviations. (B) Western blot analysis of HapR production in the same strains at the cell densities indicated. Strains with different VqmA levels are indicated as explained in the legend to panel A. The *vqmA* deletion mutant and the wt strain contained a vector control, and the strain constitutively expressing *vqmA* contained plasmid pZL3. IPTG (0.1 μM) was added to all cultures. Results are representative of three experiments.
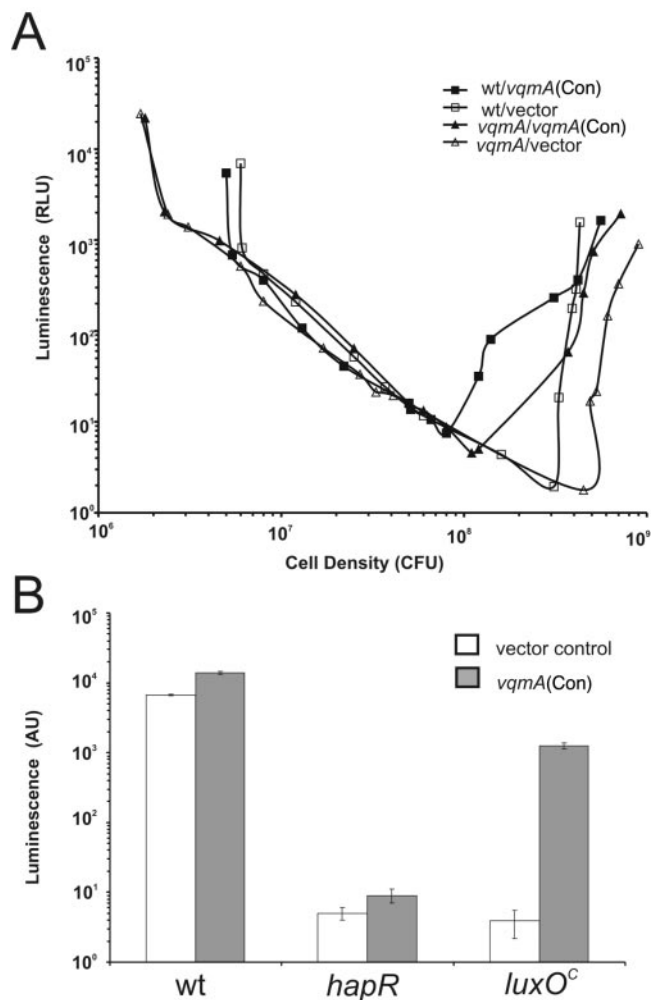

FIG. 3. VqmA activates quorum-sensing-regulated luminescence at lower cell densities and can bypass the LuxO-sRNA regulatory circuits. (A) Light production per cell in strains containing the *V. harveyi lux* operon cosmid pBB1. The wild-type strain containing either the pMal-c2x vector (wt) (open squares) or pZL3, constitutively expressing *vqmA* (solid squares), and *vqmA* mutants containing either pMal-c2x (open triangles) or pZL3 (solid triangles) were grown in LB containing 0.1 μM IPTG at 30°C. At various time points, light production and densities of cultures (in CFU) were measured. Relative luminescence units were calculated as 10^3 light units/CFU ml^{-1}. Results are representative of three experiments. (B) Effect of VqmA on light production in a mutant with constitutively active LuxO. Strains containing either pMal-c2x (vector control) (white bars) or pZL3 [*vqmA*(Con)] (gray bars) were grown overnight at 30°C in LB containing 0.1 μM IPTG, and luminescence was measured. Results are representative of three experiments ± standard deviations.

tionship between VqmA and quorum-sensing regulation, we introduced the *V. harveyi* luciferase genes into strains with different *vqmA* constructs and used light production as an indicator of quorum-sensing-dependent gene expression. Cultures were diluted, and light production per cell was measured during subsequent growth. Figure 3A shows that the wild-type strain produced light in a cell density-dependent pattern. This U-shaped curve is due to the dilution of the culture and thus a drop in the extracellular autoinducer levels to below the threshold concentration required for stimulation of *lux* expression. As cell density increases, *lux* is induced over time due to the accumulation of new autoinducers (26). Constitutive VqmA expression in either *vqmA* mutants or wild-type strains caused earlier induction of Lux activity than that observed for the wild type with a control vector. On the other hand, Lux induction was delayed in the *vqmA* deletion strain containing only a vector plasmid. In addition, overproduction of VqmA in either *luxO* mutants (displaying constitutive *lux* expression) or *hapR* mutants (no light produced) did not affect light production (data not shown). These data indicate that the quorum-sensing system is activated at lower cell densities when VqmA is present. Furthermore, VqmA proteins do not affect autoinducer production, as evidenced by the fact that all four strains

produced similar amounts of both CAI-1 and AI-2 under these conditions (data not shown).

Recent work from Bassler and colleagues reveals that when *V. cholerae* is at a low cell density, phosphorylated LuxO (LuxO-P) activates a set of small regulatory RNAs, which work together with Hfq to destabilize *hapR* mRNA (21). At high cell densities, LuxO is dephosphorylated and thus inactive. Constitutive mutations in the *luxO* allele, such as D47E (8, 13) and L104Q (32), "lock" the LuxO protein into a state mimicking LuxO-P. These constitutive LuxO mutants therefore inactivate
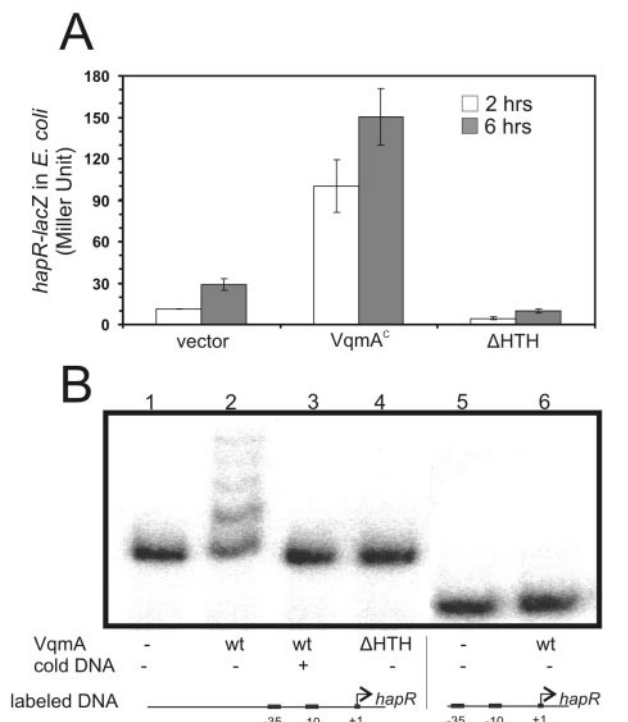
FIG. 4. VqmA regulates the *hapR* promoter directly. (A) *hapR-lacZ* expression in *E. coli*. MC4100 with either a vector control, a plasmid constitutively expressing wild-type VqmA, or a plasmid expressing VqmA lacking its DNA binding domain (ΔHTH) was inoculated 1:100 into LB in the presence of 0.1 μM IPTG and grown at 37°C for 2 h (white bars) or 6 h (gray bars). Results are representative of three experiments. Error bars, standard deviations. (B) Gel retardation assays using purified MBP-VqmA proteins. Lanes 1 to 4, labeled *hapR* promoter region; lanes 5 and 6, labeled *hapR* promoter region without upstream sequences. Lanes 1 and 5, labeled DNA only; lanes 2 and 6, 0.5 μg MBP-VqmA; lane 3, 0.5 μg MBP-VqmA with 0.1 μg unlabeled DNA; lane 4, 0.5 μg MBP-VqmA(ΔHTH). wt, wild type.
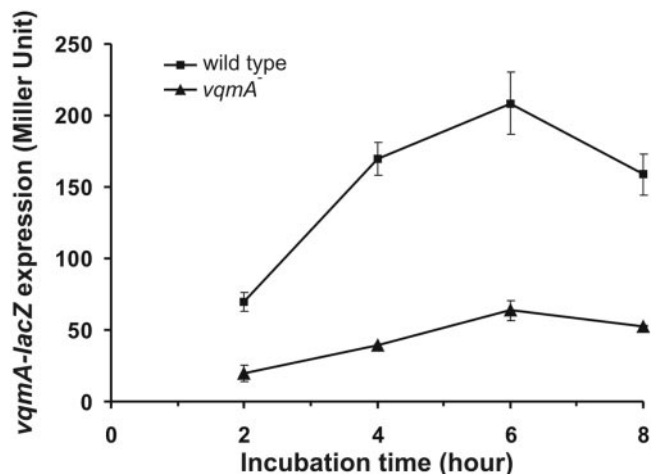

FIG. 5. VqmA is required for *vqmA* cell density-dependent expression. Wild-type (AHV302) (squares) and *vqmA* mutant (AHV301) (triangles) strains carrying a chromosomal *vqmA-lacZ* transcriptional reporter fusion were grown in LB at 37°C, and β-galactosidase activity was measured at the time point indicated. Results are representative of three experiments ± standard deviations.

the quorum-sensing system by constitutively repressing *hapR* expression. To investigate the interaction between VqmA regulation of *hapR* and the LuxO–small-RNA (LuxO-sRNA) regulatory circuits described above, we introduced pMal-c2x (vector control) or pZL3 (P$_{tac}$-*vqmA*) into the strain containing a constitutive *luxO* (L104Q) allele and measured Lux expression. Figure 3B shows that Lux was not expressed in the *luxO*(Con) mutant containing a vector control, as previously reported (32), but overexpression of VqmA induced light production more than 100-fold greater than that with the vector control. However, compared to that in wild-type strains, VqmA cannot fully recover light production in *luxO* constitutive mutants. These data suggest that VqmA can partially overcome the LuxO-sRNA inhibitory effect on *hapR* transcription, possibly by activating the production of more *hapR* mRNA. No light was detected in the *hapR* mutants, suggesting that VqmA itself cannot activate the *lux* operon.

VqmA regulates *hapR* transcription directly. Our data suggest that VqmA plays a role in quorum-sensing regulation by increasing *hapR* expression. To investigate further whether VqmA activates *hapR* transcription directly or through other regulators, we constructed a plasmid containing the *hapR* pro-

moter fused with a *lacZ* transcriptional reporter in *E. coli* and assayed for β-galactosidase activity. Compared to a vector control, VqmA increased *hapR-lacZ* expression (Fig. 4A). Deletion of the conserved helix-turn-helix DNA binding domain of VqmA completely abolished β-galactosidase activity. VqmA activation of *hapR* transcription in *E. coli* suggests that VqmA acts directly on the *hapR* promoter or, alternatively, activates a regulator gene that is conserved between *V. cholerae* and *E. coli*.

We then purified the VqmA protein as MBP fusions and performed a gel retardation assay to test whether VqmA binds directly to the *hapR* promoter DNA. The MBP-VqmA fusion protein retained the same activity as the wild-type VqmA protein, as judged by the capacity of induction of *hapR* expression in *E. coli* and in *V. cholerae* (data not shown). Two kinds of DNA fragments were labeled. The shorter fragment comprised the region of the *hapR* promoter from the predicted −35 sequences (22) to the translational start site, while the second fragment extended an additional 200 bp upstream of the −35 region. Purified MBP-VqmA retarded the mobility of the DNA fragment containing the region upstream of −35 (Fig. 4B, lane 2) but not the shorter fragment (lane 6). Addition of unlabeled target DNA abolished the retardation (lane 3), indicating that the binding of VqmA is specific and is located upstream of the −35 region. Purified MBP-VqmA lacking the helix-turn-helix DNA binding domain did not shift the *hapR* promoter DNA (lane 4), further confirming that the DNA binding domain of VqmA is essential for its activity.

The expression of *vqmA* is autoregulated. Our data demonstrated that VqmA regulates quorum sensing in *V. cholerae* by activating *hapR* expression. We were thus interested in how *vqmA* itself is expressed. To study this, we constructed two *vqmA-lacZ* chromosomal transcriptional fusions. One construct retains the intact *vqmA* gene, and the other has a disrupted *vqmA* gene (see Materials and Methods for details). Figure 5 shows that in the *vqmA*+ background, the expression

of *vqmA* increased with cell density and reached maximal activity in late log phase. In contrast, *vqmA* expression in the *vqmA* disruptant background does not increase during cell growth. These data imply that VqmA autoregulates its own promoter.

Strains carrying mutations in *vqmA* have no colonization defect. We have shown that constitutive expression of the *vqmA* genes repressed virulence gene expression in an in vitro inducing condition, while deletion of *vqmA* did not affect virulence gene expression. We examined whether VqmA affects *V. cholerae* pathogenesis by comparing the colonization capacities of the wild type and *vqmA* mutants using an infant mouse model. As expected, strains carrying a *vqmA* deletion can colonize infant mice as well as the wild-type strain (data not shown), indicating that *vqmA* is not involved in colonization, at least in the infant mouse model.

DISCUSSION

In this study, we have shown that the transcriptional regulator VqmA modulates *V. cholerae* quorum-sensing systems by directly increasing the expression of the quorum-sensing master regulator HapR. The gene encoding VqmA (VCA1078) is located on the small chromosome and may be divergently transcribed with a gene encoding pyridoxamine 5′-phosphate oxidase (VCA1079). Quorum sensing in *V. cholerae* has been shown to negatively regulate virulence gene expression in vitro by repressing the expression of *aphA*, whose gene products are required for the activation of the entire virulence regulon. Constitutive expression of VqmA induces quorum sensing early in growth and leads to repression of virulence genes. In the *vqmA* deletion mutant, quorum sensing displays a delay but is eventually fully induced. It is unclear what role VqmA plays during *V. cholerae* infection. *vqmA* is not expressed in *V. cholerae* isolated from cholera patients' stool samples (2) but is highly expressed in the rabbit ileal loop model of *V. cholerae* infection (34). Thus, we speculate that *vqmA* expression differs under different environmental conditions and affects quorum-sensing systems accordingly.

Although VqmA and other annotated LuxR family proteins may function independently of acyl homoserine lactone signals, used as quorum-sensing molecules in many other gram-negative bacteria, these proteins play regulatory roles in *V. cholerae*. In addition to VqmA, another protein in this group, VpsT (VCA0952), has been shown to serve as a positive transcriptional regulator of *vps* gene expression (4). Another noteworthy aspect of VqmA is that this protein has a conserved PAS domain (named after three proteins in which it occurs: Per, Arnt, and Sim), found widely among prokaryotes and eukaryotes (data not shown). PAS domains have been found to bind ligands and to act as sensors for light and oxygen in signal transduction (30). Although our in vitro MBP-VqmA DNA retardation assays indicated that the VqmA protein is able to bind its target promoter DNA, we cannot rule out the possibility that binding of additional ligands may enhance VqmA activity. We thus speculate that VqmA might be able to detect certain unknown environmental signals and modulate cell density-dependent cellular functions when needed.

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