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Interplay among Cyclic Diguanylate, HapR, and the General Stress Response Regulator (RpoS) in the Regulation of *Vibrio cholerae* Hemagglutinin/Protease[▽][†]

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Vibrio cholerae **secretes the Zn-dependent metalloprotease hemagglutinin (HA)/protease (mucinase), which is encoded by** *hapA* **and displays a broad range of potential pathogenic activities. Expression of HA/protease has a stringent requirement for the quorum-sensing regulator HapR and the general stress response regulator RpoS. Here we report that the second messenger cyclic diguanylic acid (c-di-GMP) regulates the production of HA/protease in a negative manner. Overexpression of a diguanylate cyclase to increase the cellular c-di-GMP pool resulted in diminished expression of HA/protease and its positive regulator, HapR. The effect of c-di-GMP on HapR was independent of LuxO but was abolished by deletion of the c-di-GMP binding protein VpsT, the LuxR-type regulator VqmA, or a single-base mutation in the** *hapR* **promoter that prevents autorepression. Though expression of HapR had a positive effect on RpoS biosynthesis, direct manipulation of the c-di-GMP pool at a high cell density did not significantly impact RpoS expression in the wild-type genetic background. In contrast, increasing the c-di-GMP pool severely inhibited RpoS expression in a ΔhapR mutant that is locked in a regulatory state mimicking low cell density. Based on the above findings, we propose a model for the interplay between HapR, RpoS, and c-di-GMP in the regulation of HA/protease expression.**

Cholera is an acute waterborne diarrheal disease caused by *Vibrio cholerae* of serogroups O1 and O139. Infecting *V. cholerae* cells adhere to the intestinal mucosa, where they express major virulence factors such as the toxin coregulated pilus (TCP) (21) and cholera toxin (CT), which is largely responsible for the profuse rice-watery diarrhea typical of this illness (12, 26). Cholera patients shed *V. cholerae* in their stools, which usually contain about 10^8 hyperinfective *V. cholerae* cells per ml (38). The *V. cholerae* cells can then survive and persist in fresh water and estuarine aquatic ecosystems to eventually gain entrance to a new host.

The Zn-dependent metalloprotease hemagglutinin (HA)/ protease (18) has been proposed to facilitate *V. cholerae* detachment from the intestinal mucosa when infecting *V. cholerae* cells reach a high density (4, 14, 43, 44). Inactivation of *hapA*, which encodes HA/protease, has been shown to enhance adherence to mucin-coated polystyrene plates (43), adherence to mucin-secreting differentiated HT29-18N2 cultured cells (4), and colonization of the suckling mouse intestine (41, 43). The mucinase activity of HA/protease (13), together with its capacity to cleave the mucin-binding adhesin GbpA at a high cell density, provides a mechanism that supports the "detachase" function attributed to this protein (25). In addition, HA/protease has been reported to perturb the paracellular barrier of cultured intestinal epithelial cells (37, 55) by acting on tight junction-associated proteins (56). In agreement with this finding, we have shown that HA/protease enhances enterotoxicity in the rabbit ileal loop model (43).

The expression of flagellar motility is required for intestinal colonization (28, 43). In addition, experiments conducted with the rabbit ileal-loop model suggest that an RpoS-dependent upregulation of motility in the stationary phase is responsible for the detachment of *V. cholerae* cells from the intestinal mucosa and their escape into the luminal fluid (40). We have shown that motility also enhances enterotoxicity (fluid accumulation) in rabbit ileal loops in which the deletion of both *hapA* and *motY* had the most prominent attenuating effect (43). The above evidences suggest that production of HA/ protease and motility could occur at one or more stages of infection to jointly promote pathogenesis and/or detachment.

The expression of HA/protease and motility are positively regulated by the cyclic AMP receptor protein (CRP) and the quorum-sensing regulator HapR (2, 22, 42). We have shown that CRP acts upstream of HapR by inducing the biosynthesis of cholera autoinducer 1 (CAI-1) to activate quorum sensing (30, 31, 42). At high cell density, HapR binds to the promoter of *hapA* to enhance protease production (52). Gene expression profiling has shown that HapR also positively affects the expression of numerous genes required for the expression of flagellar motility and chemotaxis (40, 58). Expression of HapR has clearly been shown to diminish the intracellular concentration of the second messenger cyclic diguanylic acid (c-di-GMP), an inhibitor of flagellar motility (17, 54).

HA/protease production also requires the general stress response regulator RpoS (42, 57). Although RpoS has also been shown to enhance flagellar motility and chemotaxis (40), its

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TABLE 1. *V. cholerae* strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
C7258	Wild type O1; El Tor biotype	Peru, 1991
N16961	Wild type O1; El Tor biotype; *hapR* negative	Bangladesh, 1975
AJB50	C7258 Δ*rpoS*	30
AJB51	C7258 Δ*hapR*	30
AJB52	C7258 Δ*hapR*Δ*rpoS*	This study
AJB501	C7258 Δ*luxO*	This study
AJB502	C7258 *rpoS*::pCVDRpoS-FLAG	This study
AJB503	AJB51 *rpoS*::pCVDRpoS-FLAG	This study
AJB504	AJB501 *rpoS*::pCVDRpoS-FLAG	This study
N16961-FLAG	N16961 *rpoS*::pCVDRpoS-FLAG	This study
SZS007	C7258 *luxC-lacZ* reporter	47
SZS012	SZS007 Δ*luxO*	47
HX01	SZS007 *hapR*p*	This study
HX02	SZS007 Δ*vpsT*	This study
HX03	AJB51 Δ*vpsT*	This study
AJB505	SZS007 Δ*vqmA*	This study
Plasmids		
pAT1568	PDE VieA in pBAD33	51
pAT1615	Inactive VieA(E170A) in pBAD33	51
pAT1662	DGC VCA0956 in pBAD33	51
pCMW75	DGC QrgB expression vector	54
pTT3	*rrnB* T_1T_2 transcription terminator in pUC19	44
pCVDΔRpoS2	DNA fragment carrying *rpoS* deletion in pCVD442	30
pUCΔvqmA	SacI-BamHI and BamHI-SphI DNA fragments flanking *vqmA* sequentially cloned in pUC19	This study
pCVDΔvqmA	SacI-SphI fragment harboring *vqmA* deletion in pCVD442	This study
pUCΔvpsT	SacI-BamHI and BamHI-SphI DNA fragments flanking *vpsT* sequentially cloned in pUC19	This study
pΔvpsTKm	1.2-kb BamHI fragment encoding a Km resistance cassette from pUC4K[a] in pUCΔvpsT	This study
pCVDΔvpsTKm	SacI-SphI fragment from pΔvpsTKm in pCVD442	This study
pTT3RpoS-FLAG	SphI-PstI fragment containing *rpoS* ORF in pTT3	This study
pCVDRpoS-FLAG	SphI-XbaI *rpoS*-FLAG and *rrnB* T_1T_2 fragment in pCVD442	This study
pCVD*hapR*p*	Mutant *hapR* promoter in pCVD442	This study

[a] The GenBank accession number of pUC4K is X06404.1.

role in c-di-GMP signaling in *V. cholerae* has not been established.

Cyclic di-GMP has been shown to regulate the transition between the sessile and motile lifestyles by activating biofilm formation and inhibiting motility in a broad spectrum of bacterial species (8, 19, 46, 49). The *V. cholerae* genome contains 31 genes encoding GGDEF domain family proteins, 10 genes encoding proteins with GGDEF and EAL domains, 12 genes encoding proteins with only EAL domains, and 9 genes encoding proteins with HD-GYP domains (15). The GGDEF domain family proteins have been shown to exhibit a diguanylate cyclase (DGC) activity that makes c-di-GMP from GTP. The EAL and HD-GYP domain families have been reported to exhibit a phosphodiesterase (PDE) activity that degrades c-di-GMP to GMP (15). In *V. cholerae*, overexpression of the DGC VCA0956 abolishes swimming, whereas expression of the PDE VieA enhances it (51). Transcriptional profiling has revealed that genes involved in flagellum biosynthesis, motility, and chemotaxis are repressed in response to an increase in intracellular c-di-GMP content (5).

In this study, we examined the role of c-di-GMP in the regulation of HA/protease expression. We report that c-di-GMP negatively regulates the production of HA/protease and propose a model for the interplay between c-di-GMP, HapR, and RpoS in this regulation.

MATERIALS AND METHODS

Strains, media, plasmids, and oligonucleotide primers. Strains, plasmids, and primers used in the study are listed and described briefly in Tables 1 and 2. All *V. cholerae* strains were derived from C7258 (El Tor biotype, serotype Ogawa). For HA/protease production, strains were grown in tryptic soy broth (TSB) at 37°C with agitation (225 rpm) to stationary phase. Culture media were supplemented with ampicillin (Amp; 100 μg/ml), polymyxin B (PolB; 100 units/ml), chloramphenicol (Cm; 5 μg/ml), or kanamycin (Km; 50 μg/ml) as required.

Construction of mutants. To construct a *vqmA* deletion mutant, we amplified DNA fragments flanking the *vqmA* locus (VCA1078) from C7258 genomic DNA using primer pairs VqmA645/VqmA1519 and VqmA2455/VqmA3334. The PCR products were sequentially cloned in pUC19 to yield pUCΔvqmA and confirmed by DNA sequencing. Then the chromosomal DNA fragment harboring the *vqmA* deletion was transferred to the suicide vector pCVD442 (11) to yield pCVDΔvqmA and transferred by conjugation to strain SZS007 (Table 1). Exconjugants were selected in LB agar containing Amp and PolB. Finally, the segregant AJB505, harboring the *vqmA* deletion, was isolated by sucrose selection as described previously (30). An identical strategy was used to construct Δ*vpsT* mutants, except that primer pairs VpsT690/VpsT1220 and VpsT1395/VpsT2093 were used and a Km resistance gene was inserted in place of the deleted *vpsT* DNA. The *vpsT* deletion/insertion was introduced into strains SZS007 and AJB51 (Δ*hapR*) by conjugation as described above. The Δ*luxO* strain AJB501 was constructed by allelic exchange as previously described (47) but using strain C7258 as the conjugal receptor. Similarly, a mutant with deletions of *rpoS* and *hapR* (AJB52) was constructed by conjugal transfer of the suicide vector pCVDΔRpoS2 (30) to the *hapR* mutant AJB51 followed by sucrose selection. To construct a strain defective for HapR autorepression, we amplified the *hapR* promoter from strain C7258 using primers HapR-F1 and HapR-R1. The PCR product was cloned in pUC19 and confirmed by DNA sequencing. Then we used a QuikChange site-directed mutagenesis kit (Strat-

TABLE 2. Oligonucleotide primers

Primer	Sequence (5′→3′)[a]
HapA1010	GCACGGCGTTCAGTTATGCTTGTA
HapA1551	AGGTAAAACGCGCGGTTAAACACG
HapR-F1	GCCTCTAGATGAATTTGACGAGCAAGATC
HapR-R1	GATGCATGCCTCAAACCAGACTTTGAG
HapRmut-F	CTTTAAGTAGCAAATAGCAAAATAATCATTAG
HapRmut-R	CTAATGATTATTTTGCTATTTGCTACTTAAAG
HapR589	CGCCTCAAAAACGCAAACTACAACT
HapR1046	ATGGAGTAGAAGATGCCGTGGAAC
RecA578	GTGCTGTGGATGTCATCGTTGTTG
RecA863	CCACCACTTCTTCGCCTTCTTTGA
RpoS3	GAAGCATGCCAATACCGTAACCAAA
RpoS13	GCCGAATTCGAGTGTCAGCAATAC
RpoS576	CGATTTTGAAGATGAAGCACTGGAAG
RpoS994	GCCAGATCTGTATTCGACGTTAAAC
RpoS1003	TGTTTGGTTCATCAGCGCACGTTC
RpoS1020	CTTCTGCAGTTACTTGTCGTCATCG
RpoSP189	GGCTAGCAAAGGAAGGACAAACTGT
VpsT56	CCAGATTGTTGAAAGAGGCGTTAG
VpsT252	TGCGGACAGTTTATGATGACCTCT
VpsT690	CTGCAAACACATATCGAGCTCTTCT
VpsT1220	GCAGGATCCAAGCATTCTAACGTTTA
VpsT1395	GAAGGATCCTGATGATGTTTTGACC
VpsT2093	GGGATTATGAAACGTTTGGAAAAGCC
VqmA645	GTAGAGCTCCATTCGGGTTAACTTG
VqmA1519	GAAGGATCCGAACACACAGAGGTAT
VqmA2455	GAAGGATCCCAGTAAGCAACAACGT
VqmA3334	CAAGCATGCATGAGTGGTTCCTGTT

[a] Restriction sites used for directional cloning are underlined.

agene) to introduce an A→G mutation in the *hapR* promoter using primers HapRmut-F and HapRmut-R. The mutant promoter was subcloned in pCVD442 and transferred by conjugation to strain SZS007, and segregant HX01, which retained the mutant promoter designated *hapR*p*, was isolated by sucrose selection as above. The A→G (originally described as A18G) mutation has been shown to eliminate HapR binding to its own promoter and autorepression (33).

Construction of RpoS-FLAG reporter strains. To construct a strain expressing an RpoS-tagged protein from native transcription and translation initiation signals, we amplified the *rpoS* open reading frame (ORF) lacking its stop codon from strain C7258 genomic DNA using primers RpoS13 and RpoS994 and an Advantage 2 PCR kit (BD Biosciences Clontech). The resulting PCR fragment was confirmed by DNA sequencing and cloned as an EcoRI-BglII fragment in pFLAG-CTC (Sigma-Aldrich) in frame with the FLAG epitope. The RpoS-FLAG fusion was then retrieved by PCR with primers RpoS3 and RpoS1020 and inserted into plasmid pTT3 (44) upstream of the *rrnB* T_1T_2 transcription terminator to yield pTT3RpoS-FLAG. Finally, the *rpoS*-FLAG-*rrnB* T_1T_2 cassette was transferred to the suicide vector pCVD442 to yield pCVDRpoS-FLAG. The resulting suicide vector was transferred from S17-1λpir (9) to *V. cholerae* strains C7258, AJB51 (Δ*hapR*), AJB501 (Δ*luxO*), HX03 (Δ*hapR*Δ*vpsT*), and N16961 by conjugation, and exconjugants were selected on LB plates containing Amp and PolB. Integration by homologous recombination within the *rpoS* locus was confirmed by PCR using primer RpoSP189, which anneals to the *rpoS* promoter region (not present in pCVDRpoS-FLAG), and primer RpoS1020, which anneals to the FLAG epitope. The resulting PCR products were further confirmed by DNA sequencing, and the expression of RpoS-FLAG was established by Western blot analysis.

qRT-PCR. Total RNA was isolated using an RNeasy kit (Qiagen Laboratories). The RNA samples were analyzed by quantitative real-time reverse-transcription-PCR (qRT-PCR) using an iScript two-step RT-PCR kit with SYBR green (Bio-Rad Laboratories) as described previously (30, 45). Relative expression values were calculated as $2^{-(CT\ target\ -\ CT\ reference)}$, where C_T is the fractional threshold cycle. The level of *recA* mRNA was used as a reference. Manipulation of the c-di-GMP pool did not affect *recA* mRNA levels. The following primer combinations were used: HapA1010 and HapA1551 for *hapA* mRNA, HapR589 and HapR1046 for *hapR* mRNA, RecA578 and RecA863 for *recA* mRNA, RpoS576 and RpoS1003 for *rpoS* mRNA, and VpsT56 and VpsT252 for *vpsT* mRNA. A control mixture lacking reverse transcriptase was run for each reaction to exclude chromosomal DNA contamination.

Manipulation of the c-di-GMP pool. For manipulation of the c-di-GMP pool, the PDE VieA, the inactive PDE VieA(E170A), and the soluble DGC VCA0956 were overexpressed from the pBAD promoter of the araBAD (arabinose) operon. Plasmids encoding these enzymes (Table 1) were introduced into *V. cholerae* by electroporation as described in reference 36. For studying the effect

of c-di-GMP on HA/protease production and its regulators, HapR and RpoS, *V. cholerae* transformants were grown in TSB to an optical density at 600 nm (OD_{600}) of 2.0, the onset of HA/protease expression (2). At that time, cultures were divided in halves, and one half was induced with 0.2% L-arabinose and the second half was used as a control. Cultures were then incubated at 37°C with shaking for different times up to 16 h. Alternatively, plasmid pCMW75, encoding the *V. harveyi* DGC QrgB (54), was transformed into *V. cholerae* AJB503 and N16961-FLAG, and its expression was induced as described above but with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside).

Measurement of c-di-GMP content. Manipulation of the c-di-GMP pool was confirmed by direct measurement of the c-di-GMP pool as described in reference 54. Briefly, pelleted biomass samples containing the same number of cells were washed with cold phosphate-buffered saline (PBS), pH 7.4, and extracted twice with 40% methanol–40% acetonitrile–0.1N formic acid. The supernatants from each extraction were combined and dried in an Eppendorf Vacufuge plus vacuum concentrator instrument. The extracted material was reconstituted in 30 μl of PBS, and c-di-GMP was measured by using liquid chromatography–tandem mass spectrometry (LC-MS-MS) with an Applied Biosystems API 3200 triple-quadrupole mass spectrometer instrument operating in positive turbo ion spray mode. The mass spectrometer and high-performance liquid chromatography (HPLC) were controlled with Analyst 1.4.2 software in multiple-reaction-monitoring mode. The mass/charge (m/z) transitions used for c-di-GMP were m/z 689.2 to 344, m/z 344 to 150, m/z 150 to 133, and m/z 133 to 79. The dwell time was 40 ms for all mass transitions, and the ion spray voltage was −4,500 V. For chromatography of c-di-GMP, gradients were generated with a Waters Acquity UPLC system consisting of a vacuum degasser, binary pump, and temperature-controlled autosampler. The injection volume was 10 μl, and the samples were maintained in the autosampler at 4°C. A 1.0- by 100-mm Waters Acquity UPLC BEH-C18 column was used. Mobile phases A and B were 10 mM tributylamine, pH 4.95, and methanol, respectively. The gradient was 0 to 1.0 min, 0% B; 1.0 to 2.5 min, 20% B; 2.5 to 4.0 min, 20% B; 4.0 to 7.0 min, 65% B; 7.0 to 7.5 min, 95% B; 7.5 to 9.0 min, 95% B; 9.0 to 9.1 min, 0% B; and 9.1 to 15.0 min, 0% B, and the flow rate used was 0.075 ml/min. The concentration of c-di-GMP was calculated using a standard curve for c-di-GMP (Biolog Life Science Institute, Bremen, Germany) and was standardized by biomass wet weight.

Motility measurement. Manipulation of the c-di-GMP pool was also monitored by measuring flagellar motility. To this end, three independent cultures were stabbed into LB containing 0.3% agar (swarm agar) containing Cm with and without L-arabinose, and swarm diameters were measured after the plates were incubated at 30°C for 16 h.

Western blot analysis. In all experiments, a volume containing 0.5 OD_{600} units of cells was centrifuged and the pellet was resuspended in 0.1 ml of Laemmli sample buffer (Bio-Rad Laboratories). The cell suspension was placed in a boiling-water bath for 10 min, and the cell debris was removed by centrifugation. Proteins were separated using Criterion precast 10% gels (Bio-Rad) and subsequently transferred to polyvinylidene difluoride (PVDF) membranes. Expression of RpoS-FLAG was determined with monoclonal anti-FLAG M2-peroxidase (Sigma-Aldrich) and a BM bioluminescence Western blotting kit (Roche Applied Science). In parallel, gels were directly stained with Coomassie blue as a loading control.

Enzyme assays. Production of HA/protease was measured using an azocasein assay as described previously (2). One azocasein unit is the amount of enzyme that produces an increase of 0.01 optical density unit in the assay. β-Galactosidase activity was measured as described by Miller (39), using the substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG). Specific activities are given in Miller units {$1,000[OD_{420}/(t \times v \times OD_{600})]$}, where t is reaction time and v is the volume of enzyme extract per reaction. For the determination of PDE activity, cells were disrupted by sonication in PBS containing 1 mM phenylmethylsulfonyl fluoride and the cell debris was removed by high-speed centrifugation at 4°C. Ten microliters of crude extract was added to 0.1 ml of reaction buffer consisting of 50 mM Tris (pH 8.0), 20 mM $MgCl_2$, and 5 mM bis(*p*-nitrophenyl) phosphate. The enzymatic release of *p*-nitrophenol was followed by measurement of the OD_{410} every 5 min for 60 min in a Synergy microplate reader instrument at 37°C. One unit of activity was arbitrarily defined as the amount of enzyme causing an increase of 0.01 optical density unit and was standardized by protein concentration as determined with Bradford reagent (Bio-Rad Laboratories).

Protein purification and DNA binding assays. HapR and VqmA proteins were purified using IMPACT and pMAL protein fusion and purification systems from New England BioLabs, respectively. Binding of the above proteins to the *hapR* promoters was analyzed using a DIG gel shift kit (Roche Applied Sciences). A more detailed description of this method is provided as supplemental material.

TABLE 3. Effect of PDE VieA and DGC VCA0956 induction on c-di-GMP content

Strain	c-di-GMP content (pg/mg of wet wt)[a]
C7258	3.0 ± 0.6
C7258 ΔhapR (AJB51)	11.2 ± 2.3
C7258 ΔrpoS (AJB50)	10.8 ± 0.7
C7258 ΔhapR ΔrpoS (AJB52)	22.7 ± 1.9
C7258 + VieA control	2.6 ± 0.2
C7258 + VieA induced	0.7 ± 0.1
C7258 + VCA0956 control	2.9 ± 0.8
C7258 + VCA0956 induced	180.7 ± 9.7

[a] Cultures were grown to an OD_{600} of 2.0 in TSB medium induced with L-arabinose as required; c-di-GMP content was measured after a 4-h incubation period.

RESULTS

Cyclic di-GMP coordinately regulates the expression of HA/protease and motility. HA/protease and motility are both positively regulated by HapR and RpoS. The fact that c-di-GMP inhibits motility (5) prompted us to examine the effect of this second messenger on the expression of HA/protease. A major obstacle to this end is the redundancy of proteins involved in c-di-GMP biosynthesis and degradation. An approach pioneered by other investigators has been to examine the consequences of manipulating the intracellular c-di-GMP pool (5). In this study, we chose to overexpress the PDE VieA and DGC VCA0956 to diminish and increase the c-di-GMP pool, respectively. To confirm the effect of this manipulation. we measured c-di-GMP content by LC-MS-

MS. The c-di-GMP content of C7258 grown to a high cell density was low, and as expected, the deletion of *hapR* had a positive effect (Table 3). Interestingly, the deletion of *rpoS* also positively enhanced the c-di-GMP pool, while inactivation of both *rpoS* and *hapR* had an additive effect (Table 3). Overexpression of VieA at high cell density further reduced c-di-GMP content 3.7-fold with respect to the uninduced control. This finding corresponded with an increase in PDE activity in crude extracts from 178 to 450 units/mg protein. Induction of the DGC VCA0956 resulted in a 60-fold increase in c-di-GMP content under the same conditions (Table 3). In Fig. 1A, we show that diminishing the c-di-GMP pool by overexpressing VieA enhanced HA/protease expression, while increasing c-di-GMP by overexpressing VCA0956 had the opposite effect. As expected, overexpression of the VieA(E170A) active-site mutant had no effect, indicating that the phenotypic consequence of overexpressing VieA in our El Tor biotype strain is attributable to its encoded PDE activity. Finally, cultures were stabbed into swarm agar with and without L-arabinose to confirm that changes in HA/protease production were accompanied by the expected effect on motility (Fig. 1B). These results show that both HA/protease and motility are negatively regulated by c-di-GMP.

Cyclic di-GMP affects the expression of *hapA*, *hapR*, and *rpoS* mRNAs. Since the azocasein assay measures the amount of HA/protease secreted to the culture medium, we used qRT-PCR to determine if c-di-GMP affects *hapA* transcription. As shown in Fig. 2, changes in the c-di-GMP pool exhibited the same effect on *hapA* mRNA and azocasein production, sug-
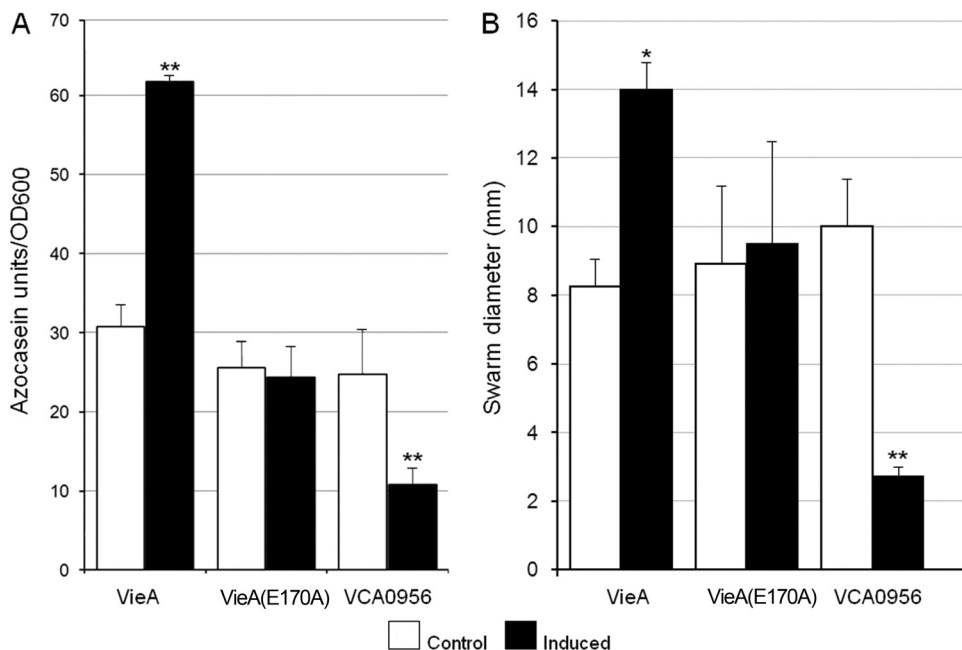

FIG. 1. Regulation of HA/protease production by c-di-GMP. (A) Production of HA/protease. Strain C7258, containing plasmids pAT1568 (VieA, EAL domain), pAT1615 [VieA(E170A), inactive EAL domain], and pAT1662 (VCA0956, GGDEF domain), was grown in TSB medium, and PDE or DGC expression was induced as described in Materials and Methods. Cultures were incubated for 16 h, and the production of HA/protease was determined. Each value represents the mean for three independent cultures. Error bars indicate standard deviations. ∗∗, $P <$ 0.01. (B) Motility assay. Overnight cultures of C7258 transformants were stabbed into swarm agar with or without (control) L-arabinose, and the plates were incubated at 30°C. Each of the resulting swarm diameters is the mean for three independent cultures. Error bars indicate standard deviations (∗, $P <$ 0.05; ∗∗, $P <$ 0.01 for comparison of induced versus control cultures).
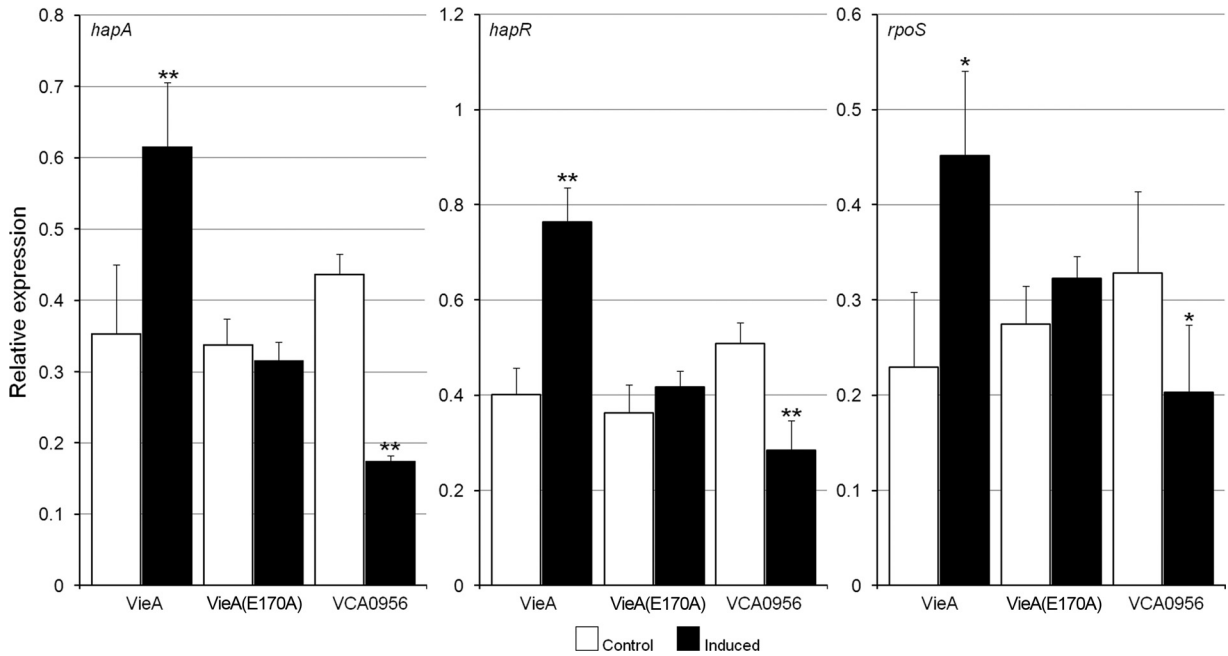
FIG. 2. Effect of manipulating the c-di-GMP pool on *hapA*, *hapR*, and *rpoS* mRNA expression. Strain C7258, containing plasmids expressing VieA, VieA(E170A), and VCA0956, was grown in TSB medium, and PDE or DGC expression was induced as described in Materials and Methods. Total RNA was extracted, and *hapA*, *hapR*, and *rpoS* mRNAs were measured by qRT-PCR. Each value represents the mean for three independent cultures. Error bars indicate standard deviations ($*$, $P < 0.05$; $**$, $P < 0.01$ for comparison of induced versus control cultures).

gesting that it acts by modulating *hapA* transcription. Given that transcription of *hapA* requires HapR and RpoS, we also examined whether manipulation of c-di-GMP content affected the expression of these regulators. As shown in Fig. 2, expression of both *hapR* and *rpoS* mRNAs was affected by c-di-GMP, exhibiting the same pattern as that observed for *hapA*, though this effect was more modest for *rpoS* mRNA.

Genetic analysis of HapR regulation by c-di-GMP. The results obtained in the qRT-PCR assay prompted us to determine if manipulation of c-di-GMP impacted HapR protein and activity. To this end, we introduced the VieA and VCA0956 expression vectors into strain SZS007, in which the chromosomal *lacZ* gene was replaced by a HapR-dependent *luxC-lacZ* transcriptional fusion (47). Manipulation of the c-di-GMP pool
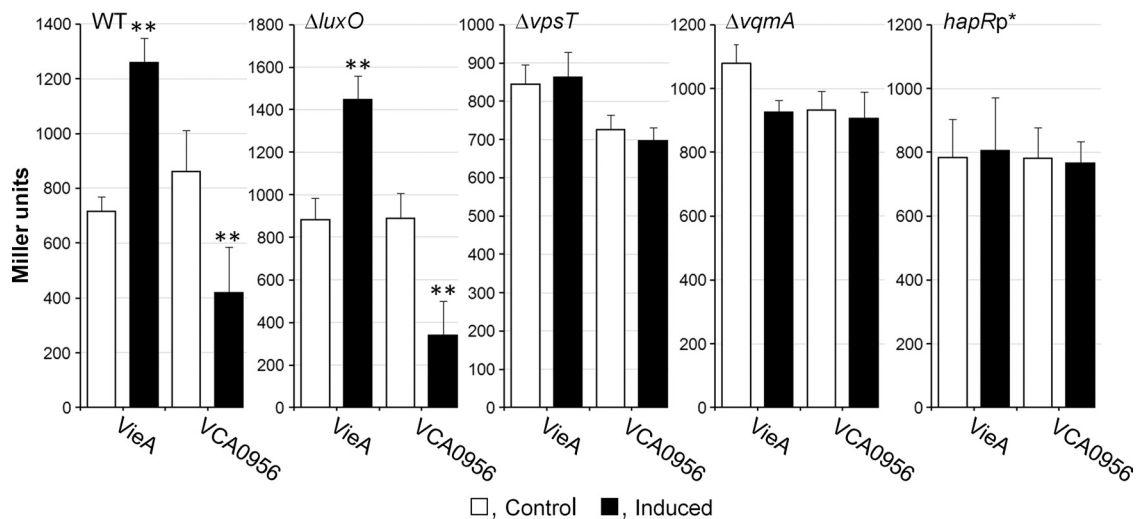

FIG. 3. Effect of c-di-GMP on HapR expression in different genetic backgrounds. Strain SZS007, containing a HapR-dependent *luxC-lacZ* fusion (WT), and the isogenic SZS012 (ΔluxO), HX02 (ΔvpsT), AJB505 (ΔvqmA), and HX01 (hapRp*) mutants were transformed with plasmids expressing VieA and VCA0956. Transformants were grown in TSB medium, PDE or DGC expression was induced as described in Materials and Methods, and β-galactosidase activity was determined. Each value represents the mean for three independent cultures. Error bars indicate standard deviations ($**$, $P < 0.01$ for comparison of induced versus control cultures).

in this strain reproduced the same regulatory pattern as that observed in the azocasein and qRT-PCR assays (Fig. 3). To determine if the effect of c-di-GMP on HapR expression involved quorum sensing, we introduced the VieA and VCA0956 expression vectors into strain SZS012, which contains a deletion of *luxO*. As shown in Fig. 3, manipulation of the c-di-GMP pool continued to modulate HapR expression in this genetic background, indicating that this effect is independent of LuxO. The LuxR-type regulator VpsT binds c-di-GMP to regulate motility (27). Therefore, we introduced a *vpsT* deletion into strain SZS007 and examined the effect of c-di-GMP on HapR expression in the mutant. As shown in Fig. 3, c-di-GMP had no effect on HapR expression in a strain that does not make VpsT. Gene expression profiling has identified VpsT as a negative regulator of HapR, induced when c-di-GMP concentrations are artificially increased (5, 6). Thus, we used qRT-PCR to determine if induction of VCA0956 enhanced VpsT under our conditions. The relative expression of *vpsT* 4 h after the induction of VCA0956 was 2.10 ± 0.33, compared to 0.36 ± 0.09 for the control ($n = 3$). Two cases of LuxO-independent regulation of HapR expression have been reported. In the first case, the LuxR-type transcriptional regulator VqmA was shown to positively regulate the *hapR* promoter at a low cell density (34). In the second case, HapR was found to negatively autoregulate its expression by binding to its own promoter (33, 48). To determine if c-di-GMP affected the above regulatory mechanisms, we constructed the *vqmA* deletion mutant AJB505 in the genetic background of the *luxC-lacZ* reporter SZS007. As shown in Fig. 3, manipulation of the c-di-GMP pool had no effect on HapR expression in a strain that does not make VqmA. Furthermore, we constructed strain HX01, which harbors a A→G mutation in the *hapR* promoter (*hapR*p*) reported to abolish HapR binding and autorepression (33). Interestingly, a single-base change in the *hapR* promoter eliminated the effect of manipulating the c-di-GMP pool on HapR expression (Fig. 3). Since both VqmA and HapR have been shown to bind specific regions within the *hapR* promoter, we also examined the possibility that c-di-GMP affects the binding of these regulators. However, c-di-GMP had no effect on HapR binding to its own promoter (see Fig. S1 in the supplemental material). In addition, we could not demonstrate binding of VqmA to the *hapR* promoter in the absence or presence of c-di-GMP, suggesting that this regulator could require an unidentified ligand other than c-di-GMP for effective binding to the *hapR* promoter or could act indirectly (see Fig. S1 in the supplemental material).

Since expression of *hapA* requires both HapR and RpoS (42), we measured the production of HA/protease in the Δ*luxO*, Δ*vpsT*, Δ*vqmA*, and *hapR*p* genetic backgrounds. Regulation of HA/protease by c-di-GMP was also found to be independent of LuxO (data not shown). Interestingly, mutations that abolished c-di-GMP regulation of HapR (Fig. 3) also eliminated its effect on HA/protease production (data not shown), even though induction of VCA0956 was found to diminish *rpoS* mRNA in the wild-type background (Fig. 2). These results suggest that the observed effect of c-di-GMP on the expression of RpoS could be an indirect consequence of c-di-GMP modulation of HapR. Thus, we further examined the role of HapR and c-di-GMP in the expression of RpoS.
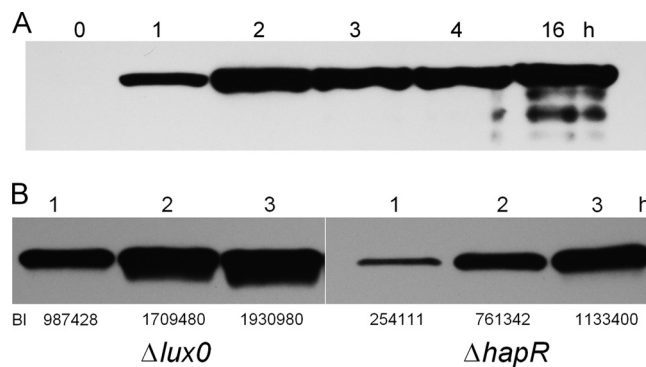

FIG. 4. Induction of RpoS in quorum-sensing mutants. (A) Strain AJB502, containing a chromosomally integrated *rpoS*-FLAG reporter, was grown to an OD_{600} of 2.0 (time zero), and samples were taken at different time points for Western blot analysis of RpoS-FLAG expression. (B) Strains AJB504 (Δ*luxO*) and AJB503 (Δ*hapR*), containing a chromosomally integrated *rpoS*-FLAG reporter, were grown to an OD_{600} of 2.0, and cells were collected at different time points for Western blot analysis of RpoS-FLAG expression. Band intensities (BI) were determined using Kodak 1D image analysis software.

Effect of HapR and c-di-GMP on the expression of RpoS protein. Since in *Escherichia coli* the expression of RpoS is subject to complex regulation at the level of transcription, translation, and protein stability (20), we devised an RpoS reporter that would allow us to monitor the expression of RpoS in *V. cholerae* at the protein level. To this end, we constructed strain AJB502, which expresses a C-terminal FLAG-tagged RpoS protein. The tagged *rpoS* allele was integrated by homologous recombination into the *rpoS* locus so that the expression of the FLAG epitope would reflect the production of RpoS from native transcription and translation initiation signals. The reporter system allowed us to determine the kinetics of RpoS expression. As shown in Fig, 4A, RpoS could be detected 1 h after the culture reached an OD_{600} of 2.0 in TSB medium. To determine if the expression of HapR could influence RpoS biosynthesis, we compared the expression of RpoS-FLAG in the isogenic Δ*luxO* (HapR constitutive) and Δ*hapR* strains. As shown in Fig. 4B, approximately 4 times less RpoS was detected in the Δ*hapR* mutant than in the Δ*luxO* strain at the 1 h time point. Expression of RpoS was approximately 50% reduced in the Δ*hapR* mutant at the later time points, suggesting that in the absence of HapR, induction of RpoS is delayed. A parallel Coomassie-stained gel was run to confirm that each well received a similar protein load (see Fig. S2 in the supplemental material).

Next, we examined the effect of directly manipulating the c-di-GMP pool on the expression of RpoS-FLAG. To this end, we grew strain AJB502, which contains the VieA and VCA0956 expression vectors, to an OD_{600} of 2.0 and added L-arabinose to diminish or increase the c-di-GMP pool, respectively. As shown in Fig. 5, although manipulation of the c-di-GMP pool affected *rpoS* expression in the qRT-PCR assay, it had a minor impact at the protein level measured using the RpoS-FLAG reporter. This result suggests that the positive effect of HapR on RpoS expression observed at high cell density (Fig. 4B) is not due to changes in c-di-GMP content.

The above result prompted us to consider if c-di-GMP could affect RpoS biosynthesis in the absence of quorum sensing.
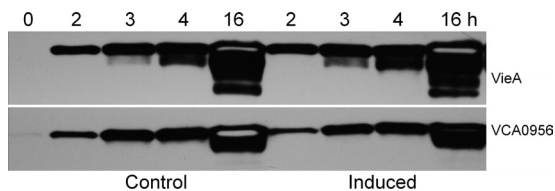
FIG. 5. Effect of c-di-GMP on RpoS expression at a high cell density. Strain AJB502, which produces *rpoS*-FLAG, was transformed with plasmids expressing VieA and VCA0956. Transformants were grown in TSB medium to an OD_{600} of 2.0 (time zero), and PDE or DGC expression was induced as described in Materials and Methods. Induction of RpoS-FLAG was determined by Western blotting.

FIG. 6. Regulation of RpoS expression by c-di-GMP in the absence of HapR. Strains AJB504 (Δ*luxO*), AJB503 (Δ*hapR*), and HX03 (Δ*hapR* Δ*vpsT*), containing the *rpoS*-FLAG reporter and the VCA0956 expression vector, as well as strains AJB503 and N16961-FLAG, containing the QrgB expression vector, were grown to an OD_{600} of 2.0 (time zero). At this stage, cultures were divided in halves; one half was induced by adding L-arabinose for VCA0956 or IPTG for QrgB, and the other half was used as a control. Samples were taken at different time points for Western blot analysis of RpoS-FLAG expression.

Thus, we compared the effect of increasing c-di-GMP on RpoS-FLAG expression in strains AJB504 (Δ*luxO*) and AJB503 (Δ*hapR*) locked in regulatory stages mimicking high and low cell densities, respectively. As shown in Fig. 6, increasing c-di-GMP in the Δ*luxO* mutant had a minor negative effect on RpoS expression. In contrast, increasing c-di-GMP in the Δ*hapR* mutant strongly inhibited RpoS expression. To exclude the possibility of VCA0956 inhibiting RpoS expression in this genetic background by a mechanism unrelated to its DGC activity, we introduced plasmid pCMW75, which encodes DGC QrgB, into strain AJB503 (Δ*hapR*). The *V. cholerae* genome does not encode a homolog of this DGC. Again, induction of QrgB by the addition of IPTG strongly inhibited expression of RpoS-FLAG (Fig. 6). We also examined the effect of c-di-GMP on RpoS expression in a Δ*hapR* Δ*vpsT* double mutant. In contrast to c-di-GMP regulation of HapR, significant c-di-GMP-mediated repression of RpoS in the strain lacking *hapR* and *vpsT* was observed (Fig. 6). Finally, we modified strain N16961, which contains a natural frameshift mutation in *hapR*, to express the RpoS-FLAG reporter. As shown in Fig. 6, over-expression of QrgB in strain N16961-FLAG resulted in a substantial delay in full RpoS induction. Taken together, our results suggest that under conditions in which HapR is not expressed, c-di-GMP negatively regulates the expression of RpoS.

DISCUSSION

In this study, we examined the role of c-di-GMP in the regulation of HA/protease, known to affect cholera pathogenesis in animal models and to contribute to live-vaccine reactogenicity (3, 43). As is the case with motility, expression of HA/protease increased upon lowering of the c-di-GMP pool and decreased upon overexpression of a DGC. This result suggests that HA/protease and motility are coordinately regulated by c-di-GMP. HA/protease and motility have been proposed to facilitate *V. cholerae* detachment from the intestinal mucosa (4, 14, 40, 43, 44). The coordinate regulation of these phenotypes by CRP, HapR, RpoS, and c-di-GMP suggests that they could be simultaneously expressed in the host to modulate pathogenesis. *V. cholerae* possesses a single polar flagellum and is unlikely to swim in a highly viscous medium (1). It has been reported that the cholera bacterium loses its flagellum in a viscous mucin-containing medium (35). We have proposed that production of extracellular proteases could facilitate preservation of the flagellum of *V. cholerae* during detachment by
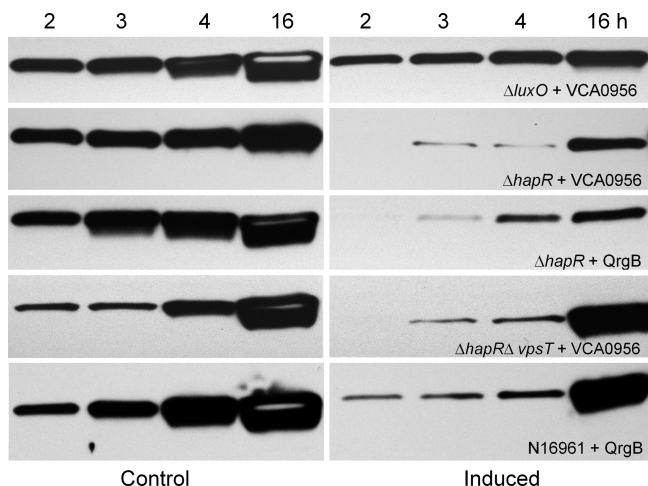
decreasing the viscosity of the medium (44). This could result from HA/protease degradation of preexisting mucin (13) and cleavage of the GbpA adhesin, which has also been reported to enhance the production of intestinal mucins (7, 25). Since it is well established that motility and low c-di-GMP content promote intestinal colonization (28, 50), the coordinate expression of HA/protease and motility could facilitate the detachment of motile-stage *V. cholerae* cells, which could readily swim toward and colonize additional sites along the gastrointestinal tract.

The expression of HapR is fine-tuned through the action of numerous regulators and regulatory loops that can function under different physiological conditions. For instance, in addition to regulation by small RNAs (sRNAs) (29, 48), HapR levels can be modulated by autorepression, VqmA, VpsT, and VpsR (6, 33, 34, 54, 58). In this study, we report that expression of HapR measured by either qRT-PCR or by using an HapR-dependent *luxC-lacZ* fusion is negatively modulated by c-di-GMP content. Our data are consistent with the finding that a deletion mutant lacking the PDE CdgC (expected to exhibit elevated c-di-GMP content) expressed a lower level of HapR (32). We found that c-di-GMP acts downstream of LuxO to negatively affect HapR expression. Deletion of *vqmA* or a single-base change in the *hapR* promoter that abolished autorepression (33) eliminated the negative effect of c-di-GMP on HapR expression. Importantly, we found that c-di-GMP regulation of HapR was eliminated by the deletion of VpsT, known to directly sense c-di-GMP (27). Thus, our data suggest that c-di-GMP may repress HapR expression indirectly by binding to VpsT and activating it (5, 27). VpsT could then act through VqmA or other regulators in a LuxO-independent manner to diminish HapR expression. We also considered the possibility that c-di-GMP directly interacts with HapR, known to contain a binding pocket for an unknown ligand (10), and/or with VqmA to regulate *hapR* expression. However, we did not

find c-di-GMP to influence binding of HapR to its own pro-
moter, nor could we demonstrate VqmA binding to this pro-
moter in the absence or presence of c-di-GMP (see the sup-
plemental material). The relationship between HapR
autorepression and HapR regulation by c-di-GMP is intrigu-
ing. Conceivably, binding of HapR to its own promoter might
be part of a more complex regulation involving additional
factors modulated by c-di-GMP content.

Deletion of *vqmA* did not significantly impact the expression
of HapR in uninduced cultures. This result was expected, given
that our studies were conducted at a high cell density, when
VqmA has no effect on *hapR* transcription (34). In contrast to
previous studies (33, 48), elimination of HapR autorepression
in strain C7258 did not result in elevated HapR levels in the
stationary phase. However, we did confirm that HapR purified
from C7258 binds to the wild-type promoter but not to the
mutant promoter resistant to autorepression (see the supple-
mental material). A possible explanation for this difference is
that the *luxC-lacZ* fusion used in our study reflects the accu-
mulation of active HapR protein, while previous studies used
hapR-lacZ and *hapR*-GFP promoter and translational fusions
(33, 48). Recently, it was shown that HapR is additionally
regulated at the level of activity (53).

Both lowering and increasing the c-di-GMP pool affected
rpoS expression in the qRT-PCR assay. To monitor RpoS
protein expression, we constructed a reporter strain expressing
RpoS-FLAG. The kinetics of RpoS-FLAG expression closely
paralleled the time course of HA/protease expression in the
same medium (2). Furthermore, induction of RpoS-FLAG
biosynthesis closely mimicked the induction of native RpoS in
genetically unmodified C7258 when a polyclonal RpoS antise-
rum was used (data not shown). Differences in *rpoS* mRNA
levels induced by c-di-GMP did not result in different RpoS
protein levels in the Western blot analysis. The lack of corre-
lation between mRNA and protein levels is not completely
unexpected in this case, due to the complex regulation of RpoS
at the levels of transcription, translation, and protein stability
(20). Consistent with c-di-GMP having no effect on RpoS lev-
els in the wild-type strain at a high cell density, elimination of
c-di-GMP modulation of HapR was sufficient to abolish its
regulation of RpoS-dependent HA/protease production. Nev-
ertheless, we did show that expression of HapR had a positive
effect on RpoS biosynthesis, though this regulation is not likely
to involve c-di-GMP signaling. This finding is in agreement
with a previous study showing that an *rpoS-lacZ* fusion exhib-
ited reduced expression in a *hapR* mutant (23). In the absence
of quorum sensing, increasing the c-di-GMP content severely
inhibited RpoS biosynthesis. Interestingly, deletion of *vpsT* in
the *hapR* generic background did not abolish c-di-GMP repres-
sion of RpoS, suggesting that c-di-GMP modulation of HapR
and RpoS involves distinct mechanisms. We suggest that ex-
pression of HapR in the wild-type genetic background could
oppose the negative effect of high c-di-GMP content on RpoS
expression in several ways. For instance, as shown above,
HapR has a positive effect on the expression of RpoS protein
at a high cell density that is independent of c-di-GMP. In
addition, HapR could repress a component of the c-di-GMP
signaling pathway that affects RpoS expression or indirectly
prevent binding of an unknown regulator activated by c-di-
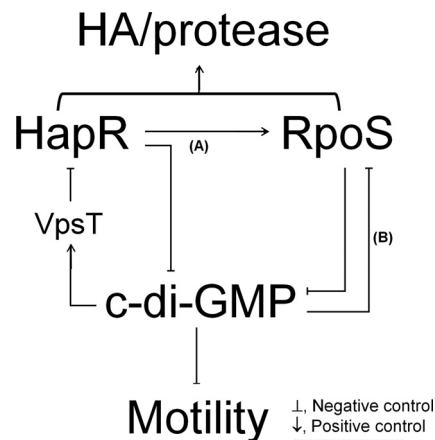GMP to the *rpoS* promoter. As expected, increasing c-di-GMP

FIG. 7. Model for the interplay between c-di-GMP, HapR, and
RpoS in the regulation of HA/protease expression. The second mes-
senger c-di-GMP negatively affects the expression of HapR. This reg-
ulation is independent of LuxO but requires the c-di-GMP binding
protein VpsT and VqmA. At a high cell density, HapR enhances RpoS
(pathway A) and both regulators diminish the c-di-GMP pool, gener-
ating a double-negative regulatory loop that further enhances the
expression of HapR, HA/protease production, and motility (see the
text for details). We propose that in the absence of quorum sensing,
c-di-GMP also inhibits the expression of RpoS (pathway B).

inhibited RpoS expression in strain N16961, which contains a
natural frameshift mutation in *hapR*. Taken together, our re-
sults suggest that c-di-GMP functions as a negative regulator of
RpoS expression when HapR is not expressed or in *V. cholerae*
strains exhibiting dysfunctional quorum-sensing systems. *V.
cholerae* strains with dysfunctional quorum-sensing systems are
very common among environmental and clinical isolates and
have been shown to arise with high frequency under laboratory
conditions (16, 24).

It has been reported that expression of HapR diminishes the
c-di-GMP pool (17, 54). In this study, we showed that expres-
sion of RpoS also contributes to lowering of the intracellular
c-di-GMP content. In *Escherichia coli*, many GGDEF and
EAL domain proteins are regulated by RpoS (19). In *V. chol-
erae*, VCA0080, VC1986 (GGDEF domain family), VC1087,
and VC2340 (HD-GYP domain family) were found to be dif-
ferentially expressed in an *rpoS* mutant (40). The elevated
c-di-GMP content observed in our Δ*rpoS* mutant is consistent
with the fact that *rpoS* mutants are less motile than the wild
type and express lower levels of numerous genes involved in
flagellar motility and chemotaxis (40, 45).

Since HapR acts to diminish the intracellular c-di-GMP pool
(17, 54), our findings suggest the occurrence of a double-
negative regulatory loop mediated by VpsT, which enhances
the expression of HA/protease at a high cell density (Fig. 7).
Briefly, (i) expression of HapR at a high cell density results in
lower c-di-GMP content (17, 54); (ii) the lowering of c-di-GMP
content further enhances HapR, generating a double-negative
regulatory loop; (iii) enhanced expression of HapR positively
impacts the biosynthesis of RpoS (pathway A); (iv) expression
of RpoS feeds into the regulatory loop by diminishing c-di-
GMP content; and (v) concurrent activation of HapR and
RpoS results in elevated expression of HA/protease and mo-
tility. Cyclic di-GMP also inhibits RpoS expression in the ab-

sence of HapR (pathway B). Pathway B could be significant under conditions in which *V. cholerae* cells could enter the stationary phase prior to activation of quorum sensing (i.e., nutrient limitation) or in strains with dysfunctional quorum-sensing systems. We propose that the above model for the coordinate expression of HA/protease and motility by HapR, RpoS, and c-di-GMP could contribute to the dissemination of colonizing *V. cholerae* cells along the gastrointestinal tract in terms of multiple cycles of detachment and recolonization.

ACKNOWLEDGMENTS

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