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The Cyclic AMP Receptor Protein Modulates Colonial Morphology in *Vibrio cholerae*[▽]

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Inactivation of the quorum-sensing regulator HapR causes *Vibrio cholerae* El Tor biotype strain C7258 to adopt a rugose colonial morphology that correlates with enhanced biofilm formation. *V. cholerae* mutants lacking the cyclic AMP (cAMP) receptor protein (CRP) produce very little HapR, which results in elevated expression of *Vibrio* exopolysaccharide (*vps*) genes and biofilm compared to the wild type. However, Δ*crp* mutants still exhibited smooth colonial morphology and expressed reduced levels of *vps* genes compared to isogenic *hapR* mutants. In this study we demonstrate that deletion of *crp* and *cya* (adenylate cyclase) converts a rugose Δ*hapR* mutant to a smooth one. The smooth Δ*hapR* Δ*crp* and Δ*hapR* Δ*cya* double mutants could be converted back to rugose by complementation with *crp* and *cya*, respectively. CRP was found to enhance the expression of VpsR, a strong activator of *vps* expression, but to diminish transcription of VpsT. Ectopic expression of VpsR in smooth Δ*hapR* Δ*crp* and Δ*hapR* Δ*cya* double mutants restored rugose colonial morphology. Lowering intracellular cAMP levels in a Δ*hapR* mutant by the addition of glucose diminished VpsR expression and colonial rugosity. On the basis of our results, we propose a model for the regulatory input of CRP on exopolysaccharide biosynthesis.

Cholera is a paradigm waterborne disease caused by *Vibrio cholerae* serogroups O1 and O139, which continue to cause seasonal outbreaks in heavily populated regions in Asia, Africa, and Latin America. *V. cholerae* is transmitted through contaminated food and drinking water as well as person to person through the fecal-oral route. The existence of an aquatic reservoir of serogroup O1 and O139 toxigenic strains has not been established. However, their capacity to survive and persist in estuarine and brackish waters is widely accepted (6, 8, 22). *V. cholerae* has been shown to alternate between a free-swimming planktonic lifestyle and biofilm communities attached to biotic and abiotic surfaces. In the biofilm lifestyle, *V. cholerae* has been found in association with phytoplankton and zooplankton (11, 12). In addition, large clumps of aggregated partially dormant *V. cholerae* cells can be detected in surface water as biofilms that resist cultivation in conventional microbiological media (7). These aggregates can be recovered as virulent *V. cholerae* cells by inoculation into rabbit ileal loops (7).

It has been shown that *V. cholerae* cells in biofilm communities are more resistant to environmental stresses and protozoan predation (11, 12, 13, 18, 31). The *V. cholerae* rugose colonial morphology variant initially described by White (27) has been shown to produces more exopolysaccharide and biofilm than the smooth colonial variant (28). Furthermore, the rugose variant has been demonstrated to be more resistant to chlorinated water (20, 21, 28) and osmotic and oxidative stresses than the smooth variant (26, 28).

The rugose colonial morphology has been demonstrated to be a highly multifactorial phenotype subject to complex regulatory mechanisms. The genes responsible for exopolysaccharide biosynthesis (*vps*) are clustered in two operons in which *vpsA* and *vpsL* are the first genes of operons I and II, respectively (28). Regulation of *vps* genes involves cyclic diguanylate signaling (16, 17, 25), the positive regulators VpsT (3) and VpsR (29), and the negative regulator CytR (10). In some El Tor biotype strains, the quorum-sensing regulator HapR negatively affects biofilm formation and rugosity by repressing the expression of *vps* genes (9, 30, 31). As a result, *hapR* mutants of these strains exhibit enhanced biofilm formation and rugose colonial morphology. In addition, a flagellum-dependent *vps* signaling cascade has been reported in some El Tor biotype strain (14). Finally, a third *vps* signaling pathway initiated by nutrient starvation has been hypothesized for strain N16961; the absence of *hapR* and *flaA* genes in this strain does not produce rugose colonies (14).

We have shown that the cyclic AMP (cAMP) receptor protein (CRP) is required for the expression of cholera autoinducer I (15). Accordingly, deletion of *crp* in *V. cholerae* C7258 (El Tor, Ogawa, 1991 Perú isolate) resulted in very low expression of the quorum-sensing regulator HapR (15, 23). The *V. cholerae* Δ*crp* mutant expressed elevated *vpsA*, *vpsL*, and biofilm compared to the wild type (15). However, although the Δ*crp* mutant expressed extremely low levels of *hapR* mRNA, it produced less *vpsA* and *vpsL* mRNA compared to an isogenic rugose Δ*hapR* mutant and displayed smooth colonial morphology. These results suggested that CRP could be required for the expression of other factors required for maximal exopo-

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TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or sequence
Strains	
C7258	Wild type, El Tor biotype (Perú isolate, 1991)
WL7258	C7258 Δ*crp* (15)
AJB51	C7258 Δ*hapR* (15)
WL51	C7258 Δ*hapR* Δ*crp* (15)
WL52	C7258 Δ*hapR* Δ*cya* (this study)
Plasmids	
pTTVpsR	1.4-kb BamHI-EcoRI DNA fragment encoding *vpsR* ORF in pTTQ18
pBADCRP7	*crp* ORF cloned in pBADHisB (23)
pCya1	0.9-kb EcoRI-BamHI DNA fragment 5′ of *cya* ORF in pUC19
pCya2	0.8-kb BamHI-XbaI DNA fragment 3′ of *cya* ORF in pUC19
pΔCya	EcoRI-BamHI and BamHI-XbaI fragments from pCya1 and pCya2 in pUC19
pΔCya-Km	1.2-kb BamHI Km^r cassette from pUC4K cloned in pΔCya
pCVDΔCya-Km	2.9-kb EcoRI/Klenow-XbaI fragment from pΔCya-Km in pCVD442
pTT-Cya	Cya ORF in pTTQ18
Primers	
CytR295	5′-CAACAGAAGCGTCGGGAGAACTC
CytR421	5′-CGCAAATTCACACGCCATCACCAT
Cya7	5′-CGGAATTCCTTGCAGGCTTATAC TCAG
Cya902	5′-CGGGATCCAGTCCATGCCGTAC AGAT
Cya1759	5′-CGGGATCCTGGTGCTGAGCAGA AATG
Cya2567	5′-GCTCTAGAGATTTAGAAAACCTGA GACG
RecA578	5′-GTGCTGTGGATGTCATCGTTGTTG
RecA863	5′-CCACCACTTCTTCGCCTTCTTTGA
VpsA434	5′-ACCACTTTGCACCTACAGATACTTC
VpsA676	5′-CGGTAGTGATCAGCGCTTGGCAA
VpsL607	5′-ACTGGGCAGGTGCAAAATGTC TATA
VpsL775	5′-AGGGGGTATCAAAAATGCTAA ACGC
VpsR75	5′-GGCTGTGTTGGAAAAAGTGG GTTG
VpsR206	5′-GGCTACTCACCAAATTCGCAATCC
VpsR78	5′-GCGAATTCCATGAGCACTCAATTC CGTA
VpsR1431	5′-GCGGATCCCAAGGTAAATCAGCA AAAC
VpsT56	5′-CCAGATTGTTGAAAGAGGCGTTAG
VpsT252	5′-TGCGGACAGTTTATGATGACCTCT

lysaccharide biosynthesis. In this study we address the relationship between CRP, HapR, and colonial morphology. To this end, we constructed isogenic Δ*crp*, Δ*hapR*, and Δ*hapR* Δ*crp* deletion mutants in the genetic background of the smooth strain C7258. The construction of these mutants (Table 1) has been described previously (15). We prepared colonies from LB agar plates for scanning electron microscopy as described earlier (28). As shown in Fig. 1A, the Δ*crp* mutant exhibited smooth colonial morphology, while the Δ*hapR* mutant AJB51 (Fig. 1B) was rugose. Furthermore, deletion of *crp* from the rugose Δ*hapR* mutant yielded a smooth Δ*hapR* Δ*crp* double mutant (Fig. 1C). This result is consistent with the finding that Δ*crp* and Δ*hapR* Δ*crp* mutants expressed less *vpsA* and *vpsL* than the isogenic Δ*hapR* mutant did (15). To confirm that the smooth colony morphology of strain WL51 (Δ*hapR* Δ*crp*) was due to deletion of *crp*, we restored the active *crp* allele by transformation with pBADCRP7 (Table 1). The transformant exhibited the rugose colonial morphology of the Δ*hapR* single mutant (Fig. 1D). Since the activity of CRP is determined by intracellular cAMP levels (4), we constructed a Δ*hapR* Δ*cya* double mutant lacking adenylate cyclase. To this end, chromosomal DNA flanking the *cya* gene was amplified using primer pairs Cya7/Cya902 and Cya1759/Cya2567. These fragments and a Km^r gene from pUC4K (GenBank accession no. X06404) were sequentially cloned in pUC19 to yield pΔCya-Km (Table 1). The *cya* deletion/insertion was transferred to pCVD442 (5) and propagated in the permissive *Escherichia coli* strain SM10λpir (19). The resulting suicide vector pCVDΔCya-Km (Table 1) was transferred to strain AJB51 by conjugation, and the Δ*hapR* Δ*cya* mutant WL52 was obtained by sucrose selection as described previously (15). We show that, similar to the Δ*hapR* Δ*crp* mutant, the Δ*hapR* Δ*cya* double mutant exhibited smooth colonial morphology (Fig. 1E). For complementation, the *cya* open reading frame (ORF) was amplified using primers Cya7 and Cya2567 and cloned in the

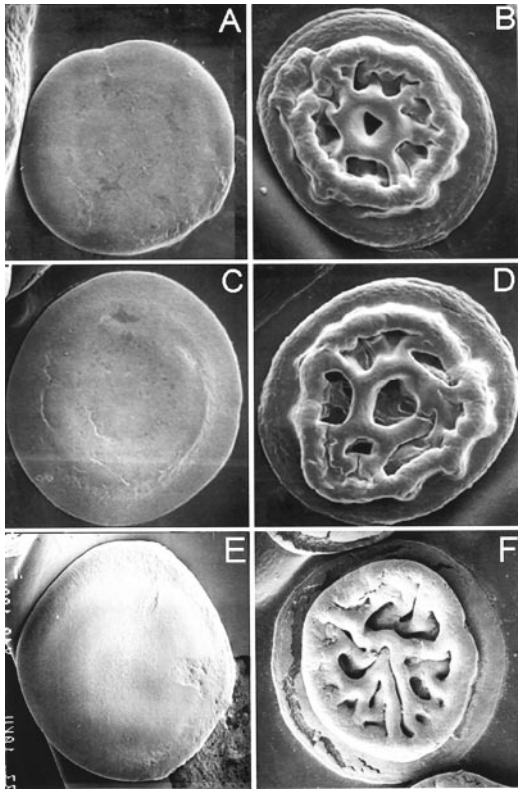

FIG. 1. Scanning electron microscopy of Δ*crp*, Δ*cya*, and Δ*hapR* colonies. (A) Strain WL7258 (Δ*crp*); (B) strain AJB51 (Δ*hapR*); (C) strain WL51 (Δ*hapR* Δ*crp*); (D) WL51 transformed with pBADCRP7; (E) WL52 (Δ*hapR* Δ*cya*); (F) WL52 (Δ*hapR* Δ*cya*) transformed with pTT-Cya. Strains were streaked onto LB agar, and colonies were allowed to develop at 30°C for 24 h. Expression of *crp* was induced by the addition of 0.02% L-arabinose. Scanning electron microscopy was performed with a magnification of ×50.

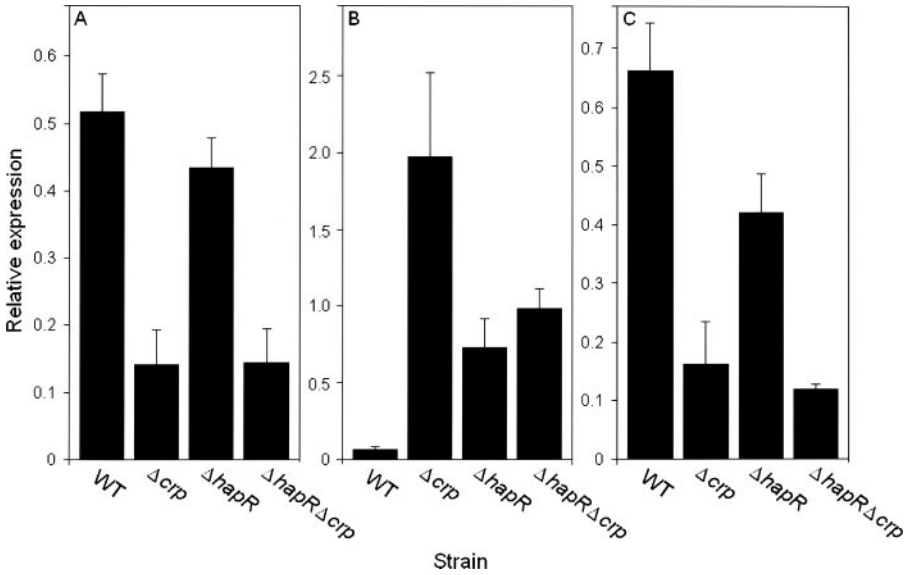

FIG. 2. Expression of regulators of rugose colonial morphology in *V. cholerae* Δ*hapR* and Δ*crp* mutants. Strains C7258 (wild type [WT]), AJB51 (Δ*hapR*), WL7258 (Δ*crp*), and WL51 (Δ*hapR* Δ*crp*) were grown in LB medium to an OD_{600} of 1.5. Abundance of mRNAs encoding *cytR* (A), *vpsT* (B), and *vpsR* (C) was determined by qRT-PCR. Results were normalized to *recA* mRNA expression. Error bars indicate the standard deviations of at least three independent cultures.

expression vector pTTQ18 (24) to yield pTT-Cya (Table 1). Transformation of the Δ*hapR* Δ*cya* strain with plasmid pTT-Cya expressing *cya* from the Tac promoter restored the rugose colonial phenotype even without the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) (Fig. 1F). These results clearly establish a role for the cAMP-CRP complex in regulating *V. cholerae* colonial variation.

We first considered the possibility of cAMP-CRP directly interacting with the *vpsA* and *vpsL* promoters. However, analysis of the DNA sequence located upstream of *vpsA* and *vpsL* using the Virtual Footprint software program (http://www.prodoric.de/vfp) did not reveal any cAMP-CRP binding site. Therefore, we hypothesized that cAMP-CRP modulation of *vpsA* and *vpsL* is due to cAMP-CRP modulation of HapR and other factors required for maximal expression of *vps* genes. To investigate the mechanisms by which cAMP-CRP affects colonial morphology, we measured the expression of several regulators of *vps* gene expression in Δ*hapR* and Δ*crp* single and double mutants using quantitative real-time reverse transcription-PCR (qRT-PCR). Because it is well established that *hapR* is expressed and functions in cells at a high cell density (9, 31), *V. cholerae* strains were grown in LB at 37°C, and cells were collected at an optical density at 600 nm (OD_{600}) of 1.5. Total RNA was isolated using the RNeasy kit (Qiagen Laboratories) combined with DNase treatment on column and qRT-PCR conducted using the iScript two-step RT-PCR kit with SYBR green (Bio-Rad Laboratories). Relative expression values (R) were calculated using the equation $R = 2^{-(\Delta C_T \text{ target} - \Delta C_T \text{ reference})}$ where C_T is the fractional threshold cycle and *recA* mRNA was used as internal reference. Gene-specific primers for *cytR*, *vpsT*, and *vpsR* used in this analysis are listed in Table 1. The Δ*crp* mutant expressed reduced levels of CytR, a repressor of biofilm formation (10) (Fig. 2A). Analysis of the DNA sequence located upstream of the *cytR* ORF using the Virtual Footprint software program revealed the presence of two sites closely matching the $TGTGAN_6TCANA$ cAMP-CRP consensus binding sites. This result suggests that cAMP-CRP could act directly at the *cytR* promoter to activate its transcription. The *cytR* promoter contains a putative binding site for HapR, and it has been suggested that HapR activates *cytR* (30). However, *cytR* mRNA levels were unaffected by the deletion of *hapR* under our experimental conditions (Fig. 2A). This discrepancy could be due to the use of different strains and to the fact that in our study, RNA was extracted from cells grown to a higher cell density (OD_{600} of 1.5). In the preceding study, cells were harvested at a different physiological stage (OD_{600} of 0.3 to 0.4) (30). This difference would be expected to impact the expression and activity of HapR and CRP, respectively.

The Δ*crp* mutant expressed elevated *vpsT* (Fig. 2B), a positive regulator of rugose colonial morphology (3). However, no cAMP-CRP binding sites were found upstream of *vpsT*, suggesting that CRP modulates expression of this gene indirectly. Deletion of *hapR* resulted in elevated *vpsT* mRNA, suggesting that HapR represses *vpsT* as reported earlier (30). It is noteworthy that the deletion of *crp* had little effect on *vpsT* expression in the Δ*hapR* background, suggesting that the CRP modulation of VpsT could be partly due to CRP modulation of HapR.

The Δ*crp* mutants expressed little VpsR (Fig. 2C), which has been reported to be a strong activator of *vpsA* and *vpsL* (29). No potential cAMP-CRP binding sites could be detected upstream of the *vpsR* coding sequence. It has been recently reported that VpsR is a stronger activator of *vps* expression than VpsT (2). Thus, the lower expression of VpsR in the Δ*hapR* Δ*crp* mutant could explain why inactivation of CRP in the Δ*hapR* background tends to diminish expression of *vpsA* and *vpsL* and convert the rugose Δ*hapR* mutant to the smooth form. This interpretation is in agreement with the finding that Δ*hapR* Δ*vpsR* double mutants display smooth colonial morphology (2). We have observed that the deletion of *crp* in the Δ*hapR* background has a more pronounced effect on *vpsL*,

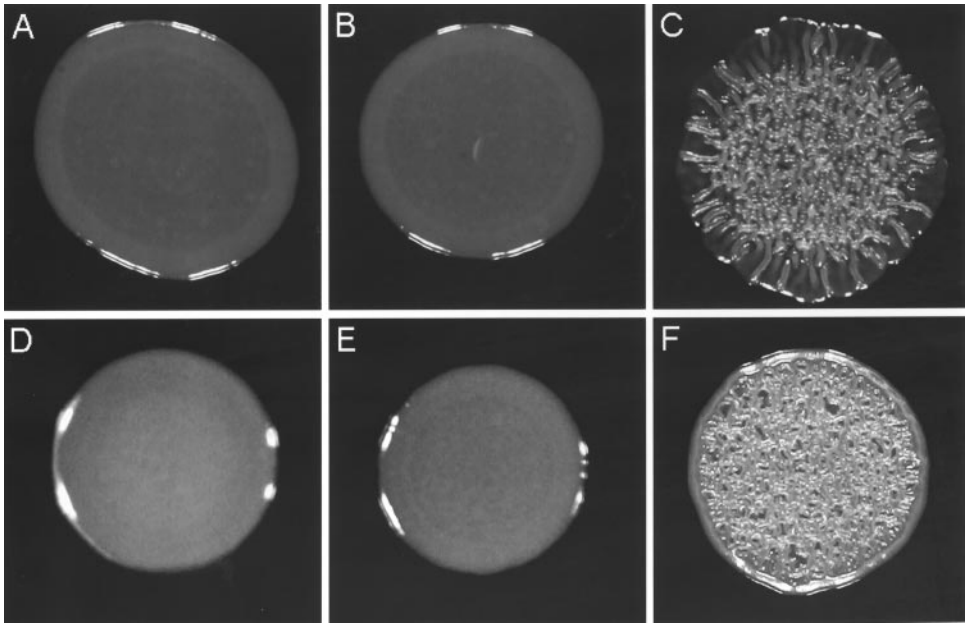

FIG. 3. Spot colonial morphology of Δ*crp* and Δ*cya* mutants expressing VpsR. Overnight cultures of strain WL51 (Δ*hapR* Δ*crp*) and WL52 (Δ*hapR* Δ*cya*) were diluted 1:200, and 2 μl of each dilution was spotted onto LB agar containing 100 μg/ml ampicillin as required. (A) Strain WL51 with empty vector pTTQ18; (B) WL51 with pTTVpsR; (C) WL51 with pTTVpsR induced with 10 mM IPTG; (D) strain WL52 with empty vector pTTQ18; (E) WL52 with pTTVpsR; (F) WL52 with pTTVpsR induced with 10 mM IPTG. Plates were incubated for 24 h at 30°C, and colonies were photographed with a Canon EOS camera with a 60-mm macro lens.

which contains a putative VpsR binding motif (30). It is noteworthy that the deletion of *crp* diminished VpsR expression in both the wild-type and Δ*hapR* backgrounds, but a negative effect on *vpsA* and *vpsL* expression was observed only in the Δ*hapR* background. We propose that the down-regulation of HapR and CytR and overexpression of VpsT in the Δ*crp* mutant partly compensates for reduced expression of VpsR resulting in a discrete increase in *vps* expression that is not big enough to switch the colonial morphology to rugose. We did not observe repression of *vpsR* by HapR in our study (Fig. 2C). In fact, the Δ*hapR* mutant expressed slightly less VpsR (Fig. 2C). These results contrast with a previous report suggesting that HapR represses VpsR in a different strain (2, 30). Strain differences and the cell density of cultures could be responsible for these discrepancies. Occurrence of strain variation is further suggested by the fact that no repression of VpsR by HapR was observed in mutants derived from strain C6706 of the El Tor biotype (9).

Since deletion of *crp* resulted in significantly lower *vpsR* expression in the Δ*hapR* background (Fig. 2C), we considered the possibility of low *vpsR* expression being responsible for the smooth colonial morphology of Δ*hapR* Δ*crp* and Δ*hapR* Δ*cya* mutants. To test this hypothesis, we used primers VpsR78 and VpsR1431 to clone *vpsR* in plasmid pTTQ18 (24). The resulting plasmid, pTTVpsR (Table 1), expresses VpsR under the control of the inducible Tac promoter. Expression of VpsR from a heterologous promoter restored colonial rugosity to Δ*hapR* Δ*crp* and Δ*hapR* Δ*cya* double mutants (Fig. 3C and F).

CRP plays a key role in carbon catabolite repression, a process by which the presence of a favorable carbon source (i.e., glucose, fructose, or sucrose) in the medium inhibits gene expression and/or activity of enzymes involved in the catabolism of other carbon sources (4). We have observed that the addition of 5% glucose to LB agar but not glycerol inhibited expression of rugose colonial morphology in the Δ*hapR* mutant AJB51 (data not shown). We hypothesized that lowering intracellular cAMP levels by the addition of glucose should abolish expression of rugose morphology by reducing VpsR expression. To test this hypothesis, we extracted RNA from cells grown with and without 5% glucose and performed qRT-PCR. As shown in Fig. 4, glucose significantly diminished expression

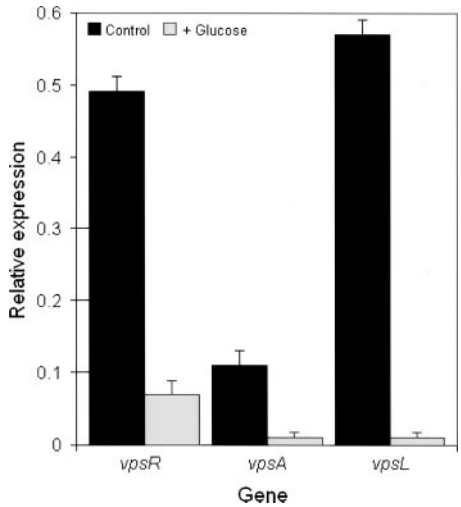

FIG. 4. Repression of VpsR by glucose. Strain WL51 (Δ*hapR*) was grown in LB (control) and LB supplemented with 5% glucose. Expression of *vpsR*, *vpsA*, and *vpsL* was determined by qRT-PCR. Error bars indicate the standard deviations of at least three independent cultures.

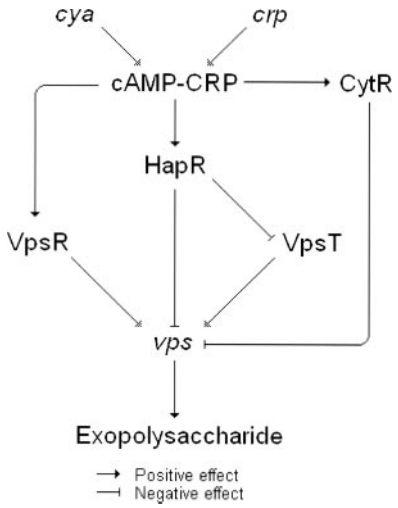

FIG. 5. Model for the regulatory input of *cya* and *crp* in exopolysaccharide biosynthesis and rugose colonial morphology.

of *vpsR*. We used primer pairs VpsA434/VpsA676 and VpsL607/VpsL775 and qRT-PCR to determine the expression of the *vpsA* and *vpsL* genes, which are located downstream of *vpsR*. As expected, glucose repression of VpsR was reflected in significantly reduced expression of *vpsA* and *vpsL* (Fig. 4). These results further confirm a role for cAMP and CRP in the modulation of *V. cholerae* colonial morphology phase. The inhibition of the rugose colonial morphology by sugars has been previously reported (1). Our results suggest that carbon catabolite repression participates among other factors in the regulation of exopolysaccharide biosynthesis.

Our results add new information to the highly complex regulatory circuitry controlling exopolysaccharide biosynthesis and colonial rugosity in *V. cholerae*. In Fig. 5, we propose a model for the regulatory input of cAMP-CRP in exopolysaccharide biosynthesis and rugose colonial morphology. Deletion of *crp* results in diminished expression of the positive regulator VpsR, which is required for expression of rugose colonial morphology (Fig. 2C). However, this event is partially compensated for by reduced expression of the negative regulators HapR (15) and CytR (Fig. 2A) and overexpression of VpsT (Fig. 2B). As a result, Δ*crp* mutants express elevated *vpsA* and *vpsL* than the wild type does (15), but this increase is not big enough to induce rugose colonial morphology. The reduced motility of Δ*crp* mutants could be a second mechanism compensating for the reduced expression of VpsR. We have shown that the Δ*crp* mutant WL7258 is less motile than the wild type and expresses reduced FlaA (15). Inactivation of *flaA* has been reported to enhance the expression of *vps* genes (14). According to our model, maximal expression of *vps* genes resulting in rugose colonial morphology occurs when the *vps* repressor HapR is eliminated by mutation, but cAMP-CRP is available to induce the positive regulator VpsR. Further studies are required to understand how cAMP-CRP regulates VpsR. We considered the possibility that cAMP-CRP modulates the expression of GGDEF and EAL domain proteins that regulate VpsR by controlling intracellular levels of cyclic diguanylate (16, 17). However, we have not found any effect of deleting *crp* on the transcription of genes *cdgA*, *mbaA*, and *cgdC* encoding GGDEF/EAL domain proteins (data not shown). These results do not rule out the possibility of cAMP-CRP influencing cyclic diguanylate levels by affecting the activity of the above proteins or modulating other GGDEF/EAL domain proteins. VpsR has been reported to be a σ^{54}-dependent activator (29). Interestingly, gene expression profiling of strain WL7258 revealed down-regulation of multiple σ^{54}-dependent genes affecting motility and C_4-dicarboxylate transport (15).

Our results clearly establish cAMP-CRP as a regulator of exopolysaccharide biosynthesis and rugose colonial morphology. The recognition of the role of cAMP-CRP in this process will facilitate further studies to determine how environmental cues influencing intracellular cAMP levels (i.e., carbon starvation) affect the ability of *V. cholerae* to adopt the rugose colonial morphology variant and resist environmental stresses.

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