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Interplay between Cyclic AMP-Cyclic AMP Receptor Protein and Cyclic di-GMP Signaling in *Vibrio cholerae* Biofilm Formation[▽]†

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Vibrio cholerae **is a facultative human pathogen. The ability of** ***V. cholerae*** **to form biofilms is crucial for its survival in aquatic habitats between epidemics and is advantageous for host-to-host transmission during epidemics. Formation of mature biofilms requires the production of extracellular matrix components, including** ***Vibrio*** **polysaccharide (VPS) and matrix proteins. Biofilm formation is positively controlled by the transcriptional regulators VpsR and VpsT and is negatively regulated by the quorum-sensing transcriptional regulator HapR, as well as the cyclic AMP (cAMP)-cAMP receptor protein (CRP) regulatory complex. Transcriptome analysis of** ***cyaA*** **(encoding adenylate cyclase) and** ***crp*** **(encoding cAMP receptor protein) deletion mutants revealed that cAMP-CRP negatively regulates transcription of both VPS biosynthesis genes and genes encoding biofilm matrix proteins. Further mutational and expression analysis revealed that cAMP-CRP negatively regulates transcription of** ***vps*** **genes indirectly through its action on** ***vpsR*** **transcription. However, negative regulation of the genes encoding biofilm matrix proteins by cAMP-CRP can also occur independent of VpsR. Transcriptome analysis also revealed that cAMP-CRP regulates the expression of a set of genes encoding diguanylate cyclases (DGCs) and phosphodiesterases. Mutational and phenotypic analysis of the differentially regulated DGCs revealed that a DGC, CdgA, is responsible for the increase in biofilm formation in the Δ*crp*** **mutant, showing the connection between of cyclic di-GMP and cAMP signaling in** ***V. cholerae.***

Vibrio cholerae, the causative agent of cholera (26), is a natural inhabitant of aquatic environments (14). Seasonal cholera outbreaks occur where the disease is endemic and can spread worldwide (14, 34). The ability of *V. cholerae* to cause epidemics is linked to its ability to survive in natural aquatic ecosystems. One important factor for environmental survival and transmission of *V. cholerae* is its ability to form biofilms (14, 60, 63). Biofilms are surface-attached microbial communities composed of microorganisms and the extrapolymeric substances that they produce (9). The biofilm mode of growth is the preferred lifestyle in the microbial world, as it enhances survival in natural settings. In addition, biofilms protect the constituent microbes from predators, such as protozoa and viruses, and from toxic compounds, such as antimicrobial agents (4, 10, 13, 36, 41, 63).

The process of biofilm development in *V. cholerae* can be divided into distinct stages: transport and attachment of bacteria to the surface, colonization of the attached surface, formation of a monolayer of cells, and synthesis of the extracellular matrix, leading to formation of a mature biofilm with a characteristic three-dimensional (3D) architecture. The *Vibrio* polysaccharide (VPS), encoded by the *vps* genes, is essential for the development of 3D biofilm structures (63). The *vps* genes are clustered in two regions on the large chromosome of *V. cholerae* O1 El Tor; the *vps*-I cluster consists of *vpsU* (VC0916) and *vpsA* to *vpsK* (VC0917 to VC0927), and the *vps*-II cluster consists of *vpsL* to *vpsQ* (VC0934 to VC0939). Recently, we identified protein components of the biofilm matrix of *V. cholerae* and showed that the RbmA, RbmC, and Bap1 proteins are also required for the formation of a wild-type biofilm. Mutants that are not able to produce these matrix proteins form biofilms that are structurally unstable (15, 16).

The regulation of biofilm formation in *V. cholerae* is complex and involves several transcriptional regulators. Two proteins that positively regulate VPS production and biofilm formation have been identified, VpsR and VpsT. Disruption of *vpsR* prevents expression of the *vps* genes and production of VPS, and it eliminates formation of typical 3D biofilm structures (61). A *vpsT* mutant exhibits reduced *vps* gene expression and biofilm-forming capacity (7). A population-density-dependent regulatory system, known as the quorum-sensing system, negatively regulates biofilm formation in *V. cholerae.* HapR is the master regulator of the quorum-sensing regulatory system, and a *hapR* mutant has increased biofilm-forming capacity (19, 62, 64). Consistent with this observation, expression of the *vps* genes, including *vpsR* and *vpsT*, is increased in the *hapR* mutant (62). In addition, a second messenger, cyclic di-GMP (c-di-GMP), which is produced by diguanylate cyclases (DGCs) containing a GGDEF amino acid motif and is degraded by phosphodiesterases (PDEs) that have EAL or HD-GYP domains (8, 46, 49), positively regulates biofilm formation in *V. cholerae* (3, 31, 56). We recently determined that *cdgA* (encoding a DGC), whose transcription is positively regulated by VpsR and negatively regulated by HapR, positively regulates biofilm formation in *V. cholerae* (2).

The cyclic AMP (cAMP)-cAMP receptor protein (CRP) regulatory complex was recently identified as a negative regulator of biofilm formation in *V. cholerae* (29, 30). These studies showed that cAMP-CRP negatively regulates *vpsL* and *vpsT*

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expression and positively regulates *vpsR* expression. Additional study showed that growth in the presence of glucose, which leads to a decrease in cellular cAMP levels, induces biofilm formation, while addition of cAMP to a growth medium leads to a decrease in biofilm formation in wild-type *V. cholerae* (23). To further evaluate the mechanism by which cAMP-CRP negatively regulates biofilm formation in *V. cholerae*, we determined whole-genome expression profiles of *cyaA* (encoding adenylate cyclase) and *crp* (encoding CRP) deletion mutants. Our analysis revealed that cAMP-CRP negatively regulates transcription of VPS biosynthesis genes and genes encoding biofilm matrix proteins. cAMP-CRP negatively regulates transcription of both *vps* genes and the genes encoding biofilm matrix proteins indirectly, through its action on *vpsR* transcription. In addition, cAMP-CRP can also negatively regulate transcription of the genes encoding biofilm matrix proteins independent of VpsR. We also determined that cAMP-CRP regulates the expression of a set of genes encoding DGCs and PDEs. Through mutational and phenotypic analysis, we showed that CdgA is largely responsible for the increased transcription of *vps* and biofilm matrix protein genes, as well as enhanced biofilm formation in a Δ*crp* mutant, revealing the connection between c-di-GMP and cAMP-CRP signaling in *V. cholerae*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. All *V. cholerae* and *Escherichia coli* strains were routinely grown aerobically in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl; pH 7.5) at 30 and 37°C, respectively, unless otherwise noted. The *E. coli* DH10B and CC118λ*pir* strains were used for DNA manipulation, while the *E. coli* S17-1λ*pir* strain was used for conjugation with *V. cholerae* A1552. Conjugation with other *V. cholerae* strains (C6706, N16961, and MO10) was carried out using the *E. coli* SM10λ*pir* strain. Agar medium contained 1.5% granulated agar (Difco), unless otherwise noted. Ampicillin, rifampin, and streptomycin were used at a concentration of 100 μg/ml, while gentamicin was used at a concentration of 50 μg/ml. We observed that Δ*cyaA* and Δ*crp* mutants had increased doubling times in LB medium at 30°C compared to the wild type (data not shown). This finding is similar to the growth defect reported for a *crp* mutant grown in LB medium at 37°C (52). To minimize the effect of reduced growth rates in our experiments, expression profiling and β-galactosidase assays were carried out with cultures grown to stationary phase.

Recombinant DNA techniques. DNA manipulations were carried out by using standard molecular techniques (47). Restriction and DNA modification enzymes were purchased from New England Biolabs. PCRs were carried out using primers purchased from Operon Technologies (Table 2) and a high-fidelity PCR kit (Roche). DNA sequencing was carried out by the UC Berkeley DNA Sequencing Facility.

Generation of in-frame deletion mutants. Deletion mutants of *V. cholerae* strains were generated by using a previously described protocol (16). The DNA sequences of the constructed deletion plasmids were verified by DNA sequencing. Primers used in the construction of the deletion plasmids are shown in Table 2.

Pellicle formation and motility assays. Pellicle formation experiments were carried out using glass culture tubes (18 by 150 mm) containing 5 ml of medium. The medium was inoculated with overnight cultures, resulting in 200-fold dilution. The tubes were incubated at 30°C without shaking for 2 days. LB soft agar plates (0.3% agar) were used to determine the motility of the bacterial strains. The diameter of each migration zone (in cm) was measured after 18 h of incubation at 30°C. Assays were repeated with at least two biological replicates.

Generation of *lacZ* transcriptional fusion constructs. *lacZ* transcriptional fusions with promoters of *rbmC* and *bap1* were constructed by cloning the PCR-amplified ~300-bp promoter regions immediately upstream of the start codons of *rbmC* and *bap1* into pRS415 (51) as described previously (15). The resulting transcriptional fusion plasmids were sequenced. The plasmids were electroporated into *V. cholerae* strains containing a *lacZ* in-frame deletion. The primers used for amplification of the promoter regions are shown in Table 2.

β-Galactosidase assays. β-Galactosidase assays were carried out by using a protocol similar to that described by Miller (38). Briefly, overnight cultures were diluted 200-fold in LB medium supplemented with ampicillin and incubated at 30°C for 10 h with shaking (200 rpm). The optical densities at 600 nm (OD_{600}) of the stationary-phase cultures were determined, and 1-ml portions of the cultures were harvested and washed with 1 ml of buffer Z (16.1 g/liter $Na_2HPO_4 \cdot 7H_2O$, 5.5 g/liter $NaH_2PO_4 \cdot H_2O$, 0.75 g/liter KCl, 0.246 g/liter $MgSO_4 \cdot 7H_2O$; pH 7.0). Cells were lysed by resuspending a cell pellet in 1 ml of buffer Z containing 0.69% β-mercaptoethanol, 0.02% cetyltrimethylammonium bromide, and 0.01% deoxycholic acid (sodium salt), followed by incubation at room temperature for 5 min. Cell lysates (100 μl) of different dilutions were pipetted into flat-bottom 96-well microtiter plates, and 20-μl portions of an *o*-nitrophenyl-β-D-galactopyranoside solution (4 mg/ml) were added, followed by incubation at 30°C until sufficient color development was observed. The reactions were stopped by adding 50 μl of 1 M Na_2CO_3, and the color intensities were measured at OD_{420} and OD_{550}. The duration of color development was noted, and the β-galactosidase activity (expressed in Miller units) was calculated as previously described (15, 38). The assays were repeated with at least two different biological replicates and eight technical replicates.

Generation of *gfp*-tagged strains and confocal laser scanning microscopy (CLSM). *V. cholerae* strains were chromosomally tagged with the gene encoding green fluorescent protein (*gfp*), using a previously described procedure (2, 15). Non-flow-cell experiments were carried out using Lab-Tek II chambered coverglass systems (Nalge Nunc) and a previously described protocol (2). Briefly, overnight cultures were diluted to obtain an OD_{600} of 0.2, and 3-ml portions of the diluted cultures were placed into chambers and incubated at 30°C for 8 h. The chambers were then washed twice with 1 ml of LB medium. Biofilms formed by non-*gfp*-tagged strains were stained for 15 min at room temperature in the dark with 1 ml of 5 μM SYTO9 (Molecular Probes). Images of the biofilms formed in the chambers were acquired using a Zeiss Axiovert 200 M laser scanning microscope. 3D images of the biofilms were reconstructed using IMARIS software (Bitplane) and were quantified using the COMSTAT program (22). Non-flow-cell experiments were carried out with at least two different biological replicates.

Biofilm formation assays. Biofilms were formed in 96-well polyvinyl chloride microtiter plates by using 100-μl portions of overnight cultures diluted to obtain an OD_{600} of 0.2. The microtiter plates were incubated at 30°C for 8 h. Crystal violet staining and ethanol solubilization were carried out as previously described (15, 63). The assays were repeated with two different biological replicates and eight technical replicates.

RNA isolation. Total RNA was isolated from *V. cholerae* strains in stationary growth phase by using a previously described protocol (62). Briefly, overnight cultures of *V. cholerae* grown in LB medium at 30°C with shaking (200 rpm) were diluted 200-fold in LB medium and incubated at 30°C for 10 h. Aliquots (2 ml) of the cultures were collected and centrifuged for 2 min at room temperature. The cell pellets were immediately resuspended in 1 ml of TRIzol (Invitrogen) and stored at −80°C. Total RNA was isolated according to the manufacturer's instructions. To remove contaminating DNA, total RNA was incubated with RNase-free DNase I (Ambion), and an RNeasy mini kit (Qiagen) was used to clean up RNA after DNase digestion.

Whole-genome expression profiling. Whole-genome expression profiling was performed by using a previously described procedure (3). A common reference RNA was used, which contained equal amounts of total RNA isolated from *V. cholerae* cells grown to stationary phase in LB medium. Normalized signal ratios were obtained with LOWESS print tip normalization using the Bioconductor packages (http://www.bioconductor.org) in the R environment (18). Differentially regulated genes were determined (with three biological and two technical replicates for each data point) using the Significance Analysis of Microarrays (SAM) software (58) with a ≥2-fold difference in gene expression and a false discovery rate (FDR) of ≤1% as cutoff values, unless otherwise noted.

qPCR. Quantitative PCR (qPCR) was carried out by first synthesizing cDNA from 1 μg of a total RNA sample using an iScript cDNA synthesis kit (Bio-Rad). The cDNA product was then diluted 1:4 with water, and 4 μl was used as a template with 12 pmol of each qPCR primer (Table 2) in a PCR performed with the Expand high-fidelity PCR system (Roche). The PCR conditions were as follows: 94°C for 2 min and then 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s and a final incubation at 72°C for 2 min. The amplified products were analyzed on a 2% agarose gel and were quantified using the ImageQuant 5.2 software (Molecular Dynamics). The intensity of each DNA band was normalized to that of the corresponding *recA* band amplified with primers RecA578 and RecA863 (29). The data presented below are from three biological replicates, and reaction mixtures containing no template or reverse transcriptase were used as negative controls.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and phenotype	Source or reference
E. coli strains		
DH10B	F^{-} *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 endA1 ara*Δ*139* Δ(*ara leu*) *7697 galU galK* λ-*rpsL* (Sm^{r}) *nupG*	Invitrogen
CC118λ*pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λ*pir*	21
S17-1λ*pir*	Tp^{r} Sm^{r} *recA thi pro* r_K^- m_K^+ RP4::2-Tc::MuKm Tn7 λ*pir*	11
SM10λ*pir*	*thi thr leu tonA lacY supE recA* (RP4-2-Tc::Mu) λ*pir*R6K Km^{r} π^{+}	54
V. cholerae strains		
FY_Vc_1	*V. cholerae* O1 El Tor A1552, wild-type variant, Rif^{r}	63
C6706	*V. cholerae* O1 El Tor C6706, wild-type variant, Sm^{r}	35
N16961	*V. cholerae* O1 El Tor N16961, wild-type variant, Sm^{r}	20
MO10	*V. cholerae* O139 MO10, wild-type variant, Sm^{r}	P. Watnick
FY_Vc_2322	Δ*cyaA*, Rif^{r}	This study
FY_Vc_2326	Δ*crp*, Rif^{r}	This study
FY_Vc_237	FY_Vc_1 mTn*7-gfp*, Rif^{r} Gm^{r}	3
FY_Vc_2448	Δ*cyaA* mTn*7-gfp*, Rif^{r} Gm^{r}	This study
FY_Vc_2451	Δ*crp* mTn*7-gfp*, Rif^{r} Gm^{r}	This study
FY_Vc_3	FY_Vc_1 Δ*lacZ*, Rif^{r}	7
FY_Vc_3756	C6706 Δ*lacZ*	This study
FY_Vc_3748	N16961 Δ*lacZ*	This study
FY_Vc_3763	MO10 Δ*lacZ*	This study
FY_Vc_2456	FY_Vc_1 Δ*cyaA* Δ*lacZ*, Rif^{r}	This study
FY_Vc_2459	FY_Vc_1 Δ*crp* Δ*lacZ*, Rif^{r}	This study
FY_Vc_3759	C6706 Δ*crp* Δ*lacZ*	This study
FY_Vc_3751	N16961 Δ*crp* Δ*lacZ*	This study
FY_Vc_3766	MO10 Δ*crp* Δ*lacZ*	This study
FY_Vc_2919	FY_Vc_1 Δ*hapR* Δ*lacZ*, Rif^{r}	This study
FY_Vc_2922	FY_Vc_1 Δ*vpsT* Δ*lacZ*, Rif^{r}	This study
FY_Vc_2874	FY_Vc_1 Δ*vpsR* Δ*lacZ*, Rif^{r}	This study
FY_Vc_344	FY_Vc_1 Δ*cdgA*, Rif^{r}	31
FY_Vc_360	FY_Vc_1 Δ*cdgA* mTn*7-gfp*, Rif^{r} Gm^{r}	31
FY_Vc_3296	FY_Vc_1 Δ*cdgA* Δ*lacZ*, Rif^{r}	This study
FY_Vc_350	FY_Vc_1 Δ*cdgB*, Rif^{r}	31
FY_Vc_987	FY_Vc_1 Δ*cdgF*, Rif^{r}	3
FY_Vc_1592	FY_Vc_1 Δ*cdgH*, Rif^{r}	Beyhan et. al., submitted
FY_Vc_956	FY_Vc_1 Δ*cdgI*, Rif^{r}	This study
FY_Vc_354	FY_Vc_1 Δ*rocS*, Rif^{r}	31
FY_Vc_152	FY_Vc_1 ΔVC0072, Rif^{r}	This study
FY_Vc_869	FY_Vc_1 ΔVC1376, Rif^{r}	This study
FY_Vc_158	FY_Vc_1 ΔVC2750, Rif^{r}	This study
FY_Vc_154	FY_Vc_1 ΔVCA0217, Rif^{r}	This study
FY_Vc_2779	FY_Vc_1 Δ*crp* Δ*hapR* Δ*lacZ*, Rif^{r}	This study
FY_Vc_2781	FY_Vc_1 Δ*crp* Δ*vpsT* Δ*lacZ*, Rif^{r}	This study
FY_Vc_2916	FY_Vc_1 Δ*crp* Δ*vpsR* Δ*lacZ*, Rif^{r}	This study
FY_Vc_3299	FY_Vc_1 Δ*crp* Δ*cdgA* Δ*lacZ*, Rif^{r}	This study
FY_Vc_3712	FY_Vc_1 Δ*crp* Δ*cdgA* Δ*lacZ* mTn*7-gfp*, Rif^{r} Gm^{r}	This study
FY_Vc_3311	FY_Vc_1 Δ*crp* Δ*cdgB* Δ*lacZ*	This study
FY_Vc_3323	FY_Vc_1 Δ*crp* Δ*cdgF* Δ*lacZ*	This study
FY_Vc_3317	FY_Vc_1 Δ*crp* Δ*cdgH* Δ*lacZ*	This study
FY_Vc_3308	FY_Vc_1 Δ*crp* Δ*cdgI* Δ*lacZ*	This study
FY_Vc_3314	FY_Vc_1 Δ*crp* Δ*rocS* Δ*lacZ*	This study
FY_Vc_3305	FY_Vc_1 Δ*crp* ΔVC0072 Δ*lacZ*	This study
FY_Vc_3318	FY_Vc_1 Δ*crp* ΔVC1376 Δ*lacZ*	This study
FY_Vc_3320	FY_Vc_1 Δ*crp* ΔVC2750 Δ*lacZ*	This study
FY_Vc_3322	FY_Vc_1 Δ*crp* ΔVCA0217 Δ*lacZ*	This study
FY_Vc_3302	FY_Vc_1 Δ*hapR* Δ*cdgA* Δ*lacZ*, Rif^{r}	This study
FY_Vc_337	FY_Vc_1 Δ*flaA*, Rif^{r}	Beyhan et. al., submitted
FY_Vc_231	FY_Vc_1 Δ*vps*-I, Rif^{r}	3
FY_Vc_3787	FY_Vc_1 Δ*crp* Δ*vps*-I, Rif^{r}	This study
FY_Vc_3411	FY-Vc_1 Δ*vps*-I Δ*vps*-II, Rif^{r}	This study
FY_Vc_3788	FY-Vc_1 Δ*crp* Δ*vps*-I Δ*vps*-II, Rif^{r}	This study
FY_Vc_102	FY_Vc_1 Δ*rbmA*, Rif^{r}	15
FY_Vc_3789	FY_Vc_1 Δ*crp* Δ*rbmA*, Rif^{r}	This study
FY_Vc_3790	FY_Vc_1 Δ*bap1*, Rif^{r}	This study
FY_Vc_3791	FY_Vc_1 Δ*crp* Δ*bap1*, Rif^{r}	This study

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Relevant genotype and phenotype	Source or reference
Plasmids		
pGP704-*sacB*28	pGP704 derivative, *mob/oriT sacB*, Ap^r	G. Schoolnik
pFY-659	pGP704-*sacB*28::Δ*vps*-II operon, Ap^r	This study
pFY-308	pGP704-*sacB*28::Δ*cyaA*, Ap^r	This study
pFY-333	pGP704-*sacB*28::Δ*crp*, Ap^r	This study
pFY-149	pGP704-*sacB*28::Δ*cdgA*, Ap^r	31
pFY-447	pGP704-*sacB*28::Δ*cdgI*, Ap^r	This study
pCC27	pGP704-*sacB*28::Δ*vpsR*, Ap^r	7
pCC2	pGP704-*sacB*28::Δ*lacZ*, Ap^r	7
pFY-252	pGP704-*sacB*28::ΔVC0072, Ap^r	This study
pFY-384	pGP704-*sacB*28::ΔVC1376, Ap^r	This study
pFY-237	pGP704-*sacB*28::ΔVC2750, Ap^r	This study
pFY-250	pGP704-*sacB*28::ΔVCA0217, Ap^r	This study
pRS415	Promoterless *lacZ* cloning vector for transcriptional fusion studies, Ap^r	51
pCC12	pRS415 *vpsL* promoter, Ap^r	7
pCC25	pRS415 *vpsT* promoter, Ap^r	7
pCC10	pRS415 *vpsR* promoter, Ap^r	7
pFY-169	pRS415 *rbmA* promoter, Ap^r	15
pFY-578	pRS415 *rbmC* promoter, Ap^r	This study
pFY-581	pRS415 *bap1* promoter, Ap^r	This study
pFY-150	pACYC177::*cdgA* operon (includes VCA0074 and VCA0075), Ap^r	31
pMCM11	pGP704::mTn*7*-*gfp*, Gm^r Ap^r	M. Miller and G. Schoolnik
pUX-BF13	oriR6K helper plasmid, *mob/oriT*, provides the Tn*7* transposition function in *trans*, Ap^r	1

RESULTS

Identification of genes differentially regulated in Δ*cyaA* and Δ*crp* mutants. cAMP-CRP negatively regulates biofilm formation in *V. cholerae*. To further understand how cAMP-CRP regulates biofilm formation, we generated in-frame *cyaA* (VC0122) and *crp* (VC2614) deletion mutants of our prototype *V. cholerae* O1 El Tor A1552 strain and performed whole-genome expression profiling of these mutants. The gene expression data were analyzed by using the SAM software and the following criteria to define significantly regulated genes, unless otherwise indicated: an FDR of ≤1% and a ≥2-fold transcript abundance difference between samples. This analysis revealed that cAMP-CRP differentially regulates transcription of a large set of genes and that the overall gene expression profiles of the Δ*cyaA* and Δ*crp* mutants are similar (see Fig. S1 in the supplemental material). Altogether, 889 genes (22.9% of the genome) and 822 genes (21.2% of the genome) are differentially regulated in the Δ*cyaA* and Δ*crp* mutants compared to the wild type, respectively. Of the 889 differentially regulated genes in the Δ*cyaA* mutant, 431 are upregulated and 458 are downregulated, whereas of the 822 differentially regulated genes in the Δ*crp* mutant, 386 are upregulated and 436 are downregulated. All the differentially regulated genes are shown in Tables S1 and S2 in the supplemental material.

In this study, our main objective was to determine how cAMP-CRP negatively regulates biofilm formation. Thus, for the genes that are differentially regulated by cAMP-CRP, we focused on two sets of genes: the genes required for biofilm matrix production and its regulation and the genes predicted to be involved in the production and degradation of c-di-GMP, as this second messenger regulates biofilm formation in *V. cholerae*.

cAMP-CRP negatively regulates transcription of the *vps*, *rbmA*, *rbmC*, and *bap1* genes. As expected for a negative regulator of biofilm formation, the levels of expression of *vps* genes and *vpsT* were higher in both the Δ*cyaA* and Δ*crp* mutants than in the wild type (Table 3). Gene expression profiling also revealed that the expression of *hapR* was decreased in both the Δ*cyaA* and Δ*crp* mutants. This finding is consistent with previously described results (29, 50). In addition to these genes, we observed that the levels of expression of the genes encoding the biofilm matrix proteins, *rbmA*, *rbmC*, and *bap1*, were higher in both the Δ*cyaA* and Δ*crp* mutants than in the wild type. To verify this finding, we monitored transcription of the genes encoding biofilm matrix proteins by using *rbmA-lacZ*, *rbmC-lacZ*, and *bap1-lacZ* fusion constructs and measuring β-galactosidase activities. In parallel, we also monitored transcription of the genes involved in VPS biosynthesis using a *vpsL-lacZ* fusion construct. As expected, *vpsL* transcription was increased in both the Δ*cyaA* mutant (205-fold) and the Δ*crp* mutant (142-fold) compared to the wild type (Fig. 1A). Transcription of the genes encoding biofilm matrix proteins was also increased in the Δ*cyaA* mutant (15-fold for *rbmA*, 36-fold for *rbmC*, and 32-fold for *bap1*) and the Δ*crp* mutant (13-fold for *rbmA*, 27-fold for *rbmC*, and 15-fold for *bap1*) compared to the wild type (Fig. 1B to D). This indicates that cAMP-CRP negatively regulates transcription of genes required for the production of both VPS and biofilm matrix proteins.

cAMP-CRP negatively regulates transcription of *vpsT* and *vpsR* in *V. cholerae* O1 El Tor A1552. To understand how cAMP-CRP negatively regulates transcription of *vps* genes and biofilm matrix protein genes, we analyzed how transcription of *vpsR* and *vpsT* is altered in Δ*cyaA* and Δ*crp* mutants. Using *vpsT-lacZ* and *vpsR-lacZ* fusion constructs, we determined that *vpsT* and *vpsR* transcription was increased in the Δ*cyaA* mutant (178-fold for *vpsT* and 4-fold for *vpsR*) and the Δ*crp* mutant (144-fold for *vpsT* and 4-fold for *vpsR*) compared to the wild type, indicating that cAMP-CRP also negatively regulates tran-

TABLE 2. Sequences of oligonucleotides used in this study

Primer	Sequence (5′–3′)
vps-II_del_A	CATGCCATGGCATGCGGCTGGTCTATGTGGCTTG
vps-II_del_B	CGAGCATAGTCCCTAGCAAGGCAACCGAAA
vps-II_del_C	GTCTTGCTAGGGACTATGCTCGCGGGTTTACTGC
vps-II_del_D	CGAGCTCGCTCGATCTTTGCCGATCACC
*cyaA*_del_A	GATCCCATGGGTTTTCCCGCTTGATTGTGT
*cyaA*_del_B	CGCGGTTGGCCTGCTGGATAGGATTGGCTTC
*cyaA*_del_C	TATCCAGCAGGCCAACCGCGTTGAAGTCTAT
*cyaA*_del_D	GATCTCTAGACAAATCGATTGATGGCGAAT
*crp*_del_A	GATCTCTAGATGAGTTTTGCGATGGATTTG
*crp*_del_B	TGATCTTGATCGCAACTGAACCTTTTACG
*crp*_del_C	TTCAGTTGCGATCAAGATCACTCGCCAAGAG
*crp*_del_D	GATCGAGCTCCCAACATGGCTTTAGCATCA
*cdgI*_del_A	CTAGCCATGGCCTCTTCGTGTCCCGGAGTATC
*cdgI*_del_B	CAACGGGTAAGGCCAAAGAATGAATCTTGC
*cdgI*_del_C	TTCTTTGGCCTTACCCGTTGCGACAAACAGT
*cdgI*_del_D	CTAGTCTAGACTCATCAAGGAATCGCATCA
VC0072_del_A	GATCTCTAGACTAGGCTAATCGAACGCTCATTCC
VC0072_del_B	GCTGCAAAGCTAACGGTTGGCTCGATAAGG
VC0072_del_C	CCAACCGTTAGCTTTGCAGCAGATTGGTGTG
VC0072_del_D	GGAGCTCGAGACTGATGCGCTCACTGAC
VC1376_del_A	CGAGCTCCAGCCCAGCATGGAGCATATC
VC1376_del_B	ACCTTTCGATACCAGCAAGGGCACAATCAC
VC1376_del_C	CCTTGCTGGTATCGAAAGGTCGAAATCGTGT
VC1376_del_D	CATGCCATGGCATGCATTCACCAGCCAACAGACG
VC2750_del_A	CATGCCATGGCAGCCAAAGAGCTCGGAG
VC2750_del_B	GCAACCGACAAACGGCAGTATGATGGC
VC2750_del_C	TTTGTCGGTTGCCCACGCGGGCAAGGC
VC2750_del_D	CGATTCTAGAGTACCAAAGGTGCGGCTC
VCA0217_del_A	GATCTCTAGACTAGTGCGCCATGTAACCAATAGA
VCA0217_del_B	GCTTTTACCGATGCGCTATTGGGTTCAACT
VCA0217_del_C	AATAGCGCATCGGTAAAAGCAGGAGAGTGA
VCA0217_del_D	GGAGCTCGGTCTTATTGATGCGGGAGCA
*rbmC*_pro F	GATCGAATTCCTAGAAAATGCTTCTTGA
*rbmC*_pro R	GATCGGATCCTTGTAAGACTCCCTTTACCT
*bap1*_pro F	GAGAGAATTCCGCCGCGTTGCTGAG
*bap1*_pro R	GAGAGGATCCGGCTTGACCTTCATCT
RecA578	GTGCTGTGGATGTCATCGTTGTTG
RecA863	CCACCACTTCTTCGCCTTCTTTGA
*cdgA*_rt F	CAAGCGATCTGGTTCTTATTCC
*cdgA*_rt R	AAAACGGCTCCAAGTCAGC
*cdgI*_rt F	GATGTGGAAAGGCAAAGAGC
*cdgI*_rt R	CTGTGCTATGCGAGTTTTGC
*rocS*_rt F	CAGGTTGCACCTCTTTACTCG
*rocS*_rt R	ACCCGTGTCGGTTATACAGC

scription of *vpsT* and *vpsR* (Fig. 2A and B). Interestingly, transcription of *vpsR* was shown to be positively regulated by cAMP-CRP in the *V. cholerae* O1 El Tor C7258 strain (30). Differences in the regulation of *vpsR* by cAMP-CRP in our prototype strain, *V. cholerae* O1 El Tor A1552, and *V. cholerae* O1 El Tor C7258 prompted us to look at *vpsR* regulation in other commonly used *V. cholerae* strains. To this end, we generated in-frame *crp* deletion mutants of *V. cholerae* strains N16961, C6706, and MO10 and introduced a reporter plasmid harboring a *vpsR-lacZ* fusion construct into these strains. We then monitored transcription of *vpsR* by measuring β-galactosidase activity (Fig. 2C). In our prototype A1552Δ*crp* strain, *vpsR-lacZ* transcription was increased 3.9-fold compared to the wild-type transcription. N16961Δ*crp* also exhibited a similar increase in transcription of *vpsR-lacZ* (3.4-fold), while MO10Δ*crp* exhibited a slight increase (1.3-fold) in *vpsR-lacZ* transcription compared to the corresponding wild-type strains. On the other hand, in C6706 and transcription of *vpsR-lacZ* in C6706Δ*crp* did not differ significantly. These results indicate that while cAMP-CRP negatively regulates *vpsR* transcription in the A1552, N16961, and MO10 (albeit slightly) strains of *V. cholerae*, there is no such regulation in strain C6706. Hence, the results are consistent with the idea that *vpsR* regulation by cAMP-CRP varies in strains of *V. cholerae*. Together, our results indicate that in our prototype strain, cAMP-CRP represses biofilm formation in *V. cholerae* by negatively regulating transcription of the genes required for VPS biosynthesis and matrix protein production, as well as the genes encoding the positive transcriptional regulators VpsT and VpsR.

***crp* is epistatic to *hapR* in the regulation of *vpsT* and *vpsR* transcription.** Biofilm formation in *V. cholerae* is negatively regulated by both HapR (19, 62, 64) and cAMP-CRP (29, 30). Since cAMP-CRP positively regulates *hapR* transcription, repression of biofilm formation by cAMP-CRP could be mediated through HapR. Thus, to better evaluate the mechanism by which cAMP-CRP and HapR negatively regulate biofilm formation, we analyzed *vpsT* and *vpsR* transcription in wild-type, Δ*crp*, Δ*hapR*, and Δ*crp* Δ*hapR* strains harboring *vpsT-lacZ* and *vpsR-lacZ* fusion plasmids (Fig. 2D and E). We observed 27- and 3.2-fold increases in β-galactosidase activities in the Δ*hapR* mutants harboring *vpsT-lacZ* and *vpsR-lacZ* fusion plasmids, respectively, compared to the wild type. These results are congruent with the results of our previous studies showing that HapR negatively regulates the expression of *vpsT* and *vpsR* in strain A1552 (2, 62). It is noteworthy that in *V. cholerae* strains C6706 and C7258 negative regulation of *vpsR* by HapR has not been observed (19, 30, 59), indicating that, similar to *vpsR* repression by cAMP-CRP, *vpsR* repression by HapR also varies in strains of *V. cholerae*. Interestingly, similar increases in the transcriptional levels of *vpsT* (210-fold in the Δ*crp* mutant and 231-fold in the Δ*crp* Δ*hapR* mutant) and *vpsR* (6.2-fold in the Δ*crp* and mutant 6.0-fold in the Δ*crp* Δ*hapR* mutant) were observed for the Δ*crp* and Δ*crp* Δ*hapR* mutants, indicating that *crp* is epistatic to *hapR* in regulating *vpsT* and *vpsR* transcription.

cAMP-CRP regulates *rbmC* and *bap1* expression both through and independent of VpsR. Formation of mature biofilms in *V. cholerae* requires production of VPS and the matrix proteins RbmA, RbmC, and Bap1. As discussed above, cAMP-CRP negatively regulates the expression of both *vps* genes and

TABLE 3. Differentially expressed genes involved in biofilm matrix production and c-di-GMP signaling in Δ*cyaA* and Δ*crp* mutants compared to the wild type[a]

Gene no.	Designation	Change (fold)	
		Δ*cyaA*/wild type	Δ*crp*/wild type
Biofilm matrix production genes			
VC0916	*vpsU*	3.76	3.35
VC0917	*vpsA*	1.51	
VC0918	*vpsB*	2.93	2.16
VC0919	*vpsC*	2.36	2.37
VC0922	*vpsF*	2.04	
VC0928	*rbmA*	5.89	5.45
VC0929	*rbmB*	2.83	3.57
VC0930	*rbmC*	21.04	8.01
VC0931	*rbmD*	2.30	
VC0932	*rbmE*	7.46	4.27
VC0933	*rbmF*	5.89	5.99
VC0935	*vpsM*	12.75	8.06
VC0936	*vpsN*	2.63	2.32
VC0937	*vpsO*	1.61	1.99
VC0939	*vpsQ*	2.73	2.84
VC0583	*hapR*	0.11	0.48
VC1888	*bap1*	7.84	6.28
VCA0952	*vpsT*	74.62	48.70
c-di-GMP signaling genes: GGDEF and EAL			
VC0072		1.91	2.53
VC0653	*rocS*		1.81
VC0658	*cdgI*	2.95	3.34
VC0703	*mbaA*	1.78	1.77
VC1934		0.42	0.44
VC2750			1.70
VCA0785	*cdgC*	1.53	
c-di-GMP signaling genes: only GGDEF			
VC1029	*cdgB*	1.88	2.17
VC1067	*cdgH*		1.58
VC1216		0.56	0.45
VC1353		0.53	
VC1367	*cdgE*	0.64	
VC1370		0.37	
VC1376			1.99
VC1599		0.44	0.40
VCA0049		0.58	
VCA0074	*cdgA*	1.61	
VCA0165		0.56	
VCA0217			1.94
VCA0697	*cdgD*	0.09	0.11
VCA0956	*cgdF*	1.90	1.87
VCA0965		0.31	0.50
c-di-GMP signaling genes: only EAL			
VC0137		1.69	1.93
VC1086		0.38	0.40
VC1211		1.58	
VC1641		1.66	2.56
VC1710		0.21	0.29
c-di-GMP signaling genes: HD-GYP			
VC1087		0.43	0.55
VC1348		0.62	
VC2340			2.57
VC2497		0.54	0.47
VCA0210			1.76
VCA0895		0.46	0.61
VCA0931		0.48	

[a] Differentially expressed genes were determined using the SAM software with a ≥1.5-fold change in gene expression and an FDR of ≤3% as the criteria.

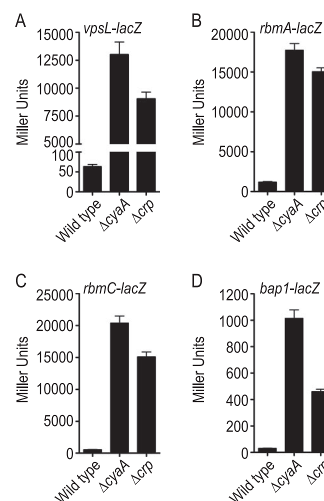

FIG. 1. cAMP-CRP negatively regulates expression of genes involved in VPS biosynthesis and biofilm matrix protein production. β-Galactosidase assays of wild-type, Δ*cyaA*, and Δ*crp* strains harboring (A) *vpsL-lacZ*, (B) *rbmA-lacZ*, (C) *rbmC-lacZ*, and (D) *bap1-lacZ* fusion constructs were performed. The data are representative of at least two independent experiments. The error bars indicate standard deviations.

genes encoding matrix proteins. However, we have a limited understanding of the mechanism by which cAMP-CRP regulates transcription of these genes and do not know whether *vps* and matrix protein genes are regulated differently by cAMP-CRP.

We previously reported that VpsR is the most downstream regulator of *vps* gene transcription and genes encoding matrix proteins in the VpsT, VpsR, and HapR regulatory circuitry (2). We wanted to determine how cAMP-CRP contributes to this regulatory circuitry. To this end, we monitored transcription of *vpsL*, *rbmC*, and *bap1* using *lacZ* transcriptional fusion constructs in the wild-type strain and Δ*crp*, Δ*vpsT*, Δ*vpsR*, Δ*crp* Δ*vpsT*, and Δ*crp* Δ*vpsR* mutants (Fig. 3). As discussed above, in the Δ*crp* strain harboring the fusion construct *vpsL-lacZ*, transcription of *vpsL* was markedly increased compared to that in the wild type. Transcription of *vpsL-lacZ* was 1.7- and 4.6-fold lower in the Δ*vpsT* and Δ*vpsR* mutants, respectively, than in the wild type (Fig. 3A). This finding is consistent with our previous report that the magnitude of regulation of *vps* gene expression by VpsR is greater than that by VpsT (2). In the Δ*crp* Δ*vpsT* double-deletion mutant, a 2.1-fold increase in *vpsL* transcription was observed compared to the wild type. This slight increase in *vpsL* transcription was likely due to decreased expression of *hapR* in the Δ*crp* genetic background. cAMP-CRP positively regulates *hapR* expression, and a decrease in *hapR* message abundance, in turn, leads to an increase in *vpsL* and *vpsR* expression (2, 62). Unlike the findings for the Δ*crp* Δ*vpsT* mutant, the *vpsL* transcription in the Δ*crp* Δ*vpsR* mutant was 3.3-fold lower than that in the wild type (similar to the Δ*vpsR* mutant), indicating that VpsR acts downstream of cAMP-CRP.

Transcription of *rbmC* and *bap1* was increased in the Δ*crp* mutant compared to the wild type. Although deletion of *vpsR* resulted in decreases in transcription of *rbmC* (7.6-fold) and *bap1* (6.8-fold), deletion of *vpsT* did not significantly alter

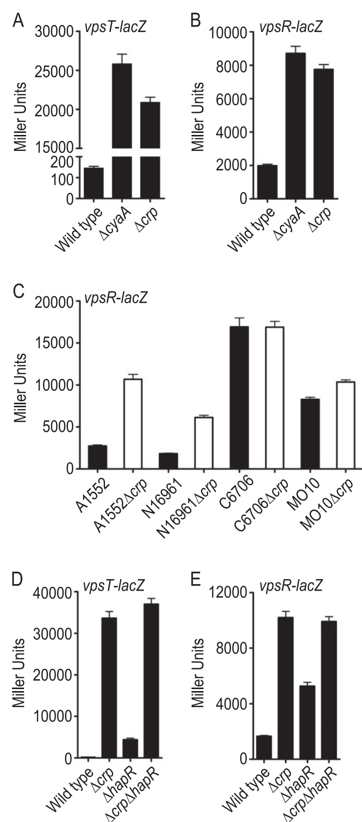

FIG. 2. cAMP-CRP negatively regulates *vpsT* and *vpsR* expression. (A and B) β-Galactosidase assays of wild-type strain A1552 and Δ*cyaA* and Δ*crp* mutants harboring (A) *vpsT-lacZ* and (B) *vpsR-lacZ* fusion constructs. (C) β-Galactosidase assays of different *V. cholerae* strains (A1552, N16961, C6706, and MO10) and Δ*crp* deletion strains harboring the *vpsR-lacZ* fusion construct. (D and E) β-Galactosidase assays of wild-type, Δ*crp*, Δ*hapR*, and Δ*crp* Δ*hapR* strains harboring (D) *vpsT-lacZ* and (E) *vpsR-lacZ* fusion constructs. The data are representative of at least two independent experiments. The error bars indicate standard deviations.

rbmC and *bap1* transcription compared to the wild type (Fig. 3B and C). This result suggests that, like the findings for *vpsL* transcription, the magnitude of transcriptional regulation by VpsR is greater than the magnitude of transcriptional regulation by VpsT for *rbmC* and *bap1.* Intriguingly, unlike the results for *vpsL* transcription, deletion of *crp* in both the Δ*vpsT* and Δ*vpsR* genetic backgrounds resulted in increased transcription of *rbmC* (16.4-fold in the Δ*crp* Δ*vpsT* mutant and 3.0-fold in the Δ*crp* Δ*vpsR* mutant) and *bap1* (8.1-fold in the Δ*crp* Δ*vpsT* mutant and 2.8-fold in the Δ*crp* Δ*vpsR* mutant) compared to the wild type. Together, these findings indicate that cAMP-CRP negatively regulates the transcription of *vps* genes and genes encoding matrix proteins in a different manner. While VpsR is the most downstream positive transcriptional regulator of *vps* gene expression, cAMP-CRP negatively regulates transcription of the genes encoding matrix proteins both through and independent of VpsR. Whether the action of cAMP-CRP is mediated by direct binding to the *rbmC* and *bap1* promoter regions or indirectly through another regulatory protein(s) remains unknown.

Since we observed that VPS biosynthesis genes and genes encoding matrix proteins are regulated differently by cAMP-CRP, we wanted to determine the contribution of VPS and biofilm matrix proteins to biofilm formation in the Δ*crp* genetic background. To this end, we generated Δ*crp* Δ*vps*-I (*vps*-I cluster deletion), Δ*crp* Δ*vps*-I Δ*vps*-II (*vps*-I and *vps*-II cluster deletion), Δ*crp* Δ*rbmA*, and Δ*crp* Δ*bap1* mutants and compared their biofilm-forming capacities to those of the wild type and Δ*crp*, Δ*vps*-I, Δ*vps*-I Δ*vps*-II, Δ*rbmA*, and Δ*bap1* single mutants. Deletion of the *vps*-I cluster or the *vps*-I and *vps*-II clusters in the Δ*crp* genetic background eliminated formation of a pellicle (biofilm formed at the air-liquid interface) (data not shown) and drastically reduced biofilm formation in both 96-well microtiter plate and non-flow-cell systems (Fig. 3D and E). Deletion of *rbmA* or *bap1* in the Δ*crp* genetic background decreased, but did not eliminate, biofilm formation. Compared to the Δ*crp* mutant biofilm, the biofilms formed by the Δ*crp* Δ*rbmA* and Δ*crp* Δ*bap1* mutants were less structured, and there were fewer mature pillars in the biofilms formed by the double-deletion mutants (Fig. 3E). Although the total biomasses and average biofilm thicknesses were not significantly different for the Δ*crp*, Δ*crp* Δ*rbmA*, and Δ*crp* Δ*bap1* mutants, the maximum biofilm thickness was greater for the Δ*crp* mutant than for the Δ*crp* Δ*rbmA* and Δ*crp* Δ*bap1* mutants (data not shown). Together, these results indicate that under the experimental conditions that we utilized, VPS production is essential for biofilm formation in a Δ*crp* genetic background, while biofilm matrix proteins have an accessory role. We do not know yet how biofilm matrix proteins function, whether they bind VPS carbohydrates or mediate cell-cell or cell-surface interactions. It is possible that the relative contributions of VPS and biofilm matrix proteins to biofilm formation are different when the organisms are tested under different environmental conditions using different surfaces.

cAMP-CRP differentially regulates the expression of a set of genes encoding proteins harboring GGDEF, EAL, and HD-GYP domains. Biofilm formation in *V. cholerae* is positively regulated by c-di-GMP (3, 31, 32, 56). Since cAMP-CRP negatively regulates biofilm formation in *V. cholerae*, we wanted to investigate if there is a connection between cAMP-CRP regulatory circuitry and c-di-GMP signaling in regulation of biofilm formation. We hypothesized that c-di-GMP levels may be increased in Δ*cyaA* and Δ*crp* mutants due to increased expression of a gene(s) encoding a key DGC or due to decreased expression of a gene(s) encoding a key PDE. Indeed, whole-genome expression profiling of Δ*cyaA* and Δ*crp* mutants revealed that genes encoding proteins predicted to exhibit DGC and PDE activities were differentially regulated in Δ*cyaA* and/or Δ*crp* mutants compared to the wild type (Table 3).

In this set of differentially regulated genes, we focused on 10 genes that encode proteins with a conserved GGDEF domain which is predicted to act as a DGC: VC0072, VC0653 (*rocS*), VC0658, VC1029 (*cdgB*), VC1067 (*cdgH*), VC1376, VC2750, VCA0074 (*cdgA*), VCA0217, and VCA0956 (*cdgF*) (referred to below as the GGDEF genes). To examine the involvement of the GGDEF genes in the regulation of biofilm formation in *V. cholerae*, we generated mutants with in-frame deletion mutations in these genes in the wild-type background, as well as in the Δ*crp* genetic background. We then analyzed the biofilm-forming capacity of each of these mutants using pellicle formation in glass culture tubes and biofilm formation in 96-well

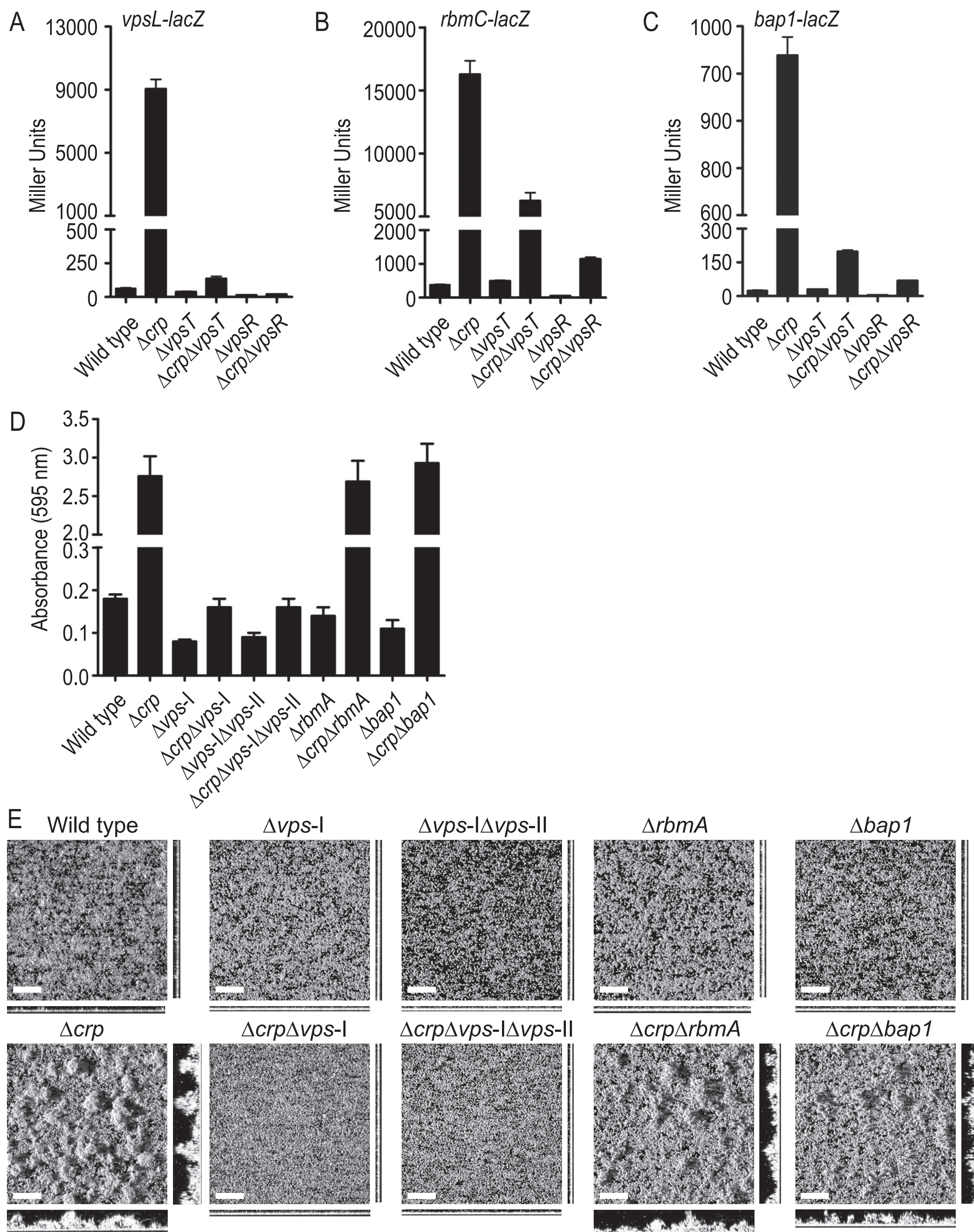

FIG. 3. Analysis of the cAMP-CRP contribution to VpsR regulation of *rbmC* and *bap1* expression. (A to C) β-Galactosidase assays of wild-type, Δ*crp*, Δ*vpsT*, Δ*crp* Δ*vpsT*, Δ*vpsR*, and Δ*crp* Δ*vpsR* strains harboring (A) *vpsL-lacZ*, (B) *rbmC-lacZ*, and (C) *bap1-lacZ* fusion constructs. The data are representative of at least two independent experiments. The error bars indicate standard deviations. (D) Quantitative comparison of biofilm formation by wild-type, Δ*crp*, Δ*vps*-I, Δ*crp* Δ*vps*-I, Δ*vps*-I Δ*vps*-II, Δ*crp* Δ*vps*-I Δ*vps*-II, Δ*rbmA*, Δ*crp* Δ*rbmA*, Δ*bap1*, and Δ*crp* Δ*bap1* strains. The data are representative of two independent experiments. The error bars indicate standard deviations. (E) Biofilms formed after 8 h of incubation at 30°C in a non-flow-cell system by the wild-type, Δ*crp*, Δ*vps*-I, Δ*crp* Δ*vps*-I, Δ*vps*-I Δ*vps*-II, Δ*crp* Δ*vps*-I Δ*vps*-II, Δ*rbmA*, Δ*crp* Δ*rbmA*, Δ*bap1*, and Δ*crp* Δ*bap1* strains. Biofilms were stained with SYTO9, and images were acquired by CLSM. The large images are images of the upper surfaces of biofilms, and the images below and to right of the large images are orthogonal views. Bars = 40 μm.

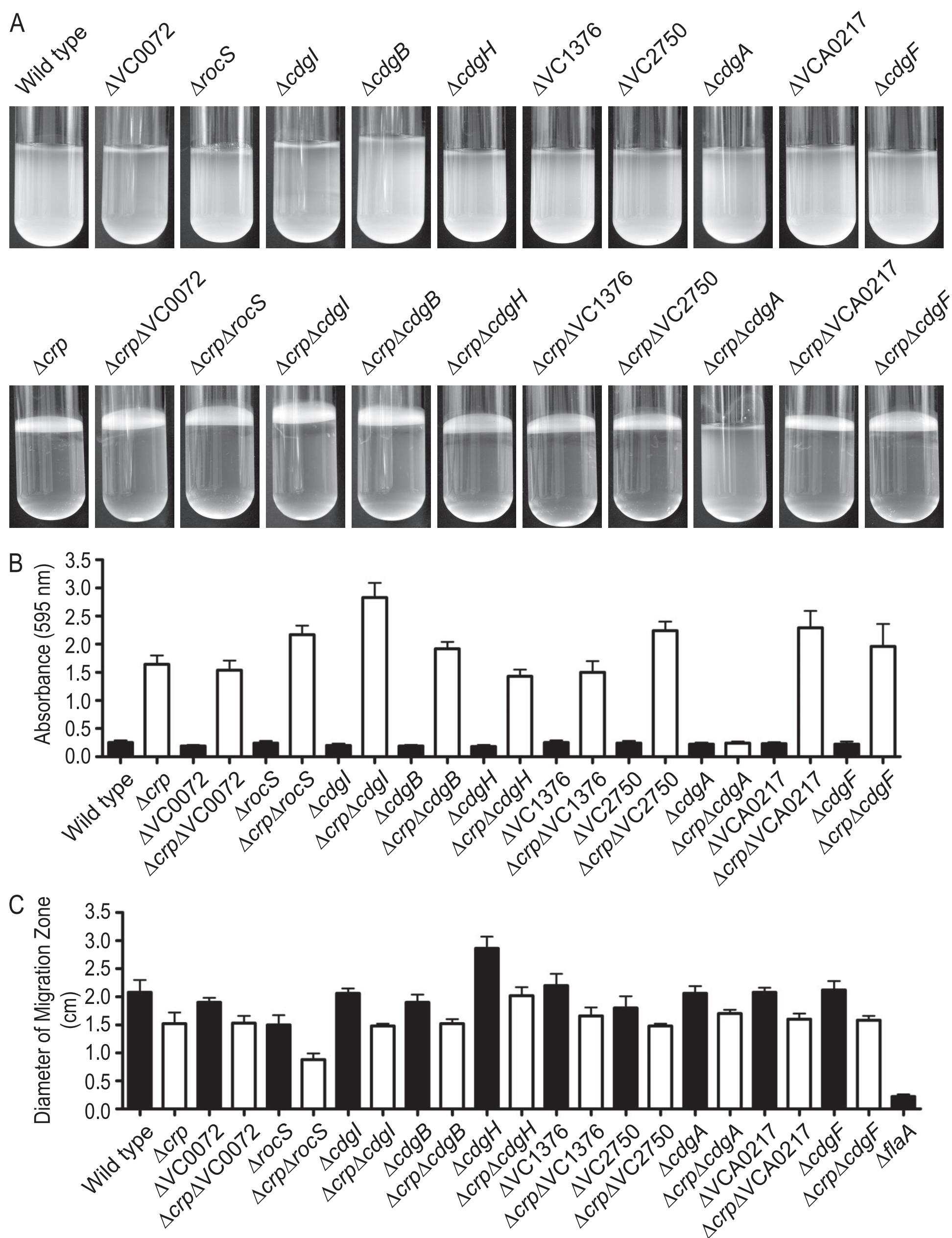

FIG. 4. Phenotypic characterization of GGDEF deletion mutants and GGDEF *crp* double-deletion mutants. (A) Pellicle formation, (B) quantitative comparison of biofilm formation, and (C) motility assays for the wild type, for Δ*crp* and GGDEF single-deletion mutants, and for mutants with GGDEF deletions generated in the Δ*crp* genetic background. The data are representative of two independent experiments. The error bars indicate standard deviations.

microtiter plates with a crystal violet staining assay. As shown in Fig. 4A and B, deletion of GGDEF genes in the wild type did not alter the pellicle and biofilm formation phenotypes. However, when the genes were deleted in the enhanced biofilm-forming Δ*crp* genetic background, only the Δ*crp* Δ*cdgA* double-deletion mutant failed to form pellicles and exhibited a reduced biofilm-forming capacity. This finding indicates that the increased pellicle- and biofilm-forming capacities of a Δ*crp* mutant are due to increased expression of *cdgA*. Interestingly, we recently reported that *cdgA* positively regulates colony rugosity and biofilm formation in *V. cholerae* (2, 31). The crystal violet staining assays also revealed that deletion of *rocS* and VC0658 (designated *cdgI* [*c*yclic *d*i-*g*uanylate I]) in the Δ*crp* genetic background further enhanced biofilm formation. Both RocS and CdgI contain conserved GGDEF-EAL domains. Such proteins can function as either DGCs or PDEs, and the enzymatic functions are commonly regulated by environmental stimuli. Although the enzymatic activities of these proteins have not been determined yet, based on biofilm-forming phenotypes, RocS and CdgI appear to function as a PDE under the experimental conditions that we utilized.

Flagellar motility is negatively regulated by c-di-GMP in *V.*

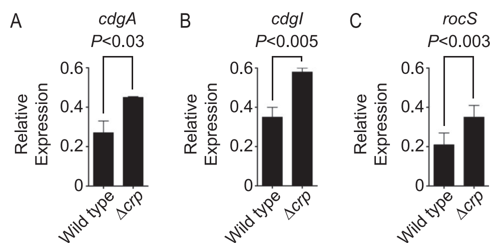

FIG. 5. qPCR analysis of *cdgA*, *cdgI*, and *rocS* message levels in the Δ*crp* mutant: quantification of relative repression of (A) *cdgA*, (B) *cdgI*, and (C) *rocS* in the wild type and the Δ*crp* mutant, normalized using *recA*. The results are from three independent biological replicates. The error bars indicate standard deviations. *P* values (two-tailed *t* test) are indicated at the top.

cholerae. Therefore, we sought to understand the effect of deletion of GGDEF genes on motility. To this end, we measured the migration zone formed by each single-deletion mutant, as well as the migration zones formed by mutants generated in the Δ*crp* genetic background, when the organisms were grown on LB soft agar plates (Fig. 4C). We also used Δ*flaA* as a control. Of the GGDEF single-deletion mutants tested, the Δ*rocS* mutant exhibited a decrease in motility and the Δ*cdgH* mutant exhibited an increase in motility compared to the wild type. These results are consistent with our previous report (31) and recent findings of Beyhan et al. (S. Beyhan et al., submitted for publication). Deletion of *crp* led to a decrease in motility, consistent with the expression profiling data showing downregulation of genes involved in flagellar biosynthesis and chemotaxis in Δ*cyaA* and Δ*crp* mutants compared to the wild type (see Fig. S1 in the supplemental material). Deletion of *crp* in individual GGDEF deletion mutants also led to further decreases in motility compared to the corresponding GGDEF single-deletion mutants. One of the mutants tested, the Δ*crp* Δ*rocS* double-deletion mutant, exhibited a further reduction in motility compared to both the Δ*crp* and Δ*rocS* single-deletion mutants, suggesting that cAMP-CRP and RocS have additive effects on motility regulation.

In summary, for the 10 GGDEF genes tested, deletion of *cdgA*, *rocS*, and *cdgI* in the Δ*crp* genetic background altered biofilm-forming phenotypes. Since we chose these genes based on their increased expression in the Δ*cyaA* and/or Δ*crp* mutant in whole-genome expression profiling experiments, we further confirmed their message abundance using qPCR. In agreement with microarray data, the message levels of *cdgA*, *rocS*, and *cdgI* were greater in the Δ*crp* mutant than in the wild type (Fig. 5).

Expression of VC1934, which encodes a protein containing an EAL domain and a nonconserved GGDEF domain, expression of VC1086 and VC1710, which encode proteins containing an EAL domain, and expression of VC1087, VC1348, VC2497, VCA0895, and VCA0931, which encode proteins containing a HD-GYP domain, were downregulated, with $\geq$1.5-fold changes and FDR of $\leq$3% in the Δ*cyaA* and/or Δ*crp* mutant (Table 3).

It is possible that a decrease in the expression of these genes encoding proteins predicted to have PDE activity (leading to a decrease in the cellular c-di-GMP level) (45, 49) may also contribute to an overall increase in the c-di-GMP level, thus giving rise to the increased biofilm-forming capacities observed for the Δ*cyaA* and Δ*crp* mutants. Regulation of the expression of these genes by cAMP-CRP in *V. cholerae* is currently under investigation.

CdgA is required for the enhanced biofilm-forming phenotype in the Δ*crp* mutant and negative regulation of *vpsL* and *rbmC* expression by cAMP-CRP. In the experiments described above we showed that while the Δ*crp* single-deletion mutant exhibits enhanced pellicle- and biofilm-forming capacities (as determined by the crystal violet staining assay), the *crp* and *cdgA* double-deletion mutant (Δ*crp* Δ*cdgA* mutant) exhibited a decrease in biofilm formation. We confirmed this finding by carrying out a CLSM analysis of biofilms formed by the wild type and the Δ*crp*, Δ*cdgA*, and Δ*crp* Δ*cdgA* mutants in a non-flow-cell system (Fig. 6A). After 8 h of biofilm development, the Δ*crp* mutant formed a thicker and more-structured biofilm, and the total biomass, average and maximum biofilm thicknesses, and substratum coverage were greater for the Δ*crp* mutant than for the wild type (Table 4). The Δ*cdgA* mutant formed biofilms with reduced total biomass, average biofilm thickness, and substratum coverage compared to the wild-type biofilms. Although deletion of *cdgA* in the Δ*crp* mutant significantly reduced biofilm formation, the Δ*crp* Δ*cdgA* double mutant formed biofilms whose total biomass, average biofilm thickness, and substratum coverage were greater than those of the biofilms formed by the Δ*cdgA* mutant (Fig. 6A and Table 4). These results indicate that the negative regulation of biofilm formation by cAMP-CRP is largely mediated by CdgA, but additional factors may also contribute to an increase in biofilm formation in the Δ*crp* mutant.

To determine if the altered pellicle- and biofilm-forming phenotypes of the Δ*crp* Δ*cdgA* double-deletion mutant were due to a reduction in the transcription of genes involved in biofilm matrix production, we carried out β-galactosidase assays with the wild-type, Δ*crp*, Δ*cdgA*, and Δ*crp* Δ*cdgA* strains harboring *vpsL-lacZ* and *rbmC-lacZ* transcriptional fusion constructs (Fig. 6B and C). While the Δ*crp* mutant exhibited increased transcription of *vpsL-lacZ* (154-fold) and *rbmC-lacZ* (37-fold), the Δ*cdgA* mutant exhibited decreases in the transcription of *vpsL-lacZ* (1.8-fold) and *rbmC-lacZ* (1.3-fold) compared to the wild type, consistent with our previous finding (obtained using gene expression profiling) that CdgA positively regulates *vps* and *rbm* gene expression (2). The Δ*crp* Δ*cdgA* double-deletion mutant exhibited a decrease in the transcription of *vpsL-lacZ* (79-fold) and *rbmC-lacZ* (7.1-fold) compared to the Δ*crp* single-deletion mutant. These results indicate that the decreased biofilm-forming capacities of the Δ*crp* Δ*cdgA* double mutant compared to the Δ*crp* mutant were due to decreased expression of genes involved in VPS biosynthesis and matrix protein production. Interestingly, in the Δ*crp* Δ*cdgA* double-deletion mutant the levels of transcription of *vpsL-lacZ* and *rbmC-lacZ* were higher than those in the wild type (1.9-fold higher for *vpsL-lacZ* and 5.1-fold higher for *rbmC-lacZ*) and in the Δ*cdgA* mutant (3.5-fold higher for *vpsL-lacZ* and 6.5-fold higher for *rbmC-lacZ*). These findings suggest that besides CdgA, other factors and processes also contribute to an increase in biofilm formation in the Δ*crp* mutant.

We also carried out a complementation assay by introducing *cdgA*, in which transcription is driven from its own promoter, in a multicopy number plasmid into the wild type and into the

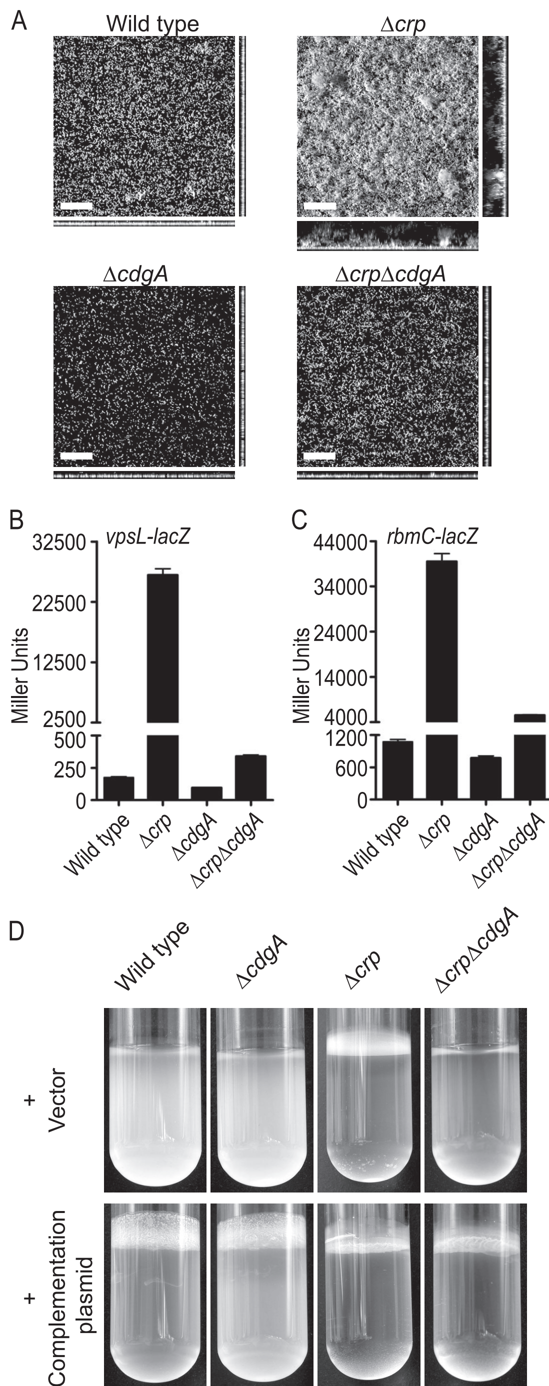

FIG. 6. Phenotypic characterization of Δ*crp*, Δ*cdgA*, and Δ*crp* Δ*cdgA* mutants. (A) Biofilms of wild-type, Δ*crp*, Δ*cdgA*, and Δ*crp* Δ*cdgA* strains formed after 8 h of incubation at 30°C in a non-flow-cell system. Images were acquired by CLSM. The large images are images of the upper surfaces of biofilms, and the images below and to right of the large images are orthogonal views. Bars = 40 μm. (B and C) β-Galactosidase assays for the wild-type, Δ*crp*, Δ*cdgA*, and Δ*crp* Δ*cdgA* strains harboring (B) *vpsL-lacZ* and (C) *rbmC-lacZ* fusion constructs. The data are representative of two independent experiments. The error bars indicate standard deviations. (D) Pellicle formation in the wild-type, Δ*crp*, Δ*cdgA*, and Δ*crp* Δ*cdgA* strains harboring the vector or the *cdgA* complementation plasmid.

TABLE 4. COMSTAT analysis for biofilms of the wild-type, Δ*crp*, Δ*cdgA*, and Δ*crp* Δ*cdgA* strains[a]

Strain	Total biomass ($\mu m^3/\mu m^2$)	Thickness (μm)		Substratum coverage
		Avg	Maximum	
Wild type	2.1 (0.4)	1.9 (0.4)	8.7 (2.8)	0.4 (0.1)
Δ*crp*	6.6 (1.6)	9.0 (1.8)	40.4 (4.4)	0.7 (0.1)
Δ*cdgA*	1.0 (0.1)	0.9 (0.1)	8.0 (1.7)	0.2 (0.02)
Δ*crp* Δ*cdgA*	1.6 (0.3)	1.3 (0.2)	9.3 (0.9)	0.4 (0.1)

[a] The values are means of data from at least six *z*-series image stacks. The numbers in parentheses are standard deviations.

Δ*cdgA*, Δ*crp*, and Δ*crp* Δ*cdgA* mutants. The Δ*cdgA* and Δ*crp* Δ*cdgA* mutants harboring the complementation plasmid exhibited increased pellicle-forming capacities compared to the same strains carrying only the vector (Fig. 6D), further confirming that CdgA is a key DGC that positively regulates biofilm formation. Although we now know many of the molecular players, it is still unclear how c-di-GMP regulates transcription of the genes involved in biofilm matrix production.

DISCUSSION

Environmental cues, such as nutrient availability, affect biofilm formation and/or dispersal (6, 9), linking carbon metabolism and biofilm formation. In *Pseudomonas aeruginosa*, the catabolite repression control protein (Crc), which regulates carbon metabolism, positively regulates biofilm formation (40), and a sudden increase in carbon substrate availability (including glucose availability) induces biofilm dispersion (48). In addition, glucose represses biofilm formation in *Bacillus subtilis* (53), as well as in *E. coli*, *Citrobacter freundii*, *Klebsiella pneumoniae*, and *Salmonella enterica* serovar Typhimurium (24). In contrast, *V. cholerae* exhibits an increase in biofilm-forming capacity when it is grown in minimal medium supplemented with 0.5% (wt/vol) glucose (23). The cAMP-CRP global transcriptional regulatory complex, which regulates carbon metabolism, positively regulates biofilm formation in *E. coli*, in which a *crp* mutation results in decreased biofilm formation (24), and in *Shewanella oneidensis*, in which a *crp* mutation results in a defect in biofilm detachment in the stop-of-flow-induced detachment response compared to the wild type (55). In contrast, a *V. cholerae* Δ*crp* deletion mutant exhibits increased biofilm formation (29), indicating that cAMP-CRP can act as either a positive regulator or a negative regulator of biofilm formation. We were, therefore, interested in further elucidating the molecular mechanism by which cAMP-CRP regulates biofilm formation in *V. cholerae*.

Using whole-genome transcriptional profiles of Δ*cyaA* and Δ*crp* mutants, we showed that cAMP-CRP regulates the expression of a large set of genes in *V. cholerae* (see Fig. S1 and Tables S1 and S2 in the supplemental material), consistent with its role as a global transcriptional regulatory complex (5, 12). It is also noteworthy that the overall transcriptional profiles of the Δ*cyaA* and Δ*crp* mutants were similar (see Fig. S1 in the supplemental material), consistent with the requirement for both cAMP and CRP in transcriptional regulation (5). More than 20% of the predicted genes in the genome of *V. cholerae* (as annotated by The Institute for Genomic Research)

are differentially regulated in both Δ*cyaA* and Δ*crp* mutants (≥2-fold change and FDR of ≤1%) compared to the wild type, and approximately equal numbers of genes are positively and negatively regulated. Consistent with the negative role of cAMP-CRP in biofilm formation, many of the genes involved in biofilm matrix production are upregulated in Δ*cyaA* and Δ*crp* mutants (Table 3). Several genes involved in pathogenesis are also upregulated, while genes involved in flagellum biosynthesis and chemotaxis are downregulated in the deletion mutants (see Fig. S1 and Tables S1 and S2 in the supplemental material).

V. cholerae wild-type biofilm formation requires the production of both VPS and matrix proteins, including RbmA, RbmC, and Bap1 (15, 16). These matrix proteins play a crucial role in maintaining the structural integrity of the biofilm. They may function as an agglutinin/adhesion in binding cells together and/or binding to VPS. They may also act as anchors for biofilm and/or cells to attach to surfaces that contain carbohydrates. As a consequence of environmental signals, *V. cholerae* may modulate the ratio of matrix proteins to VPS within a biofilm, giving rise to different degrees of biofilm rigidity and stability, which may promote detachment of single cells or cell aggregates for dispersal. It is therefore intriguing but not surprising that, although VpsR is the most downstream regulator of *vps* gene expression, cAMP-CRP can also regulate *rbmC* and *bap1* expression independent of VpsR regulation (Fig. 3).

Putative cAMP-CRP binding sites were predicted upstream of the *rbmA*, *rbmC*, and *bap1* coding regions. We are currently investigating if cAMP-CRP binds directly to these predicted binding sites. It is very intriguing that a predicted VpsR binding site (GTCTCATTACTGAGGCGT) overlaps by 11 bp the putative cAMP-CRP binding site (AACTTTGAGATGTCTC ATTACT) upstream of *rbmC*. A very plausible hypothesis is that cAMP-CRP negatively regulates *rbmC* expression by directly binding to its promoter region and, in doing so, interferes with the binding of VpsR to the upstream regulatory region of *rbmC* and thus prevents VpsR from positively regulating *rbmC* expression. Similar antagonistic functions have been reported for cAMP-CRP and AphA/AphB for *tcpPH* expression (28), as well as for HapR and VpsR for *aphA* expression (33). We are currently examining the possible antagonistic role of cAMP-CRP and VpsR in binding the *rbmC* promoter region. Using Virtual Footprint software (http://www.prodoric.de/vfp/index.php), a putative cAMP-CRP binding site was also predicted upstream of the coding region of *vpsR*, but not upstream of the coding region of *vpsT*. It is therefore possible that cAMP-CRP negatively regulates *vpsR* expression by direct binding to the upstream promoter region of *vpsR*. Since no cAMP-CRP binding sites upstream of *vpsT* have been predicted, the cAMP-CRP negative regulation of *vpsT* may be via VpsR (in addition to HapR) or by a so-far-unidentified repressor protein(s). We are currently investigating if cAMP-CRP binds directly upstream of the *vpsR* promoter region.

A glucose effect or catabolite repression has been observed in many microorganisms (5) and is a likely environmental nutritional cue for regulating biofilm formation. It has been reported that *vpsL* expression is regulated by one of the components of the phosphoenolpyruvate phosphotransferase (PTS) system, such that the accumulation of the phosphorylated form of enzyme I (EI~P) due to the absence of a PTS sugar, such as glucose, represses *vps* gene expression and leads to a reduction in biofilm formation (23). Furthermore, addition of cAMP to the growth medium, mimicking activation of adenylate cyclase (CyaA) by $EIIA^{Glu}$~P (12), represses biofilm accumulation in *V. cholerae* (23). It is noteworthy that addition of exogenous cAMP eliminated the enhanced pellicle-forming capacity of the Δ*cyaA* mutant (see Fig. S2 in the supplemental material). Consistent with this model, Δ*cyaA* and Δ*crp* mutants both exhibit increased biofilm-forming capacities. We also observed increased biofilm formation in wild-type *V. cholerae* grown in LB medium supplemented with 0.2 and 0.5% (wt/vol) glucose (data not shown). Interestingly, mannose (another PTS sugar) has also been reported to enhance biofilm formation in *V. cholerae*, likely through the PTS system (23, 27, 39). Similarly, growth on *N*-acetylglucosamine (also a PTS sugar) has been shown to promote attachment of *V. cholerae* and subsequent colonization of chitinous surfaces (37, 42). *V. cholerae* can colonize surfaces of phytoplankton and zooplankton (14, 34). Nutrients provided by mucous secretions of phytoplankton and zooplankton, as well as chitinous surfaces of zooplankton, could then facilitate or enhance biofilm formation by *V. cholerae* by providing environmental stimuli that lead to enhanced biofilm formation, thereby facilitating environmental persistence and growth of the pathogen.

The *V. cholerae* genome contains 62 genes coding for proteins containing GGDEF, EAL, and HD-GYP domains (17, 20). These proteins modulate cellular c-di-GMP levels (8, 45, 46, 49), which in turn regulate several phenotypic characteristics, including colony morphology, pellicle- and biofilm-forming capacities, and motility (3, 31, 32, 56, 57). c-di-GMP also regulates similar biological processes in several other microorganisms, including *P. aeruginosa*, *E. coli*, and *S. enterica* serovar Typhimurium (25, 43, 44). Using whole-genome expression profiles of Δ*cyaA* and Δ*crp* mutants, we identified 10 genes encoding proteins that contain a conserved GGDEF domain and are upregulated in the Δ*cyaA* and/or Δ*crp* mutant compared to the wild type (Table 3). For these genes, only deletion of *cdgA* in the Δ*crp* genetic background eliminated pellicle formation and reduced the biofilm-forming capacity (Fig. 4 and 6), indicating that the increased biofilm-forming capacity of the Δ*crp* mutant was due in part to increased expression of *cdgA*, which in turn modulated the expression of *vps* genes and genes encoding matrix proteins (Fig. 6B and C). Interestingly, we also observed increases in biofilm formation for the Δ*crp* Δ*rocS* and Δ*crp*Δ*cdgI* mutants compared to the Δ*crp*, Δ*rocS*, and Δ*cdgI* single-deletion mutants (Fig. 4B). Although both RocS and CdgI contain conserved GGDEF-EAL domains, the results of biofilm formation assays suggest that, like RocS, CdgI functions mainly as a PDE under the conditions that we utilized. The contributions of other PDEs (Table 3) to the negative regulation of biofilm formation by cAMP-CRP remain to be investigated.

Regulation of biofilm formation in *V. cholerae* is complex. We previously described the regulatory network controlling *vps* gene expression, where *vps* genes are positively regulated by VpsR, VpsT, and CdgA and negatively regulated by HapR (2). HapR also negatively regulates the expression of *vpsT*, *vpsR*, and *cdgA*. Interestingly, a recent study reported that HapR binds directly to the promoter regions of *vpsT* and *cdgA* (59). We also previously described the positive regulation of *cdgA* by

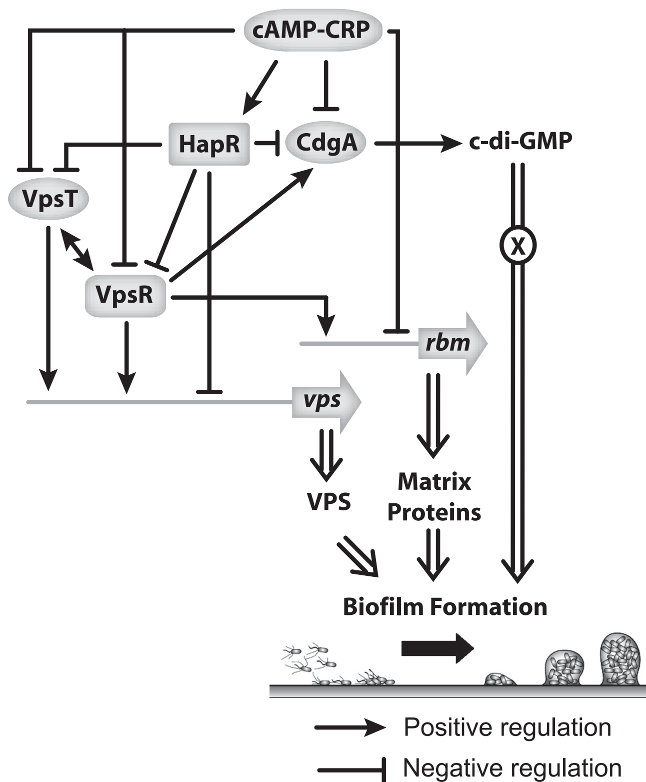

FIG. 7. Model of cAMP-CRP regulation of biofilm formation in *V. cholerae*. cAMP-CRP regulates biofilm formation at multiple levels. Expression of *vps* and *rbm* genes is negatively regulated by cAMP-CRP through positive regulation of *hapR* expression and through negative regulation of *vpsR* and *vpsT* expression. In turn, VpsR and VpsT positively regulate *vps* and *rbm* gene expression, while HapR negatively regulates *vps* and *rbm* gene expression. VpsR, VpsT, and HapR regulation of *vps* and *rbm* gene expression also involves c-di-GMP signaling, where *cdgA* expression is negatively regulated by HapR and positively regulated by VpsR and VpsT. An increase in *cdgA* transcription leads to an increase in the c-di-GMP level, which in turn could interact with an effector protein(s) to positively regulate biofilm formation. We have a very limited understanding of the link between c-di-GMP pools and the signaling that leads to biofilm formation in *V. cholerae*. In addition, cAMP-CRP may also directly regulate *vpsR*, *cdgA*, and *rbmC* expression and indirectly regulate *vpsT* expression.

VpsR and VpsT. Using Virtual Footprint software, two putative cAMP-CRP binding sites were predicted upstream of the *cdgA* coding region, suggesting that cAMP-CRP can regulate *cdgA* expression both directly by binding to the *cdgA* promoter region and indirectly through HapR.

Data obtained in this study, together with results described in other reports, indicate that cAMP-CRP regulates biofilm formation at multiple levels (Fig. 7). First, cAMP-CRP negatively regulates biofilm formation in *V. cholerae* through positive regulation of *hapR* expression, which in turn negatively regulates expression of *vps* and *rbm* (and *bap1*) genes, as well as *cdgA*, *vpsT*, and, in our strain, *vpsR*. VpsR, VpsT, and CdgA, in turn, positively regulate expression of *vps* and *rbm* (as well as *bap1*) genes. Second, cAMP-CRP may also negatively regulate expression of *vpsR*, *cdgA*, and *rbmC* expression by directly binding to their promoter regions. The connection between cAMP-CRP regulatory circuitry and c-di-GMP signaling reported here is particularly intriguing. Further characterization of this connection should provide additional insight into the wiring of the regulatory circuitry controlling biofilm formation in *V. cholerae*.

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REFERENCES

1. **Bao, Y., D. P. Lies, H. Fu, and G. P. Roberts.** 1991. An improved Tn*7*-based system for the single-copy insertion of cloned genes into chromosomes of gram-negative bacteria. Gene **109:**167–168.
2. **Beyhan, S., K. Bilecen, S. R. Salama, C. Casper-Lindley, and F. H. Yildiz.** 2007. Regulation of rugosity and biofilm formation in *Vibrio cholerae*: comparison of VpsT and VpsR regulons and epistasis analysis of *vpsT*, *vpsR*, and *hapR*. J. Bacteriol. **189:**388–402.
3. **Beyhan, S., A. D. Tischler, A. Camilli, and F. H. Yildiz.** 2006. Transcriptome and phenotypic responses of *Vibrio cholerae* to increased cyclic di-GMP level. J. Bacteriol. **188:**3600–3613.
4. **Beyhan, S., and F. H. Yildiz.** 2007. Smooth to rugose phase variation in *Vibrio cholerae* can be mediated by a single nucleotide change that targets c-di-GMP signalling pathway. Mol. Microbiol. **63:**995–1007.
5. **Botsford, J. L., and J. G. Harman.** 1992. Cyclic AMP in prokaryotes. Microbiol. Rev. **56:**100–122.
6. **Bowden, G. H., and Y. H. Li.** 1997. Nutritional influences on biofilm development. Adv. Dent. Res. **11:**81–99.
7. **Casper-Lindley, C., and F. H. Yildiz.** 2004. VpsT is a transcriptional regulator required for expression of *vps* biosynthesis genes and the development of rugose colonial morphology in *Vibrio cholerae* O1 El Tor. J. Bacteriol. **186:**1574–1578.
8. **Christen, M., B. Christen, M. Folcher, A. Schauerte, and U. Jenal.** 2005. Identification and characterization of a cyclic di-GMP-specific phosphodiesterase and its allosteric control by GTP. J. Biol. Chem. **280:**30829–30837.
9. **Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott.** 1995. Microbial biofilms. Annu. Rev. Microbiol. **49:**711–745.
10. **Davey, M. E., and G. A. O'Toole.** 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. **64:**847–867.
11. **de Lorenzo, V., and K. N. Timmis.** 1994. Analysis and construction of stable phenotypes in gram-negative bacteria with Tn*5*- and Tn*10*-derived minitransposons. Methods Enzymol. **235:**386–405.
12. **Deutscher, J., C. Francke, and P. W. Postma.** 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. Microbiol. Mol. Biol. Rev. **70:**939–1031.
13. **Donlan, R. M., and J. W. Costerton.** 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin. Microbiol. Rev. **15:**167–193.
14. **Faruque, S. M., M. J. Albert, and J. J. Mekalanos.** 1998. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. Microbiol. Mol. Biol. Rev. **62:**1301–1314.
15. **Fong, J. C., K. Karplus, G. K. Schoolnik, and F. H. Yildiz.** 2006. Identification and characterization of RbmA, a novel protein required for the development of rugose colony morphology and biofilm structure in *Vibrio cholerae*. J. Bacteriol. **188:**1049–1059.
16. **Fong, J. C., and F. H. Yildiz.** 2007. The *rbmBCDEF* gene cluster modulates development of rugose colony morphology and biofilm formation in *Vibrio cholerae*. J. Bacteriol. **189:**2319–2330.
17. **Galperin, M. Y., A. N. Nikolskaya, and E. V. Koonin.** 2001. Novel domains of the prokaryotic two-component signal transduction systems. FEMS Microbiol. Lett. **203:**11–21.
18. **Gentleman, R. C., V. J. Carey, D. M. Bates, B. Bolstad, M. Dettling, S. Dudoit, B. Ellis, L. Gautier, Y. Ge, J. Gentry, K. Hornik, T. Hothorn, W. Huber, S. Iacus, R. Irizarry, F. Leisch, C. Li, M. Maechler, A. J. Rossini, G. Sawitzki, C. Smith, G. Smyth, L. Tierney, J. Y. Yang, and J. Zhang.** 2004. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. **5:**R80.
19. **Hammer, B. K., and B. L. Bassler.** 2003. Quorum sensing controls biofilm formation in *Vibrio cholerae*. Mol. Microbiol. **50:**101–104.
20. **Heidelberg, J. F., J. A. Eisen, W. C. Nelson, R. A. Clayton, M. L. Gwinn, R. J. Dodson, D. H. Haft, E. K. Hickey, J. D. Peterson, L. Umayam, S. R. Gill, K. E. Nelson, T. D. Read, H. Tettelin, D. Richardson, M. D. Ermolaeva, J. Vamathevan, S. Bass, H. Qin, I. Dragoi, P. Sellers, L. McDonald, T. Utterback, R. D. Fleishmann, W. C. Nierman, O. White, S. L. Salzberg, H. O. Smith, R. R. Colwell, J. J. Mekalanos, J. C. Venter, and C. M. Fraser.** 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. Nature **406:**477–483.

21. **Herrero, M., V. de Lorenzo, and K. N. Timmis.** 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. J. Bacteriol. **172:**6557–6567.
22. **Heydorn, A., A. T. Nielsen, M. Hentzer, C. Sternberg, M. Givskov, B. K. Ersboll, and S. Molin.** 2000. Quantification of biofilm structures by the novel computer program COMSTAT. Microbiology **146:**2395–2407.
23. **Houot, L., and P. I. Watnick.** 2008. A novel role for enzyme I of the *Vibrio cholerae* phosphoenolpyruvate phosphotransferase system in regulation of growth in a biofilm. J. Bacteriol. **190:**311–320.
24. **Jackson, D. W., J. W. Simecka, and T. Romeo.** 2002. Catabolite repression of *Escherichia coli* biofilm formation. J. Bacteriol. **184:**3406–3410.
25. **Jenal, U., and J. Malone.** 2006. Mechanisms of cyclic-di-GMP signaling in bacteria. Annu. Rev. Genet. **40:**385–407.
26. **Kaper, J. B., J. G. Morris, Jr., and M. M. Levine.** 1995. Cholera. Clin. Microbiol. Rev. **8:**48–86.
27. **Kierek, K., and P. I. Watnick.** 2003. Environmental determinants of *Vibrio cholerae* biofilm development. Appl. Environ. Microbiol. **69:**5079–5088.
28. **Kovacikova, G., and K. Skorupski.** 2001. Overlapping binding sites for the virulence gene regulators AphA, AphB and cAMP-CRP at the *Vibrio cholerae tcpPH* promoter. Mol. Microbiol. **41:**393–407.
29. **Liang, W., A. Pascual-Montano, A. J. Silva, and J. A. Benitez.** 2007. The cyclic AMP receptor protein modulates quorum sensing, motility and multiple genes that affect intestinal colonization in *Vibrio cholerae*. Microbiology **153:**2964–2975.
30. **Liang, W., A. J. Silva, and J. A. Benitez.** 2007. The cyclic AMP receptor protein modulates colonial morphology in *Vibrio cholerae*. Appl. Environ. Microbiol. **73:**7482–7487.
31. **Lim, B., S. Beyhan, J. Meir, and F. H. Yildiz.** 2006. Cyclic-diGMP signal transduction systems in *Vibrio cholerae*: modulation of rugosity and biofilm formation. Mol. Microbiol. **60:**331–348.
32. **Lim, B., S. Beyhan, and F. H. Yildiz.** 2007. Regulation of *Vibrio* polysaccharide synthesis and virulence factor production by CdgC, a GGDEF-EAL domain protein, in *Vibrio cholerae*. J. Bacteriol. **189:**717–729.
33. **Lin, W., G. Kovacikova, and K. Skorupski.** 2007. The quorum sensing regulator HapR downregulates the expression of the virulence gene transcription factor AphA in *Vibrio cholerae* by antagonizing Lrp- and VpsR-mediated activation. Mol. Microbiol. **64:**953–967.
34. **Lipp, E. K., A. Huq, and R. R. Colwell.** 2002. Effects of global climate on infectious disease: the cholera model. Clin. Microbiol. Rev. **15:**757–770.
35. **Marsh, J. W., D. Sun, and R. K. Taylor.** 1996. Physical linkage of the *Vibrio cholerae* mannose-sensitive hemagglutinin secretory and structural subunit gene loci: identification of the *mshG* coding sequence. Infect. Immun. **64:** 460–465.
36. **Matz, C., D. McDougald, A. M. Moreno, P. Y. Yung, F. H. Yildiz, and S. Kjelleberg.** 2005. Biofilm formation and phenotypic variation enhance predation-driven persistence of *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **102:**16819–16824.
37. **Meibom, K. L., X. B. Li, A. T. Nielsen, C. Y. Wu, S. Roseman, and G. K. Schoolnik.** 2004. The *Vibrio cholerae* chitin utilization program. Proc. Natl. Acad. Sci. USA **101:**2524–2529.
38. **Miller, J. H.** 1972. Assay of β-galactosidase, p. 352–355. *In* J. H. Miller (ed.), Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
39. **Moorthy, S., and P. I. Watnick.** 2004. Genetic evidence that the *Vibrio cholerae* monolayer is a distinct stage in biofilm development. Mol. Microbiol. **52:**573–587.
40. **O'Toole, G. A., K. A. Gibbs, P. W. Hager, P. V. Phibbs, Jr., and R. Kolter.** 2000. The global carbon metabolism regulator Crc is a component of a signal transduction pathway required for biofilm development by *Pseudomonas aeruginosa*. J. Bacteriol. **182:**425–431.
41. **Parsek, M. R., and P. K. Singh.** 2003. Bacterial biofilms: an emerging link to disease pathogenesis. Annu. Rev. Microbiol. **57:**677–701.
42. **Pruzzo, C., L. Vezzulli, and R. R. Colwell.** 2008. Global impact of *Vibrio cholerae* interactions with chitin. Environ. Microbiol. **10:**1400–1410.
43. **Romling, U., and D. Amikam.** 2006. Cyclic di-GMP as a second messenger. Curr. Opin. Microbiol. **9:**218–228.
44. **Romling, U., M. Gomelsky, and M. Y. Galperin.** 2005. C-di-GMP: the dawning of a novel bacterial signalling system. Mol. Microbiol. **57:**629–639.
45. **Ryan, R. P., Y. Fouhy, J. F. Lucey, L. C. Crossman, S. Spiro, Y. W. He, L. H. Zhang, S. Heeb, M. Camara, P. Williams, and J. M. Dow.** 2006. Cell-cell signaling in *Xanthomonas campestris* involves an HD-GYP domain protein that functions in cyclic di-GMP turnover. Proc. Natl. Acad. Sci. USA **103:** 6712–6717.
46. **Ryjenkov, D. A., M. Tarutina, O. V. Moskvin, and M. Gomelsky.** 2005. Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: insights into biochemistry of the GGDEF protein domain. J. Bacteriol. **187:**1792–1798.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring, NY.
48. **Sauer, K., M. C. Cullen, A. H. Rickard, L. A. Zeef, D. G. Davies, and P. Gilbert.** 2004. Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. J. Bacteriol. **186:**7312–7326.
49. **Schmidt, A. J., D. A. Ryjenkov, and M. Gomelsky.** 2005. The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: enzymatically active and inactive EAL domains. J. Bacteriol. **187:**4774–4781.
50. **Silva, A. J., and J. A. Benitez.** 2004. Transcriptional regulation of *Vibrio cholerae* hemagglutinin/protease by the cyclic AMP receptor protein and RpoS. J. Bacteriol. **186:**6374–6382.
51. **Simons, R. W., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:**85–96.
52. **Skorupski, K., and R. K. Taylor.** 1997. Cyclic AMP and its receptor protein negatively regulate the coordinate expression of cholera toxin and toxin-coregulated pilus in *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **94:**265–270.
53. **Stanley, N. R., R. A. Britton, A. D. Grossman, and B. A. Lazazzera.** 2003. Identification of catabolite repression as a physiological regulator of biofilm formation by *Bacillus subtilis* by use of DNA microarrays. J. Bacteriol. **185:** 1951–1957.
54. **Taylor, R. K., C. Manoil, and J. J. Mekalanos.** 1989. Broad-host-range vectors for delivery of Tn*phoA*: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. J. Bacteriol. **171:**1870–1878.
55. **Thormann, K. M., R. M. Saville, S. Shukla, and A. M. Spormann.** 2005. Induction of rapid detachment in *Shewanella oneidensis* MR-1 biofilms. J. Bacteriol. **187:**1014–1021.
56. **Tischler, A. D., and A. Camilli.** 2004. Cyclic diguanylate (c-di-GMP) regulates *Vibrio cholerae* biofilm formation. Mol. Microbiol. **53:**857–869.
57. **Tischler, A. D., and A. Camilli.** 2005. Cyclic diguanylate regulates *Vibrio cholerae* virulence gene expression. Infect. Immun. **73:**5873–5882.
58. **Tusher, V. G., R. Tibshirani, and G. Chu.** 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. USA **98:**5116–5121.
59. **Waters, C. M., W. Lu, J. D. Rabinowitz, and B. L. Bassler.** 2008. Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic di-GMP levels and repression of *vpsT*. J. Bacteriol. **190:**2527–2536.
60. **Watnick, P. I., and R. Kolter.** 1999. Steps in the development of a *Vibrio cholerae* El Tor biofilm. Mol. Microbiol. **34:**586–595.
61. **Yildiz, F. H., N. A. Dolganov, and G. K. Schoolnik.** 2001. VpsR, a member of the response regulators of the two-component regulatory systems, is required for expression of *vps* biosynthesis genes and EPS^{ETr}-associated phenotypes in *Vibrio cholerae* O1 El Tor. J. Bacteriol. **183:**1716–1726.
62. **Yildiz, F. H., X. S. Liu, A. Heydorn, and G. K. Schoolnik.** 2004. Molecular analysis of rugosity in a *Vibrio cholerae* O1 El Tor phase variant. Mol. Microbiol. **53:**497–515.
63. **Yildiz, F. H., and G. K. Schoolnik.** 1999. *Vibrio cholerae* O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. Proc. Natl. Acad. Sci. USA **96:**4028–4033.
64. **Zhu, J., and J. J. Mekalanos.** 2003. Quorum sensing-dependent biofilms enhance colonization in *Vibrio cholerae*. Dev. Cell **5:**647–656.