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RESEARCH ARTICLE

Modulation of CrbS-Dependent Activation of the Acetate Switch in *Vibrio cholerae*

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ABSTRACT *Vibrio cholerae* controls the pathogenicity of interactions with arthropod hosts via the activity of the CrbS/R two-component system. This signaling pathway regulates the consumption of acetate, which in turn alters the relative virulence of interactions with arthropods, including *Drosophila melanogaster*. CrbS is a histidine kinase that links a transporter-like domain to its signaling apparatus via putative STAC and PAS domains. CrbS and its cognate response regulator are required for the expression of acetyl coenzyme A (acetyl-CoA) synthetase (product of *acs*), which converts acetate to acetyl-CoA. We demonstrate that the STAC domain of CrbS is required for signaling in culture; without it, *acs* transcription is reduced in LB medium, and *V. cholerae* cannot grow on acetate minimal media. However, the strain remains virulent toward *Drosophila* and expresses *acs* similarly to the wild type during infection. This suggests that there is a unique signal or environmental variable that modulates CrbS in the gastrointestinal tract of *Drosophila*. Second, we present evidence in support of CrbR, the response regulator that interacts with CrbS, binding directly to the *acs* promoter, and we identify a region of the promoter that CrbR may target. We further demonstrate that nutrient signals, together with the cAMP receptor protein (CRP)-cAMP system, control *acs* transcription, but regulation may occur indirectly, as CRP-cAMP activates the expression of the *crbS* and *crbR* genes. Finally, we define the role of the Pta-AckA system in *V. cholerae* and identify redundancy built into acetate excretion pathways in this pathogen.

IMPORTANCE CrbS is a member of a unique family of sensor histidine kinases, as its structure suggests that it may link signaling to the transport of a molecule. However, mechanisms through which CrbS senses and communicates information about the outside world are unknown. In the *Vibrionaceae*, orthologs of CrbS regulate acetate metabolism, which can, in turn, affect interactions with host organisms. Here, we situate CrbS within a larger regulatory framework, demonstrating that *crbS* is regulated by nutrient-sensing systems. Furthermore, CrbS domains may play various roles in signaling during infection and growth in culture, suggesting a unique mechanism of host recognition. Finally, we define the roles of additional pathways in acetate flux, as a foundation for further studies of this metabolic nexus point.

KEYWORDS *Vibrio cholerae*, acetate, two-component system, acetyl-CoA synthetase, sensor histidine kinase

Vibrio cholerae causes global pandemics of the diarrheal disease cholera, but most strains are adapted for survival in marine environments. *V. cholerae* can multiply rapidly in response to an influx of dissolved organic carbon (1, 2), or it can thrive in close association with copepods, sea birds, fish, and midge larvae (3–7). *V. cholerae* may also inhabit the gastrointestinal (GI) tracts of terrestrial arthropods, including house flies (8, 9). Investigations of molecular mechanisms underlying *V. cholerae* infection in an

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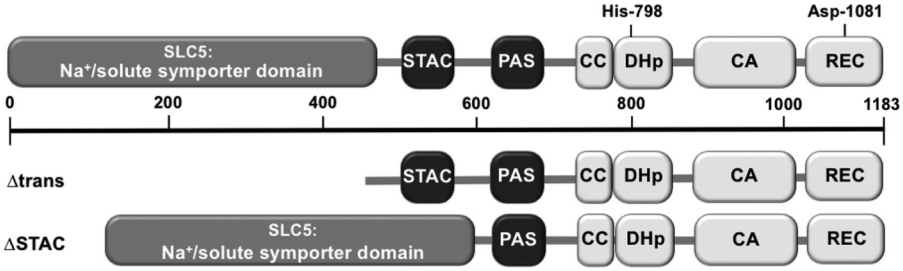

FIG 1 Structure of CrbS and deletions in *crbS* introduced into the *V. cholerae* genome. The CrbS protein consists of 1,183 amino acids, with the SLC5 Na^+/solute symporter-like domain, the STAC domain, the PAS domain, a coiled-coil (CC) region, a DHp domain, the CA domain, and the receiver (REC) domain. The conserved His and Asp residues that may contribute to phosphotransfer are indicated. The Δ*trans* construct lacks the entire SLC5-like domain, while the ΔSTAC construct lacks the STAC domain.

arthropod (*Drosophila melanogaster*) model of infection revealed unexpected roles for bacterial metabolites, including short-chain fatty acids (SCFAs), in modulating the pathogenicity of these interactions (10–12). SCFAs produced by colonizing bacteria can alter the physiology of a variety of host organisms, affecting the development of the immune system, appetite, and overall body size (13–18). In *Drosophila*, levels of the SCFA acetate in the *Drosophila* midgut are controlled by commensal *Acetobacter* bacteria (18). During *V. cholerae* infection, the pathogen's molecular mechanisms of regulating both acetate excretion and consumption are important determinants of virulence (10, 19). *V. cholerae* removes acetate from the fly midgut, causing fats to amass not in the fat body, as they do in healthy flies, but in cells lining the fly GI tract, which sensitizes the flies to killing by this pathogen (10). Removal of acetate from the surrounding medium is controlled by the expression and activity of acetyl coenzyme A (acetyl-CoA) synthetase (Acs), which converts acetate to acetyl-CoA (10, 20). In *V. cholerae* and other members of the *Vibrionaceae*, *acs* transcription is positively regulated by the CrbS/R two-component system (10, 21, 22). Due to its role in controlling *acs*, the CrbS/R pathway is necessary for *V. cholerae* infection and virulence toward *Drosophila*; without it, *V. cholerae* is virtually avirulent (10, 21). By connecting acetate metabolism to the CrbS/R two-component system, *V. cholerae* and related pathogens may link this metabolic switch to additional environmental cues.

The structure of CrbS, with several domains of unknown function, suggests that it employs a novel mechanism of transmitting information across the bacterial cell envelope (Fig. 1). The N terminus consists of a membrane-localized domain with similarity to the solute carrier 5 (SLC5) transporter family and the sodium-proline symporter PutP from *Escherichia coli*, with 13 transmembrane regions (23, 24). Directly adjacent is a STAC (SLC5- and two-component signal transduction-associated component) domain, which is thought to regulate transport through the sodium/solute symporter domain, based on structural and bioinformatics analyses (25). CrbS also carries a PAS domain; these domains are often involved in signal sensing and transmission in two-component systems (26). CrbS includes both DHp (dimerization histidine phosphotransfer) and CA (catalytic ATP binding) domains involved in the autophosphorylation of a conserved histidine residue. Finally, CrbS carries a receiver (REC) domain, with the conserved aspartate residue that receives the phosphoryl group. The presence of a REC domain implies that CrbS functions as a hybrid histidine kinase that initiates a multistep phosphorelay, but our results indicate that the receiver domain is not necessary for signaling, and it may instead act as a negative regulator (21). We have shown, however, that the phosphor-accepting His residue located in the DHp domain is required for *acs* expression, indicating that phosphorylation is associated with the "on" state of this pathway (21).

Based on the arrangement of these domains, we hypothesize that CrbS and related proteins may activate a signaling mechanism that ties transport directly to gene expression (27). By linking the putative transporter domain with the signaling domains,

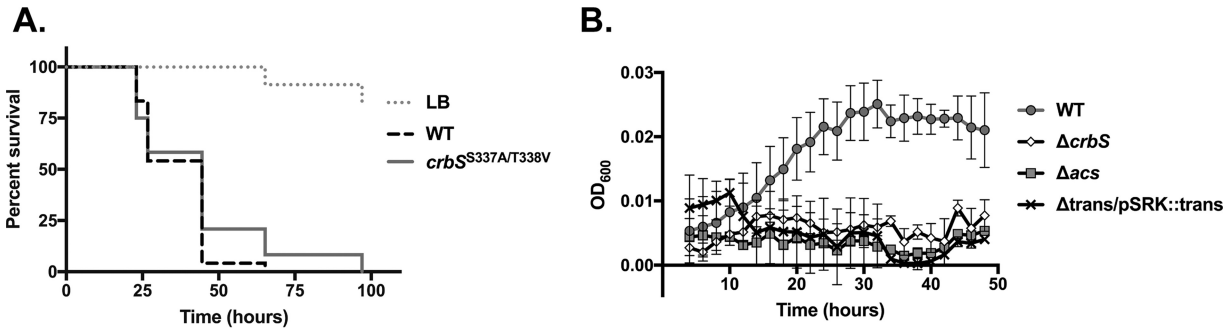

FIG 2 The CrbS transporter-like domain is needed for CrbS function, but putative Na^+ binding sites are not important. (A) Survival of *Drosophila* flies infected with *V. cholerae* strains carrying mutations in the putative CrbS Na^+ binding site (S337A/T338V) does not differ from that of flies infected with the wild-type *V. cholerae* strain ($P > 0.05$ by a log rank test). Results of this assay are representative of data from two biological replicates. (B) The transporter domain is required for CrbS function, as the strain lacking the domain is unable to grow on minimal medium with acetate. Growth of *V. cholerae* strains carrying deletions in *crbS*, *acs*, or the transporter-like domain within *crbS* was observed in M63 minimal medium supplemented with acetate (10 mM). Complementation of the transporter domain in the pSRK-Km plasmid, with induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), does not restore growth.

the sensor kinase may regulate *acs* in response to a small molecule as it is transported into the cell (27). After transporting the signal, the pathway may then immediately convert this information into a response, by directly activating the expression of a suite of genes, including *acs*. This is borne out in other bacterial species; an ortholog of CrbR in *Vibrio vulnificus*, AcsR, directly binds to and activates the *acs* promoter (22), and a binding site has been defined in the promoters of genes targeted by CrbR homologs across the gammaproteobacteria (28). Thus, we hypothesize that a similar mechanism is operative in *V. cholerae*. Therefore, the first two goals of this study were to ascertain the effects of CrbS domains on signal detection and propagation and to characterize the nature of the interaction between CrbR and the *acs* promoter in *V. cholerae*.

CrbS is necessary for *acs* transcription, but it may function as one component of a larger network of regulators controlling acetate metabolism. If so, this could support the hypothesis that CrbS regulates *acs* under novel conditions, rather than supplanting information provided by these conserved regulators. Therefore, a third goal of this study was to determine whether other conserved signaling mechanisms contribute to *acs* transcription in *V. cholerae*. Here, we took a candidate approach, based on pathways important in *E. coli* for the regulation of *acs* and acetate metabolism (20). These include the cAMP receptor protein (CRP)-cAMP system, which regulates *acs* in response to preferred carbon availability; *rpoS*, the sigma factor required for the expression of stationary-phase genes; and the *pta-ackA* pathway, which is required for acetate excretion.

Altogether, our results suggest new mechanisms of *acs* regulation that intersect with CrbS. We also show that the *in vivo* environment may alter CrbS-dependent regulation of *acs*, supporting the hypothesis that additional *in vivo* signals modulate signaling.

RESULTS

Mutational analysis of the CrbS transporter domain. CrbS includes a transporter-like domain (Fig. 1), and we began by identifying specific residues, based on homology to PutP, that may mediate signaling (24). PutP utilizes a Na^+ gradient to drive the transport of proline, and marine bacteria, including the *Vibrionaceae*, tie transport processes to Na^+ gradients (23). We aligned the *E. coli* PutP protein sequence with the CrbS transporter domain and identified several conserved residues that contribute to Na^+ transport in *E. coli*. These included Ser-340 and Thr-341 (29), which fall at residues 336 and 337 within transmembrane segment IX in *V. cholerae*. We introduced simultaneous mutations of these residues into the *V. cholerae* chromosome, and we tested the effects of these mutations on *Drosophila* survival. We observed no difference in fly survival relative to the wild-type (WT) strain, indicating that these residues are not required for signaling *in vivo* (Fig. 2A).

We next tested the hypothesis that an intact transporter domain is necessary for signaling, by excising the entire transporter domain coding sequence, including codons

for amino acids 4 to 504, from the gene in the chromosome. As expected, deletion of the transporter domain halted acetate catabolism both in culture and during infection. This deletion abrogated virulence in *Drosophila* (data not shown) and prevented growth on acetate minimal medium (Fig. 2B). These observations are consistent with one of two possible interpretations. First, the remaining protein may be unstable, as it has no means of being tethered in the membrane. Second, the protein may be blind to the presence of specific signaling molecules or conditions and therefore may be unable to initiate a signaling cascade. To test whether this phenotype can be rescued by the expression of the CrbS transporter domain alone, we overexpressed the transporter domain and observed the growth of this strain on acetate minimal medium. Overexpression of this domain was insufficient to rescue this deletion (Fig. 2B). This may be due to one of two reasons. First, the two domains may need to be physically linked in order for signaling to occur, as observed in CbrA (27). Alternatively, it is possible that the proteins are unstable or misfolded and are unable to function. These findings indicate that a functional transporter domain is necessary for CrbS signaling, due to its role in ensuring protein stability, membrane tethering, or signaling initiation and propagation.

Mutational analysis of the STAC domain. Next, we tested whether the STAC domain contributes to signaling. The STAC can comprise one of several domains in cytosolic or membrane-tethered proteins, or it can exist as a stand-alone domain (25). In proteins with SLC5-like transporter domains, the STAC is located directly adjacent to this domain. To identify CrbS residues conserved with STAC domains, we performed an alignment with other STAC-containing proteins in MUSCLE (30). The first of two G(X)XXA motifs, which likely falls at a hairpin turn in this protein family, includes the G residue alone, but the second motif is fully conserved (25). Hydrophobic amino acids, which are present at regular intervals in this domain, also align with the pattern of conservation observed in this domain family (25).

To test the effects of this domain on signaling, we engineered the removal of the STAC domain from the *crbS* gene in the chromosome, by excising amino acids 536 to 617, leaving behind no tag or scar in the protein sequence. We then introduced a plasmid carrying the *acs* promoter driving the expression of the *lux* genes (21), and we tested the effects of the STAC domain deletion on *acs* transcription. The expression level of *acs* was lower in the *crbS*ΔSTAC strain than in the wild-type strain (Fig. 3A). To determine whether this deletion may have impacted growth on acetate minimal medium, the strain was inoculated into M63 medium supplemented with 10 mM acetate. This strain was unable to grow, suggesting that lower levels of *acs* expression correlated with reduced growth on acetate minimal medium (Fig. 3B). Next, we asked whether this deletion altered virulence in *Drosophila*. Surprisingly, flies fed the *crbS*ΔSTAC strain died at a rate indistinguishable from that of flies fed the wild-type *V. cholerae* strain, in each of five independent biological replicates (Fig. 3C). This indicates that CrbS signaling is occurring *in vivo*, as a full deletion of *crbS* significantly impairs fly mortality (Fig. 3C). Furthermore, this result suggests that the deletion of a large domain of CrbS does not result in degradation of the protein during *Drosophila melanogaster* infection.

The STAC domain is not required for CrbS-dependent signaling during infection of *Drosophila*. We reasoned that the discrepancy between the lack of acetate metabolism of the *crbS*ΔSTAC strain and its ability to cause *Drosophila* mortality could be explained in three ways. First, it is possible that this mutant, despite expressing *acs* at low levels, allows for consumption of acetate at rates similar to those of the wild-type strain. Second, this strain might reduce the expression of *acs in vivo*, but another (unknown) virulence factor that compensates for the lack of *acs* expression during infection may be upregulated. Third, signaling *in vivo* may be altered relative to *in vitro* conditions, such that the *acs* expression level in the flies is higher than our *in vitro*, culture-based assays had indicated. We tested each of these hypotheses in turn.

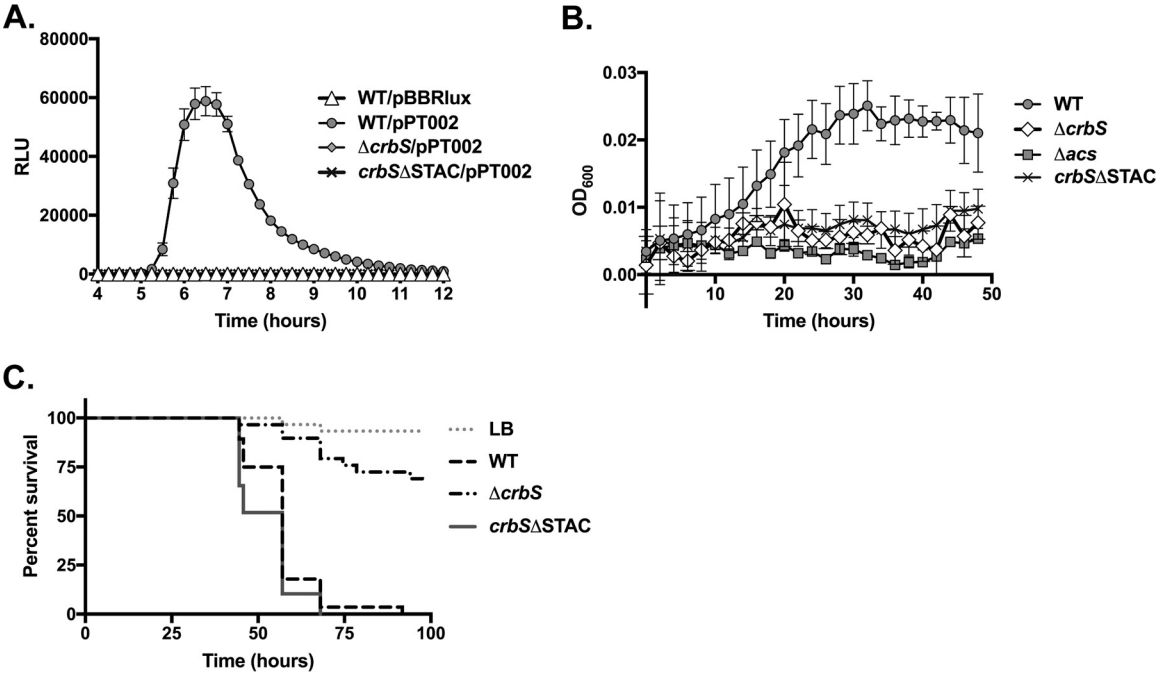

FIG 3 Effects of the CrbS STAC deletion on *acs* transcription, acetate metabolism, and fly survival. (A) Deletion of the STAC domain prevents *acs* promoter activation, similarly to the Δ*crbS* strain. For each strain, relative light units (RLU) (defined as luminescence/OD_{600} unit) from 8 wells of a 96-well plate were measured, with SIO strains carrying either the empty pBBR*lux* plasmid or the pPT002 plasmid into which the *acs* promoter was inserted. Error bars indicate standard deviations. (B) Deletion of the STAC domain prevents growth on acetate minimal medium. Growth of the SIO WT, Δ*acs*, Δ*crbS*, or *crbS*ΔSTAC strain on M63 minimal medium supplemented with 10 mM acetate was monitored every 2 h for 48 h. Average growth in at least 3 wells of a 96-well plate is shown. Error bars indicate standard deviations. (C) Survival of *Drosophila* flies infected with the SIO WT, Δ*crbS*, or *crbS*ΔSTAC strain. The survival of flies fed the *crbS*ΔSTAC strain did not differ from that of flies fed the WT strain in each of five biological replicates ($P > 0.05$ by log rank analysis), of which data from one representative experiment are depicted here.

To first examine rates of acetate consumption *in vitro*, we measured acetate concentrations in cultures inoculated with wild-type *V. cholerae*, the *crbS* mutant, or the STAC mutant. The *crbS* mutant delayed acetate consumption, but deletion of the STAC domain did not affect acetate consumption relative to the WT (Fig. 4A). Thus, a reduction in *acs* expression may not result in a measurable difference in acetate levels under these conditions. This finding argues that a low threshold of *acs* transcription may be sufficient for the removal of acetate from the medium.

Next, we tested whether the *crbS*ΔSTAC strain may be upregulating a second virulence factor that can compensate for the lack of *acs* expression. We constructed a double-deletion strain that carries the Δ*acs* and the *crbS*ΔSTAC alleles. Introducing *crbS*ΔSTAC into the Δ*acs* background did not recover the virulence of this strain (Fig. 4B). Thus, a second, unknown virulence factor cannot explain the pathogenic phenotype of the *crbS*ΔSTAC strain.

Finally, we tested whether this strain reduces *acs* expression during infection, by performing reverse transcription-quantitative real-time PCR (RT-qPCR) on RNA isolated from flies infected with the WT or *crbS*ΔSTAC *V. cholerae* strain. As controls, we also tested *acs* expression in flies infected with the Δ*crbS* strain, which should reduce *acs* expression, as well as a strain carrying a deletion in the *crbS* REC domain, which we expected to express *acs* similarly to the wild-type strain (21). We confirmed that the *acs* expression level is lower in flies carrying the *crbS* deletion (10), and we observed that *acs* expression was equivalent to that of the wild-type strain in both the STAC and REC domain deletions (Fig. 4C). Therefore, conditions in the fly gastrointestinal tract allow for *acs* expression regardless of the deletion of the CrbS STAC domain.

We reasoned that host-specific conditions could be affecting CrbS-dependent expression of *acs* via one of several mechanisms. First, the fly environment may be

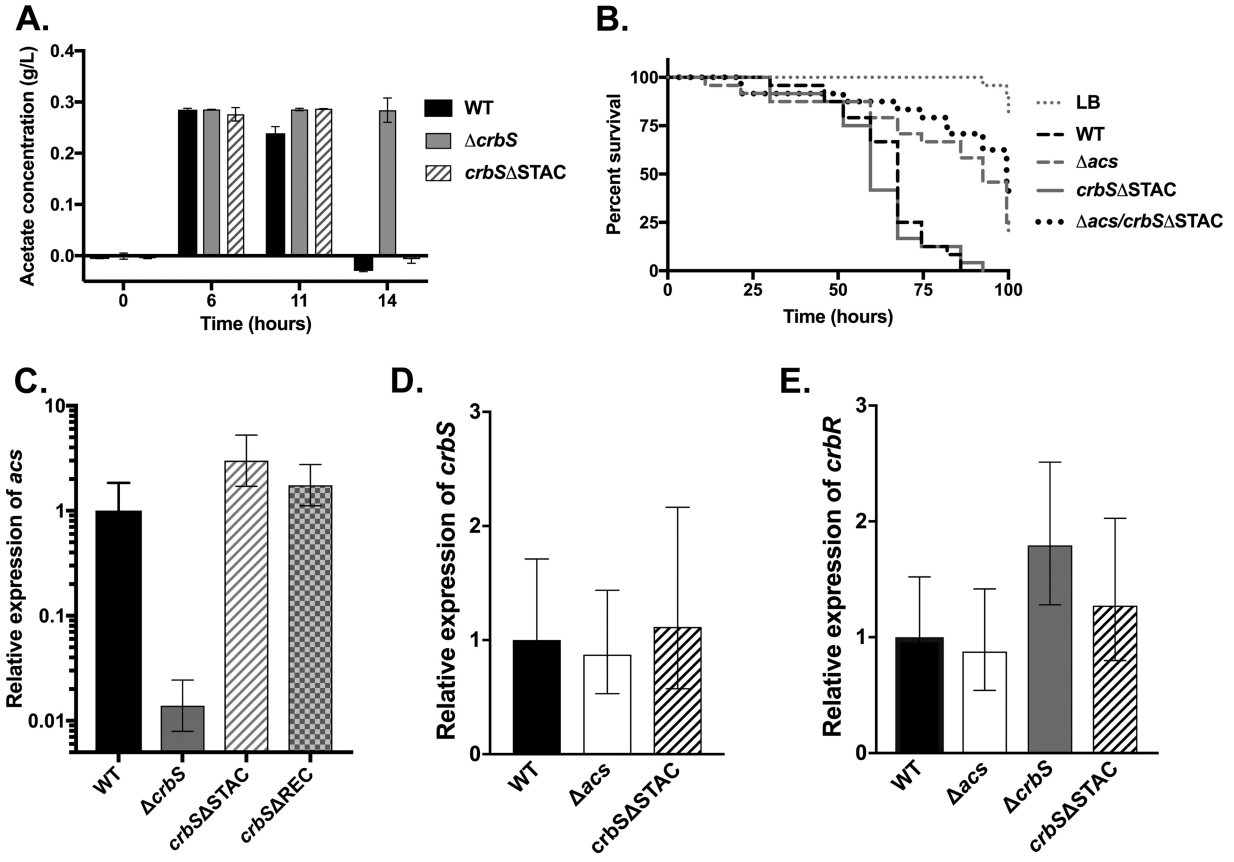

FIG 4 The CrbS STAC domain deletion does not alter acetate uptake, other virulence mechanisms, or signaling *in vivo*. (A) Acetate concentrations in medium in the SIO WT, Δ*crbS*, and *crbS*ΔSTAC strains. Both the WT and *crbS*ΔSTAC strains consumed acetate between 11 and 14 h, while the *crbS* strain was delayed. (B) Deletion of the STAC domain does not increase virulence in *Drosophila* when introduced into the Δ*acs* background ($P > 0.05$ by log rank analysis). Results of this assay are representative of data from two biological replicates. (C) During *Drosophila* infection, the expression level of *acs* in the *crbS* mutant was lower than that in the WT, but the *acs* level was not reduced in the *crbS*ΔSTAC strain. Averages and standard deviations of relative expression levels in each of three samples (with 6 to 10 flies in each sample) are shown. Expression is normalized to that of WT *V. cholerae* in flies. As an additional control, *acs* expression in the *crbS*ΔREC strain was monitored, which did not reduce *acs* expression. An identical trend was observed in a second biological replicate of this assay. (D and E) In *Drosophila*, expression of *crbS* (D) or *crbR* (E) is not altered as a result of the introduction of the STAC domain deletion. As an additional control, expression in the Δ*acs* strain was also monitored, and there was no difference, as expected.

altering the function or stability of the CrbSΔSTAC protein. Second, it is possible that the *in vivo* environment reduces the threshold level of CrbS activity necessary for maximal *acs* transcription. Third, the fly environment may be inducing increased levels of transcription of *crbS* and *crbR*, such that *acs* is fully expressed despite a reduction in the activity of the protein. To examine the latter hypothesis, we measured the expression of *crbS* and *crbR* via RT-qPCR in *V. cholerae* within *Drosophila*. We observed that the *crbS* and *crbR* genes are expressed to similar levels in the WT and *crbS*ΔSTAC strains (Fig. 4D and E). As a control, the *acs* deletion did not affect the expression of *crbS* or *crbR*. Therefore, we hypothesize that the *Drosophila* environment can alter the parameters of CrbS-dependent signaling relative to *in vitro* culture conditions.

CrbR interactions with the *acs* promoter. We hypothesize that CrbS directly couples transport to signal transduction in order to activate a maximally efficient pathway to regulate *acs* transcription. Thus, the CrbR response regulator likely binds directly to the *acs* promoter, without an intervening regulatory step. To test this hypothesis, we expressed the CrbR protein in *E. coli* together with the *acs* promoter fused to the *lacZ* gene (31). The presence of *crbR* significantly increased *lacZ* expression (Fig. 5A), consistent with *crbR* directly activating *acs* expression, as observed in *V. vulnificus* and *Pseudomonas* (22, 28).

Next, we defined the region of the *acs* promoter necessary for transcriptional activation, by taking a "promoter-bashing" approach. We fused various regions of the

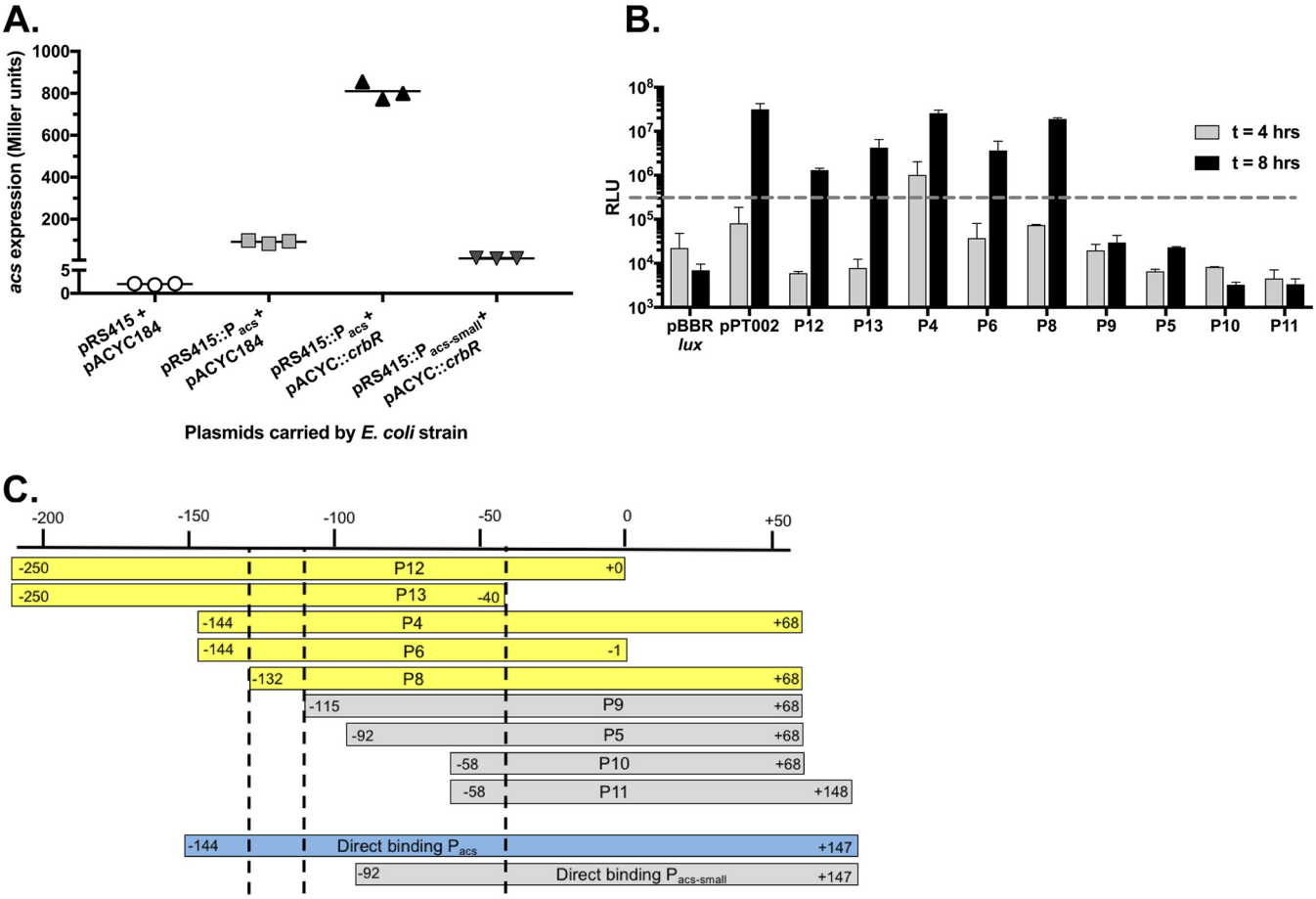

FIG 5 CrbR activates the *acs* promoter. (A) Evidence supporting a direct relationship between the CrbR response regulator and the *acs* promoter in *V. cholerae*. The gene encoding CrbR was introduced into the pACYC184 plasmid (pACYC::*crbR*) and cotransformed into *E. coli* carrying the empty pRS415 plasmid or the pRS415 plasmid with either of two segments of the *acs* promoter driving *lacZ* expression. The pRS415::P_{acs} segment extends from 144 bp upstream from the translational start site to 147 bp downstream, while pRS415::$P_{acs\text{-}small}$ begins 92 bp upstream of the translational start site, as in panel C. The introduction of the *crbR* gene to the strain carrying pRS415::P_{acs} significantly increases *acs* expression ($P < 0.0001$ by a two-tailed *t* test). These results are representative of data from five biological replicates. (B) Light production from *V. cholerae* SIO transformed with pBBR*lux* carrying the promoters indicated in panel C, or the pPT002 plasmid, which includes a 660-bp fragment of the *acs* promoter, following growth in LB medium for either 4 or 8 h. The dashed line indicates the threshold above which a promoter is designated "on." Error bars indicate standard deviations of data from duplicate samples, and the results are representative of data from two biological replicates. (C) Summary of the promoter fusion experiment indicating relative minimum promoter regions. Promoters in yellow initiated transcription in panel B, while those in gray did not. The promoter in blue initiated transcription in panel A. Numbering indicates the distance from the translational start site, as the transcriptional start site has not been defined.

acs promoter to the pBBR*lux* plasmid and measured luminescence during exponential growth. We defined an ~15-bp region necessary for expression (Fig. 5B and C), which may include the binding site targeted by CrbR. A previous study identified a putative CrbR binding site, with the primary consensus depicted as GAC(N_4)GTC (28). Within the 15-bp region that we identified lies the sequence TC**C**(TAAA)**GTC**T (boldface indicates base pairs that match with the consensus CrbR binding site), which could act as the binding target. We also created a smaller version of a putative promoter for the CrbR activity assay that lacks this sequence and observed that CrbR did not increase expression (Fig. 5A and C). Together, these results strongly suggest that CrbR directly binds to and activates the *acs* promoter, and a specific region of the promoter is necessary for mediating this interaction.

Nutrients and cAMP-CRP regulate *acs* expression. Acetate metabolism and *acs* transcription are controlled by multiple factors in *E. coli*. Closely related bacteria, including *Vibrio fischeri*, have also linked acetate metabolism to conditions that uniquely reflect their environmental settings, including the presence of chitin or glucose, and increased cell density (32, 33). In *E. coli*, glucose similarly regulates *acs*

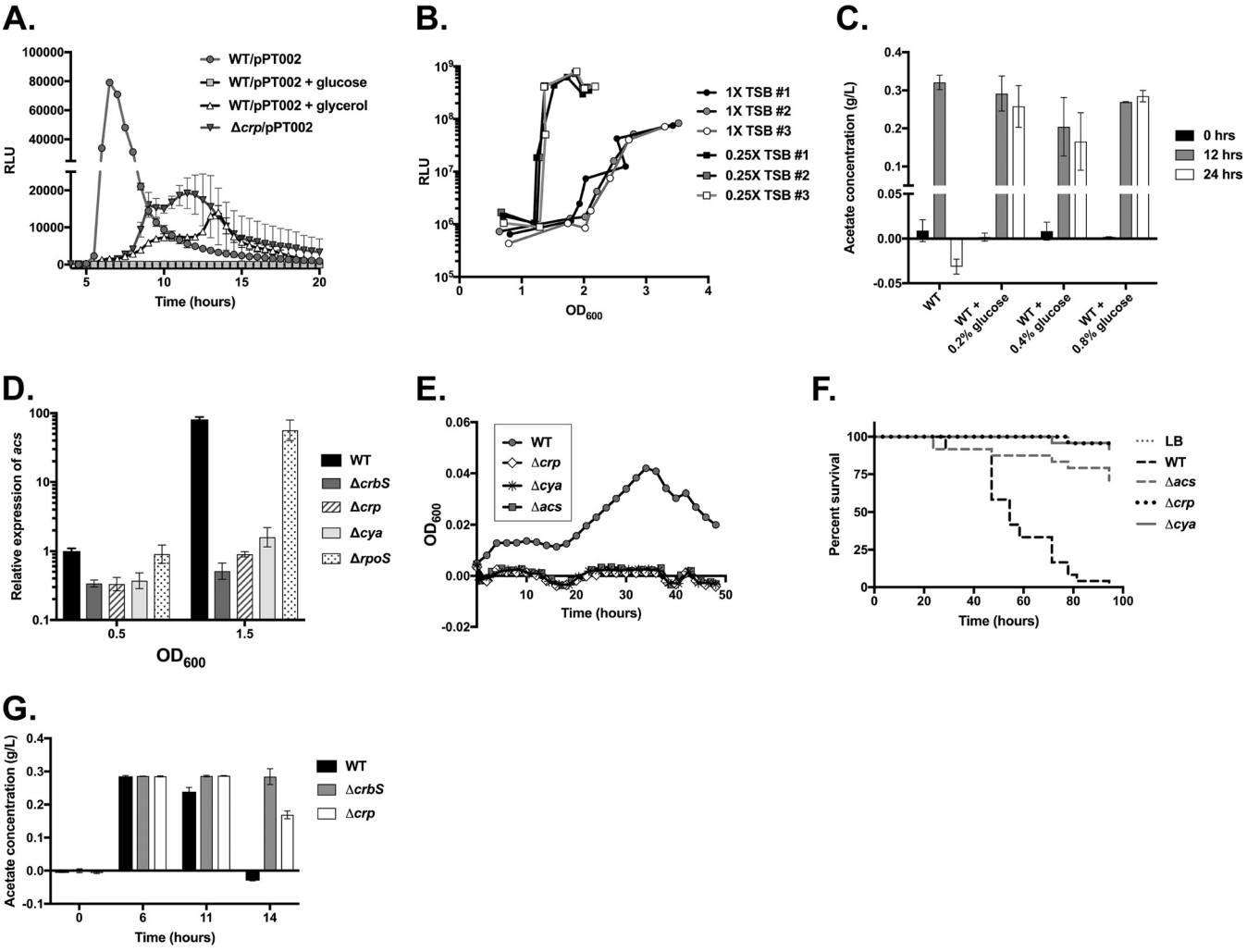

FIG 6 Sugars and CRP regulate *acs* expression. (A) Glucose, glycerol, and CRP alter *acs* promoter activity. Luminescence (RLU) driven by the *acs* promoter in the pPT002 plasmid in WT SIO, the WT supplemented with either glucose (40 mM) or glycerol (40 mM), or the Δ*crp* strain was measured. Light production from an average of 4 wells in a 96-well plate, with standard deviations indicated by error bars, is depicted. Results are representative of data from at least three biological replicates. (B) Nutrient levels, and not cell density, alter *acs* promoter activity. Luminescence (RLU) driven by the *acs* promoter in the pPT002 plasmid in WT SIO in either 1× tryptic soy broth (TSB) or 0.25× TSB was measured in culture. Each line indicates measurements taken at the indicated optical densities from an individual culture. Results are representative of data from two biological replicates. (C) Glucose halts acetate uptake. Acetate concentrations were measured in duplicate samples after 0 h, 12 h, and 24 h of growth in LB medium or LB medium supplemented with glucose (0.2%, 0.4%, or 0.8%). Results are representative of data from at least two biological replicates. (D) CRP and adenylate cyclase, but not RpoS, are required for *acs* transcription. Levels of *acs* transcripts were quantified by RT-qPCR in the Δ*crbS*, Δ*crp*, Δ*cya*, and Δ*rpoS* strains in LB medium at OD_{600} values of 0.5 and 1.5, with relative expression normalized to the expression of the WT strain at an OD_{600} of 0.5. The average expression levels of three samples, with the standard deviations, are shown. This assay is representative of data from four biological replicates for the *crp* deletion and two replicates that include the *cya* deletion. (E) CRP and adenylate cyclase are required for growth on acetate minimal medium. Growth of the WT, Δ*crp*, Δ*cya*, and Δ*acs* strains in M63 minimal medium supplemented with acetate (10 mM) was measured every 2 h for 48 h. Average values from 3 wells of a 96-well plate are depicted. Standard deviations are indicated but are often smaller than the size of the symbol. Results are representative of data from two biological replicates. (F) CRP and adenylate cyclase are required for virulence in *Drosophila*. Survival of *Drosophila* flies infected with the Δ*crp*, Δ*cya*, and Δ*acs* strains was measured over 100 h. Survival of flies that ingested the Δ*crp* and Δ*cya* strains was improved relative to those fed the WT ($P < 0.0001$ by a log rank test). For the Δ*crp* strain, results are representative of data from two biological replicates. (G) Acetate concentrations in culture were compared in the WT SIO, Δ*crbS*, and Δ*crp* strains. Acetate consumption in the Δ*crp* strain was slightly delayed at 14 h ($P = 0.0017$ by a *t* test). However, this may be explained by a growth defect in LB medium for this strain (57) (data not shown). Acetate concentrations were measured in duplicate cultures, and results are representative of data from three biological replicates.

transcription. To determine whether nutrients affect *acs* transcription in *V. cholerae*, we examined *acs* transcription from the pBBR*lux* reporter plasmid in the presence of glucose or glycerol. Transcription of *acs* was suppressed to almost undetectable levels in the presence of glucose, while glycerol had a moderate effect (Fig. 6A). To further explore the role of nutrients in *acs* transcriptional activation, we grew the strains in different concentrations of tryptic soy broth (TSB) and measured the optical density (OD) at which expression was initiated. At lower concentrations of TSB, *acs* promoter

activity was activated at earlier stages of growth, indicating that cell density is not the defining variable stimulating expression. Instead, nutrients, as well as other processes tied to nutrient levels, are an important determinant of *acs* promoter firing (Fig. 6B). We further observed that glucose prevented acetate uptake as well, demonstrating that nutrients repress acetate catabolism (Fig. 6C).

Regulation of gene expression by glucose can occur via its effects on the phosphoenolpyruvate-phosphotransferase (PTS) system, in which glucose alters concentrations of cAMP in the cell to affect CRP binding to targeted promoters. To determine whether CRP plays a role in regulating *acs* transcription, we constructed a deletion in *crp* and measured *acs* transcription from the same reporter plasmid. We observed a reduction in promoter activity, although some transcription was detectable (Fig. 6A). To confirm this finding, we also constructed an in-frame deletion in the gene encoding adenylate cyclase (*cya*). We then measured *acs* transcription via RT-qPCR and observed that *acs* transcription was similarly reduced in the both the Δ*crp* and Δ*cya* strains when cells were grown to an OD at 600 nm (OD_{600}) of 1.5 (Fig. 6D). Next, we examined growth on minimal medium supplemented with acetate and observed that neither the *crp* nor the *cya* deletion strain was capable of growing (Fig. 6E). Consistent with these findings, survival of flies fed the *crp* or *cya* mutant was significantly improved over that of flies provided the wild-type strain (Fig. 6F). Acetate consumption is either unaffected or slightly reduced in the Δ*crp* strain (Fig. 6G), indicating both that (i) a reduction in *acs* transcription is not sufficient to prevent the removal of acetate from media and (ii) CRP may control levels of another metabolite, in addition to acetate, to affect virulence in *Drosophila* (11, 12). Altogether, these findings collectively suggest that CRP regulates *acs* transcriptional activation.

Nutrients and cAMP-CRP regulate expression of *crbS* and *crbR*. We next searched the *acs* promoter for a CRP binding site and identified multiple imperfect sites with Virtual Footprint (34). We reasoned instead that CRP may be regulating the transcription of the *crbS* and *crbR* genes, and in each of these promoters, we were able to identify stronger putative CRP binding sites, using the Virtual Footprint tool (34). The *crbS* gene carries a site, GC**TGA**TTGAGT**TCA**A**A** (boldface type indicates nucleotides aligned with the consensus CRP binding site), centered at position −78.5 relative to the translational start site. The *crbR* gene carries a site, A**GG**G**A**TACAGT**TCA**G**A**, at position −88.5 relative to the translational start site. To determine whether these promoters were subject to CRP regulation, we cloned both promoters into the pBBR*lux* plasmid and characterized nutrient-dependent responses. Levels of expression from the *crbS* and *crbR* promoters were each reduced in the presence of glucose but not glycerol (Fig. 7A and B). Next, we introduced the *crbS* and *crbR* promoter fusion plasmids into the Δ*crp* strain and examined expression. Both promoters were substantially suppressed (Fig. 7C). We further confirmed this result by RT-qPCR for both the Δ*crp* and the Δ*cya* strains (Fig. 7D and E). Thus, the cAMP-CRP system regulates the transcription of the *crbS* and *crbR* genes.

We next examined the relationship between *crp* and CrbS/R expression by complementing the *crp* and *crbR* genes and measuring the expression of *acs* in culture. First, we deleted *crp* and then overexpressed the *crp* or *crbR* gene in this background. Overexpression of *crbR* in the Δ*crp* background was not sufficient to restore expression (Fig. 8A). This confirms that *crp* may be regulating *crbS* and/or *acs* independently as well. As a control, overexpression of *crp* complemented the Δ*crp* phenotype. Similarly, the expression of *crbR* could complement the Δ*crbR* phenotype. Interestingly, overexpression of *crbR* increased the expression of *acs* beyond wild-type levels, indicating that transcriptional regulation of *crbS* and *crbR* limits signaling through the pathway. Overexpression of CRP in this background has no effect on *acs*, indicating that expression requires *crbR* despite high levels of CRP (Fig. 8B).

CrbS and CrbR do not activate a positive-feedback loop. We also examined whether the *crbS* and *crbR* genes activate their own transcription to initiate a positive-feedback loop that amplifies expression. We measured luminescence driven by the *crbS*

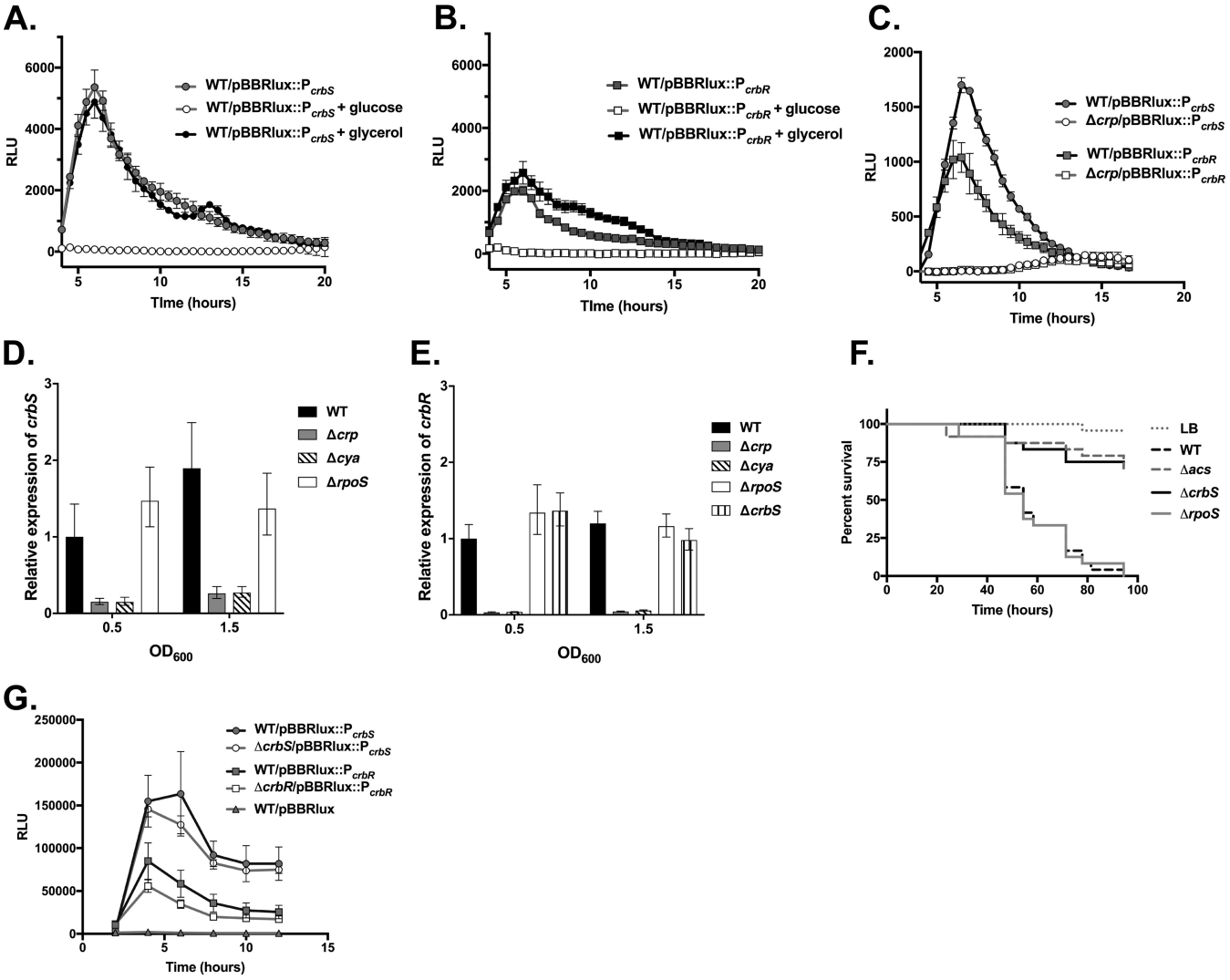

FIG 7 Glucose and CRP regulate transcription of *crbS* and *crbR*. (A and B) Luminescence driven by the *crbS* (A) or *crbR* (B) promoter is suppressed by glucose (40 mM) but not by glycerol (40 mM) in LB medium. The 461-bp *crbS* promoter fragment extended from 395 bp upstream to 66 bp downstream of the translational start codon. The *crbR* promoter fragment, 648 bp in length, extended 522 bp upstream and 126 bp downstream of the translational start codon. (C) Deletion of *crp* also impacts expression from the *crbS* and *crbR* promoters in the same assay. Average values for 3 or 4 wells of a 96-well plate with standard deviations are depicted, and results are representative of data from two biological replicates. (D and E) Both CRP and adenylate cyclase, but not RpoS, are required for expression of *crbS* (D) or *crbR* (E). *crbS* and *crbR* transcript levels were determined via RT-qPCR for the Δ*crbS*, Δ*crp*, Δ*cya*, and Δ*rpoS* strains in LB medium at OD_{600} values of 0.5 and 1.5 and normalized to the expression level of the WT strain at an OD_{600} of 0.5. The average expression levels for three samples, with the standard deviations, are shown. Expression levels in the Δ*crp* and Δ*cya* backgrounds are representative of data from three and two independent replicates, respectively. (F) Survival of *Drosophila* flies infected with the *rpoS* mutant, together with the Δ*acs* and Δ*crbS* mutants as controls. Survival of flies provided the Δ*rpoS* mutant is not different from that of flies fed the WT ($P > 0.05$ by a log rank test). (G) Deletion of *crbS* or *crbR* has little, if any, effect on *crbS* or *crbR* promoter activity, respectively. Luminescence (RLU) driven by the *crbS* or the *crbR* promoter in the pBBR*lux* plasmid was measured in culture at the indicated time points for the WT, Δ*crbS*, or Δ*crbR* strain. Averages of triplicate values, with standard deviations, are indicated. This graph is representative of data from three biological replicates.

and *crbR* promoters in the pBBR*lux* plasmid in strains lacking either the *crbS* or the *crbR* gene. We found that expression of *crbS* or *crbR* from the promoter was unaffected (Fig. 7G). We further confirmed this finding by RT-qPCR for the expression of the *crbR* gene (Fig. 7E). Therefore, transcriptional activation by CRP controls the expression of *crbS*, *crbR*, and *acs*, but there is no evidence that additional self-amplification occurs.

RpoS does not regulate *acs* transcription or virulence in *Drosophila*. Finally, acetate catabolism and *acs* expression patterns correlate with the onset of late exponential and stationary phases in batch culture. Therefore, we reasoned that the stationary-phase sigma factor, encoded by *rpoS*, may be regulating *acs* transcription, as has been observed in *E. coli* (35). We constructed an in-frame deletion of *rpoS* and

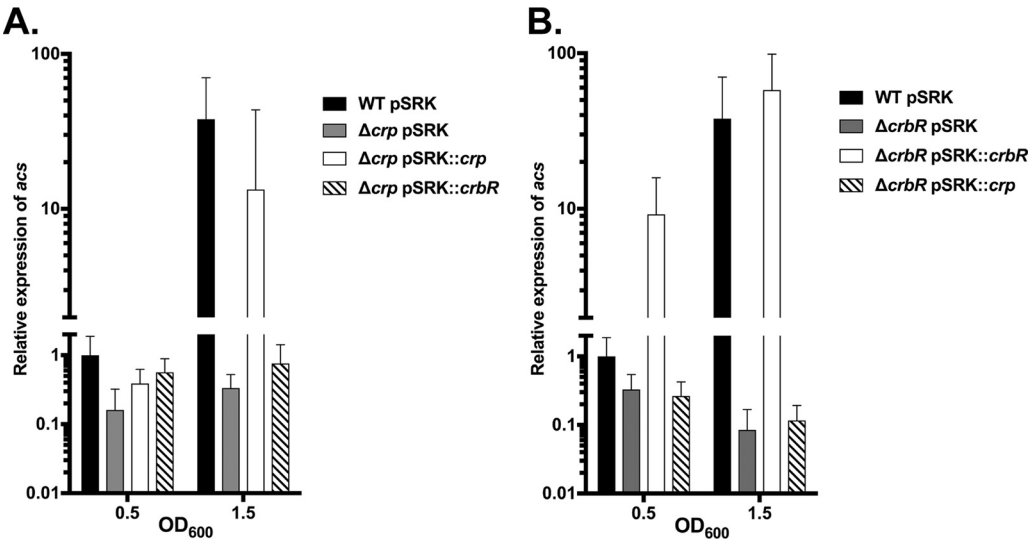

FIG 8 Expression of *acs* requires both CRP and CrbR. Expression of *acs* in strains overexpressing *crp* or *crbR* was quantified via RT-qPCR. Expression of *acs* was measured in the WT strain carrying the empty pSRK-Km (pSRK) complementation plasmid; in the Δ*crp* strain carrying pSRK, pSRK expressing *crp*, or pSRK expressing *crbR* (A); or in the Δ*crbR* background (B). All experiments were performed in the presence of 1 mM IPTG.

tested the expression of *acs* by RT-qPCR. We observed that *rpoS* has no effect on the expression of *acs*, *crbS*, or *crbR* (Fig. 6D and 7D and E). As expected, *rpoS* has no effect on *Drosophila* survival (Fig. 7F). Therefore, *rpoS* has no effect on acetate-dependent virulence or *acs* transcription in *V. cholerae*.

The *pta-ackA* system is required for excretion but not consumption of physiological concentrations of acetate in *Vibrio cholerae*. In *E. coli*, multiple pathways mediate the interconversion of acetate and acetyl-CoA. Acs is thought to act as a scavenger of low concentrations of acetate, while the Pta-AckA system may assist with assimilation of higher concentrations (20). However, excretion of acetate is controlled by the Pta-AckA system in *E. coli*. The *pta* gene catalyzes the conversion of acetyl-CoA to acetyl-phosphate (acetyl-P), while *ackA* converts acetyl-P to acetate (20).

To define the contributions of the Pta-AckA system to acetate metabolism in *V. cholerae*, we constructed deletions in the *pta* and *ackA* genes individually, and we created a third deletion that removes the two genes together from the chromosome, as they are located directly adjacent to one another in an operon. When the Pta-AckA system is deleted, there is no detectable acetate accumulation in the medium (Fig. 9A). When acetate is added exogenously to this culture, the Δ*pta-ackA* mutant consumes acetate similarly to the WT strain (Fig. 9A). Deletion of *pta* similarly abrogated acetate excretion, but deletion of *ackA* had no effect (Fig. 9B). The *V. cholerae* genome carries two genes annotated as acetate kinases; the second, VCA0235, may have a redundant function. Similarly, there is no effect of the *pta-ackA* system on growth on acetate or on *Drosophila* virulence (Fig. 9C and D). These results confirm that the Pta-AckA system is not required for consumption of acetate excreted by *V. cholerae* at these concentrations.

DISCUSSION

Bacterial survival in a wide variety of environmental contexts requires efficient regulation of metabolic fluxes in response to changing nutrient availability. *Vibrio cholerae*, a waterborne pathogen, can experience fluctuations in both the abundance and composition of carbon sources as it grows in close association with host organisms, as a biofilm on aquatic particulate matter, or as single cells in the water column. In marine ecosystems, vibrios are uniquely attuned to dividing rapidly upon encountering dissolved organic carbon sources (1, 2, 36). However, mechanisms of controlling and responding to carbon catabolism in vibrios are complex and appear to differ in

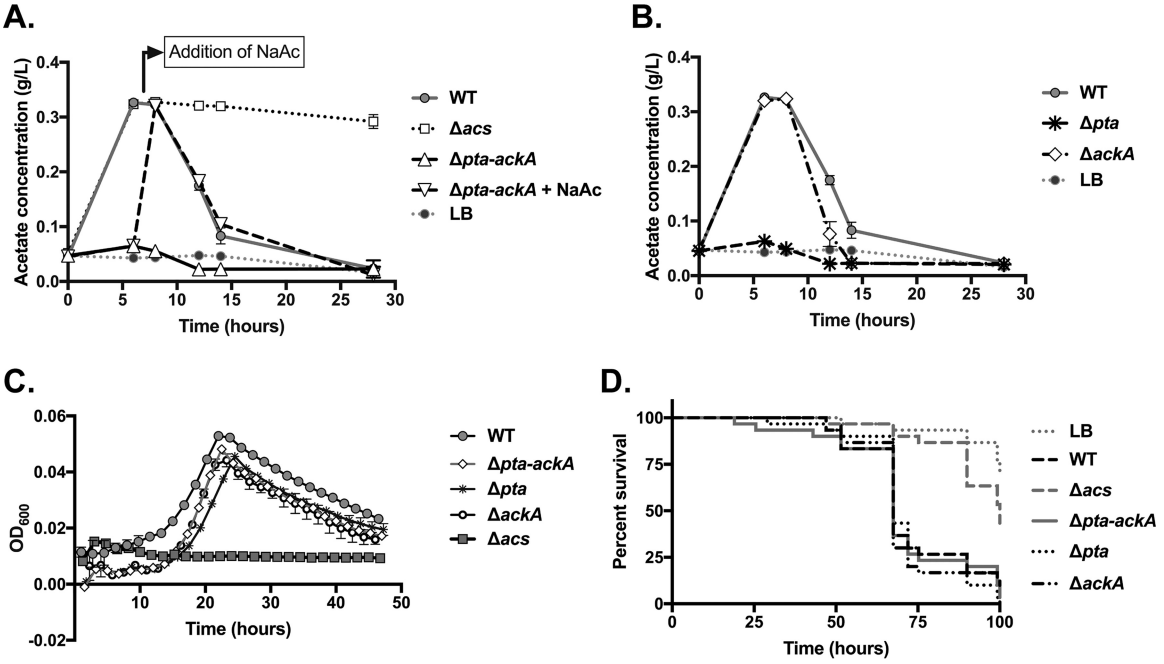

FIG 9 The Pta-AckA system is required for acetate consumption in *Vibrio cholerae*. (A) Acetate was measured in duplicate cultures of the WT, Δ*acs*, and Δ*pta-ackA* strains at the designated times. The Δ*pta-ackA* strain was incapable of excreting acetate but, upon supplementation with 5.6 mM acetate (NaAc) after the 6-h time point, was able to consume exogenous acetate. (B) In the same assay, acetate concentrations in cultures with the Δ*pta* and Δ*ackA* strains were measured. The Δ*pta* strain was unable to excrete acetate, but the Δ*ackA* strain both excreted and consumed acetate similarly to the WT strain. In both panels A and B, the concentration of acetate in uninoculated LB medium is also depicted, indicating the baseline level. Standard deviations are indicated and are often smaller than the size of the symbol. (C) Strains carrying deletions in the *pta-ackA* system are not impaired during growth on acetate minimal medium. Averages from at least 3 wells of a 96-well plate are shown; error bars depict standard deviations and are often smaller than the symbol. (D) Survival of *Drosophila* flies infected with the *pta*, *ackA*, or *pta-ackA* mutant. Survival of flies provided with these mutants does not differ from that of flies fed the WT ($P > 0.05$ by a log rank test).

significant ways from those of *E. coli* (37–40). The regulation of acetate catabolism by the CrbS/R two-component system may further differentiate carbon metabolic pathways in the *Vibrionaceae*. In this study, we define additional pathways that regulate *acs* and acetate metabolism, and we provide evidence for novel roles for the STAC domain in CrbS signaling.

The domain structure of CrbS suggests a unique mechanism of signal sensing and transduction that links transport to signaling. We took a genetic approach to our investigation of these domains, a strategy that has informed previous studies of two-component regulators and their signaling pathways (41, 42). In a first analysis of the transporter-like domain, we demonstrate that conserved residues necessary for Na^+ binding in PutP are not required for virulence, suggesting that they do not contribute to signaling *in vivo*. Deletion of the transporter domain is detrimental, though, indicating that the lack of the transporter disrupts signaling, protein function, or protein stability. This is not surprising but establishes a baseline from which future studies can investigate questions about the minimum components necessary. For example, can signaling occur if the protein is simply tethered in the membrane, or is transport necessary? Because the transporter-like domain has diverged relative to PutP, it may be more informative to take a random-mutagenesis approach to uncovering important residues for protein function. Furthermore, the substrate of the transporter domain remains elusive. This has been investigated in one homolog of CrbS, CbrA in *Pseudomonas*, which similarly carries a PutP-like transporter domain. Transport of proline, as well as other amino acids, was assessed, but no transport was detected, and no effect on signaling was observed (24). However, a link between histidine transport and signaling has been suggested (27).

We further demonstrate that the STAC domain is required for *acs* expression and *in*

vitro growth on acetate, but it does not affect signaling when *V. cholerae* is infecting *Drosophila*. We considered three possible hypotheses to explain this unexpected result. In the first, we questioned whether the ΔSTAC deletion strain was upregulating a second, unknown virulence factor that may be sufficient to kill flies. We introduced the ΔSTAC deletion into the Δ*acs* background, and this did not improve the virulence of the Δ*acs* strain alone. Therefore, an unknown virulence factor is not increasing virulence in the absence of acetate-mediated pathogenesis mechanisms. Next, we showed that acetate consumption in these strains is not significantly affected by the STAC deletion, despite low levels of *acs* transcription. This alone could be sufficient to explain our finding, although it reveals an additional issue: a discrepancy between *acs* transcription levels and acetate consumption. Clearly, acetate consumption is dependent upon *acs*, because a deletion in *acs* entirely halts acetate uptake in these strains, and a deletion in *pta-ackA* has no effect on acetate removal from the medium at these concentrations. Therefore, low levels of *acs* transcription may be sufficient for the removal of acetate from the medium. We further observe that growth on minimal medium with acetate is reduced in the *crbS*ΔSTAC strain, despite its ability to remove acetate from the medium. We hypothesize that CrbS may be further altering the expression of other genes involved in acetate metabolism that, when collectively expressed at low levels, prevent the assimilation of acetate.

Finally, we observed that the deletion of the STAC domain does not affect *acs* transcription during infection. Instead, *acs* levels are similar to those observed in the wild-type strain. This suggests that signals that activate *acs* in *Drosophila* can be detected, despite the lack of this domain. Deletion of the entire *crbS* gene reduces *acs* levels in *Drosophila*; therefore, the increase in *acs* levels cannot be ascribed to a CrbS-independent factor. We hypothesize that the STAC domain may be acting as a negative regulator of *in vivo* signals or that signaling proceeds independently of the STAC domain during infection. One alternative possibility is that the *in vivo* environment is regulating the stability of the CrbSΔSTAC proteins, providing a factor or condition that prevents degradation or maintains the membrane localization of the deletion constructs. It is also possible that the *Drosophila* environment simply lowers the threshold level of CrbS activity needed to reach wild-type levels of *acs* transcription. Thus, a future goal will be to distinguish between these alternative explanations. In each case, this finding suggests that the *Drosophila* gastrointestinal environment provides unique and specific signals to *V. cholerae*, which may be detected by CrbS.

Next, we examined whether *acs*, despite the presence of CrbS, responds to nutrient signals via conserved regulators important in other bacteria. We demonstrated that sugars reduce *acs* transcription and that deletion of CRP similarly lowered *acs* expression levels. However, our results suggest that CRP could be regulating *acs* indirectly; rather than binding directly to the *acs* promoter, CRP may instead regulate *crbS* and *crbR* transcription. Confirming that *acs* is not directly targeted by CRP will require further biochemical testing by an electrophoretic mobility shift assay and/or chromatin immunoprecipitation sequencing (ChIP-Seq). Whether or not CRP binds directly to *acs*, it is clear that the expression of *crbS* and *crbR* is subject to transcriptional control. This raises the question, then, regarding the nature of the primary signal sensed by CrbS. In one possible model, CrbS is expressed only in the absence of preferred carbon sources, and an additional signal is needed to activate the phosphorelay. Alternatively, CRP-cAMP could direct the transcription of CrbS proteins that are capable of constitutively activating CrbR, and a signal may instead suppress the phosphorelay. With either model, CRP is needed for *acs* expression via its role in controlling the expression of CrbS and CrbR. The advantage of creating a two-tiered regulatory scheme (i.e., CRP → CrbS/R → *acs*) is not yet clear but implies that additional information regarding the extracellular environment is needed to appropriately regulate *acs*.

The Pta-AckA system is the major pathway through which acetate is excreted (20). A second pathway involving pyruvate oxidase (PoxB) may contribute to

acetate excretion in *E. coli*. However, the *V. cholerae* genome lacks a *poxB* homolog, which further establishes the importance of the Pta-AckA system in this bacterium. Appropriate regulation of acetate flux is critical for virulence in multiple mammalian pathogens. Pta and AckA are required for uropathogenic *E. coli* to colonize the bladder and kidneys (43). In toxigenic strains of *V. cholerae*, *pta* is required for full expression of the toxin-coregulated pilus (TCP) operon, which is necessary for colonization of the infant mouse (44). Both *pta* and *ackA* control the expression of *toxT*, a key regulator of both TCP and cholera toxin expression, via another metabolite involved in acetate metabolism (45). Pta and AckA can also affect levels of acetyl-P, which can control acetylation states of proteins and/or phosphorylate response regulators to alter two-component signaling (20, 46, 47). We demonstrate that the Pta-AckA pathway in *V. cholerae* functions similarly to that of *E. coli*, with Pta being required for acetate excretion. Our results suggest that there is redundancy, though, as the deletion of *ackA* did not prevent the conversion of acetyl-P to acetate. The Pta-AckA system does not affect virulence in flies, consistent with our observation that the system does not affect the removal of acetate from media.

In summary, we define roles for multiple pathways in the control of acetate metabolism in *Vibrio cholerae*. We suggest possible functions for the CrbS STAC domain during signaling, and we describe an additional layer of regulation that integrates nutrient availability into CrbS-dependent *acs* transcription. Altogether, these findings indicate that information funneled through CrbS is detectable to the bacterium only at times when preferred carbon sources are absent and that the environment may affect CrbS-dependent signaling mechanisms.

MATERIALS AND METHODS

Bacterial growth and storage. Both *E. coli* and *V. cholerae* strains were routinely grown in LB-Miller (LB) medium and stored in 15% glycerol in LB medium at −80°C (Table 1). *V. cholerae* strains were derived from *V. cholerae* strain SIO, an environmental, non-O1/non-O139 nontoxigenic strain isolated from the coast of southern California (48). Antibiotics, purchased from Sigma, were added to a final concentration of 100 μg/ml unless otherwise noted.

Construction of in-frame gene deletions, point mutations, complementation constructs, and transcriptional reporter fusion plasmids. Deletions and mutations were constructed via a splicing by overlap extension (SOE)-PCR protocol, or via a Gibson protocol (New England BioLabs [NEB]), and introduced into the *V. cholerae* chromosome via conjugation and selection for double recombination events, as described previously (21). SOE constructs were generated by designing two sets of primers that amplify ~1,000 bp upstream and downstream of the region to be deleted. Primers 1 and 4 were the "exterior" primers, while the interior primers, primers 2 and 3, were designed to fall within a few amino acids of the start codon and stop codon of the gene of interest. The primers amplified a construct that generated a complete, in-frame deletion of the gene, leaving behind just the overlapping tag incorporated into primers 2 and 3, together with a small number of codons at the 5′ and 3′ ends of the gene. When mutations rather than deletions were constructed, primers 2 and 3 were designed to overlap the region that includes the mutation. The STAC domain deletion primers do not include an exogenous tag and completely overlapped one another without additional base pairs. The Gibson protocol was used to delete the *pta-ackA* operon from *V. cholerae*, and primers were designed according to the manufacturer's instructions (NEB). Following SOE-PCR with Q5 high-fidelity DNA polymerase (NEB), the PCR product was then digested with appropriate restriction enzymes and ligated into the pHC001B plasmid (49). The plasmid was transformed into DH5α λ*pir* cells, and the construct was verified by sequencing and then moved into MFD*pir* cells (50). The plasmid was then conjugated into *V. cholerae*, and transconjugants carrying the plasmid was selected for by plating on kanamycin, as described previously (21). Sucrose selection was used to identify clones that had lost the plasmid, and integration of the correct construct was verified by PCR or by sequencing.

To generate complementation constructs, the gene of interest was amplified with Q5 high-fidelity DNA polymerase (NEB) using a forward primer that incorporated an NdeI restriction site into the start codon of the gene and a reverse primer that fell after the last stop codon and incorporated a second restriction site. The resulting fragment was digested, ligated into the pSRK-Km plasmid (51), and then transformed into *E. coli* for conjugation into *V. cholerae*.

To generate reporter plasmid constructs in the pBBR*lux* plasmid (52), fragments of the respective promoters were amplified using Q5 high-fidelity DNA polymerase (NEB) with primers incorporating restriction sites, digested, and ligated into pBBR*lux*. The resulting plasmid was then introduced into *V. cholerae* by conjugation, followed by selection on ampicillin and chloramphenicol (5 μg/ml).

Measurement of transcription via luminescence assays. Transcription driven by reporter fusions to the *luxCDABE* operon was determined by measuring luminescence in a 96-well multimode plate reader (Molecular Devices). Single colonies were inoculated into LB broth with chloramphenicol (5 μg/ml), which were grown overnight at 37°C with shaking. Cultures were diluted 1:500, and multiple wells of a

TABLE 1 Strains used in this study

Strain	Description	Reference(s)
E. coli		
MFD*pir*	MG1655 RP4-2-Tc::[ΔMu1::*aac*(*3*)*IV*-Δ*aphA*-Δ*nic35*-ΔMu2::*zeo*] Δ*dapA*::(*erm-pir*) Δ*recA* Apra^r Zeo^r Erm^r	50
DH5α *λpir*	F$^-$ Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1 λpir*	58
S17-1 *λpir*	RP4-2(Km::Tn*7* Tc::Mu-1) *pro-82 λpir recA1 endA1 thiE1 hsdR17 creC510*	59
AP09	*E. coli* WM5406/pHC001B	49
AP302	*E. coli* DH5α/pSRK-Km	51, 60
AP1033	MFD*pir*/pHC001B::*crbS*$^{S337A/T338V}$	This study
AP834	MFD*pir*/pHC001B::*crbS*Δ*trans*	This study
AP1244	MFD*pir*/pSRK-Km::*trans*	This study
AP1576	MFD*pir*/pHC001B::*crbS*ΔSTAC	This study
AP1021	*E. coli*/pACYC184	31
AP1294a	TOP10/pRS415	31
AP1212	TOP10/pACYC184::*crbR*	This study
AP1221	TOP10/pRS415::P_{acs}	This study
AP1228	TOP10/pRS415::$P_{acs\text{-}small}$	This study
AP1337a	TOP10/pACYC184 and pRS415	This study
AP1298a	TOP10/pACYC184 and pRS415::P_{acs}	This study
AP1323a	TOP10/pACYC184::*crbR* and pRS415::P_{acs}	This study
AP1324a	TOP10/pACYC184::*crbR* and pRS415::$P_{acs\text{-}small}$	This study
AP1207a	S17-1/pBBR*lux*::$P_{acs\text{-}P12}$	This study
AP1211a	S17-1/pBBR*lux*::$P_{acs\text{-}P13}$	This study
AP1039	S17-1/pBBR*lux*::$P_{acs\text{-}P4}$	This study
AP1047	S17-1/pBBR*lux*::$P_{acs\text{-}P6}$	This study
AP1194a	S17-1/pBBR*lux*::$P_{acs\text{-}P8}$	This study
AP1197a	S17-1/pBBR*lux*::$P_{acs\text{-}P9}$	This study
AP1043	S17-1/pBBR*lux*::$P_{acs\text{-}P5}$	This study
AP1200a	S17-1/pBBR*lux*::$P_{acs\text{-}P10}$	This study
AP1203a	S17-1/pBBR*lux*::$P_{acs\text{-}P11}$	This study
AP547	MFD*pir*/pHC001B::Δ*crp*	This study
AP1970	MFD*pir*/pHC001B::Δ*cya*	This study
AP1968	MFD*pir*/pHC001B::Δ*rpoS*	This study
AP1719	S17-1/pBBR*lux*::P_{crbS}	This study
AP1715	S17-1/pBBR*lux*::P_{crbR}	This study
AP915	MFD*pir*/pSRKKm::*crp*	This study
AP1900	MFD*pir*/pSRKKm::*crbR*	This study
AP211	MFD*pir*/pHC001B::Δ*pta-ackA*	This study
AP1934	MFD*pir*/pHC001B::Δ*pta*	This study
AP1932	MFD*pir*/pHC001B::Δ*ackA*	This study
V. cholerae		
AP95	*V. cholerae* SIO wild type	48
AP27	SIO Δ*crbS*	21
AP218	SIO Δ*acs*	57
AP1161	*crbS*$^{S337A/T338V}$	This study
AP462	SIO/pBBR*lux*	21
AP431	SIO/pPT002	21
AP1659	SIO *crbS*ΔSTAC	This study
AP1693	SIO *crbS*ΔSTAC/pPT002	This study
AP847	SIO *crbS*Δ*trans*	This study
AP1288a	SIO *crbS*Δ*trans*::pSRK-Km	This study
AP1293a	SIO *crbS*Δ*trans*::pSRK-trans	This study
AP1986	SIO Δ*acs*/*crbS*ΔSTAC	This study
AP1268	SIO/pBBR*lux*::$P_{acs\text{-}P12}$	This study
AP1242	SIO/pBBR*lux*::$P_{acs\text{-}P13}$	This study
AP1059	SIO/pBBR*lux*::$P_{acs\text{-}P4}$	This study
AP1075	SIO/pBBR*lux*::$P_{acs\text{-}P6}$	This study
AP1264	SIO/pBBR*lux*::$P_{acs\text{-}P8}$	This study
AP1234	SIO/pBBR*lux*::$P_{acs\text{-}P9}$	This study
AP1067	SIO/pBBR*lux*::$P_{acs\text{-}P5}$	This study
AP1236	SIO/pBBR*lux*::$P_{acs\text{-}P10}$	This study
AP1238	SIO/pBBR*lux*::$P_{acs\text{-}P11}$	This study
AP555	SIO Δ*crp*	This study
AP1974	SIO Δ*cya*	This study
AP1979	SIO Δ*rpoS*	This study
AP601	SIO Δ*crp*/pPT002	This study
AP1739	SIO/pBBR*lux*::P_{crbS}	This study

(Continued on next page)

TABLE 1 (Continued)

Strain	Description	Reference(s)
AP1723	SIO/pBBR*lux*::P_{crbR}	This study
AP1980	SIO Δ*crp*/pBBR*lux*::P_{crbS}	This study
AP1982	SIO Δ*crp*/pBBR*lux*::P_{crbR}	This study
AP1763	SIO Δ*crbS*/pBBR*lux*::P_{crbS}	This study
AP1755	SIO Δ*crbR*/pBBR*lux*::P_{crbR}	This study
AP683	SIO/pSRK-Km	57
AP937	SIO Δ*crp*/pSRK-Km::*crp*	This study
AP2002	SIO Δ*crp*/pSRK-Km::*crbR*	This study
AP1996	SIO Δ*crbR*/pSRK-Km	This study
AP1906	SIO Δ*crbR*/pSRK-Km::*crbR*	This study
AP2000	SIO Δ*crbR*/pSRK-Km::*crp*	This study
AP229	SIO Δ*pta-ackA*	This study
AP1988	SIO Δ*pta*	This study
AP1992	SIO Δ*ackA*	This study

96-well plate (sterile, flat, clear-bottomed plate with black sides; Brand, Germany) were inoculated with 120 μl. Luminescence and the OD_{600} were measured over time, with incubation at 37°C and periodic shaking. Relative light units (RLU) were defined as luminescence units per OD_{600} unit.

Luminescence measured in culture was detected with a luminometer (GloMax; Promega) from aliquots of 12-ml cultures grown in 50-ml "bioreactor" conical tubes with lids containing 0.22-μm filters for gas exchange (Corning). Optical densities of cultures were measured with a spectrophotometer.

Measurement of transcription via RT-qPCR in culture. Cultures grown overnight were diluted 1:500 into 12 ml of LB broth in a bioreactor conical tube and incubated at 37°C with shaking. The OD_{600} of the growing culture was monitored in a spectrophotometer; at an OD_{600} of either 0.5 or 1.5, a 1-ml sample was removed and spun down at 6,000 relative centrifugal force (rcf) for 2 min, and the pellets were frozen in a dry ice-ethanol bath before storage at −80°C. RNA was extracted with TRIzol (Sigma) according to the manufacturer's instructions. Briefly, samples were resuspended in 1 ml of TRIzol and incubated at room temperature for 5 min. Samples were then centrifuged at 12,000 rcf for 10 min, and the supernatant was transferred to a new tube, to which 200 μl of chloroform was added. The samples were incubated at room temperature for 3 min and centrifuged at 10,000 rcf for 15 min at 4°C. The nucleic acid was precipitated from the upper aqueous phase by the addition of 500 μl of isopropanol, followed by a 10-min incubation. The RNA was pelleted by centrifugation at 20,000 rcf for 10 min at 4°C. The pellets were washed with 75% ethanol twice, dried, and resuspended in RNase-free sterile water (Ambion). The nucleic acid was treated with Turbo DNase (Ambion), incubated for 1 h at 37°C, and then treated with 20 μl of DNase deactivation reagent (Ambion). After incubation for 2 min at room temperature, the samples were centrifuged at 12,000 rcf for 2 min, and the supernatant was transferred to a new tube. The RNA concentration and quality were assessed by measuring the concentration and purity on a Nanodrop instrument and by visualizing RNA on an agarose gel.

RNA was converted to cDNA using the SuperScript IV reverse transcription kit (Invitrogen) according to the manufacturer's instructions. Each reaction mixture included 1 μl of 50 μM random hexamers, 1 μl of 10 μM deoxynucleoside triphosphates (dNTPs), and 500 ng of the RNA template in a total volume of 13 μl. The reaction mixtures were heated at 65°C for 5 min, followed by 1 min on ice. A separate mixture of 4 μl of 5× SuperScript IV buffer, 1 μl of 100 mM dithiothreitol (DTT), 1 μl of an RNase inhibitor, and 1 μl of SuperScript IV reverse transcriptase was prepared. This was combined with the RNA-hexamer solution and incubated at 23°C for 10 min, 53°C for 10 min, and 80°C for 10 min. The cDNA concentration was measured with the Nanodrop instrument, and samples were stored at −20°C.

Preparation of samples for RT-qPCR. The cDNA to be analyzed was diluted to 40 ng/μl in nuclease-free water. A solution consisting of 10 μl of iTaq SYBR green Super-Mix (Bio-Rad), 2 μl of forward and reverse primers (each at 10 μM), and 7 μl of nuclease-free water was prepared on ice. The solution was then added to a 96-well plate before 1 μl of the cDNA template was added to each well. The plate was centrifuged for 5 min at maximum speed at 4°C. Amplification was monitored with a quantitative PCR (qPCR) instrument (Agilent). Threshold cycle (C_T) values for the gene of interest were normalized to that of *clpX* (21, 53). To calculate fold changes in expression, gene expression levels were normalized to that of the WT at an OD_{600} of 0.5. Relative gene expression and experimental error were determined by calculating the ratio between the wild-type and mutant strain mean gene expression levels (54). Primers for amplification of *acs*, *crbS*, and *crbR* were designed and are listed in Table 2, and primer efficiency calculations were performed to ensure linear amplification. Primers for amplification of *clpX* in *V. cholerae* MO10 O139 were previously described (37, 53) but were adjusted to account for a mismatch with the *V. cholerae* SIO genome.

Measurement of *V. cholerae* transcription in *Drosophila* via RT-qPCR. To measure transcription in *Drosophila*, flies were provided with *V. cholerae* or sterile LB medium, as described below. To collect flies, *Drosophila* flies were anesthetized with CO_2, and live flies were transferred to an Eppendorf tube and immediately snap-frozen in a dry ice-ethanol bath, as described previously (53). Flies from each of three vials were placed into individual tubes so that each sample represents RNA taken from bacteria inside

TABLE 2 Primers used in this study

Primer	Sequence	Description[a]
ED74_VC0303Na_P1	GACAACTAGTTATCTCGGCGTGCTGTTTTT	SOE mutation of Na^+ binding site S337A/T338V, P1
ED75_VC0303Na_P2	CATGATGGATAGTGCGATAACCGCCACAAT	SOE mutation of Na^+ binding site S337A/T338V, P2
ED76_VC0303Na_P3	TCGGGGATGGTGATTGTGGCGGTTATCGCA	SOE mutation of Na^+ binding site S337A/T338V, P3
ED77_VC0303Na_P4	CATTGGATCCAACCCTGGTGGAAACTCAAA	SOE mutation of Na^+ binding site S337A/T338V, P4
FD32_delTR_P1	GACAACTAGTACTGTGGTCGGATTCTTGGT	SOE deletion of *crbS* transporter domain, P1
ED59_trans_p2	TAACGAGCGGCCGCAACCTTGCATCAGACATCCTTG	SOE deletion of *crbS* transporter domain, P2
ED60_trans_p3	TGCGGCCGCTCGTTAGCCAGCTTAAGTGAGCGTTT	SOE deletion of *crbS* transporter domain, P3
ED61_trans_p4	CATTGGATCCAAAAAGCGCGATTTGGATT	SOE deletion of *crbS* transporter domain, P4
AEP221_CrbSStrF	GACACATATGCAAGGTTGGTTGGTAATTC	Complementation of transporter domain, F
AEP222_CrbSStrR1a	GTGATGGTGCAAACGCTCACTTAAGCTGGC	Complementation of transporter domain, R
AEP230delSTACSIO1	GACAACTAGTCTGGCCGACCTTATTGATTC	SOE deletion of *crbS* STAC domain, P1
AEP231delSTACSIO2	AGACGCCTCCACAGTCACGCGACTTTGGTA	SOE deletion of *crbS* STAC domain, P2
AEP232delSTACSIO3	GTGACTGTGGAGGCGTCTGAGCTGTACG	SOE deletion of *crbS* STAC domain, P3
AEP233delSTACSIO4	CATTGGATCCCGAAACGCATTAGTCAGGAA	SOE deletion of *crbS* STAC domain, P4
IM13_acsF4IDT	CAATGTCGCTTGGCATGAAC	RT-qPCR for *acs*, F
IM14_acsR4IDT	AGCCAGAGGTGTAGAGGATAAA	RT-qPCR for *acs*, R
IM25_Qpcr_CrbS1_F	TCAGCCGTCACTCACTCAGA	RT-qPCR for *crbS*, F
IM26_Qpcr_CrbS1_R	GCAAATCACGCATCCCAACC	RT-qPCR for *crbS*, R
IM31_Qpcr_CrbR2_F	AACGGCGAGCCTTATTTCCC	RT-qPCR for *crbR*, F
IM32_Qpcr_CrbR2_R	CCTTGTATTGCTGCGGAGTCA	RT-qPCR for *crbR*, R
IM5 _clpX_qRT_SIO_F	AGAGTTCATTGGTCGTCTGCCTGT	RT-qPCR for *clpX*, F[b]
IM6_clpX_qRT_SIO_R	AACAACGCAGCATACTGTTTGGTC	RT-qPCR for *clpX*, R[b]
AEP241_CrbR_F_EcoRV	CCTTCGATATCACCTGTTGTGACGTCATGGA	CrbR activity assay, *crbR*, F
AEP242_CrbR_R_SalI	CAGGAGTCGACAGCGCGAGTAAGCAGAAATG	CrbR activity assay, *crbR*, R
AEP238_Acs_F2_Bam	CATTGGATCCCACATATCCCATCAGGCTTTC	CrbR activity assay, P_{acs}, F
AEP237_acspromP2R	ACAGGGATCCAATCCAGTCGACGATTTTGC	CrbR activity assay, P_{acs}, R
AEP239_Acs_F3_Bam	CATTGGATCCTGCTCTGACTGAGAGTTATAAACG	CrbR activity assay, $P_{acs\text{-}small}$, F
AEP237_acspromP2R	ACAGGGATCCAATCCAGTCGACGATTTTGC	CrbR activity assay, $P_{acs\text{-}small}$, R
PT48_acs_prom_P1c	GACAACTAGTCGTTTAACCAAAGGCGATCT	Promoter bashing, promoter 12, F
AEP217_acs_R1	CATTGGATCCTACGGGTTCTCCTTGTGAATT	Promoter bashing, promoter 12, R
PT48_acs_prom_P1c	GACAACTAGTCGTTTAACCAAAGGCGATCT	Promoter bashing, promoter 13, F
AEP235_acs_R5	CATTGGATCCCGCGTTTCAAACACGAGA	Promoter bashing, promoter 13, R
AEP214_acs_F2	GACAACTAGTCACATATCCCATCAGGCTTTC	Promoter bashing, promoter 4, F
AEP218_acs_R2	CATTGGATCCGTGTCGTTATCGGCATGC	Promoter bashing, promoter 4, R
AEP214_acs_F2	GACAACTAGTCACATATCCCATCAGGCTTTC	Promoter bashing, promoter 6, F
AEP217_acs_R1	CATTGGATCCTACGGGTTCTCCTTGTGAATT	Promoter bashing, promoter 6, R
AEP232_acs_F2a	GACAACTAGTGGCTTTCATCCTAAAGTCTAATTG	Promoter bashing, promoter 8, F
AEP218_acs_R2	CATTGGATCCGTGTCGTTATCGGCATGC	Promoter bashing, promoter 8, R
AEP233_acs_F2b	GACAACTAGTCTAATTGTAGAAATCCCTGCTCTG	Promoter bashing, promoter 9, F
AEP218_acs_R2	CATTGGATCCGTGTCGTTATCGGCATGC	Promoter bashing, promoter 9, R
AEP215_acs_F3	GACAACTAGTTGCTCTGACTGAGAGTTATAAACG	Promoter bashing, promoter 5, F
AEP218_acs_R2	CATTGGATCCGTGTCGTTATCGGCATGC	Promoter bashing, promoter 5, R
AEP234_acs_F3a	GACAACTAGTTCTCGTGTTTGAAACGCG	Promoter bashing, promoter 10, F
AEP218_acs_R2	CATTGGATCCGTGTCGTTATCGGCATGC	Promoter bashing, promoter 10, R
AEP234_acs_F3a	GACAACTAGTTCTCGTGTTTGAAACGCG	Promoter bashing, promoter 11, F
PT49_acs_prom_P2	CATTGGATCCAATCCAGTCGACGATTTTGC	Promoter bashing, promoter 11, R
DN07_pVC2702_F	GACAACTAGTGCTAATCTAAGCGAGCTGCAA	CrbR promoter in pBBR*lux* F
DN08_pVC2702_R	CATTGGATCCAAAGCATCCAGCGAGTCC	CrbR promoter in pBBR*lux* R
DN15_PcrbS_3_F	GACAACTAGTTCAACTACTGCATGGGGTCA	CrbS promoter in pBBR*lux* F
DN16_pcrbS_3_R	CATTGGATCCCACGCAATCAAAAACAGCAC	CrbS promoter in pBBR*lux* R
MS37_P1	GACAACTAGTCCAGATGCCCGGTACGTTTAC	SOE deletion of *crp*, P1[c]
MS33_P2	GGTTTCGCGAGAACAGCCGTGAAAGAAACCACTCTAGTG	SOE deletion of *crp*, P2[c]
MS34_P3	CACTAGAGTGGTTTCTTTCACGGCTGTTCTCGCGAAACC	SOE deletion of *crp*, P3[c]
MS39_P4	CATTCTCGAGTGCATCAACTCCTACAAGAAG	SOE deletion of *crp*, P4[c]
CP12_delACIg_SOE_P1	GACAGCGGCCGCTCGAGCTCAAGGCGTTTTAG	SOE deletion of *cya*, P1
CP13_delACIg_SOE_P2	TAACGAGCGCCCGCAAAATAGTGCGGGAATGAGGTG	SOE deletion of *cya*, P2
CP14_delACIg_SOE_P3	TGCGGGCGCTCGTTATATTCCAAGCCTTCGCAAGT	SOE deletion of *cya*, P3
MS51_ACIg_SOE4	CATTGGATCCCGGTTTTACCCACACCAGAG	SOE deletion of *cya*, P4
IM21_delRpos1	GACAACTAGTCCAGCACCAGTCTCTGGATT	SOE deletion of *rpoS*, P1
IM22_delRpos2	TAACGAGCGGCCGCATTTGGTTACGGTATTGCTGACA	SOE deletion of *rpoS*, P2
IM23_delRpos3	TGCGGCCGCTCGTTAGCGCTGTTTAACGTCGAAT	SOE deletion of *rpoS*, P3
IM24_delRpos4	CATTGGATCCCGGGTATTCACATCCAAGGT	SOE deletion of *rpoS*, P4
CP09_VC2702_SOEcomp_P1	GACACATATGGACTCGACCTACACCATC	Complementation of *crbR*, F
ED92_VC2702_compR	CATTGGATCCGCAGAAATGAGCGAGTGGAT	Complementation of *crbR*, R
MS52_CRP_comp1	GACACATATGGTTCTAGGTAAACCTCAAACC	Complementation of *crp*, F
MS53_CRP_comp2SIO	CATTGGATCCACGATACGGGTTATCGGG	Complementation of *crp*, R

(Continued on next page)

TABLE 2 (Continued)

Primer	Sequence	Description[a]
CP15_ptadeletion_SOE_P1	CATTGGATCCCGGTATTCGTCGCTACGGCATGC	SOE deletion of *pta*, P1
CP16_ptadeletion_SOE_P2	TAACGAGCGGCCGCAGAGACCCATGCTCACGCTGGTCA	SOE deletion of *pta*, P2
CP17_ptadeletion_SOE_P3	TGCGGCCGCTCGTTAACGGCTATCCAAGCGGGTCAAG	SOE deletion of *pta*, P3
CP18_ptadeletion_SOE_P4	GACAACTAGTGCAATTTGCTGCAGAATCTGGCCGT	SOE deletion of *pta*, P4
CP19_acka_delSOE_P1	CATTGGATCCGACAAATAGCCTACGCGGCAGCC	SOE deletion of *ackA*, P1
CP20_acka_delSOE_P2	TAACGAGCGGCCGCACTTAGACATGAGTGACTACCTGT	SOE deletion of *ackA*, P2
CP21_acka_delSOE_P3	TGCGGCCGCTCGTTAGATACTGCTCGTCTGACCAACAT	SOE deletion of *ackA*, P3
CP22_acka_delSOE_P4	GACAACTAGTGAGTACGGCCAGCTTCATCTACTGGT	SOE deletion of *ackA*, P4
MMS17_pta-ackA_GP1	CATTGGATCCCGAAACGCATTAGTCAGGAA	Gibson deletion of *pta-ackA*, P1
MMS18_pta-ackA_GP2	TTCTGCTCTTGACCCGCTTCGTCAACAACTGCGAACTT	Gibson deletion of *pta-ackA*, P2
MMS19_pta-ackA_GP3	AAGTTCGCAGTTGTTGACGAAGCGGGTCAAGAGCAGAA	Gibson deletion of *pta-ackA*, P3
MMS20_pta-ackA_GP4	GGCGGCCGCTCTAGAAGATGCAGGGTTTCGTGACTG	Gibson deletion of *pta-ackA*, P4

[a]F, forward; R, reverse.
[b]See references 37 and 53.
[c]See reference 57.

~6 to 10 live flies. Samples were stored at −80°C until *Drosophila* flies were homogenized with a sterile pestle in TRIzol, and RNA was isolated according to the procedure described above.

Measurement of growth on acetate minimal medium. *V. cholerae* was streaked for single colonies and grown overnight in LB medium with appropriate antibiotics, if necessary. Next, 1 ml of each culture grown overnight was centrifuged at 15,000 rcf, and the pellet was resuspended in 1 ml of M63 minimal medium (Amresco). The resuspended cells were inoculated into M63 minimal medium supplemented with 10 mM sodium acetate, diluting the sample to reach an initial OD_{600} of 0.01 when measured in a standard 1-ml cuvette. Subsequently, 120 μl of each dilution was added to each of at least 3 wells of a sterile, flat, clear-bottomed, 96-well plate (Falcon) and incubated for 48 h at 37°C with periodic shaking inside a plate reader (Molecular Devices), during which the OD_{600} was monitored.

Determination of acetate concentrations in culture. Single colonies were inoculated into 1.5-ml cultures grown overnight and incubated with shaking at 37°C. After 16 h, 0.5 ml of the bacterial culture was inoculated in 50 ml of LB broth in an Erlenmeyer flask, and flasks were inoculated in duplicate for each assay. Soon after inoculation, and at successive time points, a 1-ml sample from each flask was aliquoted into an Eppendorf tube and centrifuged at 10,000 rcf for 30 s. The supernatant was filtered with 0.2-μm syringe filters, heated at 80°C for 5 min to denature excreted enzymes or toxins, and stored at −20°C for future analysis. The OD_{600} associated with each time point was also recorded. The samples were analyzed with an acetic acid assay kit (catalog number K-ACETRM; Megazyme) according to the manufacturer's instructions, with either LB medium or buffer used as a blank. If LB medium was used as the reference point, the values are relative to LB medium, and if buffer was used as a blank, the values include a small amount of acetate present in LB medium.

***Drosophila* survival assays.** *Drosophila* survival assays were performed as previously described (10, 21, 55), with ~10 male flies feeding on LB broth provided in a cellulose acetate matrix supplemented with a culture of *V. cholerae* grown overnight and diluted 1:10, in triplicate. Fly survival was monitored daily, and survival curves were analyzed in GraphPad Prism with the log rank test.

CrbR activity assay in *E. coli*. To test whether CrbR regulates *acs* transcription by binding directly to the promoter of *acs* (P_{acs}), regions of the *acs* promoter were amplified with Q5 high-fidelity DNA polymerase (NEB) and ligated into pRS415, and the CrbR gene was amplified with Q5 high-fidelity DNA polymerase (NEB) and ligated into the pACYC184 vector, according to methods described previously (31). The plasmids were cotransformed in *E. coli* TOP10 cells (NEB). CrbR-dependent expression of the *acs* promoter was demonstrated with β-galactosidase assays. In preparation for these assays, *E. coli* was first streaked for single colonies onto LB medium with the appropriate antibiotics (chloramphenicol at 5 μg/ml and/or ampicillin at 100 μg/ml) and incubated at 37°C overnight for inoculation into cultures grown overnight. Following overnight growth, cultures were then diluted 1:500 into 3 ml of LB medium in triplicate with appropriate antibiotics and incubated at 37°C with aeration until an OD_{600} of between 0.4 and 0.6 was reached. β-Galactosidase assays were performed according to a protocol derived from methods described previously (56).

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