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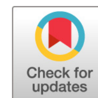

# A Putative Acetylation System in *Vibrio cholerae* Modulates Virulence in Arthropod Hosts

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**ABSTRACT** Acetylation is a broadly conserved mechanism of covalently modifying the proteome to precisely control protein activity. In bacteria, central metabolic enzymes and regulatory proteins, including those involved in virulence, can be targeted for acetylation. In this study, we directly link a putative acetylation system to metabolite-dependent virulence in the pathogen *Vibrio cholerae*. We demonstrate that the *cobB* and *yfiQ* genes, which encode homologs of a deacetylase and an acetyltransferase, respectively, modulate *V. cholerae* metabolism of acetate, a bacterially derived short-chain fatty acid with important physiological roles in a diversity of host organisms. In *Drosophila melanogaster*, a model arthropod host for *V. cholerae* infection, the pathogen consumes acetate within the gastrointestinal tract, which contributes to fly mortality. We show that deletion of *cobB* impairs growth on acetate minimal medium, delays the consumption of acetate from rich medium, and reduces virulence of *V. cholerae* toward *Drosophila*. These impacts can be reversed by complementing *cobB* or by introducing a deletion of *yfiQ* into the Δ*cobB* background. We further show that *cobB* controls the accumulation of triglycerides in the *Drosophila* midgut, which suggests that *cobB* directly modulates metabolite levels *in vivo*. In *Escherichia coli* K-12, *yfiQ* is upregulated by cAMP-cAMP receptor protein (CRP), and we identified a similar pattern of regulation in *V. cholerae*, arguing that the system is activated in response to similar environmental cues. In summary, we demonstrate that proteins likely involved in acetylation can modulate the outcome of infection by regulating metabolite exchange between pathogens and their colonized hosts.

**IMPORTANCE** The bacterium *Vibrio cholerae* causes severe disease in humans, and strains can persist in the environment in association with a wide diversity of host species. By investigating the molecular mechanisms that underlie these interactions, we can better understand constraints affecting the ecology and evolution of this global pathogen. The *Drosophila* model of *Vibrio cholerae* infection has revealed that bacterial regulation of acetate and other small metabolites from within the fly gastrointestinal tract is crucial for its virulence. Here, we demonstrate that genes that may modify the proteome of *V. cholerae* affect virulence toward *Drosophila*, most likely by modulating central metabolic pathways that control the consumption of acetate as well as other small molecules. These findings further highlight the many layers of regulation that tune bacterial metabolism to alter the trajectory of interactions between bacteria and their hosts.

**KEYWORDS** *Drosophila melanogaster*, *Vibrio cholerae*, acetate, acetyl-CoA, acetyl-CoA synthetase, acetylation, carbon metabolism, cholera, posttranslational modification

Bacteria finely tune their responses to various environmental conditions by modulating fluxes of nutrients through metabolic pathways, effectively balancing their energy needs with the biosynthetic demands associated with rapid cellular growth.

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Metabolic fluxes can be regulated most effectively by altering the concentrations of specific enzymes in the cell, but more immediate responses can be achieved by simply switching enzymes "on" and "off" via reversible covalent modifications, such as phosphorylation or acetylation, at key catalytic or allosteric residues. In bacteria, phosphorylation has been studied in the greatest detail because of its central role in mediating signaling via two-component signal transduction systems. However, acetylation similarly controls the activity of a wide variety of proteins in diverse bacterial lineages (1, 2). Acetylation targets specific lysine residues at the ε-amino group and alters one or more residues within a single protein. As bacterial cells progress from exponential- to stationary-phase growth, a surprisingly large proportion of the proteome undergoes acetylation (3–6). Although the impact of this modification on the vast number of proteins is unknown, acetylation affects the activity of specific proteins that, in turn, impact nutrient flux through metabolic pathways. These proteins include acetyl-CoA synthetase (Acs), an enzyme that converts acetate to acetyl-CoA as cells reach stationary phase, during a process termed the "acetate switch" (1). Acetate is first excreted by bacteria during rapid growth. Several theories have been proposed to explain this observation. Recently, a study suggested that growth rate can be maximized by optimally partitioning the proteome between respiratory and fermentative pathways (7). Many other studies have linked acetic acid production to ″overflow″ metabolism or more specific needs regarding recycling of metabolic intermediates (1). Flipping this switch by inducing Acs allows bacteria to consume the excreted acetate as other carbon sources are depleted (1).

Acs is regulated by acetylation in bacteria and eukaryotes (8–10). In *Salmonella*, acetylation of a conserved lysine residue, Lys609, regulates the activity of the Acs protein, although other acetylated lysine sites have been identified (5, 10). Lys609 is acetylated and inactivated via a specific acetyltransferase, YfiQ/PatZ/Pka in *Salmonella* (here referred to as YfiQ), a member of the GCN5-like acetyltransferase (GNAT) family (11). Deacetylation, which reactivates Acs, primarily depends on CobB, a sirtuin-like protein that functions as an $NAD^+$-dependent deacetylase (10). In *Bacillus subtilis*, a second deacetylase, AcuC, activates Acs as well (12). Acetylation can also occur nonenzymatically; many proteins carry lysine residues that are susceptible to an alternative, direct acetylation mechanism requiring acetyl-phosphate (acetyl-P) (5, 6). Altogether, these acetylation mechanisms target a significant number of proteins in the cell, many of which are situated within central metabolic pathways (2, 5, 6, 13). As a result, acetylation may indirectly mediate the close relationships between bacteria and multicellular organisms that are contingent upon the exchange of metabolic products.

Members of the *Vibrio* genus are marine bacteria that readily adjust to both free-living and host-associated states. In at least two lineages, regulation of acetate metabolism is critical for the development and stability of their interactions with host organisms. In *Vibrio fischeri*, the bioluminescent symbiont of the Hawaiian bobtail squid, *Euprymna scolopes*, Acs promotes colonization of the juvenile squid (14, 15). In *Vibrio cholerae*, the human pathogen responsible for historical and ongoing global cholera pandemics, Acs is required for virulence in a *Drosophila melanogaster* model of infection (16). Consumption of acetate via Acs by *V. cholerae* contributes to the misregulation of triglyceride storage in the fly, resulting in intestinal steatosis, which is necessary for *V. cholerae*-mediated mortality (16). In *V. cholerae*, *acs* transcription is activated by the CrbS/R two-component signal transduction pathway (16, 17). To further define the regulatory network that modulates levels of acetate during colonization and infection, we determined whether genes encoding putative acetyltransferase and deacetylase enzymes in *V. cholerae* alter acetate metabolism and the infection processes. Here, we demonstrate that CobB and YfiQ, but not AcuC, contribute to the regulation of acetate consumption and virulence in a *Drosophila* model, suggesting that acetylation is an important regulator of host-microbe interactions in this global pathogen.

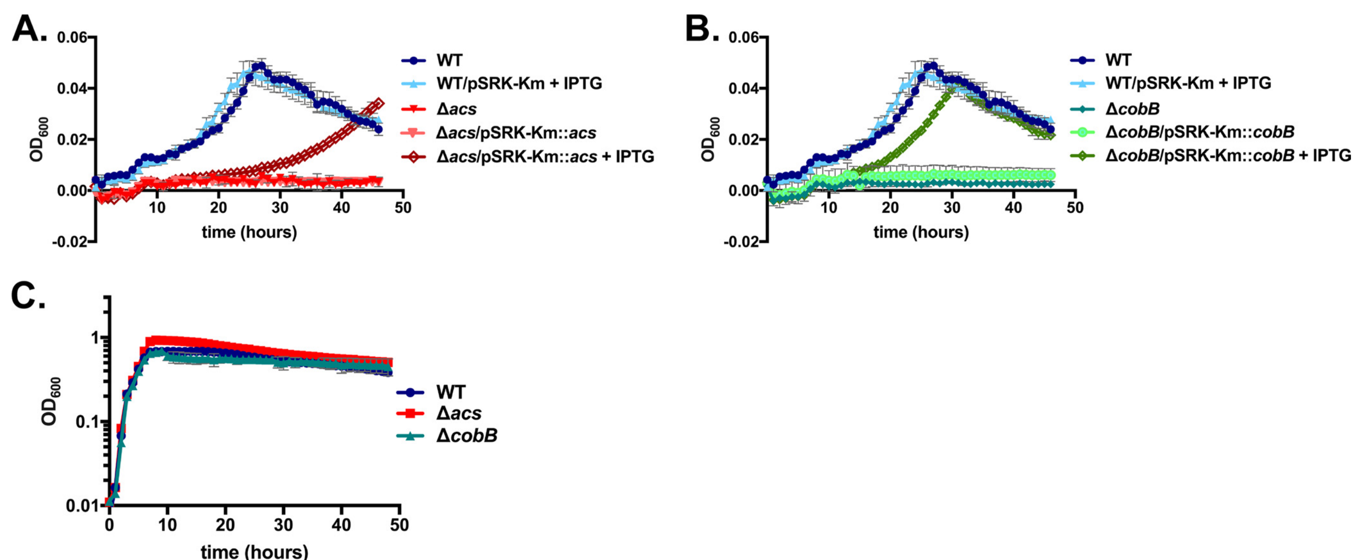


**FIG 1** Deletion of *V. cholerae* SIO *cobB* prevents growth on acetate minimal medium but not on LB broth. (A and B) *V. cholerae* SIO Δ*acs* (A) and Δ*cobB* (B) strains cannot grow on M63 minimal medium supplemented with 10 mM acetate as a sole carbon source, but the addition of the pSRK-Km complementation plasmids carrying the *acs* and *cobB* genes, respectively, together with induction with 1 mM IPTG, restores growth. Optical densities were measured once every hour over 46.5 h, and values from triplicate wells of a single experiment are plotted. (C) In LB broth, *V. cholerae* SIO Δ*acs* and Δ*cobB* grow at similar rates, and to similar optical densities, relative to the wild-type (WT) strain. Optical densities were measured once every hour over 48 h, and values from duplicate wells of a single experiment in a 96-well plate are plotted. In each panel, error bars depict standard deviations. At some time points, the symbols fully obscure the error bars and therefore are not shown.

## RESULTS

**Identification and deletion of the *V. cholerae* CobB and YfiQ genes.** In order to assess whether an acetylation system similar to those of *Escherichia coli* and *Salmonella* exists in *V. cholerae*, we first identified putative *yfiQ* and *cobB* genes in the *V. cholerae* genome via the BLAST algorithm. CobB, an $NAD^+$-dependent protein deacetylase, belongs to the sirtuin family of proteins, which deacetylate a number of different target proteins with wide-ranging biological effects in bacteria, archaea, and eukarya (18). The putative *V. cholerae* deacetylase protein, encoded by the VC1509 locus, and the *E. coli* CobB protein share 66% identity and 79% similarity across 88% of the protein, with the catalytic His110 residue being conserved in *V. cholerae* (19). The *Salmonella* YfiQ acetyltransferase protein and the putative *V. cholerae* acetyltransferase protein (VCA0574) share 55% identity and 73% similarity across all but the first four amino acids. The high levels of identity at the amino acid level, including the conservation of key residues required for enzymatic function, strongly suggest that the proteins may share a both functional and evolutionary relationships. Therefore, we refer to these genes as *cobB* and *yfiQ* in *V. cholerae*, respectively.

**CobB is required for acetate utilization.** Next, we engineered an in-frame deletion in the putative *cobB* gene in *V. cholerae* SIO. Because CobB may function as a deacetylase, we predicted that the deletion may increase the proportion of metabolic enzymes that are acetylated, including those involved in acetate catabolism, such as Acs. As a result, the cells may be unable consume acetate. To test this hypothesis, we inoculated both the *acs* mutant and the *cobB* mutant into minimal medium with acetate as the sole carbon source. After ~48 h, the *cobB* deletion mutant grew little on acetate in comparison to the wild-type strain, mimicking the phenotype of the *acs* deletion (Fig. 1A and B). Inducing the expression of *cobB* from the pSRK-Km plasmid with isopropyl-β-D-thiogalactopyranoside (IPTG) restored growth on acetate, indicating that the lack of *cobB* is solely responsible for this phenotype (Fig. 1B). Inducing the expression of *acs* partially complemented the growth of the Δ*acs* mutant (Fig. 1A). Both the *acs* mutant and the *cobB* mutant grew similarly to the wild-type strain in LB broth, demonstrating that these mutations do not result in a generalized growth defect in rich medium (Fig. 1C).

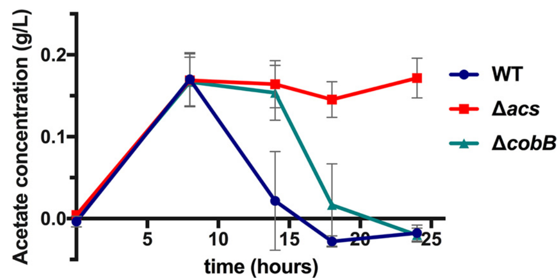


**FIG 2** Deletion of *V. cholerae* SIO *cobB* delays, but does not halt, acetate consumption. Acetate concentrations relative to those in uninoculated LB broth were measured over time. Here, results from three biological replicates, each performed in duplicate, are depicted. Acetate concentrations in cultures of wild-type SIO and SIO Δ*cobB* strains are significantly different from one another after 14 h ($P = 0.0022$) and 18 h ($P = 0.0087$) of growth (by a Mann-Whitney test). Error bars depict standard deviations.

To determine whether the defect in growth on acetate resulted from an inability to remove acetate from the medium, we directly measured acetate concentrations in LB broth over time. We observed that deleting *acs* or *cobB* did not impact acetate excretion; by 8 h, acetate concentrations rose to levels similar to those observed in the wild-type strain (Fig. 2). This is consistent with observations in *E. coli* in which the *acs* mutant also accumulates acetate to the same extent as the wild type (20). The wild-type *V. cholerae* strain flipped the acetate switch after 8 h of growth, and all acetate was removed from the medium prior to 20 h postinoculation (Fig. 2). In contrast, acetate consumption by the *acs* mutant was halted entirely. Deletion of *cobB*, however, delayed but did not halt the initiation of acetate removal in rich medium (Fig. 2).

**CobB modulates *V. cholerae* virulence in *Drosophila*.** Because *cobB* alters acetate utilization during growth in culture, we hypothesized that this gene may be necessary for *V. cholerae* virulence during infection of *Drosophila*. In this model system, *V. cholerae* pathogenicity is dependent upon the colonization of specific regions of the gastrointestinal (GI) tract and manipulation of host metabolism (16, 21–24). In order to test whether *cobB*-dependent alteration of acetate metabolism is relevant *in vivo*, we fed this mutant to adult *Drosophila* flies in a standard oral infection assay (25). Ingestion of wild-type *V. cholerae* is lethal to *Drosophila* over the course of 48 to 96 h, while a strain carrying a deletion in *acs* is significantly less lethal to flies despite populating the GI tract to the same level as the wild type (16). This phenotype is observed in strains of both environmental and clinical origins, independent of whether they carry genes that encode cholera toxin and toxin-coregulated pilus (16, 17). Deletion of *cobB* significantly delayed fly mortality relative to the wild-type strain ($P < 0.0001$), but it did not render the strain avirulent (Fig. 3A). To ensure that the *cobB* deletion did not reduce levels of bacteria in the flies, *Drosophila* flies infected with these bacterial strains were homogenized at 24 and 48 h postinfection, and numbers of CFU per fly were determined by plating. The *cobB* and *acs* mutations did not reduce bacterial numbers in the flies, as expected ($P > 0.05$ by a Mann-Whitney test) (Fig. 3B).

**YfiQ, a putative acetyltransferase, counteracts the effects of CobB *in vitro* and *in vivo*.** While CobB removes acetyl groups from proteins, the addition of acetyl moieties to specific residues by acetylation can occur both via enzyme-dependent processes mediated by acetyltransferases and via the accumulation of acetyl-P, which can nonenzymatically acetylate target proteins (5, 6). To determine whether YfiQ, the putative acetyltransferase, functions to counteract the effects of CobB, we engineered an in-frame deletion in the *yfiQ* gene in both the wild-type *V. cholerae* strain and the Δ*cobB* strain. To ensure that background mutations did not affect the phenotype, we also created the same strain by introducing the Δ*cobB* allele into the Δ*yfiQ* background. We then tested these three strains (Δ*yfiQ*; Δ*cobB* into the Δ*yfiQ* background, designated Δ*cobB*→Δ*yfiQ*; and Δ*yfiQ*→Δ*cobB* as the reverse construction) for their ability to grow on acetate minimal medium. Relative to the wild-type strain, deletion of *yfiQ* did not impact growth on acetate (Fig. 4A). Introduction of the *yfiQ* deletion into the Δ*cobB*

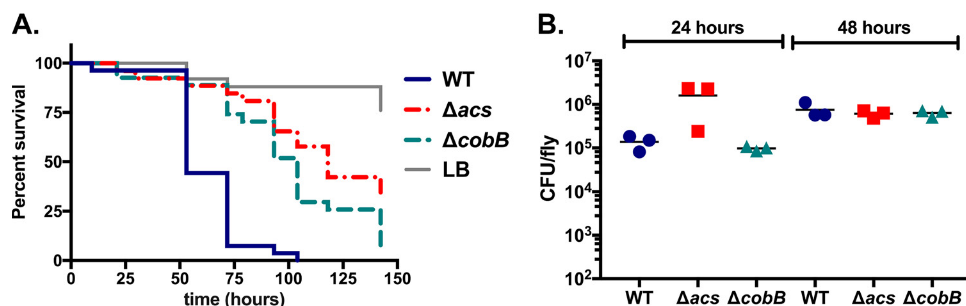


**FIG 3** Deletion of *V. cholerae* SIO *cobB* significantly improves survival of *Drosophila* without appreciably affecting colonization. (A) Survival of flies fed the *V. cholerae* wild-type SIO, SIO Δ*cobB*, or SIO Δ*acs* strain, or uninoculated LB broth as a control, was monitored over 143 h. This assay was performed with *V. cholerae* strains added to triplicate vials, with 8 to 10 flies per vial. This result is representative of data from five separate trials. Flies fed the SIO Δ*cobB* or SIO Δ*acs* strain survived significantly longer than did flies fed the wild-type strain ($P < 0.0001$ by a log rank test). (B) Colonization of flies infected with the *V. cholerae* SIO wild-type, SIO Δ*cobB*, or SIO Δ*acs* strain. The assay was performed in triplicate vials, and all surviving flies were collected after 24 and 48 h of infection, homogenized, and plated on selective medium. The bacterial loads of flies infected with the mutant strains were not significantly different from bacterial loads of those infected with the wild-type strain ($P > 0.05$ by a Mann-Whitney test).

background (and vice versa) restored the ability of the strain to grow on acetate without affecting growth on LB broth (Fig. 4A and B). These results are consistent with the deletion of *yfiQ* preventing acetylation and subsequent inactivation of one or more key enzymes required for growth on acetate minimal medium, including Acs. In this background, CobB, the putative deacetylase, would have little effect on acetylation levels or enzyme activity, as the deletion of *yfiQ* would ensure that acetylation is minimal, and targeted enzymes would be maximally active.

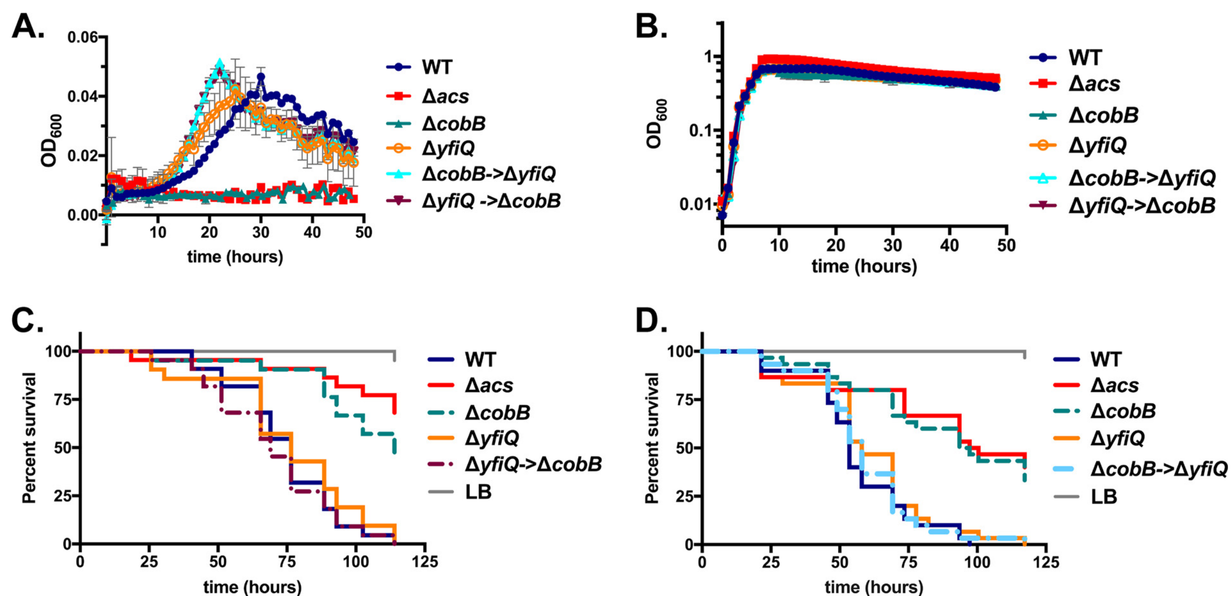


**FIG 4** Introduction of the complete deletion of *yfiQ* restores both virulence in *Drosophila* and growth on acetate minimal medium to the *V. cholerae* Δ*cobB* strain. (A) Deletion of *yfiQ* does not impact growth on minimal medium supplemented with acetate (10 mM), with growth being similar to that of the wild-type strain. Introduction of the Δ*yfiQ* mutation restores growth to the strain carrying the deletion in Δ*cobB*, as both the Δ*yfiQ*→Δ*cobB* and the Δ*cobB*→Δ*yfiQ* strains grow similarly to the wild-type strain in acetate minimal medium. (B) In LB broth, all strains grow similarly to the wild type. (C) YfiQ does not significantly alter virulence of *Drosophila* ($P > 0.05$). In seven independent trials, deletion of *yfiQ* significantly increased virulence in one assay ($P = 0.0003$), reduced virulence in a second assay ($P = 0.0143$), and did not alter virulence in the remaining five assays, including the representative assay presented here. When infected with the Δ*yfiQ*→Δ*cobB* strain, fly mortality is increased significantly relative to the strain carrying the Δ*cobB* deletion alone ($P < 0.0001$ by a log rank test). This result was reproducible in five independent trials. (D) When flies were infected with the Δ*cobB*→Δ*yfiQ* strain, virulence was similarly restored relative to the strain carrying the single *cobB* deletion ($P < 0.0001$ by a log rank test).

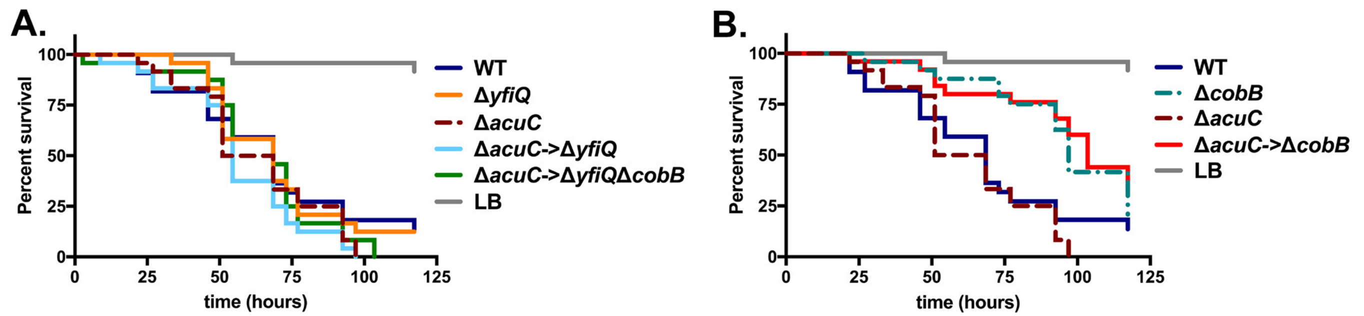


**FIG 5** Deletion of the putative deacetylase AcuC does not alter virulence in *Drosophila*. (A) Survival of flies fed the wild-type, Δ*yfiQ*, Δ*acuC*, Δ*acuC* Δ*yfiQ*, or Δ*acuC* Δ*yfiQ* Δ*cobB* strain. Deletion of *acuC* did not alter fly survival relative to the wild type in each of two separate assays, one of which is represented here ($P > 0.05$ by a log rank test). (B) Survival of flies fed the wild-type, Δ*cobB*, Δ*acuC*, or Δ*acuC* Δ*cobB* strain. Deletion of *acuC* in the Δ*cobB* strain did not alter survival relative to the Δ*cobB* deletion alone in each of two separate assays ($P > 0.05$ by a log rank test).

To determine whether *yfiQ* function is relevant *in vivo*, we infected *Drosophila* flies with the Δ*yfiQ*, Δ*yfiQ*→Δ*cobB*, and Δ*cobB*→Δ*yfiQ* strains. Based on our results presented above, we hypothesized that the deletion of *yfiQ* might reduce the proportion of acetylated Acs proteins (or other metabolic enzymes), which could, in turn, increase acetate consumption and virulence toward *Drosophila*, resulting in more rapid mortality of flies. However, the Δ*yfiQ* allele did not consistently alter virulence; in five of seven independent assays, the rate of survival of flies fed this strain was similar to that of flies fed the wild-type strain, while flies died slightly more quickly in one assay, and in the last assay, the flies died more slowly (Fig. 4C and D). A phenotype for YfiQ was observed in the Δ*cobB*→Δ*yfiQ* strain, which killed flies more quickly than did the strain carrying the Δ*cobB* deletion alone ($P < 0.0001$ in each of five independent assays) (Fig. 4C). In a confirmatory experiment, introduction of the Δ*yfiQ* allele into the Δ*cobB* background also restored virulence to the Δ*cobB* strain ($P > 0.05$ compared to the wild type and $P < 0.0001$ compared to the Δ*cobB* strain in one assay) (Fig. 4D). This is consistent with the hypothesis that the acetylation system is operational in *V. cholerae* and relevant during *in vivo* infection.

**AcuC, a second putative deacetylase, does not alter acetate-dependent virulence.** The activity of Acs in *Bacillus subtilis*, a Gram-positive soil bacterium, is controlled by two deacetylases, SrtN, its sirtuin-like deacetylase, and an additional deacetylase, AcuC (12). In the *B. subtilis* genome, the *acuC* gene is adjacent to the *acsA* gene (12). *V. cholerae* carries a homolog of AcuC at locus VC2042, but it is not colocalized with *acs* or other genes involved in altering acetylation. The two proteins are 31% identical and 43% similar across 294 amino acids. To determine whether *acuC* similarly regulates acetate metabolism in *V. cholerae*, we introduced an in-frame deletion of this gene into the wild-type, Δ*cobB*, Δ*yfiQ*, and Δ*yfiQ*→Δ*cobB* backgrounds and monitored its effects on *Drosophila* survival. We observed that the deletion of *acuC* alone did not alter the mortality of *Drosophila* flies (Fig. 5A). When the Δ*acuC* mutation was introduced into the Δ*cobB* background, survival was not further decreased, indicating that redundancy with *cobB* was not masking its phenotype (Fig. 5B). We further observed no impact on survival in the Δ*yfiQ* or Δ*yfiQ*→Δ*cobB* background, as expected (Fig. 5A). Therefore, *acuC* does not affect Acs activity or acetate metabolism during infection of *Drosophila*.

**Point mutations in Acs modulate Acs activity.** In order to further define the effects of CobB on Acs, we identified point mutations in Acs that might prevent acetylation. We hypothesized that the introduction of these mutations into a *cobB* deletion strain could support higher rates of acetate consumption, which could then improve growth on acetate minimal medium. We began by engineering specific mutations in conserved Acs residues shown by previous studies in *E. coli* and *Salmonella* to modulate acetylation. First, we replaced the conserved catalytic lysine residue K609 in the chromosomal copy of *acs* with arginine, which is thought to mimic a nonacetylated lysine (26). We reasoned that this mutation could result in one of two outcomes.

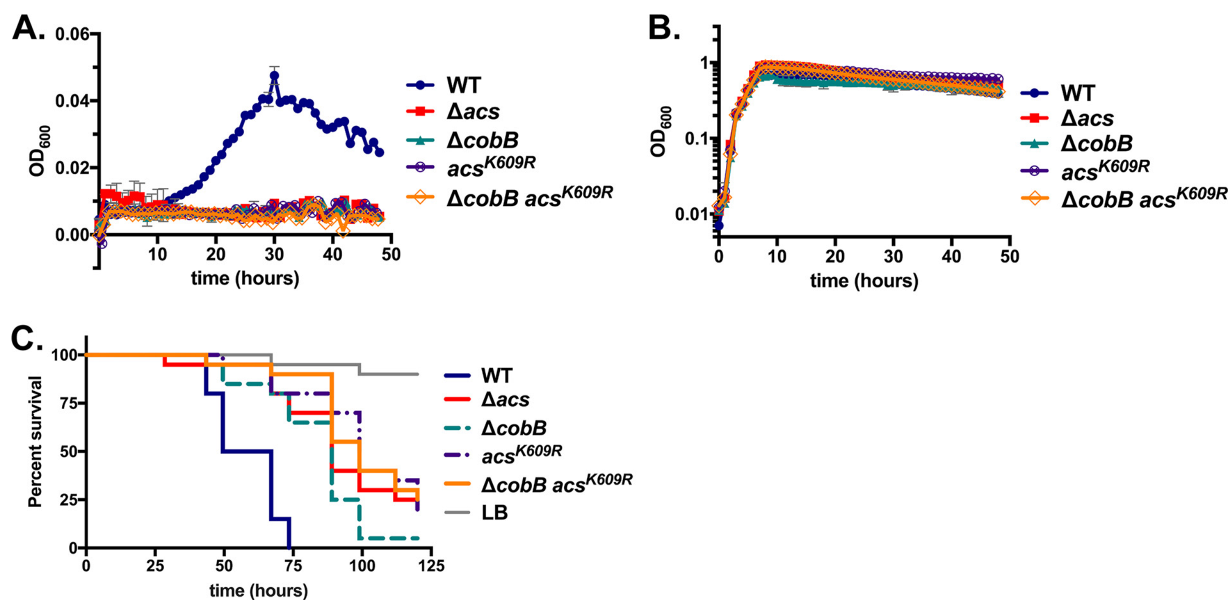


**FIG 6** Mutation of lysine 609 abrogates Acs activity. (A) Mutation of the putative catalytic residue of Acs, K609, to arginine entirely halts growth on M63 minimal medium supplemented with 10 mM acetate. The introduction of this mutation has no effect on the growth of the Δ*cobB* strain. (B) Mutations of *acs* have no effect on growth on LB broth, consistent with previous findings. (C) Introduction of the $acs^{K609R}$ mutation into the wild-type strain reduces fly mortality ($P < 0.0001$ by a log rank test). The introduction of the same mutation into the Δ*cobB* strain does not restore virulence but rather further reduces virulence ($P = 0.018$ by a log rank test). These results are representative of data from three independent trials.

First, it is possible that this mutation simply reduces the enzymatic activity of Acs, since it may interrupt the catalytic site. Alternatively, this mutation may prevent acetylation while allowing for some level of catalytic activity. We observed that the $Acs^{K609R}$ mutation completely abrogated growth on acetate minimal medium, suggesting that it primarily reduced the protein's catalytic activity (Fig. 6). To test the possibility that the mutation may prevent acetylation while permitting a low level of activity, we introduced this mutation into the Δ*cobB* strain, reasoning that the K609R mutation may increase Acs activity to a level slightly higher than that observed in the *cobB* mutant alone. However, growth of the Δ*cobB* $Acs^{K609R}$ deletion strain on acetate minimal medium was negligible and identical to those of the $Acs^{K609R}$, Δ*acs*, and Δ*cobB* strains (Fig. 6A). Thus, introducing the K609R mutation did not increase Acs activity, nor did it overcome the acetate consumption defect observed in the *cobB* deletion. The K609R mutation did not affect growth on rich LB broth, suggesting that it did not confer a generalized growth defect (Fig. 6B). Next, we tested whether this mutation altered pathogenicity during *Drosophila* infection. We observed that the K609R mutant mimicked the virulence of the *acs* mutant ($P > 0.05$ in three independent assays), further supporting the hypothesis that it abrogates the function of Acs (Fig. 6C). In addition, the introduction of the K609R mutation into the Δ*cobB* background did not increase virulence but instead further reduced virulence relative to the single mutation in *cobB* alone (Fig. 6C). These results are each consistent with the conclusion that the mutation simply reduced the ability of Acs to carry out its primary catalytic function, regardless of its effect on the acetylation state. However, confirmation of this conclusion awaits direct biochemical testing with a purified Acs protein carrying this mutation.

Next, we introduced a mutation in Acs at residue 641 that changed the Leu residue to Ala. In *Salmonella*, this mutation restores growth on acetate minimal medium to a strain that also carries deletions in *cobB* and *pta*, because the $Acs^{L641A}$ mutant prevents YfiQ from acetylating Acs (27). This permits growth on acetate despite the absence of CobB, which is otherwise needed to deacetylate and activate Acs (27). (In *Salmonella*, deletion of *pta* in addition to *cobB* is necessary to prevent background growth on acetate minimal medium. However, we observed that deletion of *cobB* alone is sufficient to halt the growth of *V. cholerae* on acetate [Fig. 1], suggesting that *pta* does not

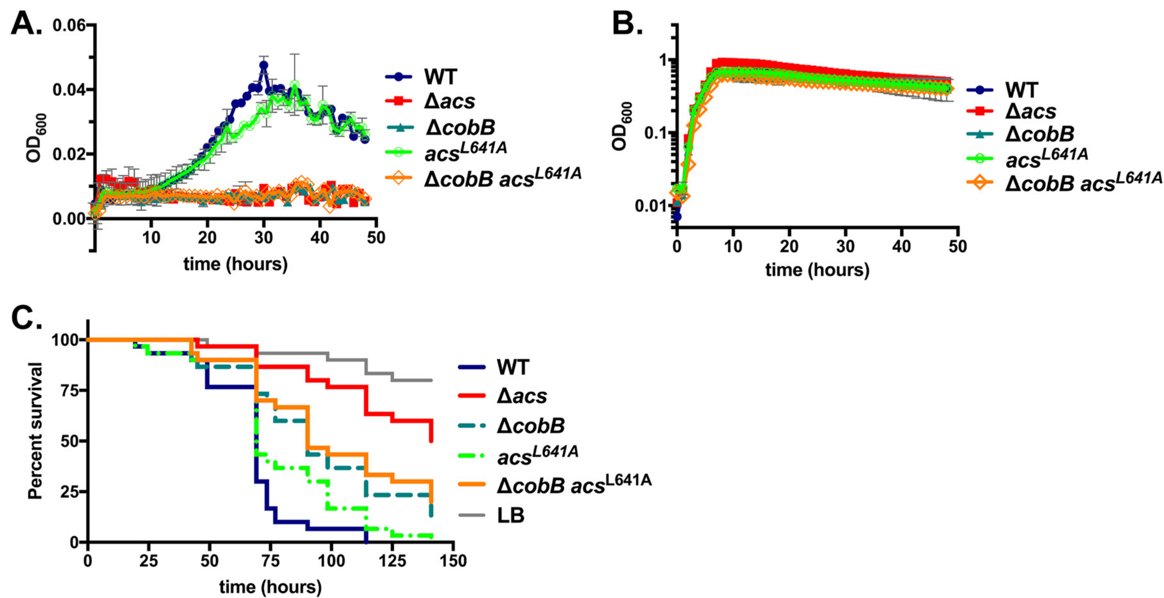


**FIG 7** Mutation of Leu641 to Ala does not restore acetate metabolism in the Δ*cobB* strain. (A) Mutation of Leu641 does not severely impede growth on M63 minimal medium supplemented with 10 mM acetate. The introduction of this mutation has no effect on the growth of the Δ*cobB* strain. (B) Mutations of *acs* have no effect on growth on LB broth, consistent with previous findings. (C) Introduction of the *acs*$^{L641A}$ mutation into the wild-type strain slightly reduces fly mortality ($P = 0.0260$ by a log rank test), consistent with findings in four of five independent trials. The introduction of the same mutation into the Δ*cobB* strain does not affect the virulence of the Δ*cobB* mutant ($P > 0.05$ by a log rank test), which was again observed in four of five independent trials.

play a role in the consumption of acetate at these concentrations, as reported previously for *E. coli* [1].) In *Salmonella*, the observation that the *cobB* deletion can be suppressed by the Acs$^{L641A}$ mutation alone, without additional mutations in other enzymes in the acetate assimilation pathway, supports the conclusion that the YfiQ/CobB system may modulate acetate metabolism primarily via its regulation of Acs activity, as opposed to any effects that it may have on other catabolic enzymes.

To determine whether the mutation of a single residue of Acs similarly alters CobB-mediated growth of *V. cholerae* on acetate, we engineered the same Leu-to-Ala substitution at residue 641 of Acs by replacing the wild-type copy with the *acs*$^{L641A}$ allele on the chromosome. As with the Acs$^{K609R}$ mutant, the mutation of Leu641 to Ala could affect *V. cholerae* Acs function in several ways. The mutation could generate an enzyme that is functional but entirely blind to acetylation. In this scenario, we would expect that introducing the Acs$^{L641A}$ mutation into the Δ*cobB* background would restore the ability of this strain to assimilate acetate, grow on acetate minimal medium, and fatally infect *Drosophila* flies. This would be most evident if both YfiQ and CobB are primarily acting through Acs to regulate acetate catabolism. If mutation of Leu641 has no effect on the acetylation of Acs, then we would expect the mutation to have no effect on the *cobB* phenotype. On the other hand, this residue could play an alternative role in Acs function or stability, and its mutation could affect Acs activity through some means other than acetylation.

The Acs$^{L641A}$ mutation did not affect growth on LB medium and only minimally affected on growth on acetate, suggesting that the mutation does not significantly impair Acs function (Fig. 7). Next, we tested whether this mutation improves the growth of the Δ*cobB* strain on acetate minimal medium. We observed that the Δ*cobB acs*$^{L641A}$ strain grew similarly to the *cobB* deletion alone; that is, the mutant was unable to grow on acetate minimal medium but grew to wild-type levels on LB medium (Fig. 7). This finding is consistent with each of two possible conclusions: (i) that the introduction of the L641A point mutation has no effect on Acs function or acetylation because its role in YfiQ binding is not conserved or (ii) that conferring resistance to acetylation upon Acs via the introduction of the L641A mutation is insufficient to alter acetate removal, because CobB may be acting multifactorially to control acetate metabolism.

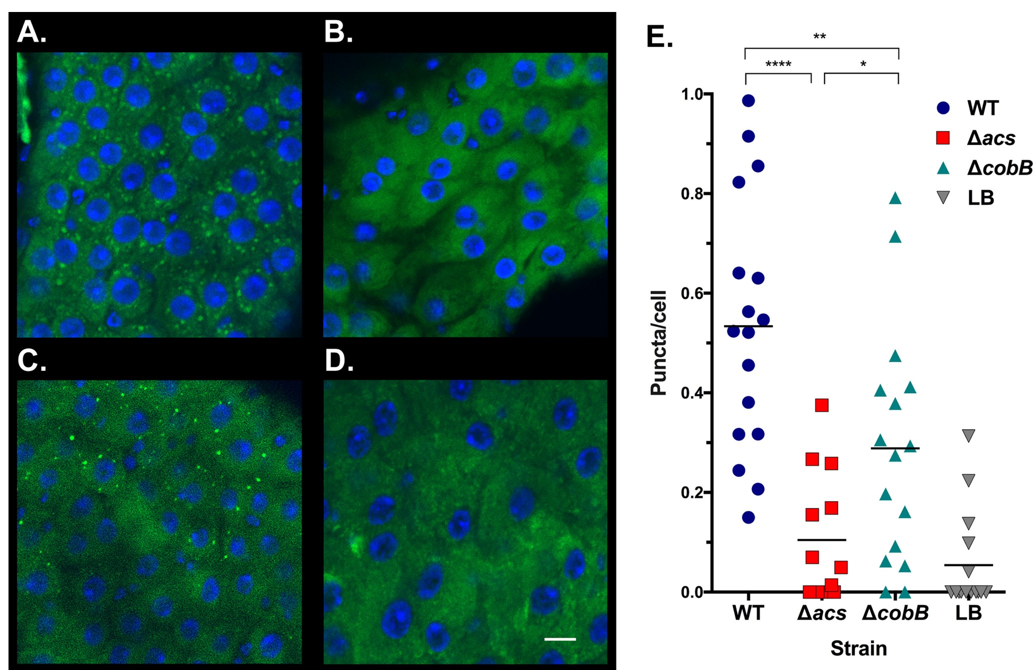


**FIG 8** CobB alters accumulation of triglycerides in the *Drosophila* gastrointestinal tract. (A to D) Representative images of *Drosophila* GI tracts infected with the *V. cholerae* wild-type (A), Δ*acs* (B), or Δ*cobB* (D) strain or provided sterile LB broth (D); visualized by confocal microscopy; and stained with DAPI and BODIPY. Bar, 10 $\mu$m. (E) Quantification of triglyceride puncta stained with BODIPY 493/503 in midguts of *Drosophila* flies infected with the *V. cholerae* wild-type, Δ*acs*, or Δ*cobB* strain or sterile LB broth, with the levels of significance indicated (****, $P < 0.0001$; **, $P = 0.0060$; *, $P = 0.0127$). These results were representative of data from three trials comparing the wild-type and Δ*cobB* strains, two of which included the Δ*acs* strain.

To determine whether *Drosophila* infection assays might more sensitively detect slight alterations in virulence caused by the L641A mutation, we fed these strains to flies and monitored survival. The L641A mutation alone reduced virulence in 4 of 5 independent assays compared to the wild-type strain ($P < 0.05$). Introducing this L641A mutation into the Δ*cobB* strain background increased virulence significantly in only one of five assays, although a nonsignificant trend was observed in a second assay ($P = 0.0687$). Therefore, we cannot conclude that the Leu641 residue modulates virulence or acetylation in any way (Fig. 7). Interestingly, the amino acids directly adjacent to Leu641 are not highly conserved between *Salmonella enterica* and *V. cholerae*, suggesting that this residue may not function similarly in the two homologous proteins, and the mutation may have no effect on YfiQ binding or interactions.

**Effect of the CobB deletion on triglyceride storage in the fly.** In order to confirm that the *cobB* gene is affecting fly survival via its manipulation of acetate or other metabolites, we examined the GI tracts of flies for effects on the storage of lipids. When fed LB broth in the absence of infection, flies accumulate and store triglycerides in the fat body (16). However, consumption of acetate by *V. cholerae* causes triglyceride droplets to amass in cells lining the GI tract (16). This is directly related to *V. cholerae*-dependent processes that remove acetate and other metabolites during infection (16, 21). In order to determine whether *cobB* similarly affects fat storage, we fed flies wild-type *V. cholerae*, LB medium alone, or the strains carrying mutations in *cobB* or *acs* for 48 h. We then removed the GI tracts, performed staining with BODIPY dye, and quantified puncta of triglycerides in cells (21). Consistent with previous findings, infection with the wild-type strain drastically increased the number of puncta of accumulating triglycerides relative to those in uninfected flies, while the *acs* mutant maintained reduced triglyceride storage (16) (Fig. 8). The *cobB* strain exhibited an intermediate phenotype. In two of three experimental replicates, the *cobB* mutant significantly reduced triglyceride storage relative to the wild type, and the trend was

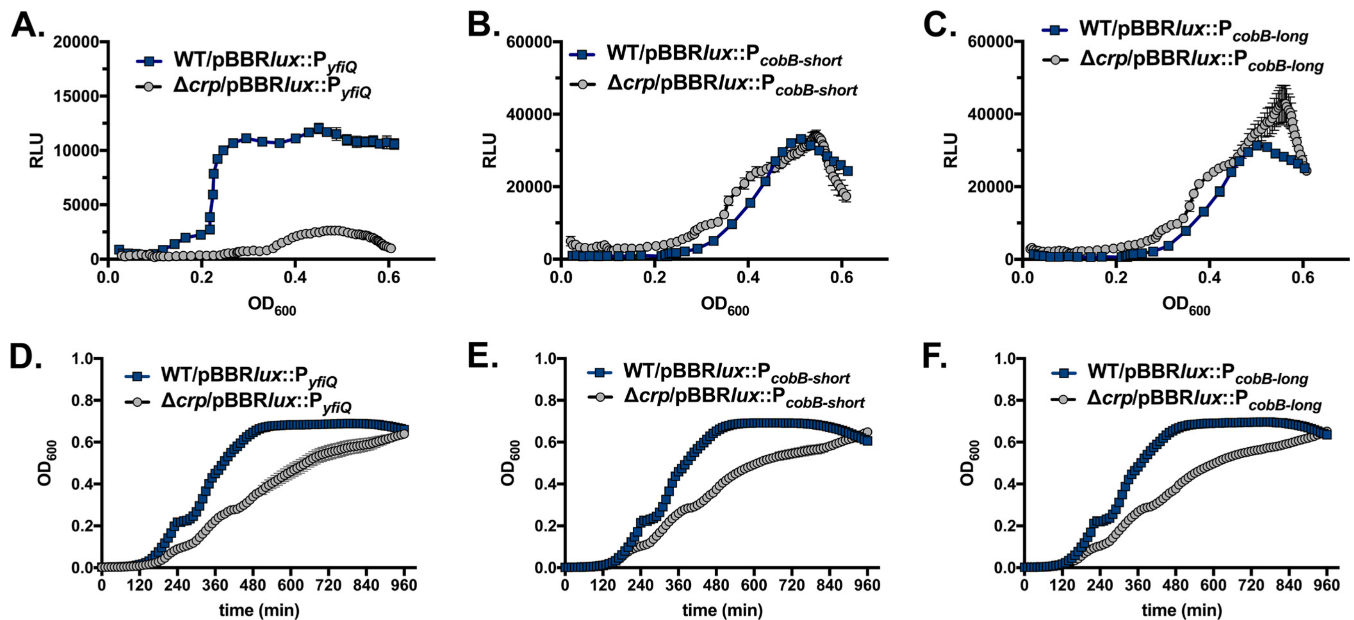


**FIG 9** CRP regulates transcription of *yfiQ* but not *cobB*. (A to C) The promoter of *yfiQ* (A) and both a short region (B) and a longer region (C) of the *cobB* promoter were cloned into the pBBR*lux* plasmid and conjugated into *V. cholerae* wild-type and *V. cholerae* Δ*crp* bacteria. Bioluminescence was monitored in a multimode plate reader over 1,000 min and is depicted as relative light units (RLU), defined as luminescence normalized to the $OD_{600}$. Due to the growth defect of the *crp* mutants, the *y* axis depicts the $OD_{600}$, and RLU are shown over the time period during which the cultures were at an $OD_{600}$ of 0.02 to 0.60. The *yfiQ* promoter is strongly induced, but the deletion of *crp* prevents the expression of *yfiQ*. In contrast, the deletion of *crp* does not affect expression from the *cobB* promoter fragments. (D to F) The Δ*crp* strain exhibits a growth defect as cells approach stationary phase. Growth of the WT and Δ*crp* strains carrying the designated promoters was monitored in the multimode plate reader by measurement of $OD_{600}$.

similar but did not reach significance in a third trial (Fig. 8). In comparison to the *acs* mutant, infection with the Δ*cobB* strain increased triglyceride storage in the enterocytes significantly (Fig. 8). These results suggest that the *cobB* gene alters bacterial metabolism, which in turn affects the accumulation of lipids in the fly GI tract in a manner that hastens mortality.

**Transcriptional regulatory mechanisms of *yfiQ* are conserved in *V. cholerae*.** In order to place the acetylation system in a broader regulatory context in *V. cholerae*, we tested whether this system is subject to conserved regulatory controls. In *E. coli* and *Salmonella*, the *yfiQ* gene is transcriptionally regulated by the cAMP-cAMP receptor protein (CRP) system, but *cobB* expression levels change little across different phases of growth (28). To test whether the cAMP-CRP system similarly activates *yfiQ* but not *cobB* in *V. cholerae*, we cloned the putative promoters of *yfiQ* and *cobB* into the pBBR*lux* plasmid and conjugated the plasmids into both wild-type *V. cholerae* and *V. cholerae* carrying a deletion in *crp*. We tested two regions of the *cobB* promoter because of the presence of a poorly conserved putative *crp* binding site. The site was included in the longer fragment but was excluded from the small promoter fragment. We then monitored both growth and luminescence continuously in a 96-well multimode plate reader. Deletion of *crp* significantly reduced luminescence driven by the promoter of *yfiQ*, but *crp* had no effect on expression driven by the *cobB* promoters (Fig. 9). Therefore, CRP activates the *yfiQ* promoter, but *cobB* is controlled independently of CRP. The latter result also serves as a control to confirm that the CRP-cAMP system is not required for regulating other metabolic pathways that feed into the luminescence reaction. We identified a putative CRP binding site [TGCGA($N_6$)TCATA] within the *yfiQ* promoter region that differs from the consensus sequences of *E. coli* CRP [TGTGA($N_6$)TCACA] and *V. cholerae* CRP [GTGA($N_6$)TCAC] at two nucleotides (29–32). This binding site is centered 225 bp upstream of the translational start site of the *yfiQ* gene. A putative $\sigma^{70}$ bacterial promoter, with −35 and −10 regions, was predicted using BPROM (Softberry, Inc.), which places the putative transcriptional start site roughly 120 bp upstream of the translational start and ~105 bp down-

stream of the putative CRP binding site. Altogether, these findings support the hypothesis that the CRP-cAMP system activates the transcription of *yfiQ* but not *cobB*. This is consistent with a previous whole-genome microarray transcriptome analysis of *V. cholerae* O1 El Tor A1552, which identified *yfiQ* as being downregulated in strains lacking CRP (33), although a second microarray approach with another *V. cholerae* El Tor strain carrying a *crp* deletion did not identify *yfiQ* as a target (34).

## DISCUSSION

Acetylation, one means of posttranslational regulation in bacteria, alters the structures of a wide variety of proteins across bacterial proteomes. Studies in *E. coli* and *Salmonella*, together with findings from other diverse bacteria, are revealing that acetylation mechanisms, alongside the enzymes targeted by this modification, are well conserved across evolutionary time scales (2, 5, 6). However, the biological and physiological impacts of acetylation-dependent regulation on protein function, particularly in the context of host-microbe interactions, remain unclear. The *Vibrio cholerae*-*Drosophila* model system is uniquely suited for the study of the effects of this posttranslational modification *in vivo*. In this system, *V. cholerae* virulence is not toxin mediated, but rather, pathogenesis is dependent upon the regulation of metabolites in the GI cavity by the pathogen (16, 21). In this work, we have shown that *Vibrio cholerae* genes likely involved in protein acetylation are critical for the proper regulation of acetate metabolism and pathogenesis in this arthropod model of infection.

The gene encoding CobB in *V. cholerae* is remarkably well conserved with its orthologs in *E. coli* and *Salmonella*, strongly suggesting that a functional relationship also exists. Although this conclusion awaits confirmation via biochemical and proteomic approaches, our findings are consistent with this hypothesis. Deletion of *cobB* prevents the growth of *V. cholerae* on acetate as a sole carbon source and slows the removal of acetate from rich media. This result is consistent with the idea that the *cobB* deletion increases the acetylation stoichiometry of key enzymes, such as Acs, that are involved in acetate catabolism. However, deleting *cobB* does not entirely halt acetate consumption, while deleting *acs* itself completely prevents both acetate consumption as well as growth on acetate minimal medium. This discrepancy is consistent with several conclusions. As one possibility, *cobB* may acetylate multiple enzymes within the acetate assimilation pathway. In this case, acetate assimilation may be halted in the Δ*cobB* strain because multiple enzymes are highly acetylated and locked in inactive states, reducing flux through this catabolic pathway such that growth cannot be supported. It is also possible that the Δ*cobB* strain may continue to remove acetate from the medium because (i) a second deacetylase can restore Acs activity or (ii) acetylation may not fully inactivate Acs. To examine a role for a second deacetylase, we introduced a deletion in *acuC*, a putative deacetylase that acts on Acs in *B. subtilis* (12). However, we observed no effect on *Drosophila* survival in any strain background, suggesting that the second deacetylase, if present in *V. cholerae*, is not AcuC. Our results, explained below, lead us to favor the conclusion that CobB targets multiple proteins controlling acetate assimilation, although these scenarios are not mutually exclusive.

To provide additional evidence for a role for CobB in acetylation, we tested whether the effects of its mutation could be rescued by the introduction of a deletion of *yfiQ*, the putative acetyltransferase, in the Δ*cobB* background. We hypothesized that the removal of YfiQ would prevent acetylation, essentially locking the targeted enzymes in their most highly active state. In this background, deletion of *cobB*, the deacetylase, would have no effect on enzyme activity. Indeed, the *yfiQ* deletion alone does not affect growth on acetate, but introducing the *yfiQ* deletion into the Δ*cobB* background restores the ability of this strain to grow on acetate minimal medium, suggesting that the two genes regulate an identical target(s). We observed a similar effect during infection of *Drosophila*: deletion of CobB reduces mortality, while the introduction of the YfiQ mutation restores virulence to this strain. Interestingly, the *yfiQ* deletion alone

does not increase fly mortality. We have found few mutations that increase lethality in strongly virulent strains, suggesting that there is a maximum rate at which *V. cholerae* can infect *Drosophila*, perhaps as a result of an infection bottleneck (16, 22).

Next, we performed a series of experiments with the goal of linking the effects of CobB and YfiQ on acetate metabolism to their impacts on Acs. We first introduced the K609R mutation into Acs at the putative catalytic site. Not surprisingly, this mutation appears to have inactivated the enzyme; none of our results were consistent with an effect on acetylation. As an alternative possibility, this mutation may have generated an Acs protein that cannot be acetylated by CobB but could halt growth via a different mechanism. In *Salmonella*, overexpression of Acs alleles that are highly active and resistant to acetylation reduces the energy charge of the cell by overproducing AMP, a by-product of the reactions that convert acetate to acetyl-CoA (35). The reduction in energy charge, in turn, impairs growth (35). If this is the case in *V. cholerae*, then we can expect the $acs^{K609R}$ mutation, as well as the same mutation introduced into the Δ*cobB* background, to phenocopy the Δ*yfiQ* deletion, because acetylation cannot reduce Acs activity in these strains. Instead, we found that the phenotypes of the Δ*yfiQ* mutation resemble those of the wild-type strain in fly survival assays and growth assays, consistent with the idea that reduced levels of acetylation in the Δ*yfiQ* strain do not impair growth on acetate in *V. cholerae*. However, strains carrying the $acs^{K609R}$ or the Δ*cobB* $acs^{K609R}$ mutations cannot grow on acetate, and fly survival is improved, indicating that acetate consumption is reduced. Therefore, it is more likely that this mutation halts enzymatic activity, rather than impairing acetylation and reducing energy charge, to impact growth.

Next, we introduced the L641A mutation, which prevents acetylation in *Salmonella* Acs, into *V. cholerae* Acs. This mutation was unable to rescue acetate catabolism in a Δ*cobB* strain, suggesting that (i) the role of this site in YfiQ binding may not be conserved or (ii) acetylated sites in other enzymes important for acetate assimilation are also controlled by CobB. In the latter case, it would be impossible to isolate mutations in Acs alone that confer growth on acetate because they prevent acetylation. Because the results from these targeted mutation studies yielded multiple interpretations, we attempted to search for alleles of Acs that are capable of resisting acetylation. We created a pool of random mutations in *acs*, introduced these mutations into a Δ*cobB* Δ*acs* strain, and selected for those mutations capable of suppressing the growth defect of the Δ*cobB* strain on acetate minimal medium (27). In *Salmonella*, acetylation-resistant alleles of Acs were sufficient to complement the growth of a Δ*cobB* Δ*acs* Δ*pta* strain (27). Ultimately, we did not identify Acs mutants capable of conferring growth to a Δ*cobB* Δ*acs* strain on acetate minimal medium (data not shown). One of several possible explanations for this result is that the *V. cholerae* CobB enzyme deacetylates multiple enzymes, as it does in *E. coli* (3, 5, 19, 36), and the activity of these enzymes may be required for acetate assimilation. This is also consistent with our previous finding that the Δ*cobB* strain delays acetate consumption but cannot grow on acetate as a sole carbon source. Altogether, we hypothesize that CobB deacetylates Acs to promote acetate consumption, but it is CobB's ability to modify other enzymes, together with its alteration of Acs, that may be important for growth on acetate minimal medium. However, further biochemical and mass spectrometry analyses are needed to confirm this conclusion, as other evidence suggests that the enzymatic acetylation system may in fact target a smaller number of enzymes than proposed previously (37).

The molecule acetyl-P also directly acetylates lysine residues nonenzymatically, and we questioned whether this system could be functioning similarly in *V. cholerae*. In *E. coli*, pools of acetyl-P can be modulated via mutations in the *pta* and *ackA* genes (5, 6). The Pta protein removes the CoA group from acetyl-CoA to generate acetyl-P, which AckA then converts to acetate. In *V. cholerae*, the *pta* and *ackA* genes are located in an operon. However, *V. cholerae* also encodes a second *ackA* gene, on chromosome II. Deletion of *pta* was sufficient to prevent acetate accumulation in the medium, as expected, but deletion of the *ackA* gene directly adjacent to *pta* did not affect acetate

excretion (61). This result argues that the two *ackA* genes encode functionally redundant proteins, and both *ackA* genes must be deleted from the *V. cholerae* genome in order to genetically probe the role of acetyl-P in the acetylation-dependent control of metabolism. However, our results suggest that the CobB-YfiQ system is the primary mechanism through which acetylation modifies enzymes involved in acetate metabolism, as the deletion of *yfiQ* entirely suppressed the deletion of *cobB*. Thus, YfiQ is likely responsible for acetylating enzymes within this pathway, which may include Acs.

Because the CobB and YfiQ enzymes modulate metabolism *in vivo*, the regulatory mechanisms controlling their expression could, in turn, play an important role in metabolite-dependent virulence. In *Salmonella*, the *cobB* and *yfiQ* genes are expressed to a greater extent during exponential phase than during stationary phase, and different carbon sources affected their transcription (3). In *E. coli*, however, *yfiQ* is upregulated by CRP, a global regulator that controls gene transcription in response to cAMP levels, which rise in the cell as other preferred carbon sources are depleted (28). Our evidence confirms that *yfiQ* is similarly regulated by CRP in *V. cholerae*, despite contradictory findings in previous microarray studies (33, 34). We identified a putative CRP binding site in the *yfiQ* promoter, but a global chromatin immunoprecipitation sequencing (ChIP-Seq) analysis did not detect significant CRP binding in that location (32). Therefore, it is possible that CRP indirectly regulates *yfiQ*. Nevertheless, our results support a model in which depletion of preferred carbon sources and entry into stationary phase result in two countervailing regulatory mechanisms: (i) *acs* expression is increased, which flips the acetate switch and increases the flux of acetate through catabolic pathways, and (ii) CRP is activated, which subsequently increases *yfiQ* expression. High levels of YfiQ may then acetylate and inactivate the newly expressed enzymes required for acetate catabolism, including Acs. This multilayered regulatory strategy may precisely control flux through these metabolic pathways in response to nutrient availability and the energy state of the cell (28). In *V. cholerae*, *acs* is upregulated as cells enter stationary phase, but its expression is controlled predominantly via CrbS/R, a two-component signal transduction system that is absent from the *E. coli* and *Salmonella* genomes (16, 17). Our evidence suggests that CRP further activates the transcription of *crbS* and *crbR*, as well as *acs* (61). Thus, CRP activates the expression of multiple mechanisms that increase *acs* transcription but reduce Acs activity.

Acetylation modifies a sizeable proportion of the proteome in a number of bacterial pathogens, including *Vibrio parahaemolyticus*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *V. cholerae*, but these modifications have been directly linked to virulence only in *Salmonella* and *E. coli* (38, 39). Acetylation regulates the expression of *Salmonella* type III secretion system 1 by controlling the stability and DNA binding capability of a transcriptional regulator (40, 41). A second transcriptional regulator contributing to *Salmonella* virulence, PhoP of the PhoP/Q two-component system, is also subject to acetylation, which specifically reduces its DNA binding affinity (42). The *E. coli* transcriptional regulator RscB, which is involved in flagellar biosynthesis, cell division, and capsule biosynthesis, is susceptible to acetylation, which reduces its transcriptional capability (19, 43, 44). Acetylation may mediate the acid tolerance response in *Salmonella*, during which highly acidic conditions downregulate *yfiQ* to alter the tricarboxylic acid (TCA) cycle consumption of acetyl-CoA, stabilizing intracellular pH and improving survival (45). Because *Salmonella* must persist through the acidic environment of the stomach to reach the GI tract and spread to other organs such as the spleen, this mechanism may contribute to virulence *in vivo*, although this hypothesis has not been tested directly (45). *Salmonella* persistence specifically in macrophages is aided by a toxin-antitoxin system that alters the acetylation state of aminoacyl-tRNAs, which, in turn, lowers rates of protein synthesis to induce the "persister" phenotype (46, 47). In addition to targeting the aminoacyl-tRNAs, the acetylase toxin also directly modifies its cognate antitoxin protein, which then increases the activity of the toxin (46). In *E. coli*, chemotaxis may be regulated by acetylation of the CheY response regulator (48). These mechanisms highlight the myriad ways in which acetylation can affect pathogenesis in *E. coli* and *Salmonella*. We hypothesize

that the *V. cholerae cobB* and *yfiQ* genes mediate virulence by directly regulating metabolite levels in the fly gut, based upon the observation that infection with the *cobB* deletion strain significantly reduced the storage of triglycerides in the enterocytes lining the fly GI tract. Previous studies have linked these changes in fat storage to reductions of both acetate and succinate levels (16, 21). Therefore, these genes may be modulating acetate alone, or both acetate and succinate as well as other active metabolites, in the context of the *Drosophila* GI tract. However, these examples highlight the potential for acetylation to affect a broad range of processes that could alter virulence in many ways.

A very recent study reported that the proteome of *V. cholerae* V52, a strain pathogenic to humans, is subject to acetylation (39). A number of global regulators important for both virulence and metabolic regulation are acetylated. These regulators include the AphB and TcpP transcription factors that control the expression of cholera toxin and toxin-coregulated pilus, via ToxT. However, *V. cholerae* SIO lacks the *ctxAB* and *tcp* gene clusters, and virulence in this strain appears to be entirely contingent upon the regulation of metabolite consumption. Intriguingly, Acs in *V. cholerae* strain V52 is acetylated, but not at the conserved lysine, as in *Salmonella* and *E. coli* (39). This observation is consistent with our own results in which mutation of $Acs^{K609}$ or $Acs^{L641}$, when introduced into a Δ*cobB* background, did not improve acetate metabolism or virulence *in vivo*, suggesting that these mutations could not prevent acetylation. The identification of alternative acetylation sites, however, implies that acetylation may be modifying Acs activity in *V. cholerae* by a novel mechanism. The physiological effects of these novel Acs acetylation sites on acetate metabolism and virulence should be confirmed and investigated via genetic, biochemical, and *in vivo* approaches.

Finally, the global metabolic regulator CRP was also subject to acetylation in *V. cholerae* V52 (39). We have confirmed that CRP is important for the regulation of *yfiQ* transcription. If CRP is acetylated by YfiQ to dampen its activity, this could enact a negative-feedback loop in which CRP activates expression of *yfiQ*, which then suppresses CRP function. However, the functional consequences of these specific acetylation sites in *V. cholerae* pathogenesis, survival, metabolism, and ecology await further investigation. Together, these studies on acetylation in *V. cholerae* reveal that this posttranslational modification is widespread within the proteome, has broad functional consequences for metabolic regulation, and is likely controlled, at least in part, by the *cobB* and *yfiQ* genes that we have identified here.

Bacterial modulation of metabolites can alter host health, physiology, and development. Alteration of succinate levels by the GI microbiota affects glucose homeostasis in mice (49), and microbiota-dependent alteration of acetate levels can have broad effects on host health (50, 51). A positive correlation between short-chain fatty acid levels and recovery from cholera has been observed for children (52), suggesting a possible link between metabolism and disease. Therefore, mechanisms of global regulation that modulate bacterial metabolic fluxes can drastically alter the effects of microbial colonization on the host. Because acetylation-dependent control of metabolism is widely conserved, the genes involved in regulating acetylation could impact host-microbe interactions across a diversity of systems.

## MATERIALS AND METHODS

**Bacterial strains and growth conditions.** Bacterial strains used in this study are listed in Table 1. All experiments were performed in *V. cholerae* strain SIO, a nontoxigenic non-O1/non-O139 strain isolated just off the coast of San Diego, CA. *Vibrio cholerae* bacteria were incubated with shaking at 37°C with appropriate antibiotics in LB-Miller broth (Fisher), unless otherwise noted. *V. cholerae* bacteria were plated onto LB-Miller agar and stored at −80°C in 15% glycerol. Kanamycin was added to a final concentration of 100 μg/ml.

**Bacterial mutant construction and complementation.** The deletion of the *acs* gene was constructed by using Gibson assembly (New England BioLabs) according the manufacturer's instructions. All other deletion and mutation constructs were generated by using a standard splicing by overlap extension (SOE) PCR protocol, as described previously for the environmental *V. cholerae* strain SIO (17). All primers are listed in Table 2. Altogether, deletions were constructed in *acs* (VC0298), *cobB* (VC1509), *yfiQ* (VCA0574), and *crp* (VC2614) (locus tags according to *V. cholerae* O1 El Tor N16961 assignments). To perform the SOE protocol, ~750 to 1,100 bp on either side of the gene deletion or modification were

**TABLE 1** Strains and plasmids 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$^r$ | 54 |
| DH5α λ*pir* | F$^-$ Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1* λ::*pir* | 58 |
| S17-1 λ*pir* | RP4-2(Km::Tn*7* Tc::Mu1) *pro-82* λ*pir recA1 endA1 thiE1 hsdR17 creC510* | 59 |
| AP09 | *E. coli* WM5406/pHC001B | 53 |
| AP207 | *E. coli* MFD*pir*/pHC001B::Δ*acs* | This study |
| AP1083 | *E. coli* MFD*pir*/pHC001B::Δ*cobB* | This study |
| AP1392 | *E. coli* MFD*pir*/pHC001B::Δ*yfiQ* | This study |
| AP302 | *E. coli* DH5α/pSRK-Km | 55, 56 |
| AP1870 | *E. coli* MFD*pir*/pSRK-Km::*cobB* | This study |
| AP1350 | *E. coli* MFD*pir*/pHC001B::$acs^{K609R}$ | This study |
| AP1351 | *E. coli* MFD*pir*/pHC001B::$acs^{L641A}$ | This study |
| AP1782 | *E. coli* S17-1 λ*pir*/pDN001 (pBBR*lux*::$P_{yfiQ}$) | This study |
| AP2027 | *E. coli* MFD*pir*/pCP001 (pBBR*lux*::$P_{cobB-long}$) | This study |
| AP2023 | *E. coli* MFD*pir*/pCP002 (pBBR*lux*::$P_{cobB-short}$) | This study |
| AP547 | *E. coli* MFD*pir*/pHC001B::Δ*crp* | This study |
| AP2006 | *E. coli* MFD*pir*/pHC001B::Δ*acuC* | This study |
| *V. cholerae* | | |
| AP95 | *V. cholerae* strain SIO; wild type | 60 |
| AP218 | *V. cholerae* strain SIO Δ*acs* | This study |
| AP1150/1151 | *V. cholerae* strain SIO Δ*cobB* | This study |
| AP1401a | *V. cholerae* strain SIO Δ*cobB*/pSRK-Km | This study |
| AP1882 | *V. cholerae* strain SIO Δ*cobB*/pSRK-*cobB* | This study |
| AP1415 | *V. cholerae* strain SIO Δ*yfiQ* | This study |
| AP1427 | *V. cholerae* strain SIO Δ*cobB*→Δ*yfiQ* (*cobB* into *yfiQ*) | This study |
| AP1862 | *V. cholerae* strain SIO Δ*yfiQ*→Δ*cobB* (*yfiQ* into *cobB*) | This study |
| AP1346 | *V. cholerae* strain SIO $acs^{L641A}$ | This study |
| AP1348 | *V. cholerae* strain SIO Δ*cobB* $acs^{L641A}$ | This study |
| AP1344 | *V. cholerae* strain SIO $acs^{K609R}$ | This study |
| AP1345 | *V. cholerae* strain SIO Δ*cobB* $acs^{K609R}$ | This study |
| AP431 | *V. cholerae* strain SIO/pPT002 (pBBR*lux*::$P_{acs}$) | 17 |
| AP462 | *V. cholerae* strain SIO/pBBR*lux* | 17 |
| AP1787 | *V. cholerae* strain SIO Δ*crp*/pDN001 (pBBR*lux*::$P_{yfiQ}$) | This study |
| AP1797 | *V. cholerae* strain SIO/pDN001 (pBBR*lux*::$P_{yfiQ}$) | This study |
| AP2033 | *V. cholerae* strain SIO/pCP001 (pBBR*lux*::$P_{cobB-long}$) | This study |
| AP2029 | *V. cholerae* strain SIO/pCP002 (pBBR*lux*::$P_{cobB-short}$) | This study |
| AP2039 | *V. cholerae* strain SIO Δ*crp*/pCP001 (pBBR*lux*::$P_{cobB-long}$) | This study |
| AP2035 | *V. cholerae* strain SIO Δ*crp*/pCP002 (pBBR*lux*::$P_{cobB-short}$) | This study |
| AP2041 | *V. cholerae* strain SIO Δ*acuC* | This study |
| AP2049 | *V. cholerae* strain SIO Δ*acuC* Δ*cobB* | This study |
| AP2048 | *V. cholerae* strain SIO Δ*acuC* Δ*yfiQ* | This study |
| AP2061 | *V. cholerae* strain SIO Δ*acuC* Δ*yfiQ* Δ*cobB* | This study |

amplified with Q5 high-fidelity DNA polymerase (New England BioLabs). The inner primers included an ~18- to 21-bp region of overlap. For each of the gene deletions, the overlap sequence was 5′-TGCGG CCGCTCGTTA-3′, while primers for specific gene mutations included an overlapping region encompassing the point mutation being constructed. Primers outside the construct incorporated restriction enzyme sites, which are designated in Table 2. The PCR products were digested and ligated into the pHC001B plasmid (53). The resulting plasmids carrying the deletion constructs were transformed into *E. coli* DH5-α λ*pir* cells or MFD*pir* cells (54), and the construct was sequenced. The correct plasmid was then conjugated from MFD*pir* cells into *V. cholerae*. Selection for double recombinants was accomplished by kanamycin and sucrose selection, as described previously (17). Integration of the correct construct was verified by PCR or sequencing. To generate the complementation plasmids in pSRK-Km carrying the *cobB* gene or the *acs* gene (55, 56), the gene was amplified by PCR using a forward primer that incorporated an NdeI site into the start codon. The PCR fragment was digested, ligated into the pSRK-Km vector, and transformed into *E. coli* SM10 λ*pir*. The plasmid was then conjugated into *V. cholerae* via standard procedures.

**Growth assays.** To monitor growth on acetate minimal medium, *V. cholerae* was streaked for single colonies, inoculated into LB medium supplemented with appropriate antibiotics (if necessary), and incubated with shaking overnight at 37°C. The following day, cells were centrifuged at maximum speed for 3 min, the supernatant was removed, and the cells were resuspended in an equal volume of M63

**TABLE 2** Primers used in this study

| Primer | Sequence[a] | Description |
|---|---|---|
| KL10_SIOdelcobB_P1 | GACAACTAGTTGGCGGCTTTAAGTGGTCTA | SOE deletion of *cobB* with SpeI |
| KL11_SIOdelcobB_P2 | ***TAACGAGCGGCCGCA***GCCTGCTCCAGTCAGTATCA | SOE deletion of *cobB* with tag |
| KL12_SIOdelcobB_P3 | ***TGCGGCCGCTCGTTA***GCGAAAGAGAGTGCAGC | SOE deletion of *cobB* with tag |
| KL13_SIOdelcobB_P4 | CATTGGATCCCAAAATTGCGGGCATCATGG | SOE deletion of *cobB* with BamHI |
| KL48_SIOdelyfiQ.3_P1 | GACAACTAGTCCACCGCACAAAAGTAGGAT | SOE deletion of *yfiQ* with SpeI |
| KL49_SIOdelyfiQ.3_P2 | ***TAACGAGCGGCCGCA***GGGTCTAAGCAGTTGATTCAA | SOE deletion of *yfiQ* with tag |
| KL50_SIOdelyfiQ.3_P3 | ***TGCGGCCGCTCGTTA***TTTGCCGTCGATATTCATT | SOE deletion of *yfiQ* with tag |
| KL51_SIOdelyfiQ.3_P4 | CATTGAGCTCAGGAGAGAGCATTGTTAAGTCCA | SOE deletion of *yfiQ* with SacI |
| KL44_AcsL641A_P1 | GACAACTAGTTGTATGGACCACTGGCGAAT | SOE mutation of Acs$^{L641A}$ with SpeI |
| KL45_AcsL641A_P2 | TTGTGCTTTTTCGGCAAT**CGC**GCGGTCAACCACGCTTGG | SOE mutation of Acs$^{L641A}$ complete overlap |
| KL46_AcsL641A_P3 | CCAAGCGTGGTTGACCGC**GCG**ATTGCCGAAAAAGCACAA | SOE mutation of Acs$^{L641A}$ complete overlap |
| KL47_AcsL641A_P4 | CATTGGATCCATTTCCAGTTCTGCGATGCC | SOE mutation of Acs$^{L641A}$ with BamHI |
| KL40_AcsK609R_P1 | GACAACTAGTTGTATGGACCACTGGCGAAT | SOE mutation of Acs$^{K609R}$ with SpeI |
| KL41_AcsK609R_P2 | CAAAATACGGCGCATAAT**TCT**ACCTGAACGGGTTTTCGG | SOE mutation of Acs$^{K609R}$ complete overlap |
| KL42_AcsK609R_P3 | CCGAAAACCCGTTCAGGT**AGA**ATTATGCGCCGTATTTTG | SOE mutation of Acs$^{K609R}$ complete overlap |
| KL43_acsK609R_P4 | CATTGGATCCGGCACCTAAACCGCAAATCA | SOE mutation of Acs$^{K609R}$ with BamHI |
| MS37_delVC2614_P1 | GACAACTAGTCCAGATGCCCGGTACGTTTAC | SOE mutation of *crp* with SpeI |
| MS33_delVC2614_P2 | GGTTTCGCGAGAACAGCCGTGAAAGAAACCACTCTAGTG | SOE mutation of *crp* complete overlap |
| MS34_delVC2614_P3 | CACTAGAGTGGTTTCTTTCACGGCTGTTCTCGCGAAACC | SOE mutation of *crp* complete overlap |
| MS39_delVC2614_P4 | CATTCTCGAGTGCATCAACTCCTACAAGAAG | SOE mutation of *crp* with XhoI |
| DN05_pYfiQ_F | GACAACTAGTCACTGTCTGGAGCTTTGACAAC | Cloning of *yfiQ* promoter with SpeI |
| DN06_pYfiQ_R | CATTGGATCCCAAACGGCAGGATAATAGGG | Cloning of *yfiQ* promoter with BamHI |
| MMS13_acs_GP1 | actcactatagggccccccCGGCTACCACATTCGTTACG | Gibson deletion of *acs*, primer 1 |
| MMS14_acs_GP2 | GGCAATCAGGCGGTCAACCGGATAAATATGGGCTTCAC | Gibson deletion of *acs*, primer 2 |
| MMS15_acs_GP3 | GTGAAGCCCATATTTATCCGGTTGACCGCCTGATTGCC | Gibson deletion of *acs*, primer 3 |
| MMS16_acs_GP4 | ggcggccgctctagaaCGCGGTTAGATTGCAGATGT | Gibson deletion of *acs*, primer 4 |
| DN01_pCobB_F | GACAACTAGTCCAAGATGGTGAGATAGCGAAT | Cloning of *cobB* long promoter with SpeI |
| DN02_pCobB_R | CATTGGATCCGTGTCGCCACATCTTCGATT | Cloning of *cobB* promoters with BamHI |
| CP37_pCobB_shortF | GACAACTAGTGGAGCCATTTCAATTTGGAT | Cloning of *cobB* short promoter with SpeI |
| GR09_VC2042_delSOE_P1 | CATGGATCCGCCAGTCTCTTTATGAAAAGC | SOE deletion of *acuC* homolog, with BamHI |
| GR10_VC2042_delSOE_P2 | ***TAACGAGCGGCCGCA***GATCATTTTGCTCTGCTCTGG | SOE deletion of *acuC* homolog, with tag |
| GR11_VC2042_delSOE_P3 | ***TGCGGCCGCTCGTTA***TGAGGCGAAATTATGACCGTA | SOE deletion of *acuC* homolog, with tag |
| GR12_VC2042_delSOE_P4 | GACAACTAGTTTACGCAGTGCATACAACG | SOE deletion of acuC homolog, with SpeI |

[a]Restriction sites are underlined, the tag (additional base pairs) that enables overlapping PCR for gene deletions is in boldface italic type, and primers that create point mutations include the specific mutation are in boldface type. Primers that overlap each other completely are indicated. For Gibson mutation, overlap with the plasmid is indicated in lowercase type.

minimal medium (Amresco). The resuspended cells were then inoculated into M63 medium supplemented with 10 mM acetate to an initial optical density at 600 nm ($OD_{600}$) of 0.01. From this dilution, 120 $\mu$l was added to each of at least three wells in a sterile, flat-bottomed, 96-well plate and incubated for 48 h at 37°C with shaking in a multimode plate reader (Molecular Devices), while the $OD_{600}$ was monitored every hour. Individual measurements were averaged, and results of assays representative of data from at least two independent replicates are shown.

**Acetate concentration in medium.** To monitor acetate concentrations in culture, *V. cholerae* was streaked for single colonies and grown overnight for 16 h. From this culture, 500 $\mu$l was inoculated into 50 ml of LB broth in a 125-ml Erlenmeyer flask in duplicate. At the appropriate times, the $OD_{600}$ of the culture was measured using a spectrophotometer (catalog number 6320D; Jenway), and a 1-ml sample was removed, centrifuged, filter sterilized, and heat treated at 80°C for 20 min, according to the manufacturer's instructions. Samples were stored at −20°C until they were analyzed with an acetic acid assay kit (catalog no. K-ACETRM; Megazyme), and acetate was measured according to the manufacturer's instructions.

**Fly survival and colonization.** *V. cholerae* strains were streaked for single colonies from glycerol stocks, grown overnight in LB broth, and then diluted 1:10 and added to a cellulose acetate *Drosophila* vial closure (Genesee Scientific) at the bottom of a standard fly vial. Between 6 and 10 flies were added to each vial, with each assay including at least two vials, and most often three vials, per strain. All survival assays were repeated on at least two separate occasions, unless otherwise indicated. Statistical significance of fly survival curves was measured with the log rank assay implemented in GraphPad Prism (GraphPad, La Jolla, CA). To assess colonization, live flies were removed from vials after 24 and 48 h, homogenized in 300 $\mu$l of phosphate-buffered saline (PBS), serially diluted, and plated onto LB agar. After overnight incubation at 37°C, plates with colonies numbering between 30 and 300 were counted to assess CFU per milliliter. To ensure that only *V. cholerae* colonies were counted, flies infected with sterile LB medium were similarly homogenized and plated, and no colonies were apparent on these plates after 24 h of incubation.

**Triglyceride storage determination.** To measure triglycerides, flies were infected with *V. cholerae* for 48 h. The midgut was then dissected in PBS and fixed in 4% paraformaldehyde in PBS with 0.2% Triton X-100 (PBS-T) for 1 h at room temperature or overnight at 4°C. Midguts were then washed three times

in PBS-T, followed by incubation in 1 μg/ml BODIPY 493/503 dye (ThermoFisher/Molecular Probes) and 1 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) in PBS-T for 45 min (21). Midguts were then washed three times in PBS-T and mounted in Vectashield. Slides were stored at 4°C prior to visualization on a Nikon C2 confocal microscope. Puncta were quantified with Fiji, using images taken with a 60× objective, and images were coded so that the researcher performing the quantification was blind as to the bacterial strain with which the fly had been infected. In each of three independent trials, two to four images from each of at least five to six midguts harvested from flies infected with either wild-type *V. cholerae* SIO or *V. cholerae* Δ*cobB* or uninfected flies were quantified. Images were similarly quantified for the *V. cholerae* Δ*acs* mutant in two of these three independent trials. To assess puncta per cell, the number of puncta was divided by the number of nuclei (as assessed by DAPI) in the image.

**Measurement of *yfiQ* and *cobB* promoter activities.** In order to assess whether the *yfiQ* promoter is controlled by *crp*, a 581-bp segment of the *yfiQ* promoter was cloned into the pBBR*lux* plasmid (57). Because the transcriptional start site was unknown, the segment was selected to extend 423 bp upstream and 158 bp downstream of the putative start codon. The *yfiQ* gene is not predicted to fall within an operon, as the gene upstream of *yfiQ*, VCA0573, is transcribed in the opposite direction. The resulting plasmid, pDN001, was introduced into both the wild-type *V. cholerae* SIO strain and a strain carrying a deletion in *crp* by conjugation from *E. coli*.

Similarly, in order to assess whether the *cobB* promoter is controlled by *crp*, two segments of the *cobB* promoter were each cloned into the pBBR*lux* plasmid. The longer of the two was selected to extend 442 bp upstream and 106 bp downstream of the putative start codon. However, a poorly conserved *crp* binding site was detected within this region. To create a promoter lacking this site, a second promoter segment was designed to reach 235 bp upstream and 106 bp downstream of the putative start codon, covering a total of 341 bp. The resulting plasmids, pCP001 and pCP002, respectively, were introduced into both the wild-type *V. cholerae* SIO strain and a strain carrying a deletion in *crp* by conjugation.

To detect bioluminescence, single colonies were inoculated into LB broth with chloramphenicol (5 μg/ml), which were grown for not more than 14 h at 37°C with shaking. The cultures were then diluted 1:500 into fresh LB broth with chloramphenicol, and 120 μl was added to sterile, black, flat, clear-bottomed, 96-well plates that isolate luminescence from adjacent wells (BRAND GMBH + CO KG, Germany). Strains were incubated overnight with shaking, while luminescence and absorption were measured in succession with a 96-well multimode plate reader (Molecular Devices).

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