Cra and cAMP Receptor Protein Have Opposing Roles in the Regulation of fruB in Vibrio cholerae

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ABSTRACT The Gram-negative bacterium Vibrio cholerae adapts to changes in the environment by selectively producing the necessary machinery to take up and metabolize available carbohydrates. The import of fructose by the fructose-specific phosphoenolpyruvate (PEP) phosphotransferase system (PTS) is of particular interest because of its putative connection to cholera pathogenesis and persistence. Here, we describe the expression and regulation of fruB, which encodes an EIIA-FPr fusion protein as part of the fructose-specific PTS in V. cholerae. Using a series of transcriptional reporter fusions and additional biochemical and genetic assays, we identified Cra (catabolite repressor/activator) and cAMP receptor protein (CRP) as regulators of fruB expression and determined that this regulation is dependent upon the presence or absence of PTS sugars. Cra functions as a repressor, downregulating fruB expression in the absence of fructose when components of PTSFru are not needed. CRP functions as an activator of fruB expression. We also report that Cra and CRP can affect fruB expression independently; however, CRP can modulate cra expression in the presence of fructose and glucose. Evidence from this work provides the foundation for continued investigations into PTSFru and its relationship to the V. cholerae life cycle.

IMPORTANCE Vibrio cholerae is the causative agent of cholera disease. While current treatments of care are accessible, we still lack an understanding of the molecular mechanisms that allow V. cholerae to survive in both aquatic reservoirs and the human small intestine, where pathogenesis occurs. Central to V. cholerae’s survival is its ability to use available carbon sources. Here, we investigate the regulation of fruB, which encodes a protein central to the import and metabolism of fructose. We show that fruB expression is controlled by the transcriptional regulators Cra and CRP. This work contributes toward a clearer understanding of how carbon source availability impacts the physiology and, potentially, the persistence of the pathogen.

KEYWORDS CRP, Cra, FPr, FruR, PTS, Vibrio cholerae, fructose

Caused by the facultative pathogen Vibrio cholerae, cholera disease is estimated to affect three to five million people each year and is characterized by profuse, watery diarrhea, resultant dehydration, and hypovolemic shock (1, 2). While current intravenous and oral rehydration treatments are effective in treating the disease, approximately 1.3 billion people across 51 countries are still at risk for infection due to gaps in health infrastructure (1, 3, 4). The transmission of V. cholerae is most frequently attributed to malfunctioning or inadequate sanitation systems, as well as the lack of clean water sources (1, 3). Important to this transmission is the ability of V. cholerae to adapt to conditions in both the human small intestine, where pathogenesis occurs, and aquatic reservoirs, where the bacteria spread between contaminated water sources (5, 6). In order to survive in both niches, V. cholerae must sense the carbon sources currently available and produce the necessary metabolic machinery to convert available
carbon sources to utilizable energy currency (7). The phosphoenolpyruvate (PEP) phosphotransferase system (PTS), a phosphotransfer cascade conserved across many bacterial species and responsible for carbon uptake and phosphorylation for downstream metabolism, is thought to play a role in *V. cholerae*’s ability to survive in multiple environments (7).

The PTS phosphocascade begins with the transfer of a phosphate group from PEP to EI, a constitutively expressed cytoplasmic protein (8, 9). Next, the phosphate group is passed from EI to histidine protein (HPr or the HPr homolog FPr), another cytoplasmic component (8, 9). From HPr or FPr, the phosphate group is then passed to a number of carbohydrate-specific domains, referred to as EIIA and EIIB (8, 9). Intake of the carbohydrate across the membrane then occurs via the EIIC domain, a carbohydrate-specific transmembrane protein (8, 9). The carbohydrate is concomitantly phosphorylated with this transport step (8, 9). Because carbon specificity lies within the domains of the EII protein, bacteria often have multiple EII proteins, one or more for each carbon source which enters the cell through the PTS (9). Moreover, synthesis of EII proteins is typically induced in the presence of its respective carbon source (8). *V. cholerae* contains 25 PTS components, including 13 distinct EIIC domains (10). Recently, the carbohydrate specificity of each EIIC transporter was defined; together, the 13 proteins are able to transport fructose, GlcNAc, (GlcN)2, glucose, mannitol, mannose, MurNAc, and sucrose into the cell via the PTS (11).

Here, we focus on one of these PTS sugars, fructose. *V. cholerae* is entirely dependent on the PTS system for the uptake of fructose, in contrast to the existence of multiple non-PTS glucose transporters (7, 11). PTS\textsuperscript{Fru}-specific components in *V. cholerae* are encoded by genes located at two distinct loci. First, VC1826 encodes an EIIABC fusion protein that is capable of both fructose and mannose transport (7, 11). VCA0516, VCA0517, and VCA0518 make up the second locus encoding PTS\textsuperscript{Fru} proteins (12). VCA0518 (fruB) encodes the fructose-specific EIIA domain and FPr, which exist as fusion proteins. VCA0517 (fruK) encodes 1-phosphofructokinase, which is responsible for phosphorylating fructose-1-phosphate to fructose-1,6-bisphosphate following uptake and phosphorylation of fructose into the cell. VCA0516 (fruA) encodes the fructose-specific EIIB and EIIC domains, which exist as fusion proteins. Experimental evidence suggests that in *V. cholerae*, the proteins encoded by fruBKA play the primary role in fructose transport and that FPr is preferred over HPr in PTS transport and phosphorylation of fructose (7, 11).

Relevant to the life cycle of *V. cholerae*, fruB (encoding EIIA-FPr, herein shorted to FPr), along with other PTS components, is upregulated when the bacteria enter their viable but nonculturable state, suggesting that the uptake of specific carbon sources may be important for the survival of these bacteria (13). fruB is also induced during infection of a mouse model of cholera, and ΔfruB mutants demonstrated a 3-fold defect during a colonization assay of infant mice (14). FPr and HPr, moreover, are involved in a signaling cascade that allows the phosphorylation state of EI to impact biofilm formation in growth conditions involving glucose (7). Thus, FPr, along with other PTS\textsuperscript{Fru} components, may be particularly important as *V. cholerae* transitions between environments containing high or low concentrations of fructose. A clearer understanding of fruB expression and its regulation in response to changes in carbon source availability would shed light on the persistence of the pathogen.

Here, we investigated the roles of two global transcription factors, Cra and cAMP receptor protein (CRP), in regulating fruB expression in *V. cholerae* in various carbon sources. We demonstrate that Cra represses fruB transcription in the absence of fructose, likely by acting near the 10 hexamer of the fruB promoter, while CRP activates fruB expression in the absence of glucose, working farther upstream in the promoter. Our data indicate that the two regulators can work independently to control the production of FPr depending on carbon source availability, although CRP can repress cra expression in some growth conditions.
RESULTS

Identification of the fruB transcription start site. Before beginning to dissect how fruB is regulated, we first confirmed that fruB, fruK, and fruA are all induced by the presence of fructose using a combination of transcriptome sequencing (RNA-Seq) and quantitative reverse transcription-PCR (qRT-PCR). RNA for RNA-Seq was extracted from bacteria cultured in 1× M9 supplemented with 0.4% (wt/vol) fructose or glucose and grown to an OD₆₀₀ of ~0.3. (B) fruA, fruK, and fruB expression in a wild-type strain grown in LB supplemented with 0.4% (wt/vol) fructose or an equivalent volume of water. Cultures were grown to an OD₆₀₀ of ~0.3, and then RNA was extracted, purified, and treated with DNase I. qRT-PCR was performed on each total RNA sample using SYBR green and gene-specific primers. RNA amounts were determined using standard curves and then normalized to an endogenous control (4.5S RNA). Bars represent means from biological replicates.

We then set out to determine the transcription start site (TSS) of fruB using 5’ RACE (rapid amplification of cDNA ends) (15). RNA was extracted from a WT strain cultured in Luria-Bertani (LB) broth only or LB broth supplemented with fructose. Similar to our RNA-Seq results, all three transcripts were expressed at high levels in fructose medium, and transcript levels decreased in the absence of fructose (Fig. 1B). From both RNA-Seq and qRT-PCR data, we concluded that fruB, fruK, and fruA are each induced and upregulated in fructose medium. A more thorough analysis of the RNA-Seq data, which was done as part of a larger study, will be presented elsewhere.

Out of 24 total sequences analyzed across 2 separate 5’ RACE experiments, we observed the fruB TSS (notated as fruB TSS-2) to lie 133 nucleotides (nt) upstream of the fruB start codon in 5 sequences (position +109 relative to TSS-1, described below; Fig. 2; Fig. S1 and S2). In the 19 remaining samples, we were unable to determine the fruB TSS because reverse transcription of fruB’s 5’ untranslated region (UTR) stopped...
prematurely downstream of the fruB start codon. We also performed 5’ RACE with RNA extracted from a WT strain cultured in minimal medium supplemented with glucose, using the same workflow. In glucose medium, we observed the fruB TSS to lie in roughly the same location as in fructose growth conditions, 133 nt upstream of the fruB start codon, in 5 of 12 sequences analyzed (Fig. S3). As in fructose medium, failed reverse transcription reactions prevented us from determining the TSS in all 12 samples. The similarities between our 5’ RACE results from fructose and glucose media suggest the location of the fruB TSS is not dependent on carbon source when samples are grown in minimal media.

Interestingly, the TSS identified here (fruB TSS-2) differs from the TSS identified by Papenfort et al. (notated as fruB TSS-1), who reported the V. cholerae fruB TSS to be 241 bp upstream of the fruB start codon (108 nt upstream of TSS-2) (Fig. 2) (16). In their work, Papenfort and colleagues used differential RNA sequencing (dRNA-Seq) to identify TSSs throughout the V. cholerae genome (16). RNA used in dRNA-Seq was extracted from WT strains cultured in LB without additional carbon sources present (16). It is possible that differences in minimal versus rich media affect the site of transcription initiation. Analysis of our RNA-Seq data points to the 5’ end of fruB ranging from roughly the +24 to +74 sites, relative to TSS-1. Therefore, it is also possible that posttranscriptional processing of fruB transcripts may occur.

To reconcile the difference between fruB TSS-1 and TSS-2, we constructed the P_{fruB} transcriptional reporter, which contains a portion of the fruB promoter region fused to the 5’ end of the E. coli lacZ gene. Reporters were then inserted into the lacZ gene in the V. cholerae genome to disrupt endogenous lacZ expression. Exact coordinates included in each fusion are listed next to the respective arrow. Putative −10 and −35 sites in the fruB promoter are depicted by black bars. Putative binding sites (BSs) for Cra and CRP are depicted by gray bars.
we identified through 5′ RACE (i.e., the +109 site [Fig. 2]). Despite our 5′ RACE data, we postulated that TSS-1 was the more likely start site, as it was the only one of the two that had an identifiable −10 site, which was predicted using BPROM and PromoterHunter and previously determined consensus matrices from both E. coli and V. cholerae (16–18). Using LacZ assays, we then measured transcriptional activity from the P
fruB
reporter in a number of PTS carbon sources in both rich (LB) and minimal (M9) media. In both rich and minimal media, we observed the largest amount of β-galactosidase activity ("LacZ activity") when the medium was supplemented with fructose (Fig. 3A and B). In media supplemented with glucose, mannitol, or, as a control, water, LacZ activity from the P
fruB
reporter was statistically lower than with fructose as the added carbon source (P < 0.05, Tukey’s multiple-comparison test). These results indicate that the P
fruB
reporter contains at least one intact TSS, as well as a corresponding transcriptional promoter. The P
fruB
reporter must also contain regions responsible for regulation of
fruB
expression, allowing for at least 8-fold (and up to 37-fold) induction in the presence of fructose (Fig. 3A and B).

In addition to the P
fruB
reporter, we also constructed the P
fruB
_null reporter, which spans +11 to +116 of the fruB promoter region (Fig. 2). The P
fruB
_null reporter lacks fruB TSS-1 but includes putative −10 and −35 hexamers and the +1 site for fruB TSS-2. When we measured LacZ activity, we observed a significant difference in transcriptional activity between the P
fruB
_null and P
fruB
reporters in both of the tested growth conditions (P < 0.05, Tukey’s multiple-comparison test) (Fig. 3C). Furthermore, no significant difference was observed when comparing transcriptional activity from the null reporter in fructose and water growth conditions. These results suggest the P
fruB
reporter contains elements necessary for transcription—and induction in the presence of fructose—while the P
fruB
_null reporter lacks such sites. We concluded that fruB TSS-1, and not fruB TSS-2, is the point at which fruB transcription begins.


fruB expression is fine-tuned by available carbon sources. To further probe the dependence of fruB expression on the presence of fructose, we conducted LacZ assays with the P
fruB
reporter in which cultures were prepared with a mixture of carbon sources, combining fructose and either glucose or mannitol in a range of concentrations. In mixtures of fructose and glucose, LacZ activity from the P
fruB
reporter decreased 4-fold when an equal amount of glucose (0.2% wt/vol of 0.4% wt/vol total supplemental
sugar) was included in the culture ($P < 0.05$, Tukey’s multiple-comparison test) (Fig. 4A). In mixtures of fructose and mannitol, LacZ activity from the $P_{fruB}$ reporter followed a more linear pattern (Fig. 4B). As a higher percentage of mannitol was included in cultures, LacZ activity significantly decreased ($P < 0.05$, Tukey’s multiple-comparison test), although the fold activity reductions were not as high as observed in cultures containing mixtures of fructose and glucose. Similar patterns were observed in analogous Western blots (Fig. S4). These patterns are likely due to the preference for glucose over other carbon sources in bacterial metabolism; in Gram-negative bacteria, carbon catabolite repression regulates metabolic pathways to promote the breakdown of desirable sugars like glucose before others like fructose and mannitol (8, 9, 19). Regardless of carbon source preference, these LacZ assays illustrate the dependence of $fruB$ expression on the presence of fructose: a higher percentage of fructose in the system correlates with higher $fruB$ expression.

$fruB$ expression is regulated by Cra and CRP. We next turned our attention to identifying the protein regulators that control induction of $fruB$ in the presence of fructose. Observations from other Gram-negative bacteria provided a starting point from which to evaluate possible regulators of $fruB$ in *V. cholerae*. Lying directly adjacent to the $fruBKA$ locus in *V. cholerae*, VCA0519 encodes a LacI-GalR family transcriptional regulator referred to both as FruR (fructose repressor) and Cra (catabolite repressor/activator) (Fig. 2) (12). In *E. coli*, Cra is considered a global transcriptional regulatory protein that affects the metabolism of 36 different carbon sources and represses $fruB$ expression (20–24). Alongside Cra, the 3′,5′-cyclic AMP (cAMP) receptor protein (CRP) is another global regulator that affects $fruB$ expression in *E. coli* and *Salmonella enterica* serovar Typhimurium by activating the gene’s transcription (25, 26). In *V. cholerae*, moreover, a $crp$ mutant strain exhibited decreased expression of $fruB$ and $fruA$, along with decreased fructose metabolism (27, 28).

We first investigated Cra as a potential regulator of $fruB$ in *V. cholerae*. We repeated our RNA-Seq analysis using a $\Delta cra$ mutant. In the absence of cra, expression of all three transcripts increased in glucose medium, indicating that Cra represses $fruB$, $fruK$, and $fruA$ expression in the presence of glucose and providing preliminary evidence that Cra represses $fruB$ in nonfructose media (Fig. 1A). To further examine this relationship, we evaluated FPr protein levels in WT and $\Delta cra$ strains in fructose, glucose, and

**FIG 4** $fruB$ expression is carbon source dependent. $fruB$ promoter activity in a mixture of carbon sources as measured by mean $A_{420}/$minute$ \times \text{OD}_{600}$ from the $P_{fruB}$-lacZ transcriptional fusion. Bacteria were initially cultured in $1 \times$ M9 plus 0.4% maltose overnight and back diluted into fresh media supplemented with the indicated carbon sources the following day. Cultures were prepared with the indicated percentage (wt/vol) of fructose and brought to a total 0.4% (wt/vol) sugar with additional glucose (A) or mannitol (B). After reaching an OD$_{600}$ of 1.0, cultures were lysed and incubated with ONPG substrate solution for 1 h, from which $A_{420}/$minute was measured and normalized to OD$_{600}$. For each carbon source, cultures were grown in biological triplicate and measured in technical triplicate. Technical triplicates were averaged during data analysis. Bars represent means from biological triplicates. Within each panel, bars with different letters indicate mean values that are significantly different from each other ($P < 0.05$, Tukey’s multiple-comparison test).
mannitol growth conditions (Fig. 5A). Similar to the expression patterns observed in our RNA-Seq results, FPr levels were highest in media supplemented with fructose and decreased 100-fold in nonfructose media. In the absence of Cra, FPr levels increased compared to the WT strain across all culture conditions, supporting the role of Cra as a repressor of fruB in nonfructose media. Moreover, these data provide additional evidence that the transcriptional regulator Cra specifically affects fruB transcription and such effects are reflected at the protein level as well.

We also evaluated the relationship between CRP and FPr, given evidence which implicates CRP as an activator of fruB expression (27, 28). To do so, we measured FPr levels in WT and Δcrp strains when LB cultures were supplemented with fructose or an equivalent volume of water (Fig. 5B). Consistent with our previous findings, FPr levels were highest in cultures supplemented with fructose. In the absence of CRP, FPr levels in both fructose and nonfructose conditions decreased 3- to 6-fold (∗P < 0.05, Sidak’s multiple-comparison test), suggesting that CRP activates fruB expression in both growth conditions.

To confirm that Cra and CRP are regulators of fruB transcription, we used the PfruB reporter to measure fruB expression in a series of LacZ assays. In these assays, we measured LacZ activity from the PfruB reporter in strains lacking either the endogenous cra or crp loci. In an effort to probe Cra- and CRP-mediated fruB regulation independently, these strains (herein referred to as “mutant strains”) also lacked the endogenous locus of the other regulator (i.e., the cra mutant strain also lacked the endogenous crp locus and was compared to a strain that possessed cra but still lacked crp). To rule out polar effects, we also measured LacZ activity from a complementation strain that possessed a plasmid containing the cra or crp gene under the control of an inducible promoter (pJML05::cra or pTrc99A::crp).

In assessing the activity of Cra, the deletion of cra resulted in 2- and 38-fold increases in LacZ activity when the strains were grown in fructose and nonfructose media, respectively (∗P < 0.05 by Tukey’s multiple-comparison test) (Fig. 6A). In both
growth conditions, LacZ activity in the complementation strain (Δcra Δcrp P_\text{fruB}-\text{lacZ} pJML05::cra) was significantly less than LacZ activity in the mutant strain containing an empty vector (Δcra Δcrp P_\text{fruB}-\text{lacZ} pJML05; P < 0.05 by Tukey’s multiple-comparison test) (Fig. 6A). We conclude that Cra represses fruB expression by directly or indirectly affecting some portion of the fruB promoter included in the P_\text{fruB} reporter.

The crp mutant strain, on the other hand, produced 5-fold lower LacZ activity (P < 0.05, Tukey’s multiple-comparison test) in both fructose and nonfructose growth conditions compared to the “WT strain” containing the endogenous crp locus (Fig. 6B). Also in both growth conditions, LacZ activity in the complementation strain (Δcra Δcrp P_\text{fruB}-\text{lacZ} pTrc99A::crp) was 3-fold higher than the mutant strain containing an empty vector (Δcra Δcrp P_\text{fruB}-\text{lacZ} pTrc99A) (Fig. 6B), suggesting partial complementation. Overall, these results support a role for CRP in activating fruB transcription in both fructose and nonfructose conditions by acting, directly or indirectly, on some portion of the fruB promoter included in the P_\text{fruB} reporter. Collectively, data presented in Fig. 6 also indicate that Cra and CRP can work independently of the other to regulate fruB expression.

**Determination of Cra and CRP regulatory sites.** Having established Cra and CRP as transcriptional regulators of fruB expression using the P_\text{fruB} reporter, we then constructed additional transcriptional reporter fusions to identify regulatory sites for each regulator in the fruB promoter. In constructing these reporters, we considered the locations of putative regulatory sites for Cra and CRP to act directly on the promoter, based...
on computational predictions and their similarities to consensus sequences in *E. coli* and *V. cholerae*. BPROM identified only one putative Cra site starting at position −12 in relation to fruB TSS-1 (Fig. 2 and Fig. S1). Moreover, the sequence of this putative site, TGAATC-GATTCA, aligns well with the palindromic Cra consensus sequence previously identified in *E. coli*, TGAAAC-GTTTCA (20–23, 29). To evaluate the validity of this predicted regulatory site, we constructed the P_{fruB_{min}} reporter, which spans −50 to +10 of the fruB promoter region and is smaller than the P_{fruB} reporter (Fig. 2). We then measured LacZ activity from the P_{fruB_{min}} reporter strain grown in the presence of fructose, and this induction was reduced to 1.2-fold when cra was absent (*P* < 0.05, Tukey’s multiple-comparison test) (Fig. 7A). These observations suggest that the promoter, as well as a TSS, are still intact within the P_{null_{min}} reporter. In the cra mutant strain, LacZ activity from the P_{null_{min}} reporter increased in both growth conditions compared to the WT strain (*P* < 0.05, Tukey’s multiple-comparison test), indicating that Cra-mediated regulation is still taking place within the 60 nt of the fruB promoter included in the P_{null_{min}} reporter and is responsible for the majority of the induction observed from this miniaturized promoter. This region includes the sequence that spans the predicted −10 and

**FIG 7** Cra acts near the predicted −10 and −35 sites of the fruB promoter, while CRP acts farther upstream. (A) fruB promoter activity from P_{null_{min}} transcriptional reporter in wild-type, Δcra, and Δcrp strains. (B) fruB promoter activity from P_{null_{crp}} transcriptional reporter in wild-type and Δcrp strains. Strains were initially grown in LB and back diluted into fresh cultures supplemented with 0.4% (wt/vol) fructose, glucose, or an equivalent volume of water. After reaching an OD_{600} of 1.0, cultures were lysed and incubated with ONPG substrate solution for 1 h, from which A_{420}/minute was measured and normalized to OD_{600}. For each carbon source and strain, cultures were grown in biological replicates and measured in technical triplicate. Technical triplicates were averaged during data analysis. Bars represent means from biological replicates. Within each panel, bars with different letters indicate mean values that are significantly different from each other (*P* < 0.05, Tukey’s multiple-comparison test).
that CRP may repress increases in LacZ activity in the conditions, LacZ activity from the P

We then measured LacZ activity from this reporter in WT and crp mutant strain compared to the WT (P < 0.05, Tukey’s multiple-comparison test). This is inconsistent with CRP functioning as a transcriptional activator (Fig. 5B and 6B). Our transcriptional reporters are integrated, in an antisense direction, into the native lacZ gene in the V. cholerae genome, which is activated by CRP (data not shown). We postulate that the observed changes in LacZ activity when comparing P

To account for promoter regions through which CRP activates fruB expression, we used the search tool Virtual Footprint, which predicted a CRP site lying 61 nt upstream of the fruB promoter, which lies 60 nt upstream of the fruB TSS (25). Additionally, the sequence of this putative site, TGTGC-GTCTGA-TCATA, is in good agreement with the CRP box motif previously identified in E. coli, TGTGA-NNNNN-TCACA (25).

To validate or invalidate this predicted site, we constructed the P

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postulate that in the presence of fructose, CRP prevents unnecessary, excess synthesis of Cra, and in glucose conditions, CRP may “rein in” Cra to allow for some basal levels of FPr to be synthesized such that the bacteria are primed to metabolize fructose once it is available.

**DISCUSSION**

Important to the survival of *V. cholerae* is its ability to respond to changes in carbohydrate availability, depending on its current environment (7). The series of carbohydrate-specific PTSs encoded in the *V. cholerae* genome is believed to be crucial to this process; PTS components are thought to be selectively produced in order to transport and phosphorylate available carbon sources into the bacteria for metabolism (7, 10, 11). Here, we describe the transcriptional regulation of *fruB*, which encodes the FPr component of PTSFru, in an effort to further dissect how *V. cholerae* responds to changes in carbohydrate availability. We show that *fruB* is expressed at the highest levels in fructose media, which is consistent with data collected during a similar study in *Pseudomonas putida*, the only other Gram-negative bacterium that, to our knowledge, has been used to study *fruB* using a transcriptional reporter system (32). Expression of *fruB* is controlled by both Cra and CRP, which impact transcription through different regions of the *fruB* promoter in response to the absence and presence of fructose. The proximal location of *fruK* and *fruA* to *fruB*, and their parallel induction by fructose, further suggests that the three genes may be coregulated by Cra and CRP.

Using the P*fruB* reporter, we showed that the *fruB* TSS lies approximately 241 nt upstream of its start codon, in line with previously published dRNA-Seq data from Papenfort and colleagues (Fig. 2 and 3; see Fig. S1 in the supplemental material) (16). This conflicts with the TSS we determined by 5’ RACE, which lies 133 nt upstream of the *fruB* start codon (Fig. 2; Fig. S1 and S2). Interestingly, the intergenic transcript IGR4 (107 nt) lies directly between these two sites, spanning almost the entire length between the two markers (Fig. S1). IGR4 was first identified in a massively parallel sequencing experiment which aimed to uncover novel small RNAs (sRNAs) with regulatory roles in *V. cholerae* (33). Because of its proximity to the *cra* promoter region, IGR4 was hypothesized to be a putative cis-acting sRNA, imparting regulation through extensive binding to the *cra* promoter (34). However, Western blotting probing for Cra in a strain overexpressing IGR4 suggests the small transcript has no regulatory effect on Cra levels (Fig. S7). One possible explanation for the range of 5’ ends observed in
our RNA-Seq data, our aberrant 5’ RACE results, as well as the existence of IGR4, is the occurrence of posttranscriptional processing of the fruB transcript by an endonuclease. RNase E, arguably the most prominent RNase in E. coli, and YbeY are both promising candidates for cleavage of fruB mRNA; these two endonucleases have also been shown to target transcripts in V. cholerae (13–38).

Using PfruB_min and PfruB_crp reporters, we identified sites through which Cra and CRP influence fruB transcription, possibly through direct interactions with the fruB promoter; the likely locations of −10 and −35 hexamers of the fruB promoter were also mapped (Fig. 7A and B). The sequences of predicted regulator binding sites identified here agree well with previously determined consensus sequences from E. coli (Fig. S1) (20–23, 25, 29). In the V. cholerae genome, the −10 hexamer shows a distinct consensus sequence, TANAAT (spanning −12 to −7 sites), and highly conserved bases at positions −11 and −7 (adenine and thymine, respectively) are maintained in the sequence of the −10 hexamer we predict here, CAGTAT (16). While the manuscript was under review, a separate study was published that demonstrated the binding of Cra to the putative Cra binding site we included in the PfruB_min-lacZ construct (Fig. 2 and Fig. S1) (39). Interestingly, the authors propose that in some growth conditions, Cra may recruit RNA polymerase to the fruB promoter (39). These results, combined with those presented in this work, open up intriguing possibilities for further interrogation of the nuances of fruB expression throughout the V. cholerae life cycle.

In E. coli, Cra is frequently described as a pleiotropic regulator, involved in modulating the expression of a large number of genes, including mtlA, which encodes the EIIMTL protein (22). While Cra-mediated repression of the fru operon in V. cholerae closely mimics the same process in E. coli, it is unclear if a similar Cra regulon exists in V. cholerae. Western blotting probing for the PTSMTL transporter MtlA in WT and Δcra strains suggests the V. cholerae Cra regulon may not include the same targets as in E. coli; MtlA levels did not vary between WT and mutant strains in V. cholerae, although Cra has been shown to repress MtlA in E. coli (20) (Fig. S8). Our RNA-Seq data further confirm this finding. Thus, although Cra regulates fruB expression in both E. coli and V. cholerae, their regulons likely contain divergent targets.

One peculiarity in our description of Cra’s activity in the fruB promoter is the repressor’s individual expression pattern. LacZ activity from the PcrA reporter and analogous Western blotting probing for Cra indicate that cra is expressed at the highest levels in fructose media, just like fruB (Fig. 8 and Fig. S8), and in the absence of fructose, Cra levels are low. This particular expression pattern is not intuitive given our observations of Cra activity. In fructose media, Cra is expressed at the highest levels when its activity as a repressor is expected to be minimal. In nonfructose media, Cra is expressed at much lower levels but is active in repressing fruB. Similar patterns of expression and activity have been observed in V. cholerae for the transcriptional regulator MtlR, which represses the genes encoding PTSMTL in the absence of the sugar alcohol mannitol (40). In the case of Cra, fructose-1-phosphate (F1P) may provide an explanation for these unexpected activity patterns. We propose a model that when fructose is imported into the cell, it is first phosphorylated to F1P as it crosses the inner bacterial membrane and is then phosphorylated again once inside by 1-phosphofructokinase (the protein product of fruK) to become fructose-1,6-bisphosphate (FBP) (12). In E. coli, F1P has been shown to bind Cra and significantly inhibit Cra’s DNA binding ability (41). Considering the similarities between E. coli cra and its V. cholerae homolog, we hypothesize F1P likely plays a similar role in V. cholerae and could help to explain why Cra is inactive in fructose media, even when expressed at relatively high levels. The enzymatic activity of FruK, which converts F1P to FBP, may initiate a feedback loop that controls Cra activity; FruK’s activity essentially removes the effector molecule (i.e., F1P) which dampens Cra activity. When the concentration of F1P decreases, Cra becomes active and represses fruB transcription, which may, in turn, also decrease expression of fruK, consequently increasing the concentration of F1P. As this concentration increases, Cra becomes inactive, fruB expression increases, and the cycle repeats itself.
MATERIALS AND METHODS

Strains and culture conditions. All strains used in this study are described in Table S1 in the supplemental material. The wild-type V. cholerae strain used in this study, from which all subsequent strains were constructed, was the O1 biovar El Tor N16961 tcpA mutant strain. The tcpA mutant is highly attenuated for virulence and was used for safety purposes. Unless otherwise denoted, “wild type” refers to the N16961 ΔtcpA strain.

All strains were streaked onto Luria-Bertani (LB) plates with the appropriate antibiotics and incubated at 37°C for 12 to 16 h. Liquid cultures were prepared by inoculating 2 ml LB broth or 1 × M9 minimal medium containing one or more carbon sources (totaling 0.4% [wt/vol]) with individual colonies. All cultures were supplemented with the appropriate antibiotics at the following concentrations: streptomycin (Sm) at 100 μg/ml and carbenicillin (Cb) at 50 to 100 μg/ml. Cultures prepared in 1 × M9 minimal medium were also supplemented with 0.1% (wt/vol) trace metals (5% MgSO4, 0.5% MnCl2, 0.5% FeCl3, and 0.4% nitritotriacetic acid). Unless otherwise stated, cultures were incubated at 37°C for 12 to 16 h, with shaking at 250 rpm.

Mutant strain construction. V. cholerae strains harboring chromosomal mutations were constructed as follows. A plasmid bearing the desired mutation was constructed in the allelic exchange vector pCVD442 via splicing by overlap extension (SOE) PCR. Two roughly 600-bp DNA fragments flanking the region of interest were amplified by PCR using F1/R1 and F2/R2 primer pairs (Table S2). These fragments were annealed together and then amplified by PCR using the F1 and R2 primers. The final PCR product was assembled via Hi-Fi DNA assembly (New England BioLabs) with the pCVD442 backbone, which was prepared using the appropriate pCVD_F and pCVD_R primers (Table S2). The resultant plasmid was propagated in E. coli DH5α·pir and then transformed into E. coli SM10·pir before being conjugated into V. cholerae. Successful conjugates were selected from one round of growth in LB broth with streptomycin, and the resultant colonies were plated on sucrose medium to screen for successful vector dissemination. Sucose-resistant colonies were screened for the desired mutation by PCR with the F0 and R0 primers and confirmed by sequencing (Eurofins).

Transcriptional reporter construction. To assemble the lacZ transcriptional fusion reporters, we generated a series of derivatives of the pJL1::lacZ(EC) plasmid, which contains the ribosome binding site (RBS) and coding sequence of E. coli lacZ homolog (lacZ) in the antisense orientation (42). In each derivative plasmid, we inserted the desired portion of the fruB promoter directly upstream of the lacZ(EC) RBS. To construct derivative plasmids for Pnu and Pnut_min reporters, DNA fragments containing the desired portion of the fruB promoter were amplified by PCR using forward insert and reverse insert primer pairs (Table S2). The derivative plasmid was then assembled via Hi-Fi DNA assembly (New England BioLabs) with the amplified DNA fragment and the pJL1::lacZ(EC) backbone, which was amplified by PCR using forward vector and reverse vector primers (Table S2). To construct derivative plasmids for Pnut_min and Pnut_max reporters, desired double-stranded DNA (dsDNA) fragments of the fruB promoter (gBlocks) were ordered from Integrated DNA Technologies, and plasmids were assembled via Hi-Fi DNA assembly as described for Pnut and Pnut_min reporters. The exact coordinates of the fruB promoter region included in each fusion are indicated in Table S1.

Derivative plasmids were then propagated in E. coli DH5α·pir, and correct assembly of the plasmid was checked by sequencing using LIU126 and LIU127 primers, which flank the site at which regions of the fruB promoter were inserted into pJL1::lacZ(EC) (Table S2). Plasmids were then transformed into E. coli SM10·pir before being conjugated into V. cholerae, using the sucrose-screening method described above.

Complementation plasmid construction. All plasmids were constructed using the Hi-Fi master mix (New England BioLabs) to assemble DNA fragments. Plasmid pTrc99a:cpp was obtained using PCR fragments amplified using primers LIU152-153 (to amplify the pTrc99a backbone) and LIU154-155 (to amplify cpp from the V. cholerae genome). Vector pJML05 was created by replacing the Ptc promoter in the pTrc99a backbone with the PllacO-1 promoter. This was accomplished using primers LIU476-477 to amplify the backbone of pTrc99a and DNA oligonucleotide LIU480. Plasmid pJML05:crp was assembled using PCR products derived by using primers LIU652-653 (to amplify the backbone of pJML05) and LIU654-655 (to amplify crp from the V. cholerae genome). Plasmid pJML05:IGR4 was assembled from PCR products obtained using primers LIU494-495 (to amplify the backbone of pJML05) and LIU496-497 (to amplify the IGR4 sequence from the V. cholerae genome). All constructs were confirmed by sequencing.

RNA-Seq experiments and analysis. For each combination of strain and growth sample, RNA samples in biological duplicate were prepared. Cells from LB agar plates were used to inoculate 2 ml of 1 × M9 minimal medium supplemented with 0.4% glucose or fructose (wt/vol). Overnight cultures were diluted into 2 ml fresh 1 × M9 medium with 0.4% glucose or fructose (starting optical density at 600 nm [OD600] ~0.05) and grown to an OD600 of ~0.3. The entire culture was harvested (8,000 g, 5 min, 4°C), and the RNA was purified from the cells using the Direct-zol RNA miniprep kit (Zymo Research) following the manufacturer’s instructions. Eluted samples were treated with DNase at 37°C for 30 min, twice, using Turbo DNA-free (Ambion) according to the manufacturer's protocol. RNA integrity was initially analyzed by agarose gel electrophoresis. Additional RNA integrity analysis, rRNA depletion, cDNA synthesis, and library preparation and sequencing were performed by Quick Biology (Pasadena, CA). Paired-end, 150-bp sequences were generated for 10 million reads per sample.

Fastq files were mapped to the V. cholerae biovar El Tor strain N16961 genome (NCBI accession number GCA_000006745.1) using the BWA-MEM algorithm in BWA version 0.7.12 (43). Aligned reads were counted with htseq-count version 0.11.2 (44) with the intersection-strict argument. Counts were
imported into R version 3.6.2 (45), and differential expression was assessed with DEseq2 version 1.26.0 (46).

**General total RNA extraction.** RNA for 5' RACE and qRT-PCR was extracted from overnight *V. cholerae* cultures that were back diluted and grown to mid-log phase (OD\textsubscript{600} ~0.3). Total RNA was extracted using the Direct-zol RNA miniprep kit (Zymo) following the manufacturer’s instructions. For qRT-PCR, eluted samples were treated with DNase at 37°C for 30 min using Turbo DNA-free (Ambion) according to the manufacturer’s protocol.

**qRT-PCR.** Determination of relative expression levels was performed on total RNA using the Stratagene MX3005P system, the Brilliant II SYBR green qRT-PCR master mix kit (Agilent), and primers specific to *fruB*, *fruK*, *fruA*, and 4.5S (Table S2 in the supplemental material). The reactions were set up in 96-well optical reaction plates and contained 1 × Brilliant SYBR green qPCR master mix, 30 nM ROX reference dye, each primer at 100 nM, 100 ng RNA, and 1 μl RT/RNase block enzyme mixture in a 25-μl reaction mixture. The following conditions were used for cDNA synthesis and PCR: 30 min at 50°C, 10 min at 95°C, and 40 cycles of 30 s at 95°C and 1 min at 60°C (Agilent). MxPro QPCR software (v. 4.10) was used to determine threshold cycle (Ct) values for each reaction, and relative RNA concentrations were calculated from the Ct values by comparison to standard curves. All transcript levels were normalized to a 4.5S RNA endogenous control. No signals were detected in no-template controls and no-reverse transcriptase (RT) controls.

**5' RACE.** 5' rapid amplification of cDNA ends (RACE; Invitrogen) was performed according to the manufacturer’s instructions. Gene-specific primers (GSPs) (Table S2) were designed to anneal to the coding region of the gene of interest (i.e., *cra* or *fruB*) in order to synthesize cDNA and amplify the upstream region from extracted RNA samples. Amplified PCR products were introduced into the pCR4-TOPO vector using the TOPO TA cloning kit (Invitrogen). Plasmids were then isolated and sequenced using M13 forward and M13 reverse primers (Eurofins).

**Transcriptional reporter assays.** All transcriptional reporter assays were performed using strains containing a lacZ(Ec) gene construct that was inserted into the endogenous lacZ gene in order to disrupt native lacZ expression. Bacterial samples were taken from liquid cultures which were back diluted and grown to late log phase (OD\textsubscript{600}). Technical replicates were averaged during data analysis and statistical analyses were performed using GraphPad Prism (version 7) software.

**Western blotting.** For FPr-FLAG and MtaA-FLAG analysis, cell pellets were prepared from 2 ml back-diluted cultures grown to mid-log phase (OD\textsubscript{600} ~0.3). Following centrifugation at 8,000 × g for 5 min at 4°C, pellets were resuspended in LB or 1 × M9 medium, mixed 1:4 in SDS sample buffer (250 mM Tris-HCl [pH 6.8], 10% SDS, 50% glycerol, 10% β-mercaptoethanol, and 0.5% orange G), and heated at 95°C for 10 min. Samples were loaded onto an SDS containing 4 to 20% Tris gel (Bio-Rad) and run at 200 V for 25 min. Proteins were then transferred to a nitrocellulose membrane using a Trans-Blot Turbo transfer system (7 min at 1.3 A; Bio-Rad). Revert total protein stain (Li-Cor), a near-infrared fluorescent membrane stain, was then used to stain all protein on the membrane following the manufacturer’s instructions. Revert stain was detected at 700 nm using an Odyssey imager (Li-Cor). The membrane was then incubated with a dilution of primary antibody (1:5,000 of rabbit anti-FLAG [Abcam]) for 1 h, followed by incubation with a dilution of secondary antibody (1:10,000 of IR800-conjugated goat anti-rabbit immunoglobulin [Li-Cor]) for 30 min. Signal was visualized at 800 nm using an Odyssey imager (Li-Cor), and ImageStudio software (version 5; Li-Cor) was used to quantify fluorescent signal and normalize values to Revert total protein measurements.

For Cra-hemagglutinin (HA) analysis, cell pellets were prepared from 50-ml back-diluted cultures grown to mid-log phase (OD\textsubscript{600} ~0.3). Pellets were lysed with B-PER bacterial protein extraction reagent (Thermo Scientific) in the presence of DNase I (Thermo Scientific) following the manufacturer’s instructions. Following protein extraction, the same methods used for FruB-FLAG and MtaA-FLAG analysis (described above) were used. During the immunodetection steps, membranes were incubated with rabbit anti-HA antibody (Abcam) in a 1:1,000 dilution for 1 h, followed by a 30-minute incubation with IR800-conjugated goat anti-rabbit immunoglobulin (Li-Cor) antibody in a 1:6,667 dilution.

**Data availability.** RNA-Seq data were deposited in the GEO database with accession number GSE164298.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1, PDF file, 1.7 MB.**
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C.B., S.P., and J.M.L. performed all of the experiments with assistance from D.M.S.; C.B. and J.M.L. wrote the paper, with editorial assistance from all authors. J.M.L. conceived and supervised the study.

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REFERENCES

1. Harris JB, LaRocque RC, Qadri F, Ryan ET, Calderwood SB. 2012. Cholera. Lancet 379:2466–2476. https://doi.org/10.1016/S0140-6736(12)60436-X.
2. Kaper JB, Morris JE, Levine MM. 1995. Cholera. Clin Microbiol Rev 8:48–86. https://doi.org/10.1128/CMR.8.1.48-86.1995.
3. Deen J, Mandlik A, Livny J, Robins WP, Ritchie JM, Mekalanos JJ, Waldor MK. 2011. RNA-seq-based monitoring of gene expression changes of viableVibrio choleraeO1 non-O1 pathogen. PLoS Negl Trop Dis 5:e1000330. https://doi.org/10.1371/journal.pntd.0000330.
4. Ali M, Nelson AR, Lopez AL, Sack DA. 2015. Updated global burden of cholera. Lancet 385:2000–2006. https://doi.org/10.1016/S0140-6736(15)60265-x.
5. Houot L, Chang S, Absalon C, Watnick PI. 2010. Vibrio choleraephosphoenolpyruvate phosphotransferase system control of carbohydrate transport, biofilm formation, and colonization of the gormouse mouse intestine. Infect Immun 78:1482–1494. https://doi.org/10.1128/IAI.01356-09.
6. Galinier A, Deutscher J. 2017. Sophisticated regulation of transcriptional factors by the bacterial phosphoenolpyruvate: sugar phosphotransferase system. J Mol Biol 429:773–789. https://doi.org/10.1016/j.jmb.2017.02.006.
7. Deutscher J, Francke C, Postma PW. 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. Microbiol Mol Biol Rev 70:939–1031. https://doi.org/10.1128/MMBR.00024-06.
8. Houot L, Chang S, Pickering BS, Absalon C, Watnick PI. 2010. The phosphoenolpyruvate phosphotransferase system regulatesVibrio choleraebiofilm formation through multiple independent pathways. J Bacteriol 192:3055–3067. https://doi.org/10.1128/JB.00213-10.
9. Hayes CA, Dalia TN, Dalia AB. 2017. Systematic genetic dissection of PTS inVibrio choleraunovels a glucose transporter and a limited role for PTS during infection of a mammalian host. Mol Microbiol 104:568–579. https://doi.org/10.1111/mmi.13646.
10. Heidelberg JF, Eisen JA, Nelson WC, Clayton RA, Gwinn ML, Dodson RJ, Haft DH, Hickey EK, Peterson JD, Umayam G, Gill SR, Nelson KE, Read TD, Tettelin H, Richardson D, Ermolaeva MD, Vamathevan J, Bass S, Qin H, Dragoi I, Sellers P, McDonald L, Utterback T, Fleischmann RD, Nierman WC, White O, Salzberg SL, Smith HO, Colwell RR, Mekalanos JJ, Venter JC, Fraser CM. 2000. DNA sequence of both chromosomes of the cholera pathogenVibrio cholerae. Nature 406:477–483. https://doi.org/10.1038/35020000.
11. Xu T, Cao H, Zhu W, Wang M, Du Y, Yin Z, Chen M, Liu Y, Yang B, Liu B. 2018. RNA-seq-based monitoring of gene expression changes of viable but non-culturable state ofVibrio choleraeinduced by cold seawater. Environ Microbiol Rep 10:594–604. https://doi.org/10.1111/1758-2229.12685.
12. Mandlik A, Livny J, Robins WP, Ritchie JM, Mekalanos JJ, Waldor MK. 2011. RNA-Seq-based monitoring of infection-linked changes inVibrio cholerae gene expression. Cell Host Microbe 10:165–174. https://doi.org/10.1016/j.chom.2011.07.007.
13. Frohman MA, Dush MK, Martin GR. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc Natl Acad Sci U S A 85:8998–9002. https://doi.org/10.1073/pnas.85.23.8998.
14. Papenfort K, Förster NU, Cong J-P, Sharma CM, Bassler BL. 2015. Differential RNA-seq ofVibrio choleraeidentifies the VqmR small RNA as a regulator of biofilm formation. Proc Natl Acad Sci U S A 112:E766–E775. https://doi.org/10.1073/pnas.1502031112.
15. Sacco C, Gutiérrez-Grau K, de Lorenzo V. 2013. Cra regulon in Vibrio vulnspecies: molecular insights and biotechnological applications. Microbiol Mol Biol Rev 77:537–561. https://doi.org/10.1128/MMBR.00024-10.
16. Cheng J, Zhu J, Wang J, Jiang Y, Zhang J, Guo S, He W, Cui D, Feng S, He Q. 2018. Cra regulon in Vibrio vulnspecies: molecular insights and biotechnological applications. Microbiol Mol Biol Rev 77:537–561. https://doi.org/10.1128/MMBR.00024-10.
phosphotransferase system of *Pseudomonas putida*. Environ Microbiol 15:121–132. https://doi.org/10.1111/j.1462-2920.2012.02808.x.

33. Liu JM, Livny J, Lawrence MS, Kimball MD, Waldor MK, Camilli A. 2009. Experimental discovery of sRNAs in *Vibrio cholerae* by direct cloning, SS/strRNA depletion and parallel sequencing. Nucleic Acids Res 37:e46. https://doi.org/10.1093/nar/gkp080.

34. Waters LS, Storz G. 2009. Regulatory RNAs in bacteria. Cell 136:615–628. https://doi.org/10.1016/j.cell.2009.01.043.

35. Carpousis AJ. 2007. The RNA degradosome of *Escherichia coli*: an mRNA-degrading machine assembled on RNase E. Annu Rev Microbiol 61:71–87. https://doi.org/10.1146/annurev.micro.61.080706.093440.

36. Mackie GA. 2013. RNase E: at the interface of bacterial RNA processing and decay. 1. Nat Rev Microbiol 11:45–57. https://doi.org/10.1038/nrmicro2930.

37. Davis BM, Waldor MK. 2007. RNase E-dependent processing stabilizes MicX, a *Vibrio cholerae* sRNA. Mol Microbiol 65:373–385. https://doi.org/10.1111/j.1365-2958.2007.05796.x.

38. Vercruysse M, Köhrer C, Davies BW, Arnold MFF, Mekalanos JJ, RajBhandary UL, Walker GC. 2014. The highly conserved bacterial RNase YbeY is essential in *Vibrio cholerae*, playing a critical role in virulence, stress regulation, and RNA processing. PLoS Pathog 10:e1004175. https://doi.org/10.1371/journal.ppat.1004175.

39. Yoon C-K, Kang D, Kim M-K, Seok Y-J. 2021. *Vibrio cholerae* FruR facilitates binding of RNA polymerase to the fru promoter in the presence of fructose 1-phosphate. Nucleic Acids Res 49:1397–1410. https://doi.org/10.1093/nar/gkab013.

40. Byer T, Wang J, Zhang MG, Vather N, Blachman A, Visser B, Liu JM. 2017. MtrR negatively regulates mannitol utilization by *Vibrio cholerae*. Microbiology (Reading) 163:1902–1911. https://doi.org/10.1099/mic.0.000559.

41. Folly BB, Ortega AD, Hubmann G, Bonsing-Vedelaar S, Wijma HJ, van der Meulen P, Millas-Argetis A, Heinemann M. 2018. Assessment of the interaction between the flux-signaling metabolite fructose-1,6-bisphosphate and the bacterial transcription factors CggR and Cra. Mol Microbiol 109:278–290. https://doi.org/10.1111/mmi.14008.

42. Zhang MG, Liu JM. 2019. Transcription of cis antisense small RNA MtIS in *Vibrio cholerae* is regulated by transcription of its target gene, mtlA. J Bacteriol 201:e00178-19. https://doi.org/10.1128/JB.00178-19.

43. Li H. 2013. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. ArXiv 1303.3997. https://arxiv.org/abs/1303.3997.

44. Anders S, Pyl PT, Huber W. 2015. HTSeq—a Python framework to work with high-throughput sequencing data. Bioinformatics 31:166–169. https://doi.org/10.1093/bioinformatics/btu638.

45. R Core Team. 2020. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

46. Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol 15:550. https://doi.org/10.1186/s13059-014-0550-8.