RNA helicase-regulated processing of the *Synechocystis* rimO–crhR operon results in differential cistron expression and accumulation of two sRNAs

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The arrangement of functionally-related genes in operons is a fundamental element of how genetic information is organized in prokaryotes. This organization ensures coordinated gene expression by co-transcription. Often, however, alternative genetic responses to specific stress conditions demand the dis-coordination of operon expression. During cold temperature stress, accumulation of the gene encoding the sole Asp–Glu–Ala–Asp (DEAD)-box RNA helicase in *Synechocystis* sp. PCC 6803, *crhR* (slr0083), increases 15-fold. Here, we show that *crhR* is expressed from a dicistronic operon with the methylthiotransferase *rimO/miaB* (slr0082) gene, followed by rapid processing of the operon transcript into two monocistronic mRNAs. This cleavage event is required for and results in destabilization of the *rimO* transcript. Results from secondary structure modeling and analysis of RNase E cleavage of the *rimO–crhR* transcript in vitro suggested that CrhR plays a role in enhancing the rate of the processing in an auto-regulatory manner. Moreover, two putative small RNAs are generated from additional processing, degradation, or both of the *rimO* transcript. These results suggest a role for the bacterial RNA helicase CrhR in RNase E-dependent mRNA processing in *Synechocystis* and expand the known range of organisms possessing small RNAs derived from processing of mRNA transcripts.

Differential expression of operon gene members is a major player in gene expression regulation in bacteria (1, 2). An operon is defined as a collation of two or more functional genes under the control of the same promoter that are transcribed as a primary polycistronic transcript (3). Gene clustering into operons is generally associated with their coordinated expression, involvement in functionally-related processes, and in some instances physical interactions between the encoded proteins (2). This organization provides the ability to rapidly acclimate to new growth conditions by regulating expression of a protein complex through the generation of equimolar levels of each operon member (1, 2). However, numerous studies indicate that whereas genes within an operon typically exhibit similar patterns of expression, co-expression can be lost in response to alteration of environmental conditions (4). Disco-ordinate operon expression can result from a number of mechanisms that alter transcript stability (5, 6) associated with RNA cleavage (7, 11, 12), the formation of secondary structures (8, 9), or the binding of sRNAs (10). Regulation due to sRNA–mRNA binding can be either dependent on RNA chaperones, such as the host factor for phage Qβ (Hfq)3 (11), or chaperone-independent (10). Polycistronic transcripts can also be subject to processing events, initially by an endonuclease, such as RNase E, whose endonucleolytic cleavage generates monocistronic transcripts that subsequently undergo maturation by either 5′–3′- or 3′–5′-exonuclease trimming, as demonstrated in *Bacillus subtilis* (13) and *Escherichia coli* (14), respectively. The processing event can differentially alter transcript stability of operon members, either positively or negatively (15, 16). Using RNA-Seq, Conway et al. (17) observed differential expression of polycistronic genes in 43% of the operons in *E. coli*. Differential regulation of operon expression is thus complex and widespread.

Operon expression has been associated with cellular responses to environmental stresses, including temperature fluctuations (18, 19). The coordination of gene expression in response to temperature downshift is in part governed by co-expression of a group of operons, with genes within an operon responding more similarly to cold-shock conditions than monocistronic genes randomly selected from the genome (18). A major impact of low temperature involves thermodynamic stabilization of RNA secondary structure that inhibits RNA

*3 The abbreviations used are: Hfq, host factor for phage Qβ; Csp, cold-shock protein; crhR<sub>trim</sub>, truncated crhR; CrhR, cyanobacterial RNA helicase redox; DEAD, Asp–Glu–Ala–Asp; Hik, sensor histidine kinase; RACE, rapid amplification of cDNA; RimO, ribosomal protein S12 methylthiotransferase; nt, nucleotide; PAA, polyacrylamide; ssRNA, single-stranded RNA; Tricine, N[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; RCA, rolling circle amplification; FWD, forward; RNA-Seq, RNA sequencing.
reorganization and thus function. Stabilized RNA can be relieved by RNA chaperones whose expression is frequently regulated by low temperature, including members of the cold-shock protein (Csp) (20) and RNA helicase (21, 22) gene families. RNA helicases rearrange RNA secondary structure via duplex unwinding and/or annealing, frequently involving sRNA metabolism thereby regulating gene expression (23–25).

Cyanobacteria are Gram-negative bacteria that perform oxygenic photosynthesis and fix atmospheric carbon dioxide and thus have immense biotechnological and bioremediation potential. In response to low temperature, they induce a number of canonical cold-stress genes, including RNA chaperones belonging to the RNA-binding protein and RNA helicase families (26–28). Although a diverse variety of mechanisms are known to regulate gene expression in response to temperature downshift, the molecular basis of crhR activation is not completely understood in these organisms (29, 30). In the model cyanobacterium, *Synechocystis* sp. PCC 6803 (from here *Synechocystis*), the sensor histidine kinase, Hik33, regulates a subset of cyanobacterial cold-stress genes that excludes crhR and other classic cold-inducible genes (30–32), whereas a cold-specific σ factor has not yet been reported in cyanobacteria (33). Thus, an as-yet-unknown mechanism regulates the temperature-dependent expression of Hik33-independent genes. Many cold-stress genes are expressed in operons in *Synechocystis*, which encodes a total of 425 predicted operons (2). Although processing of mRNAs is one mechanism by which expression of multicistrionic operons can be differentially regulated, the extent of RNA processing and its impact on protein expression is poorly understood. Only a few examples of differential cistron regulation due to RNA processing have been reported in cyanobacteria: the nitrogenase operons of *Anabaena variabilis* ATCC 29413 and two mixed sRNA protein–coding gene operons in *Synechocystis* (34–38).

We have previously shown that the product of the *Synechocystis* slr0083 gene, the RNA helicase CrhR, auto-regulates its own expression through a complex network of interactions at the post-transcriptional level involving temperature-induced alterations of both transcript and protein stability (39, 40). Here, we provide evidence that the *Synechocystis* rimO–*crhR* operon is expressed as a dicistronic transcript that is rapidly processed into monocistronic transcripts having different stabilities. The mechanism is auto-regulatory, as CrhR RNA helicase activity is required for the RNA-processing event, and potentially catalyzed by RNase E, which correctly cleaves the polycistronic transcript *in vitro*. The results reveal a novel mechanism by which bacteria can differentially regulate suboperonic gene expression in response to temperature shift that involves auto-regulatory, RNA helicase-dependent RNA processing that generates transcripts having divergent functions and stabilities.

**Results**

**Genetic organization of the rimO–crhR region in *Synechocystis* sp. PCC 6803**

The *Synechocystis* slr0083 gene encodes the DEAD-box RNA helicase, CrhR, whose expression is regulated by the redox status of the electron transport chain (41, 42). Upstream of the crhR gene is a hypothetical gene, slr0082, whose putative protein product has 38% identity with RimO, a ribosomal protein S12 methylthiotransferase (UniProtKB P0A4E1), and 29% identity with the parologous MiaB, a tRNA methylthiolase (UniProtKB P0AEI1) from *E. coli* (43, 44). The genomic organization of WT and two crhR mutants, a partial deletion mutant, crhR<sub>TR</sub> (39), and a complete deletion mutant, ΔcrhR (40), used in this study is shown in Fig. 1. We utilized the two crhR mutants in this analysis as the crhR<sub>TR</sub> strain allows us to track expression and processing of the operon in the absence of RNA helicase activity, whereas the ΔcrhR strain allows analysis of rimO expression in the absence of crhR.
RNA helicase-mediated RNA processing

A slr0082-slr0083 PCR amplicons

B slr0082-slr0083 operon and slr0083 transcript amplification in wild type cells

C slr0082-slr0083 transcript amplification in crhR mutants

Figure 2. rimO–crhR operon verification. A, RT-PCR with the primers ARRR10, within the rimO (slr0082) ORF, or LPF45, between the −110 primary transcript initiation site and crhR (slr0083) ORF, in conjunction with ARRR1, within the crhR ORF, was used to verify the presence of operon transcripts and crhR transcripts, respectively. Diagram is not to scale. B, operon (ARRR10–ARRR1) and crhR (LPF45–ARRR1) transcript-specific primer pairs were used to perform RT-PCR on total RNA isolated from WT cells subjected to the indicated temperature treatments. C, operon-specific RT-PCR (ARRR10–ARRR1) was performed on RNA extracted from WT, crhRΔrimO and ΔcrhR cells. Amplification from genomic DNA and in the absence of target DNA is shown as controls. The ethidium bromide–stained gel is shown.

Detection of full-length operon transcripts

Our previous study of crhR expression suggested crhR was transcribed as a monocistronic transcript whose expression is auto-regulated by the functional CrhR gene product (39). RT-PCR was performed to determine whether the tandemly-encoded genes, rimO and crhR, are expressed as an operon. The expected RT-PCR–amplified products are shown in Fig. 2A. A 1,178-bp product spanning the rimO and crhR genes, amplified across the transcription start site located at position −110 nt, was detected in WT cells in addition to the 774-bp product corresponding to the crhR transcript (Fig. 2B), confirming expression of rimO–crhR as an operon. Abundance of both amplicons was increased with low temperature, consistent with the previously observed pattern of crhR expression (39). Likewise, detection of an amplified PCR product spanning rimO–crhR and the proposed processing site in the crhRTR strain confirmed the presence of an operon transcript in the crhRTR mutant (Fig. 2C). RT-PCR products were not detected in the ΔcrhR strain under any condition, as this strain does not contain the ARRR1 primer-binding site within crhR (Fig. 2C).

CrhR inactivation results in deregulated expression of the rimO–crhR operon

The enhanced accumulation of the crhR transcript is transient in response to cold stress, returning to basal levels after ~3 h (39). To further examine expression of genes in this operon, transcript abundance of the upstream rimO ORF was determined in WT and crhRTR strains (Fig. 3). In WT cells, rimO expression was extremely transient and unstable, with minor amounts of the 1,465-nt monocistronic rimO transcript, and a major stable 550-nt transcript was detected (Fig. 3A). A significant level of degraded rimO transcript was also detected, indicative of the rapid turnover of this transcript. Transient accumulation of rimO mRNA followed a similar pattern as that observed for crhR (39); however, the rate of accumulation and return to basal levels were significantly accelerated (Fig. 3, A and C). In contrast, detection of full-length truncated operon rimO–crhRTR (2,280 nt) and monocistronic rimO (1,465 nt) transcripts in the crhRTR mutant strain indicated that the rimO–crhR genes are expressed as an operon. Also reminiscent of crhR expression, the initial rate of accumulation for rimO is similar in both WT and the crhRTR mutant for the first 20 min (Fig. 3B), suggesting that the initial cold sensing and response is not affected by crhR inactivation. In contrast, and similar to accumulation of the crhR transcript (39), the subsequent repression of rimO accumulation depended on functional CrhR, as the transient nature of the accumulation of rimO transcripts was not observed in response to cold stress in the crhRTR mutant (Fig. 3B). In addition, crhR mutation altered rimO transcript abundance, as significantly-enhanced levels of the truncated rimO–crhRTR transcript (2,280 nt) plus significant accumulation of randomly-degraded transcripts, including a stable 550-nt product, were detected in the crhRTR mutant (Fig. 3, B and C). A similar expression pattern was observed in the ΔcrhR strain, in which a transcript corresponding to the remaining 1,685 nt of the operon after insertion of the kanamycin cassette used to delete the entire crhR ORF was detected (Fig. 4). Again, significant degradation products, including the 550-nt transcript, were also detected. Stabilization of the rimO and full-length operon transcripts in the two crhR mutant strains suggests that CrhR is associated with regulating transcription, processing, and/or degradation of the operon transcript.

rimO transcript half-life is not affected by temperature downshift or crhR mutation

The observed differences in transcript accumulation between WT and crhRTR suggest that CrhR is potentially involved in the degradation of rimO mRNA. We therefore determined
whether crhR mutation affected rimO transcript half-life. In WT cells, the rimO transcript half-life is temperature-regulated, with half-lives of 2.5 and 6.0 min observed at 30 and 20 °C, respectively (Fig. 5A). A similar pattern was observed in crhR\textsubscript{TR} cells; there was only a marginal alteration in rimO transcript half-life at both warm and cold temperatures: 5.0 and 7.5 min at 30 and 20 °C, respectively (Fig. 5B). The results imply that turnover of rimO mRNA is temperature-regulated by a process that does not involve a CrhR-dependent RNA degradation pathway.

Accumulation of a stable RNA from the rimO ORF

To further characterize the effect of CrhR RNA helicase activity on the temperature-regulated expression of the rimO ORF, WT and crhR\textsubscript{TR} cells were exposed to temperatures ranging from 10 to 35 °C for 1 h (Fig. 6). In WT cells, a relatively constant accumulation of the stable 550-nt transcript from the rimO ORF was detected at all temperatures (Fig. 6A). This is reflective of the results shown in Fig. 3 where only the 550-nt transcript was detectable after 1 h of cold stress. In contrast, in crhR\textsubscript{TR} cells, the rimO ORF probe detected both the rimO–crhR\textsubscript{TR} operon (2,280 nt) and rimO (1,465 nt) monocistronic transcripts, plus a smear of shorter transcripts, including the relatively constant accumulation of the 550-nt transcript (Fig. 6B). Overall transcript abundance in crhR\textsubscript{TR} cells increased progressively as temperature decreased to 15 °C and then decreased at 10 °C. A similar pattern of rimO ORF transcript accumulation was observed when the experiment was performed by acclimation of a single WT or crhR\textsubscript{TR} culture to progressively decreasing temperatures (Fig. S1). As in Fig. 4, the results show a significant difference in temperature-dependent transcript accumulation between WT and mutant crhR\textsubscript{TR} strains, suggesting a role for CrhR in regulation of rimO expression in addition to the previously described regulation of its own transcript.

Accumulation of a stable RNA from the rimO 5’ UTR

Expression of the dicistronic operon was further analyzed by determining expression of the rimO 5’ UTR, previously mapped to start at position –143 nt (52). Northern blot analysis using a 5’ UTR–specific probe (Fig. 1) detected a 75-nt stable transcript that accumulates in both WT and crhR\textsubscript{TR} cells in a temperature-dependent manner (Fig. 7A). Minor levels of longer products were progressively detected as the temperature decreased (Fig. 7A). Again, enhanced accumulation of the rimO 5’ UTR transcripts was observed in the crhR\textsubscript{TR} mutant, indicating a CrhR-dependent effect on rimO 5’ UTR processing or degradation (Fig. 7B). It was of interest to examine the reduc-
tion in the response of *Synechocystis* *crhR* TR cells to 10 °C, as shown in Fig. 6 in more detail. In WT cells, decreasing accumulation of a 75-nt transcript and slight amounts of longer transcripts were detected by the *rimO* 5′ UTR probe over time at 10 °C (Fig. S2A). In *crhR* TR cells, a relatively constant accumulation of the 75-nt transcript was detected at all time points following exposure to 10 °C (Fig. S2B). In addition, and in contrast to WT cells, enhanced accumulation of longer transcripts was progressively observed in the *crhR* TR mutant (Fig. S2B).

In silico prediction of potential mRNA targets for the 75-nt *rimO* UTR-derived sRNA identified candidates that included gene products associated with tetrapyrrole metabolism (Fig. S3 and Table S1). Some of these candidates have predicted inter-
PAA gel and probed with a 90-nt riboprobe targeting the strongly-protected fragment corresponding to the entire probe presence of a promoter upstream of the EcoRI site in also identified a transcript 5
ployse protection and primer extension assays were em-
the presence of light/H11002 or/H11032
Fig. 1
Determination of transcript 5
action energies and interaction sites consistent with known mechanisms of regulation.

Determination of transcript 5’ ends in the rimO–crhR region

Previous Northern blot analysis (39) and the RT-PCR data presented above suggested that operon expression involved an RNA-processing site between the rimO and crhR cistrons in addition to the transcription start site located at position −110 nt with regard to the crhR initiation codon. Therefore, S1 nuclease protection and primer extension assays were employed to address all existing transcript 5’ ends in this region. Both procedures identified the transcript start at position −110 or −111 nt in cells grown in the light (photoautotrophic) and in the presence of light + 5 mM glucose (photomixotrophic conditions) to visualize potential alterations in the transcript start site use in response to growth conditions (Fig. 8, A and B). A strongly-protected fragment corresponding to the entire probe was detected by S1 nuclease protection (Fig. 8A), indicating the presence of a promoter upstream of the EcoRI site in rimO (see Fig. 1). The crhR transcript was not detected by primer extension from cells grown in the dark (Fig. 8B). Primer extension also identified a transcript 5’ end 140 nt upstream of the crhR

RNA helicase-mediated RNA processing

To initiate analysis of the operon-processing mechanism, 5’ rapid amplification of cDNA (RACE) was utilized to identify the 5’ ends of the stable crhR ORF transcripts that accumulate in the crhR/TR mutant at 20 °C (39). The crhR/TR inactivation strategy should have resulted in a 785-nt truncated crhR mRNA, but previous Northern blot analysis (39) also revealed three larger transcripts which, combined with the results presented above, indicate that they originate from co-transcription with the upstream rimO gene as a dicistronic message. Using 5’ RACE-RPA, the 5’ ends of the longest and shortest transcripts were mapped on RNA isolated from crhR/TR cells grown under photoautotrophic conditions at 20 °C. RACE identified that the 2,280-nt rimO–crhR/TR transcript initiated 143 nt upstream of the rimO ORF and extended to the PmlI site within the crhR ORF from which the 5’ RACE priming was initiated. Detection of this transcript therefore verifies that rimO–crhR is expressed as a dicistronic operon in crhR/TR cells grown at 20 °C. The shortest mRNA identified was an 815-nt transcript that started 138 nt upstream of the crhR start codon, within the 3’ end of the rimO ORF. A clone containing the shorter 785-nt transcript originating from the primary transcription start site at −110 was not detected in the 5’ RACE experiment. Extensive attempts to map the 5’ ends of the ~1,500- and ~1,300-nt transcripts were not successful, possibly due to their reduced abundance. The identified nucleotides corresponding to the 3’ termini of rimO and the 5’ start of the crhR transcripts are summarized in Fig. 8, C and D. A summary of the transcripts detected in WT and crhR/TR mutant cells is shown in Fig. 9.

Identification of a putative RNase E processing mechanism

Because the data above suggested that the transcript initiat-
−138 nt upstream of crhR is likely a processed transcript, the sequence and predicted structure of the RNA surrounding the −138-nt site were analyzed to identify a processing mechanism (Fig. 10A). The secondary structure of a 41-nt fragment (genomic position 2,887,481–2,887,521) was predicted using the RNAfold web server (45). The resulting structure has a 3-bp hairpin within the 5’ sequence, and it contains the observed potential processing site at −138 nt, as well as the −140-nt site identified by primer extension within an unpaired region (Fig. 10A). The short upstream hairpin and the presence of a uridy-
late-rich region immediately downstream of the predicted pro-
cessing sites are consistent with the sequence and structure elements previously identified as required for RNase E cleavage in Salmonella enterica (46) and in Synechocystis within the psba 5’ UTR (47) and the crRNA maturation site in a CRISPR–Cas subtype III–Bv system (48). This sequence and structure conservation prompted us to experimentally test cleavage of this RNA by RNase E.

RNase E cleavage assays were performed in vitro on the 41-nt rimO–crhR RNA fragment shown in Fig. 10B. After incubation with the recombinant Synechocystis RNase E, this RNA was processed into major products with lengths of 26/27 and minor products at 35/36 ± 1 nt (Fig. 10B, left panel). To prove that the fragments were cleaved from the 5’ end, the 3’ end of the 41-nt RNA was labeled with Cy3 and used as a substrate in the RNase
The resulting Cy3-labeled products were shorter than 26 nt, confirming the 26/27- and 35/36-nt fragments originated from the 5' end of the transcript (Fig. 10B, right panel). Comparison of the RNase E–treated RNA to a sample of the same 41-nt transcript incubated with hydroxide confirmed that the cleavage of the rimO–crhR RNA fragment by RNase E occurred in a site-specific manner, whereas the CRISPR3 repeat as substrate (48) was included as a control for RNase E activity and showed the expected cleavage fragments (Fig. 10B, left panel). The CRISPR3 control consists of a 36-nt 5'-monophosphorylated synthetic RNA oligonucleotide with a sequence identical to the repeats within the CRISPR3 repeat-spacer array of Synechocystis. RNase E cleaves the 22 and 23 nt from the 5' end of these repeats in vitro and in vivo as the major maturation endonuclease for this CRISPR–Cas system (48).

E cleavage assay. The resulting Cy3-labeled products were shorter than 26 nt, confirming the 26/27- and 35/36-nt fragments originated from the 5’ end of the transcript (Fig. 10B, right panel). Comparison of the RNase E–treated RNA to a sample of the same 41-nt transcript incubated with hydroxide confirmed that the cleavage of the rimO–crhR RNA fragment by RNase E occurred in a site-specific manner, whereas the CRISPR3 repeat as substrate (48) was included as a control for RNase E activity and showed the expected cleavage fragments (Fig. 10B, left panel). The CRISPR3 control consists of a 36-nt 5'-monophosphorylated synthetic RNA oligonucleotide with a sequence identical to the repeats within the CRISPR3 repeat-spacer array of Synechocystis. RNase E cleaves the 22 and 23 nt from the 5’ end of these repeats in vitro and in vivo as the major maturation endonuclease for this CRISPR–Cas system (48).

The RNase E–generated 26-nt cleavage product produced from the rimO–crhR RNA fragment is consistent with the location of the identified 138-nt processing site and demonstrates that it is suitable for RNase E–dependent RNA processing. The sequence and structural features (Fig. 10A) at this site GU ↓ U ↓ U as well as at the minor 35/36 site (CU ↓ A ↓ UU)
are consistent with the strong preference for a uridine in the +2 position as identified previously by Chao et al. (46). Also, the minor 35/36-nt products resulted from cleavage consistent with known RNase E substrate specificities, although they do not match the identified transcript termini in vivo. These products may represent either additional processing sites that were not identified during the 5' RACE or sites that are cleaved by RNase E in vitro but not in vivo. The sequence of the −140-nt putative processing site, AA ↓ GGU, lacks the uridine in the +2-position and was not confirmed in the RNase E assay, indicating that it may be processed by a different endoribonuclease.

**Discussion**

Here, evidence is provided for a suboperonic gene regulatory mechanism in which RNase E–dependent processing gener-
The respective transcripts, is shown in transcription, RNA processing and consequences for the regulation of maintaining co-transcription of idly degraded. The proposed scenario will result in efficient ribonucleases, leaving the vulnerable RNA processing, are consistent with protection of versus crhR RNA-Seq evidence suggested that nant RNase E with an RNase E consensus motif that is cleaved by recombi-

crH RNA helicase activity. RNA processing occurs at a site is auto-regulatory as the rate of RNA processing is enhanced by...ingly, although CrhR is not necessary and sufficient, processing...operate monocistronic mRNAs with differing half-lives. Interest-

ates differential expression of monocistronic transcripts from the dicistronic rimO–crhR operon in Synechocystis. Thus, the discoordinate expression of the rimO–crhR operon varies from that observed for operons in other bacterial systems, in which member transcripts are equally expressed (4) or where expression decreases with distance from the 5′ end (49, 50). Previous RNA-Seq evidence suggested that rimO and crhR, encoding a methylthiotransferase and the DEAD-box RNA helicase CrhR, are potentially expressed from a major promoter upstream of rimO (51), whereas rimO and crhR were also detected as monocistronic transcripts (36). These analyses, however, failed to explain how the observed differential accumulation of rimO versus crhR monocistronic transcripts was achieved. Here, we show that the dicistronic message is rapidly processed to generate monocistronic mRNAs with differing half-lives. Interestingly, although CrhR is not necessary and sufficient, processing is auto-regulatory as the rate of RNA processing is enhanced by CrhR RNA helicase activity. RNA processing occurs at a site with an RNase E consensus motif that is cleaved by recombi-

nent RNase E in vitro. Predicted secondary structures at the 5′ and 3′ termini of the crhR and rimO transcripts, generated by RNA processing, are consistent with protection of crhR from ribonucleases, leaving the vulnerable rimO transcript to be rapidly degraded. The proposed scenario will result in efficient generation of cistron transcripts with differing stability while maintaining co-transcription of rimO and crhR. A model outlining regulation of rimO–crhR operon expression, including transcription, RNA processing and consequences for the respective transcripts, is shown in Fig. 11.

rimO–crhR is expressed as a dicistronic operon

Evidence for the dicistronic expression of rimO and crhR was provided by Northern, 5′ RACE, and RT-PCR results, indicating that expression of rimO and crhR is complex, involving transcription from at least two promoters. In WT cells, the promoter upstream of rimO produces a primary dicistronic transcript during cold stress that is rapidly processed into two monocistronic transcripts. The second transcription start site is located between rimO and crhR, at the previously identified –110-position (41, 51), and presumably generates basal levels of CrhR at all temperatures.

RNA processing generates monocistronic transcripts with differing fates

Here we show that in WT cells, both rimO and crhR are primarily detectable as monocistronic transcripts with minimal levels of the full-length dicistronic message detected. In the absence of CrhR RNA helicase activity, stabilized, full-length operon transcripts extensively accumulate, in addition to transcripts that correspond to either the rimO or crhR monocistronic ORFs. This observation is crucial, as it indicates the following: (i) CrhR RNA helicase activity is associated but not absolutely required for the RNA processing event, and (ii) the full-length operon transcript is stable until the processing event occurs. The latter point is associated with our observation that the two monocistronic transcripts have significantly disparate half-lives. The WT half-lives are dramatically affected by tem-

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Figure 11. Model of rimO–crhR processing and transcript stability. The RNA-processing region can fold into the two structures shown. As transcription proceeds, the CrhR DEAD-box RNA helicase could bind and stabilize the double-stem loop RNA, acting as a protein scaffold for recruitment of the endonuclease and/or unwinding of the stem-loop to convert the cleavage site into ssRNA required for RNase E cleavage. Alternatively, the cleavage site sequence could also be masked by base pairing with the crhR coding sequence forming a dupplex structure that could be removed either by translation or CrhR binding and unwinding in a positive feedback mechanism, as described above. Subsequently, after processing by RNase E, stable RNA structures at the termini of crhR, as well as translating ribosomes protect the transcript from degradation by ribonucleases, similar to suggestions by Dar and Sorek (15) for the programmed mRNA decay in E. coli. The rimO transcript is rapidly degraded from a destabilized 3′ terminus created by the RNA-processing event. Additional rimO transcript processing and/or degradation generates two stable sRNAs of 75 and 550 nt.
Table 1

Predicted RNA structures at termini of processed transcripts

The sequences of the processed transcripts of rimO and crhR were used to predict RNA structures with the RNAfold Web Server (RRID:SCR_008550). The sequence and structure for 45 nt at the 3′ terminus of each transcript were extracted from the predicted structure of the fully-processed transcript. For structures at the 5′ terminus of the crhR transcript, the extracted sequence and structure were from the processing site to the start codon of crhR. The Gibbs free energy calculation is based on the extracted sequence and structure. Structures are shown in dot–parentheses notation.

| Gene      | Terminus | RNA sequence and structure | ΔG  |
|-----------|----------|---------------------------|-----|
| −138-nt processing site |          |                           |     |
| rimO (sbr0082) | 3′       | CCGACGAUUAUGACCGUACGGUUAUGACCCGGAGGAGGCCUAAGG | −4.7 |
| crhR (sbr0083) | 5′       | UUUUUUGCACUAUGAACUAGGGGAAUGCUGCGUGUAAUUGA | −23.1 |
| crhR (sbr0083) | 3′       | GGAAAAAAUAAUCUCUCAGAACAAAGACCCUGGAGGGGAGUUUA | −17.0 |
| −140-nt processing site |          |                           |     |
| rimO (sbr0082) | 3′       | CACCGACGAUUAUGACCGUACGGUUAUGACCCGGAGGAGGCCUA | −4.7 |
| crhR (sbr0083) | 5′       | GGUUUUUGCACUAUGAACUAGGGGAAUGCUGCGUGUAAUUGA | −23.1 |
| crhR (sbr0083) | 3′       | GGAAAAAAUAAUCUCUCAGAACAAAGACCCUGGAGGGGAGUUUA | −17.0 |

half-lives of 6 and 2.5 min and crhR >30 and 10 min at 20 and 30 °C, respectively (39). This is in distinct contrast to the observation that the rimO or crhR half-lives were not dramatically altered by crhR inactivation, suggesting that CrhR is not directly involved in further degradation of these monocistronic RNAs. Rather, the enhanced accumulation and stabilization of the full-length operon transcript in the crhR mutant indicate that RNA processing selectively destabilizes the rimO transcript, a process that is associated with functional CrhR RNA helicase activity.

Although not all sites suitable for cleavage by RNase E in vitro are significant in vivo (52), processing at the −138 site in rimO–crhR potentially is critical for the observed differential stability of the rimO and crhR transcripts. After processing, the upstream gene product in the rimO–crhR operon is predicted to have a relatively unstructured 3′ terminus, the most stable structure (ΔG = −4.7 kcal mol⁻¹) consisting of a single 3-bp hairpin with a 5-nt loop (Table 1) within the final 45 nt of the transcript. This unstructured 3′ end potentially allows 3′–5′-exonucleases to efficiently access and perform the observed rapid degradation of the rimO transcript. In contrast, the 3′ terminus of the downstream gene product, encoding crhR, is predicted to have a more stable (ΔG = −17.0 kcal mol⁻¹) hairpin with an 11-bp stem and a 10-nt loop, which potentially confers protection from 3′–5′-exonucleases. In addition, three stable stem-loop structures, with a combined ΔG of −23.1 kcal/ mol, are predicted to form at the 5′ end of the processed crhR transcript (Table 1). These structures likely perform the same protective function as the 5′ structures identified by Dar and Sorek (16) in processed E. coli transcripts, and perhaps extend to protection from the homolog for the 5′–3′-exonuclease, RNase J, in Synechocystis (53).

Interestingly, further processing and/or degradation of the rimO–crhR operon generates two candidate sRNAs that originate from the 5′ UTR and the coding sequence of rimO. It should be noticed that the processing events that generate these stable transcripts do not appear to be regulated by CrhR activity. A function cannot be proposed for these sRNAs yet. However, computational analyses predicted mRNAs encoding enzymes involved in pigment biosynthesis, signaling proteins, and others as possible targets indicating potentially interesting regulatory relationships (Fig. S3 and Table S1). NsiR4 illustrates the possible functional relevance of such sRNAs because cells lacking it are out-selected during periods of low nitrogen, consistent with its targets in the machinery controlling nitrogen assimilation (54). In addition, a 98-nt sRNA, Nc117, originating from the sbr0550–sbr0551 intergenic spacer was described by Pei et al. (55) Because there is not a transcription start site in this region, Nc117 derives from the 5′ UTR of sbr0551, encoding RNase J. Hence, this arrangement is similar to the situation with the 75-nt sRNA described here as originating from the 5′ UTR of sbr0082. Overexpression of Nc117 led to an improved tolerance to exogenous biofuel stress by an unknown mechanism (55), further supporting the biological functionality of such transcripts in Synechocystis. Moreover, hundreds of candidate sRNAs derived from processing of mRNA-coding sequences and 3′ UTRs have been identified in E. coli (56, 57). Overall, our data further extend the range of bacterial lineages that host this new class of sRNAs, suggesting they perform an important role in the regulation of gene expression in cyanobacteria.
Similar examples of RNA processing associated with differential suboperonic gene expression were reported for the two nitrogenase gene clusters in the cyanobacterium, *A. variabilis* ATCC 29413 (33–37). Processing of the *nifBSLHDKEN-1* gene cluster, immediately upstream of *nifH1*, generates an extended transcript half-life for *nifH1*, *nifD1*, and *nifK1* relative to the other nitrogenase genes (37). This effect is associated with multiple processing events and two stem-loop structures upstream of *nifH1*, which convey stability to the processed *nifH1* transcript (38). Although details of the processing mechanism were not identified in these studies, the findings clearly resemble the mechanism investigated here that regulates the discoordinate expression of the *rimO–crhR* operon.

**Role for the CrhR RNA helicase in rimO–crhR transcript processing**

*In vitro* cleavage of the *rimO–crhR* transcript at the −138-nt site upstream of *crhR* is consistent with an RNase E-dependent processing mechanism, as both the RNA sequence, GG ↓ UUU, which also conserves the strong preference for a uridine in the +2-position, and the secondary structure in the vicinity of the processing site fit the “ruler and cut” mechanism for RNase E which also conserves the strong preference for a uridine in the processing site. CrhR could rearrange this RNA secondary structure to generate ssRNA required for RNase E cleavage (40). The process is auto-regulatory, as activity of the *crhR* ORF is translated into monocistronic mRNAs with divergent half-lives.

**rimO–crhR co-transcription and cold stress**

The genes *rimO* and *crhR* are syntenic in many cyanobacteria (61), consistent with the idea that genomic context can indicate functional relatedness (62). It is therefore tempting to speculate that co-transcription of *rimO* and *crhR* may indicate that both proteins function in enhancing cellular fitness during cold stress. In support of this proposal, CrhR has been shown to co-precipitate with polysomes and RNA degradosome components (58). Furthermore, DEAD-box RNA helicase association with cold adaptation is widespread in bacteria, including the cold-induced *E. coli* helicases, CsdA and SrmB, that function in assembly of the large ribosomal subunit (63, 64), formation of a cold-adapted RNA degradosome (65), and translation initiation (66).

In conclusion, we provide evidence that *rimO–crhR* is expressed as an operon whose transcript is rapidly processed by RNase E into monocistronic mRNAs with divergent half-lives. The process is auto-regulatory, as activity of the *srl0083*-encoded DEAD-box RNA helicase, CrhR, is associated with, but not absolutely required, for processing. Further research is required to discern the role CrhR performs in the cleavage event and its potential to regulate additional operons in *Synechocystis*. As well, the function of the two stable by-products of the *rimO–crhR* operon processing remains to be determined. RNA helicase catalysis of differential operon cistron expression is likely a more common mechanism for regulation of gene expression than previously anticipated.

**Experimental procedures**

**Cyanobacterial strains and growth conditions**

Bacterial strains and plasmids used in this study are provided in Table 2. *Synechocystis* was maintained on BG-11 agar supplemented with 10 μM Tricine, pH 8.0, and 0.3% sodium thiosulfate (39). Two *crhR* mutant strains, a partial *crhR* deletion mutant created by insertion of a spectinomycin cassette (*crhR*) (39) and a *crhR* complete deletion mutant, created by insertion of a kanamycin cassette (Δ*crhR*) (40), were grown as

### Table 2

| Strain or plasmid | Relevant characteristic(s) | Source, Ref., or application |
|-------------------|----------------------------|-----------------------------|
| **Synechocystis strains** | | |
| Wildtype | Glucose-tolerant, nonmotile strain | |
| *crhR* | *crhR*: spectinomycin cassette; C-terminal deletion of CrhR | 58 |
| Δ*crhR* | Replacement of the complete *crhR* ORF with a kanamycin resistance cassette | 40 |
| **E. coli strains** | | |
| DH5α | F−Φ80lacZΔM15Δ (lacZYA-argF) U169 recA1 endA1 hsdR17 (rK−, mK+) phoA supE44 l-thi-1 | Laboratory collection |
| **Plasmids** | | |
| pMON 36456 | *E. coli–Synechocystis* hybrid cloning vector, Gen® | 75 |
| pBS CrhR | 3.02-kb *Synechocystis* genomic fragment containing 1.4-kb *crhR* ORF and flanking regions cloned in pBluescript KS+ | 41 |
| pProbeR | pBS with a 93-bp HincII–SacII internal fragment of the *crhR* ORF, riboprobe production | 39 |
described below. The previously reported ΔcrhR strain 2-76 (67) was designated as crhR<sub>TR</sub> to differentiate it from the complete deletion mutant, ΔcrhR. Antibiotics were included as required, spectinomycin and streptomycin (50 µg ml<sup>-1</sup> of each) for crhR<sub>TR</sub>, and kanamycin (50 µg ml<sup>-1</sup>) for ΔcrhR. Photoautotrophic cultures were grown in liquid BG-11 at 30 °C with continuous shaking (150 rpm) and bubbling with humidified air at an illumination of 50 µmol photons m<sup>-2</sup> s<sup>-1</sup> (41). Mixotrophic cultures were grown for an extended period in BG-11 supplemented with glucose (5 mM) to allow acclimation (42). Cold stress was induced in mid-log phase cells at 20 °C for the indicated times. For temperature gradient and transcript half-life analyses, stress was induced in mid-log phase cells at 20 °C for the indicated times prior to RNA manipulation.

**RNA manipulation**

Total RNA was extracted from *Synechocystis* cells treated with an equal volume of 3% phenol/ethanol at the stated growth temperature as defined previously using glass bead lysis and extensive phenol/chloroform extraction (68). RNA samples were resolved in either formaldehyde 1.2% agarose gels for *rimO* transcript analysis or 8 µL urea-8% PAA gels for detecting the *rimO* 5′ UTR. Resolved RNA was either capillary-blotted overnight from agarose gels using 2× SSC or electro-transferred from PAA gels using semi-dry transfer (Tyler) to Hybond-N membranes (Amersham Biosciences). Transferred RNA was UV cross-linked to the membrane with a Stratalinker UV cross-linker model 1800 (120 mJ cm<sup>-2</sup>) for the detection of the *rimO* 5′ UTR transcript, or a 93 nt HincII–SacII fragment internal to the *crhR* ORF from pBS-probeR (Table 2). Oligonucleotides used to locate the riboprobes are indicated in Table 3. The constitutively-expressed transcripts, *rnpB* (primers *rnpBr* and *rnpBl*; Table 3) or 16S rRNA, were utilized as a control for RNA loading. Transcript half-life was determined in the presence of 400 µg ml<sup>-1</sup> rifampicin to inhibit *de novo* mRNA synthesis. Transcript sizes were estimated using either the high-range or low-range RiboRuler RNA ladders (Thermo Fisher Scientific). Hybridized membranes were exposed to X-ray film for 3–5 exposures for each biological replicate. The exposure providing the best representation, depicting a compromise between over- and underexposure, was used for quantification to mitigate potential exposure issues. Quantification of transcript level was performed using *rnpB* abundance as an internal control using ImageJ software version 1.45 S (National Institutes of Health), as described previously (39, 69). Ethidium bromide–stained gel images are also provided as a control for equal RNA loading (Fig. S4). A minimum of two biological repeats was performed for each experiment and representative data shown.

**Identification of operon transcripts**

Total RNA was extracted from cyanobacterial cultures (39) and assessed qualitatively using an RNA Nano Bioanalyzer (Agilent Genomics) and quantitatively by Qubit fluorometry version 2.0 (Thermo Fisher Scientific). RNA was treated twice with RNase-free DNase I (Ambion), and residual contaminating DNA was assessed by the lack of 16S rRNA amplification using the universal bacterial primer pair 27F and 1492R (70). RNA samples were reverse-transcribed using random hexamer primers and Superscript III reverse transcriptase (Invitrogen). PCR amplifications were performed in HF buffer with standard reaction conditions for Phusion HS II (Thermo Fisher Scientific) for 25 cycles. The resulting cDNA libraries (20 ng) were used as templates for the RT-PCR and controls consisting of 50 ng of WT *Synechocystis* genomic DNA and Milli-Q water. Reaction products were separated on a 1.5% agarose-0.5× TBE gel, stained with ethidium bromide, and imaged on a UV transilluminator (CellBioSciences AlphaImager HP). Images shown were inverted.

**Primer extension and S1 nuclease protection assays**

Primer extension and S1 nuclease protection assays were performed as described by Ausubel et al. (71, 72). Poly nucleotide kinase (PNK) (Roche Applied Science) was used to end-
**RNA helicase-mediated RNA processing**

Label primers GWO42 and GWO45 (Table 3) with $\gamma^{32}$P$\cdot$ATP (Amersham Biosciences). For primer extension, AMV-RTase (Promega) was used to extend the $^{32}$P-GWO42 primer bound to total *Synechocystis* RNA (30 μg). dsDNA probes for 5’ nuclease protection assays were produced by PCR from pBS-Crhr (45) using the pBS forward primer (M13 FW/D) and $^{32}$P-GWO45. The GWO45/M13 FW/D probe was annealed to total RNA (15–30 μg) by heat denaturation at 85 °C for 10 min followed by slow cooling to 37 °C over 2 h. 5’ S1 nuclease (Amersham Biosciences) digestion was performed for 30 min at 37 °C. Primer extension and 5’ nuclease samples were separated on 6% DNA sequencing gels, and signals were detected by autoradiography for 3–5 days at −80 °C with an intensifying screen.

### 5’ RACE-RAcE, inverse PCR, and DNA sequencing

The 5’ ends of the transcripts were determined using 5’ RACE-RAcE analysis as described by Polidoros et al. (73). Extracted total RNA, isolated from WT cells grown under photoautotrophic conditions at 20 °C, was treated with RNase-free DNase I (0.2 units per 1 μg of RNA) (Invitrogen) for 15 min at 37 °C. Treated RNA was purified by extensive phenol/chloroform extraction, LiCl (2 m) precipitation, and finally ethanol precipitation and dissolved in RNase-free water (Invitrogen). RNA was tested for genomic DNA contamination on the basis of the absence of PCR amplification of 16S rRNA, using universal bacterial 16S rDNA primers 27F and 1492R (Promega) was used to extend the $^{32}$P-GWO45 primer bound (Amersham Biosciences). For primer extension, AMV-RTase (E assay). Briefly, a DNA template was produced by annealing the ssDNA primers RNaseE_CrhrF and RNaseE_CrhrR (Table 3). The substrate RNA was transcribed with T7 RNA polymerase (Thermo Fisher Scientific), treated with RNA 5’ polyphosphatase (Epicenter), and phosphorylated with T4 polynucleotide kinase (Thermo Fisher Scientific). The 3’ end labeling of the transcript was performed with Cy3 dye (Thermo Fisher Scientific). As a control, a 5’-monophosphorylated RNA oligonucleotide was synthesized that matched the sequence of the CRISPR3 repeat, a previously analyzed substrate for RNase E (48). A codon-optimized and C-terminally His-tagged version of the *Synechocystis* RNase E-encoding gene *sbrl1129* was expressed, and the enzyme was purified as recombinant protein as described previously (48). Cleavage reactions were performed at 30 °C for 30 min in 10 μl of RNase E cleavage buffer (25 mM Tris-HCl, pH 8.0, 60 mM KCl, 5 mM MgCl2, 100 mM NH4Cl, 0.1 mM EDTA) and quenched with the addition of 2× RNA loading dye (95% formamide, 0.025% SDS, 0.025% bromphenol blue, 0.025% xylene cyanol FF 0.5 mM, EDTA, pH 8.0). Following a 5-min denaturation at 95 °C, reactions (80 ng of RNA) were separated on an 8 x 8 cm–2% PAA gel, stained with SYBR Gold Nucleic Acid Gel Stain (Thermo Fisher Scientific; 1:10,000), and visualized with a Laser Scanner Typhoon FLA 9500 (GE Healthcare; excitation 473 nm, emission filter long-pass blue ≥510 nm, or excitation 532 nm, emission filter BPG1 560–580 nm in case of Cy3-labeled RNA, photomultiplier value 450 or 500).

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