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The LovK-LovR Two-Component System Is a Regulator of the General Stress Pathway in *Caulobacter crescentus*

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A conserved set of regulators control the general stress response in *Caulobacter crescentus*, including σ^T, its anti-σ factor NepR, the anti-anti-σ factor PhyR, and the transmembrane sensor kinase PhyK. We report that the soluble histidine kinase LovK and the single-domain response regulator LovR also function within the *C. crescentus* general stress pathway. Our genetic data support a model in which LovK-LovR functions upstream of σ^T by controlling the phosphorylation state and thus anti-anti-σ activity of PhyR. Transcription of *lovK* and *lovR* is independently activated by stress through a mechanism that requires *sigT* and *phyR*. Conversely, *lovK* and *lovR* function together to repress transcription of the general stress regulon. Concordant with a functional role of the LovK-LovR two-component system as a negative regulator of the general stress pathway, *lovK-lovR*-null mutants exhibit increased cell survival after osmotic stress, while coordinate overexpression of *lovK* and *lovR* attenuates cell survival relative to that of the wild type. Notably, *lovK* can complement the transcriptional and cell survival defects of a *phyK*-null mutant when *lovR* is deleted. Moreover, in this same genetic background, σ^T-dependent transcription is activated in response to osmotic stress. This result suggests that flavin-binding LOV (light, oxygen, or voltage) histidine kinases are competent to perceive cytoplasmic signals in addition to the environmental signal blue light. We conclude that the PhyK-PhyR and LovK-LovR two-component signaling systems coordinately regulate stress physiology in *C. crescentus*.

Two-component signal transduction systems (TCS) (40), alternative sigma (σ) factors (14, 15), and "one-component" regulators (43) control transcription in bacteria in response to environmental perturbation. While these regulatory proteins generally function independently of one another, the alphaproteobacteria encode a novel pathway that combines features of TCS and alternative σ regulation to control transcription under a range of stress conditions (3, 8, 13, 25). At the core of this system is the PhyR protein, which contains a σ-like domain positioned N-terminally to a TCS receiver domain (9, 12). PhyR functions to integrate signals from a transmembrane stress sensor histidine kinase (25) to affect transcription via an extracytoplasmic-function (ECF) σ factor (3, 8, 13). PhyR itself does not function as a true sigma factor. Rather, phospho-PhyR activates an EcfG-family (39) σ factor through its direct interaction with the anti-σ^{EcfG} protein NepR (3, 8, 13, 22, 25) (Fig. 1A).

Orthologs of PhyR, NepR, and σ^{EcfG} are broadly conserved in the alphaproteobacteria. These genes are most often found together in a chromosomal stress response locus where *phyR* is adjacent to, and in the opposite orientation to, a *nepR-ecfG* operon (Fig. 1B) (8, 11, 37, 39). In many but not all cases, a gene encoding a sensory histidine kinase is found at this chromosomal locus, suggesting a possible functional role as a stress sensor. This was recently confirmed in *Caulobacter crescentus*: PhyK, a transmembrane histidine kinase (Fig. 1B) encoded three genes away from *phyR*, mediates stress-dependent transcription and cell survival and can phosphorylate PhyR *in vivo* (25). In *C. crescentus*, the ortholog of *ecfG* known as *sigT* regulates adaptation to osmotic and oxidative stress (2) and carbon limitation (5); *nepR* and *phyR* function in the same pathway as *sigT* (17, 25).

We have identified a second two-component system, known as LovK-LovR, that has a regulatory role in the *C. crescentus* general stress-signaling pathway. The sensor histidine kinase LovK binds a flavin mononucleotide (FMN) cofactor (33) via an N-terminal LOV (light, oxygen, and voltage) domain (16) and has the capacity to perceive both blue light and changes in the reductive state of its environment (33, 34). LovK and LovR are encoded from a single locus on the *C. crescentus* chromosome (Fig. 1C) (34). Herein, we present evidence that transcription of *lovK* and *lovR* is independently upregulated by the general stress sigma factor σ^T. When coordinately overexpressed, LovK and LovR globally repress PhyK-PhyR-σ^T-dependent transcription and reduce the capacity of the cell to survive extended exposure to high-osmotic-strength medium. Deletion of *lovK*, *lovR*, or the entire *lovK-lovR* locus results in derepression of σ^T-dependent transcription and increased cell survival after acute osmotic stress. Epistasis analysis of single and double *lovK* and *lovR* mutants in Δ*sigT*, Δ*phyR*, and Δ*phyK* backgrounds provides support for a model in which the LovK-LovR two-component system functions upstream of σ^T by controlling the concentration of phospho-PhyR (PhyR∼P). We thus conclude that the LovK-LovR two-component system both regulates and is regulated by the PhyK-PhyR-σ^T stress response system.

MATERIALS AND METHODS

General growth conditions. *Escherichia coli* strains were cultured in LB liquid medium or grown on LB agar (15 g/liter) at 37°C supplemented with antibiotics as appropriate at the following concentrations: kanamy-

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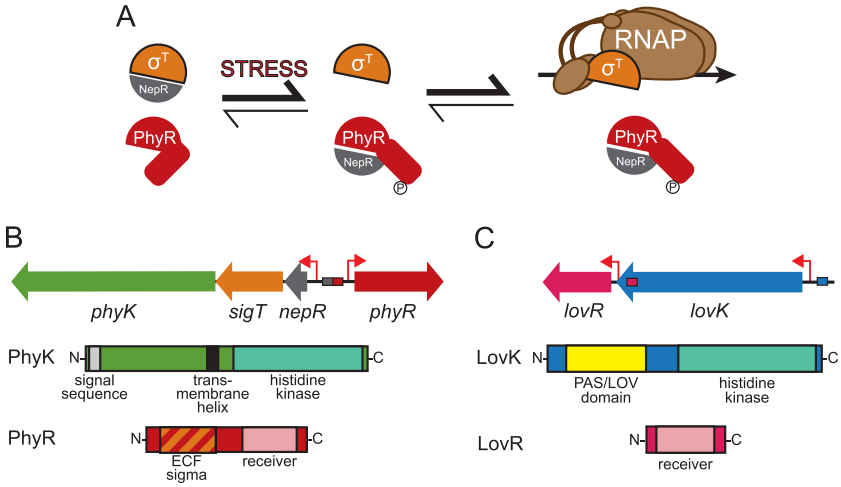

FIG 1 (A) Model of the alphaproteobacterial PhyR-NepR-ECF σ partner switching system that regulates general stress response. When phosphorylated under stress conditions, PhyR activates an EcfG-family sigma factor (σ^T) by titrating away the anti-σ factor NepR. σ^T is then released to interact with RNA polymerase and DNA to regulate transcription. (B) Genes in the *C. crescentus* general stress response locus with the protein domain structures for the sensor histidine kinase PhyK (CC_3474/CCNA_03588) and the response regulator PhyR (CC_3477/CCNA_03591). PhyK is an integral membrane sensor histidine kinase; PhyR is a chimeric receiver protein, possessing an amino-terminal ECF σ-like domain (orange and red hatching) and a carboxy-terminal receiver domain (pink). SigT (σ^T) (CC_3475/CCNA_03589) is an EcfG-family σ factor, and NepR (CC_3476/CCNA_03590) is its anti-σ factor. (C) The *lovK-lovR* chromosomal locus, with protein domain structures for each gene product. LovK (CC_0285/CCNA_00287) is a cytoplasmic histidine kinase with a flavin mononucleotide (FMN)-binding PAS/LOV domain (yellow); LovR (CC_0284/CCNA_00286) is a single-domain receiver (pink). Red arrows mark predicted transcriptional start sites. EcfG-regulatory sites, classified as group ECF15 motifs by Staron and colleagues (39), are shown as small boxes colored according to the genes they are predicted to regulate.

cin, 50 μg/ml; tetracycline, 12 μg/ml; and spectinomycin-streptomycin, 50 μg/ml–30 μg/ml. *C. crescentus* colonies were grown on peptone-yeast extract (PYE)-agar plates (6) (15 g agar/liter) at 30°C. Liquid *C. crescentus* cultures were grown in M2 defined medium (6) supplemented with 0.15% xylose (M2X) as the carbon source. To induce expression from P_{van}, 500 μM vanillate (final concentration) was added to all cultures in experiments, including those with P_{van}-*lovK* bearing strains. Antibiotics for selection in *C. crescentus* were used at the following concentrations: kanamycin, 5 μg/ml (liquid) and 25 μg/ml (solid); tetracycline, 1 μg/ml (liquid) and 2 μg/ml (solid); and spectinomycin-streptomycin, 25 μg/ml–5 μg/ml (liquid) and 100 μg/ml–5 μg/ml (solid).

Plasmid construction. *C. crescentus* DNA was amplified from colonies using KOD Xtreme hot-start polymerase (EMD Biosciences/Novagen). Reaction mixtures were supplemented with 5% dimethyl sulfoxide (DMSO). Restriction sites for cloning were added to the ends of the primers (see Table S1 in the supplemental material for primer sequences). All plasmids were cloned in *E. coli* Top10 (Invitrogen, Carlsbad, CA). The sequences of all cloned products were confirmed in the target plasmids.

Transcriptional reporter plasmids were generated by PCR amplifying ~500 bases upstream of *lovK*, *lovR*, or *sigU* and ligating this fragment into the EcoRI and HindIII sites upstream of *lacZ* in pRKlac290. Similarly, transcriptional fusions to confirm regulated genes identified by Affymetrix microarray were generated by cloning putative regulated promoters into the KpnI and HindIII sites upstream of *lacZ* in pRKLac290. To generate a xylose-inducible *phyR* overexpression plasmid, the *phyR* open reading frame was amplified with its stop codon and cloned into the NdeI and EcoRI sites of pMT585 (42). Deletion alleles of the *lovK-lovR* locus and the *phyK* gene were generated by directionally cloning approximately 500 bp upstream and downstream of each locus as well as the first and last several codons of each into the multiple cloning site of pNPTS138 to yield in-frame null alleles of each. To generate the *lovK-lovR* complementation plasmid, this locus, including *lovK*, *lovR*, and approximately 500 bp on either side of the genes to maintain endogenous transcriptional control, was PCR amplified and cloned into the EcoRI site of pMT862 (42). The resulting plasmid was integrated into the *C. crescentus* chromosome at the *vanR* locus. In all cases, the reannotated start site of *lovK* described previously (34) was used as the basis for cloning. The exact limits of the cloned regions are defined by the primers listed in Table S1 in the supplemental material.

Strain construction. pRKlac290-based transcriptional reporter plasmids were conjugated into *C. crescentus* strains by triparental mating (6) using the *E. coli* helper strain FC3 (see Table 1 for strains). Briefly, triparental matings were performed by mixing the donor *E. coli* strain, the helper strain, and the *C. crescentus* recipient strain in a 1:1:5 ratio. Mixed cells were incubated nonselectively on solid PYE for 12 to 24 h. *C. crescentus* cells containing the desired plasmid were then selected on solid PYE containing nalidixic acid (20 μg/ml) to counterselect against *E. coli* and a plasmid-appropriate antibiotic. All other plasmids were purified from *E. coli* and electroporated into *C. crescentus* strains. Electroporations were performed using a Bio-Rad MicroPulser using the manufacturer's settings for *E. coli*.

The Δ*lovK-lovR* and Δ*phyK* in-frame deletion strains were constructed using a double recombination strategy (36). Each pNPTS138-derived allele-replacement plasmid was transformed into *C. crescentus* by electroporation. Primary integrants were selected on PYE-Kan plates. Overnight growth in nonselective liquid medium followed by growth on solid PYE supplemented with 3% sucrose allowed identification of clones in which the plasmid had been excised in a second recombination event. PCR was used to confirm chromosomal deletions in kanamycin-sensitive clones.

Stress survival assays. Cultures were inoculated from freshly grown colonies on solid PYE medium in minimal medium (M2X) supplemented with vanillate as appropriate and grown overnight to saturation (optical density at 600 nm [OD_{660}] ≥ 0.8). Cultures were then diluted to an OD_{660} of 0.025 with fresh medium and allowed to outgrow for approximately 16 h to ensure uniformly saturated cultures. Cultures were diluted once more to an OD_{660} of 0.05, separated into equal volumes in two culture tubes, and grown for 1 h at 30°C in a roller prior to stress. To induce osmotic stress, 150 mM sucrose (final concentration) was added to one tube and an equivalent volume of water was added to the paired control (unstressed) culture as previously described (2). CFU in both the stressed and unstressed cultures were enumerated. Cell survival was measured as the

TABLE 1 Strains

Strain	Genotype	Reference or source
C. crescentus		
FC19	CB15 (wild type)	32
FC423	CB15 *vanR*::pMT528 *xylX*::pMT585	34
FC438	CB15 *vanR*::pMT528-*lovK xylX*::pMT585-*lovR*	34
FC799	CB15 Δ*phyR*	17
FC626	CB15 *xylX*::pMT585-*phyR*	This study
FC879	CB15/pRKlac290-P_{lovK}	This study
FC892	CB15 Δ*sigT*/pRKlac290-P_{lovK}	This study
FC896	CB15 Δ*phyR*/pRKlac290-P_{lovK}	This study
FC880	CB15/pRKlac290-P_{lovR}	This study
FC893	CB15 Δ*sigT*/pRKlac290-P_{lovR}	This study
FC897	CB15 Δ*phyR*/pRKlac290-P_{lovR}	This study
FC642	CB15/pRKlac290-P_{sigU}	This study
FC1628	CB15 *vanR*::pMT528 *xylX*::pMT585/pRKlac290-P_{sigU}	This study
FC1630	CB15 *vanR*::pMT528-*lovK*/pRKlac290-P_{sigU}	This study
FC1629	CB15 *xylX*::pMT585-*lovR*/pRKlac290-P_{sigU}	This study
FC1631	CB15 *vanR*::pMT528-*lovK xylX*::pMT585-*lovR*/pRKlac290-P^{sigU}	This study
FC1708	CB15 Δ*lovKR vanR*::pMT528 *xylX*::pMT585/pRKlac290-P_{sigU}	This study
FC1709	CB15 Δ*lovKR vanR*::pMT528-*lovK*/pRKlac290-P_{sigU}	This study
FC1710	CB15 Δ*lovKR xylX*::pMT585-*lovR*/pRKlac290-P_{sigU}	This study
FC1711	CB15 Δ*lovKR vanR*::pMT528-*lovK xylX*::pMT585-*lovR*/pRKlac290-P_{sigU}	This study
FC1697	CB15 *vanR*::pMT528-*lovK xylX*::pMT585-*lovR*(*D57A*)/pRKlac290-P_{sigU}	This study
FC1698	CB15 *vanR*::pMT528-*lovK*(*H180A*) *xylX*::pMT585-*lovR*/pRKlac290-P_{sigU}	This study
FC1699	CB15 *vanR*::pMT528-*lovK*(*H180A*) *xylX*::pMT585-*lovR*(*D57A*)/pRKlac290-P_{sigU}	This study
FC1188	CB15 Δ*lovK*/pRKlac290-P_{sigU}	This study
FC1189	CB15 Δ*lovR*Ω/pRKlac290-P_{sigU}	This study
FC1190	CB15 Δ*lovKR*/pRKlac290-P_{sigU}	This study
FC455	CB15 Δ*lovK*	34
FC301	CB15 Δ*lovR*Ω	34
FC792	CB15 Δ*lovKR*	This study
FC1411	CB15 Δ*lovKR vanR*::pMT862-P_{lovK}-*lovK-lovR*	This study
FC820	CB15 Δ*sigT*/pRKlac290-P_{sigU}	This study
FC1637	CB15 Δ*sigT* Δ*lovKR*/pRKlac290-P_{sigU}	This study
FC1632	CB15 Δ*sigT vanR*::pMT528 *xylX*::pMT585/pRKlac290-P_{sigU}	This study
FC1634	CB15 Δ*sigT vanR*::pMT528-*lovK*/pRKlac290-P_{sigU}	This study
FC1633	CB15 Δ*sigT xylX*::pMT585-*lovR*/pRKlac290-P_{sigU}	This study
FC1635	CB15 Δ*sigT vanR*::pMT528-*lovK xylX*::pMT585-*lovR*/pRKlac290-P_{sigU}	This study
FC814	CB15 Δ*phyR*/pRKlac290-P_{sigU}	This study
FC1681	CB15 Δ*phyR* Δ*lovKR*/pRKlac290-P_{sigU}	This study
FC1684	CB15 Δ*phyR vanR*::pMT528 *xylX*::pMT585/pRKlac290-P_{sigU}	This study
FC1685	CB15 Δ*phyR vanR*::pMT528-*lovK*/pRKlac290-P_{sigU}	This study
FC1686	CB15 Δ*phyR xylX*::pMT585-*lovR*/pRKlac290-P_{sigU}	This study
FC1687	CB15 Δ*phyR vanR*::pMT528-*lovK xylX*::pMT585-*lovR*/pRKlac290-P_{sigU}	This study
FC674	CB15 Δ*sigT*	17
FC1586	CB15 Δ*sigT* Δ*lovKR*	This study
FC1244	CB15 Δ*sigT vanR*::pMT528 *xylX*::pMT585	This study
FC1247	CB15 Δ*sigT vanR*::pMT528-*lovK xylX*::pMT585-*lovR*	This study
FC1701	CB15 Δ*phyK*/pRKlac290-P_{sigU}	This study
FC1703	CB15 Δ*phyK vanR*::pMT528 *xylX*::pMT585/pRKlac290-P_{sigU}	This study
FC1704	CB15 Δ*phyK vanR*::pMT528-*lovK*/pRKlac290-P_{sigU}	This study
FC1705	CB15 Δ*phyK xylX*::pMT585-*lovR*/pRKlac290-P_{sigU}	This study
FC1706	CB15 Δ*phyK vanR*::pMT528-*lovK xylX*::pMT585-*lovR*/pRKlac290-P_{sigU}	This study
FC1738	CB15 Δ*phyK vanR*::pMT528-*lovK*(*H180A*)/pRKlac290-P_{sigU}	This study
FC1702	CB15 Δ*phyK* Δ*lovKR*/pRKlac290-P_{sigU}	This study
FC1741	CB15 Δ*phyK* Δ*lovKR vanR*::pMT528 *xylX*::pMT585/pRKlac290-P_{sigU}	This study
FC1742	CB15 Δ*phyK* Δ*lovKR vanR*::pMT528-*lovK*/pRKlac290-P_{sigU}	This study
FC1743	CB15 Δ*phyK* Δ*lovKR xylX*::pMT585-*lovR*/pRKlac290-P_{sigU}	This study
FC1744	CB15 Δ*phyK* Δ*lovKR vanR*::pMT528-*lovK xylX*::pMT585-*lovR*/pRKlac290-P_{sigU}	This study
FC1746	CB15 Δ*phyK* Δ*lovKR vanR*::pMT528-*lovK*(*H180A*)/pRKlac290-P_{sigU}	This study
FC1688	CB15 Δ*phyK*	This study

(Continued on following page)

TABLE 1 (Continued)

Strain	Genotype	Reference or source
FC1692	CB15 Δ*phyK vanR*::pMT528-*lovK*	This study
FC1689	CB15 Δ*phyK* Δ*lovKR*	This study
FC1725	CB15 Δ*phyK* Δ*lovKR vanR*::pMT528-*lovK*	This study
E. coli		
FC3	MT607/pRK600	7
FC55	DH10B/pNPTS138	M. R. K. Alley
FC54	S17-1/pRKlac290	10
FC339	TOP10/pMT585 (pXGFPC-2)	42
FC338	TOP10/pMT528 (pVCHYC-1)	42
LS4270	TOP10/pMT862 (pVCERC-2)	42
FC634	TOP10/pRKlac290-P_{sigU}	This study
FC872	TOP10/pRKlac290-P_{lovK}	This study
FC873	TOP10/pRKlac290-P_{lovR}	This study
FC1651	TOP10/pRKlac290-P_{cc_0201}	This study
FC1652	TOP10/pRKlac290-P_{cc_0280}	This study
FC1645	TOP10/pRKlac290-P_{cc_0559}	This study
FC1646	TOP10/pRKlac290-P_{cc_1189}	This study
FC1654	TOP10/pRKlac290-P_{cc_3466}	This study
FC1649	TOP10/pRKlac290-P_{cc_3473}	This study
FC1659	TOP10/pRKlac290-P_{cc_3147}	This study
FC1215	TOP10/pMT585-*phyR*	This study
FC364	TOP10/pMT585-*lovR*	34
FC533	NEB5α/pMT585-*lovR*(*D57A*)	34
FC432	NEB5α/pMT528-*lovK*	34
FC490	TOP10/pMT528-*lovK*(*H180A*)	34
FC1456	Mach1/pMT862-P_{lovK}-*lovK-lovR*	This study

$CFU_{stressed}/CFU_{unstressed}$ ratio and normalized to the mean wild-type survival ratio. To assess the statistical significance of the survival differential between wild-type and mutant strains, cell survival ratios were log transformed and subjected to one-way analysis of variance (ANOVA) followed by a multiple comparison posttest. For oxidative stress, cells were treated as described above prior to the addition of stress. Instead of sucrose, hydrogen peroxide was added to a final concentration of 0.2 mM. CFU were enumerated after 1 h of treatment, and cell survival was calculated in the same manner as described above.

β-Galactosidase Assays. Strains bearing transcriptional reporter plasmids were inoculated from freshly grown colonies into M2X medium containing 1 μg/ml tetracycline and vanillate as appropriate. To assay regulation of *lovK*, *lovR*, or *sigU* promoter activity, overnight cultures were diluted to an OD_{660} of 0.025 with fresh medium, allowed to outgrow for approximately 16 h to ensure uniformly saturated cultures, and diluted once more to OD_{660} of 0.1 with fresh medium. This culture was then split into equal volumes in two culture tubes and grown for 1 h at 30°C and shaking. As above, one tube was subjected to osmotic stress by the addition of 150 mM sucrose (final concentration) and an equivalent volume of water was added to the paired control (unstressed) culture. Cultures were incubated at 30°C with shaking for 4 h before β-galactosidase activity was measured. To validate basal regulation of genes detected by microarray analysis, strains bearing transcriptional reporter plasmids were inoculated from freshly grown colonies into M2X supplemented with vanillate. Cultures were grown as described above and, upon dilution to an OD_{660} of 0.1, outgrown for 4 h before β-galactosidase activity was assayed. β-Galactosidase activity was measured colorimetrically (28). Briefly, 600 μl of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) and an excess of *o*-nitrophenyl-β-D-galactopyranoside were added to 200 μl of chloroform-permeabilized cells. Upon development of a yellow color, the reaction was stopped by the addition of 1 ml of 1 M sodium carbonate, and absorbance was measured at 420 nm on a Spectronic Genesys 20 spectrophotometer (ThermoFisher Scientific, Waltham, MA). Miller units were calculated as (A_{420} of reaction mixture × 1,000)/(A_{660} of cells × t × v), where t is reaction time in minutes and v is volume of cells used in ml. Data in the figures are from three independent biological replicates grown in parallel on the same day.

Affymetrix transcriptome profiling. (i) Growth conditions. To identify genes basally regulated by PhyR, two independent cultures each of a strain overexpressing *phyR* (FC626), a strain with *phyR* deleted (FC799), and the wild type (FC19) were inoculated from freshly grown colonies into M2X medium. Cells were grown in a roller at 30°C overnight, diluted to an OD_{660} of 0.05, and grown for ~4 h to a final OD_{660} of 0.25. To assess genes regulated by overexpression of *lovK* and *lovR*, two independent cultures each of an empty-vector control (FC423) and a *lovK-lovR* coordinate overexpression strain (FC438) (34) were grown from fresh colonies. Cells were grown under the conditions described above except that the medium was supplemented with 500 μM vanillate; *lovR* expression was constitutively induced by xylose, the carbon source in M2X medium. Cells were harvested at an OD_{660} of 0.25 for RNA purification.

(ii) RNA isolation and hybridization conditions. RNA was isolated by TRIzol (Invitrogen, Carlsbad, CA) extraction. Briefly, 5 ml of culture was centrifuged for 1 min at top speed to pellet the cells, supernatant was removed, and the pellet was resuspended in 1 ml of TRIzol. A standard protocol for RNA isolation using TRIzol was followed, complete with DNase I digestion (4). Briefly, after addition of TRIzol and chloroform, nucleic acid in the aqueous layer was isopropanol precipitated overnight at −80°C followed by a 30-min centrifugation at 16,000 × *g*. The ethanol-washed and air-dried nucleic acid pellet was resuspended in 50 μl of nuclease-free water (IDT, Coralville, IA). RNase-free DNase I (1 μl; Ambion, Austin, TX) was added to the sample and incubated at room temperature for 2 h to remove any residual DNA. The nucleic acid in this digested sample was then acid phenol-chloroform (Ambion, Austin, TX) extracted, ethanol precipitated at −80°C overnight, and centrifuged at 16,000 × *g* to produce a DNA-free RNA pellet. RNA integrity was checked on a Bioanalyzer (Agilent, Santa Clara, CA), and RNA concentration was

determined by UV spectrophotometry using a Shimadzu UV-1650 spectrophotometer (Kyoto, Japan).

Ten micrograms of each RNA sample was processed to produce single-strand cDNA, and RNA was removed using 1 N NaOH. cDNA was column purified, fragmented using DNase I (GE Life Sciences, Piscataway, NJ), and end-labeled using GeneChip labeling reagent (P/N 900542; Affymetrix, Santa Clara, CA). Labeled cDNA was hybridized to GeneChip CauloHi1 according to the GeneChip expression analysis technical manual (Affymetrix, Santa Clara, CA). After hybridization for 16 h at 50°C, arrays were washed using protocol PRO-GE-W52-V3 and stained on a GeneChipFluidics station (Affymetrix) according to the GeneChip expression analysis technical manual. The arrays were scanned using an Affymetrix GeneChip 3000 7G scanner, and CEL intensity files were generated by GCOS (GeneChip operating software) v. 1.4.

Microarray data accesion numbers. Array data have been deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo) under series accession numbers GSE21205 and GSE21204.

RESULTS

Transcription of *lovK* and *lovR* is independently controlled by σ^T and PhyR in response to osmotic stress. *C. crescentus* LovK is a cytoplasmic sensor histidine kinase that has the capacity to perceive multiple environmental signals via a bound FMN cofactor (33, 34). *lovK* is separated by 19 bp from the single-domain receiver gene, *lovR* (Fig. 1C), which is under positive transcriptional control of PhyK, PhyR, and σ^T (i.e., the general stress response system) (2, 25). Canonical σ^{EcfG}-type regulatory sequences (39) are found upstream of both *lovK* and *lovR* (Fig. 1C), suggesting that σ^T may independently regulate each gene. The σ^{EcfG} regulatory motif upstream of *lovR* (**GGAAC**CTGGAACCCGCGTGGT **CGTT**; boldface indicates the conserved −35 and −10 sites) is located within the 3′ end of *lovK* and is centered 58 bp upstream of the predicted *lovR* start codon. The σ^{EcfG} motif upstream of *lovK* (**GGAAC**TCGACCGGATCAGATA**CGTT**) is centered 43 bp upstream of the reannotated (26, 34) *lovK* start codon (Fig. 1C).

Prior to this study, it was not known whether transcription of the *lovK* sensor kinase was regulated by the general stress system. We first conducted a microarray transcriptional profiling experiment comparing Δ*phyR* (FC799), wild-type (FC19), and *phyR* overexpression (FC626) strains. This *C. crescentus phyR* transcriptome analysis differs in three ways from what was recently reported by Lourenço and colleagues (25). First, none of the cells in our analysis were subjected to explicit stress, i.e., the experiment was designed to evaluate basal regulation by PhyR. Second, a different microarray platform was utilized. Third, we manually analyzed individual probes on the array in the region of *lovK* to better quantify expression from the reannotated *lovK* gene sequence. Our array data provide evidence that basal transcription of both *lovK* and *lovR* is regulated by PhyR (Table 2; also, see Table S2 in the supplemental material). This result places the genes encoding the LovK-LovR system under positive transcriptional control of the general stress pathway in *C. crescentus*.

To validate these microarray data and to determine if *lovK* and/or *lovR* transcription is regulated by σ^T and PhyR in response to stress, we generated transcriptional reporters by fusing the region upstream of each gene to *lacZ* (see Table S2 in the supplemental material for primers). β-Galactosidase activity was measured in wild-type, Δ*sigT*, and Δ*phyR* genetic backgrounds in the presence or absence of osmotic stress (150 mM sucrose). The data show that an active promoter is present upstream of each gene; thus, both *lovK* and *lovR* can be independently regulated (Fig. 2). Transcription from both promoters increases significantly in response to osmotic stress (one-way ANOVA, $P < 0.001$; Dunnett's posttest, $P < 0.01$). Moreover, both basal transcription and stress-dependent regulation of these genes require *sigT* and *phyR* (Fig. 2) (one-way ANOVA, $P < 0.001$; Dunnett's posttest, $P < 0.01$). From this, we conclude that transcription of *lovK* and *lovR* is σ^T/PhyR dependent and stress regulated.

LovK and LovR are negative regulators of genes in the general stress pathway. Given that two-component systems often regulate transcription, we sought to determine the transcriptional effect of coordinate *lovK* and *lovR* overexpression and to evaluate the possible function of these genes in the σ^T-PhyR general stress pathway. Using the CauloHi1 Affymetrix GeneChip system, we compared the transcriptional profile of a wild-type empty vector control strain (FC423) and a strain coordinately overexpressing *lovK* and *lovR* from vanillate- and xylose-inducible promoters, respectively (FC438). Coordinate overexpression of *lovK* and *lovR* results in the repression (≥2-fold) of 48 genes, nine of which are substantially reduced (>10-fold) compared to the vector control (see Table S2 in the supplemental material). Among the most highly repressed transcripts in this data set are *phyR* and the *nepR-sigT* operon, each of which is regulated by the general stress sigma factor σ^T (2, 17, 25). The majority of the transcripts repressed by overexpression of *lovK* and *lovR* are regulated by σ^T and/or PhyR (Table 2); 58% of transcripts repressed ≥2-fold, 72% of transcripts repressed ≥3-fold, and 92% of transcripts repressed ≥5-fold are known to be controlled by σ^T and/or PhyR. This regulatory overlap provides evidence that the LovK-LovR two-component system has a repressive effect on the general stress pathway.

The set of transcripts enhanced by coordinate *lovK-lovR* overexpression is smaller (Table 2; also, see Table S2 in the supplemental material). Twenty-four transcripts (not including *lovK* and *lovR*) are at least 2-fold more abundant in cells overexpressing *lovK* and *lovR*. Only 4 genes are >3-fold more abundant; no genes exhibit an increase of 5-fold or more (see Table S2 in the supplemental material). Three transcripts activated by *lovK-lovR* overexpression (CC_0933, CC_2610, and CC_3147) are also activated to a similar extent by deletion of *phyR* and/or deletion of *sigT* (Table 2). To assess the quality of these array data, we validated regulation of genes in this set using promoter-*lacZ* fusions; regulation of *sigU* is described below, and regulation of seven other genes is described in the supplemental material (see Fig. S1).

Repression of σ^T-dependent transcription requires both *lovK* and *lovR* and the conserved phosphorylation sites on each. The data presented above provide evidence for a regulatory connection between the LovK-LovR two-component system and the general stress signaling pathway in *C. crescentus*. We sought to test (i) whether both LovK and LovR are required for transcriptional repression of σ^T-dependent genes, (ii) the effect of overexpression of *lovK* and *lovR* on σ^T-dependent transcription under a known activating stress condition, and (iii) whether the conserved phosphorylation sites on LovK and LovR are required for the observed transcriptional repression. To address these questions, we utilized a reporter plasmid in which the known σ^T-dependent promoter P_{sigU} (2) was transcriptionally fused to *lacZ*. We evaluated β-galactosidase activity from this fusion in strains individually overexpressing either *lovK* or *lovR*, coordinately overexpressing both, and in an empty vector control strain. In all cases, cells were cultured under continuous expression induction conditions (i.e., in

TABLE 2 LovK-LovR, PhyR, and σ^T regulate an overlapping set of genes[a]

Locus	Annotated function	Regulation result			Figure showing validation[e]
		lovKR++[b]	Δ*phyR*[c]	Δ*sigT*[d]	
Down-regulated genes					
CC_0163	EPS associated protein	2.6	3.5	A	
CC_0201	OmpA-family outer membrane lipoprotein	4.7	3.0	F-A, L	S1
CC_0280	Conserved cell surface protein	35	22	F, A, L	S1
CC_0284	LovR, receiver protein	NA	2.0	F, A, L	2
CC_0285	LovK, cytoplasmic sensor histidine kinase	NA	2.8	F	2
CC_0447	Beta-*N*-acetylhexosaminidae		3.6	A	
CC_0501	Hypothetical protein	4.4		L	
CC_0554	Conserved hypothetical protein	3.5	3.0		
CC_0555	FixC-family flavoprotein dehydrogenase	2.8	2.1		
CC_0556	Conserved hypothetical protein, catalase	3.2	4.3	L	
CC_0557	Conserved hypothetical protein	3.4	3.1	L	
CC_0558	Putative outer membrane lipoprotein	4.9	(1.8)		
CC_0559	DPS-family DNA stress protection protein	2.6	2.0	F	S1
CC_0747	OmpA-family outer membrane lipoprotein	8.2	4.7	A, L	
CC_0938	CsbD-related stress response protein	2.8	2.0	A, L	
CC_1178	GsbI general stress protein	54	16	L	
CC_1179	Hypothetical protein	8.9	3.1	A, L	
CC_1189	CESA-like glycosyltransferase	36	3.4	F	S1
CC_1356	MucR-family transcriptional regulator	32	92	A, L	
CC_1532	Conserved hypotethical protein	65	13	A, L	
CC_2383	GalE, UDP-glucose 4-epimerase	2.6	4.6		
CC_2384	PssZ, polyisoprenylphosphate hexose-1-phosphotransferase	4.0	4.7		
CC_2549	SOUL domain heme-binding protein	2.0	3.3	A, L	
CC_2883	SigU, ECF-family sigma factor	104	6.2	A, L	3
CC_3225	PAS-family sensor histidine kinase	2.5	(1.7)	A, L	
CC_3466	CsbD-related stress response protein	29	14.7	F, L	S1
CC_3473	Entericidin family protein	5.3	3.0	F, L	S1
CC_3474	PhyK, transmembrane sensor histidine kinase	4.0	5.9		
CC_3475	SigT, ECF-family sigma factor	6.4	6.0	A, L	
CC_3476	NepR, anti-sigma factor protein	2.3	2.0	A, L	
CC_3477	PhyR, stress regulatory protein	10	36[f]	H, L	
CC_3618	Mannose-1-phosphate guanylyltransferase	2.4	2.0		
Upregulated genes					
CC_0933	Ros-MucR-superfamily transcriptional regulator	2.1	2.5	A	
CC_2610	Hemolysin-type calcium binding protein	2.1		A	
CC_3147	TonB-dependent outer membrane receptor	5.0	3.6	F, A	S1

[a] Transcripts regulated >2-fold in at least two transcriptome analyses. The complete set of genes regulated by LovK-LovR or PhyR is presented in Table S2 in the supplemental material.
[b] Fold change compared to empty vector control strain. NA, not applicable.
[c] Fold change compared to *phyR* overexpression strain. Values in parentheses are for two transcripts weakly regulated by *phyR* which are regulated by LovK-LovR and by *sigT*.
[d] Observation of *sigT*-dependent regulation in reference 2 (A), reference 17 (H), reference 25 (L), or this work (F).
[e] Transcriptional regulation of indicated genes was confirmed using promoter-*lacZ* fusions. Data are presented in the figure listed.
[f] Calculated from probes complementary to sequences in the 5′ UTR of *phyR* not present in the overexpression construct.

M2-xylose medium supplemented with vanillate) in either the presence or absence of osmotic stress. In the absence of stress, overexpression of *lovK* does not have a significant effect on σ^T-dependent transcription; overexpression of *lovR* results in a 2.5-fold decrease in transcription (one-way ANOVA, $P < 10^{-4}$; post-test, $P < 0.01$) (Fig. 3A). Coordinate overexpression of both *lovK* and *lovR* is required to strongly repress reporter transcription to a level that is comparable to what we observed by microarray ($P < 0.001$) (Fig. 3A). Osmotic stress conditions induce σ^T-dependent transcription as expected in the wild-type vector control strain. While stress-dependent induction of transcription is weakly attenuated in strains overexpressing either *lovK* or *lovR*, coordinate overexpression of both genes nearly abolishes osmotic stress-induced transcription from the reporter plasmid ($P < 0.001$) (Fig. 3A).

These data show that overexpression of either *lovK* or *lovR* is insufficient to fully repress σ^T-dependent transcription with or without explicit stress; the strong repressive effect of coordinate *lovK* and *lovR* overexpression provides genetic evidence that these two genes function together in the same pathway. We note that overexpression of *lovR* alone does result in partial repression of σ^T-dependent transcription in both the presence and absence of osmotic stress. However, this experiment was conducted in an otherwise wild-type genetic background; i.e., endogenous *lovK* was present. To test if the partial repression of P_{sigU} transcription resulting from *lovR* overexpression requires *lovK*, we repeated this

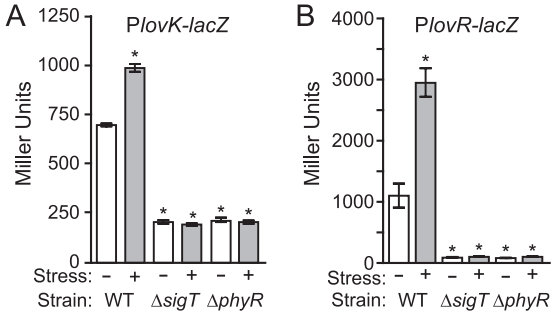

FIG 2 Independent regulation of *lovK* and *lovR* transcription by σ^T-PhyR. The presence of a functional promoter in the regions directly upstream of *lovK* or *lovR* start codons was evaluated by measuring β-galactosidase activity from *lacZ* transcriptional fusions. To assess stress-dependent regulation, cultures were exposed to 150 mM sucrose for 4 h (unstressed cultures in white; stressed cultures in gray). (A) Transcription from a P_{lovK}-*lacZ* fusion was measured in wild-type (WT), Δ*sigT*, and Δ*phyR* backgrounds. (B) Transcription from a P_{lovR}-*lacZ* fusion was measured in wild-type (WT), Δ*sigT*, and Δ*phyR* backgrounds. β-Galactosidase activities are means ± standard errors of the means (SEM) from two independent experiments ($n = 6$; *, one-way ANOVA, $P < 0.0001$; Dunnett's posttest for comparison to wild type, $P < 0.001$).

experiment in a Δ*lovK-lovR* genetic background (Fig. 3B). In the absence of native *lovK* and *lovR*, coordinate overexpression of both *lovK* and *lovR* strongly attenuates σ^T-dependent transcription (P_{sigU}); however, overexpression of *lovR* has no effect on basal P_{sigU} transcription compared to an empty vector control strain (Fig. 3B). We conclude that LovR alone is insufficient to affect σ^T-dependent transcription.

We next tested whether the repressive effect of the LovK-LovR system on σ^T-dependent transcription requires the conserved histidine phosphorylation site in LovK (H180) or the conserved phosphoacceptor aspartate (D57) in LovR. In these experiments, we monitored P_{sigU}-*lacZ* transcription in strains coordinately overexpressing (i) wild-type *lovK* and *lovR*(*D57A*), (ii) *lovK*(*H180A*) and wild-type *lovR*, or (iii) *lovK*(*H180A*) and *lovR*(*D57A*). None of these combinations of *lovK* and *lovR* alleles are capable of fully repressing transcription from P_{sigU} (Fig. 3C). The *lovR*(*D57A*) allele in combination with wild-type *lovK* or *lovK*(*H180A*) does not affect transcription from P_{sigU}. Overexpression of *lovK*(*H180A*) and wild-type *lovR* results in modest repression (Fig. 3C) similar to that seen when wild-type *lovR* is overexpressed alone (Fig. 3B and C). Together, these data indicate that the conserved histidine phosphorylation site in LovK and the conserved phosphoacceptor aspartate in LovR are required for repression of transcription from the σ^T-dependent promoter P_{sigU}.

Deletion of *lovK* and/or *lovR* enhances σ^T-dependent transcription. The data presented above suggest a model in which LovK and LovR function together as negative regulators of σ^T-dependent transcription. One prediction of this model is that deletion of this two-component system will result in derepression of σ^T-dependent transcription. To test this prediction, we evaluated transcription from P_{sigU}-*lacZ* in wild-type, Δ*lovK*, Δ*lovR*, and Δ*lovK-lovR* genetic backgrounds. Indeed, deletion of *lovK*, *lovR*, or both resulted in a modest (~30%) but reproducible (one-way ANOVA, $P < 0.001$; Dunnett's posttest, $P < 0.01$) enhancement of basal transcription from our σ^T-dependent reporter plasmid (Fig. 4). Upon stress insult, we observed elevated transcription in all four genetic backgrounds. Moreover, Δ*lovK*, Δ*lovR*, and

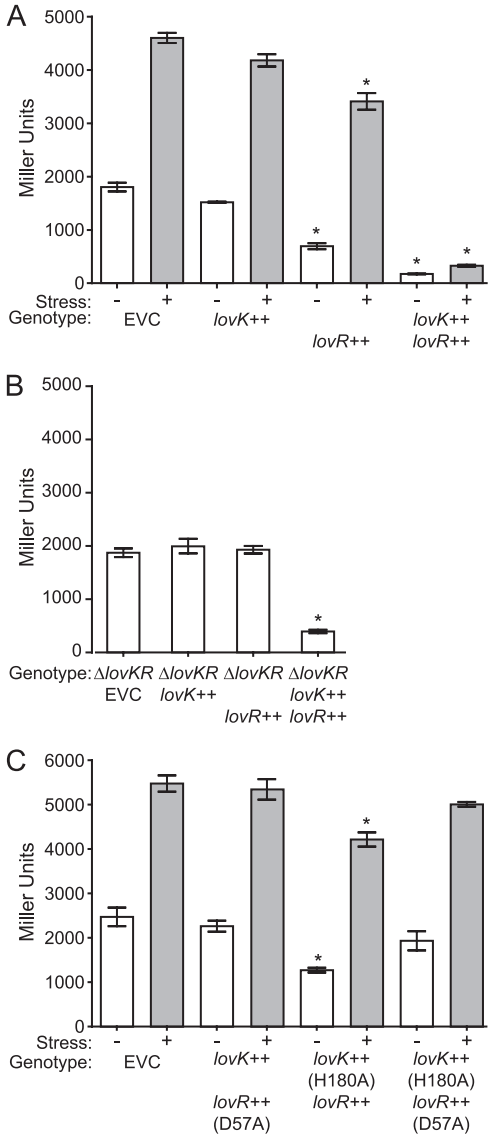

FIG 3 Coordinate overexpression of *lovK* and *lovR* strongly represses σ^T-dependent transcription. σ^T-dependent transcription under stress and nonstress conditions was evaluated by measuring β-galactosidase activity from the σ^T reporter P_{sigU}-*lacZ* (2) in unstressed cultures (white bars) and in cultures under osmotic stress (gray bars). (A) Transcription from P_{sigU}-*lacZ* was measured in a wild-type background upon overexpression of *lovK* or *lovR* or coordinate expression of both *lovK* and *lovR*. (B) Transcription from P_{sigU}-*lacZ* was measured in a Δ*lovK-lovR* background upon overexpression of *lovK* or *lovR* or coordinate expression of both genes. (C) Transcription from P_{sigU}-*lacZ* was measured in a wild-type background overexpressing *lovK* and *lovR* alleles in which the conserved histidine and aspartate phosphorylation sites, respectively, were mutated to alanine. For each panel, biological replicates of each strain were grown and assayed simultaneously; means ± SEM are presented ($n = 3$; *, one-way ANOVA, $P < 0.0001$; Dunnett's posttest for comparison to wild type, $P < 0.001$). EVC, empty vector control.

Δ*lovK-lovR* strains continue to exhibit higher levels of transcription than the wild type (one-way ANOVA, $P < 0.001$; Dunnett's posttest, $P < 0.001$) (Fig. 4). While the transcriptional effect of *lovK* and/or *lovR* deletion is modest, this result is consistent with a model in which the LovK-LovR two-component system has a repressive effect on transcription of genes in the general stress regu-

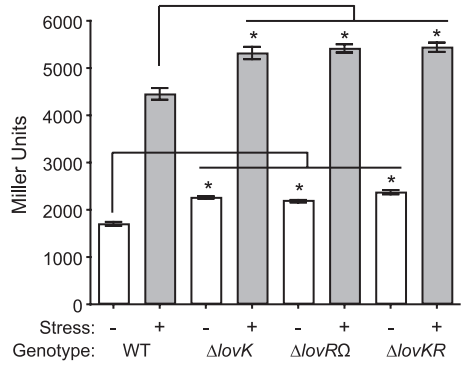

FIG 4 Derepression of σ^T-dependent transcription upon deletion of *lovK* and/or *lovR*. σ^T-dependent transcription was evaluated as for Fig. 3 in strains bearing deletions at the *lovK-lovR* locus under unstressed (white) and osmotic stress (gray) conditions. Data are means ± SEM ($n = 3$) for cultures grown and assayed in parallel; (*, one-way ANOVA, $P < 0.001$; Dunnett's posttest for comparison to wild type, $P < 0.01$).

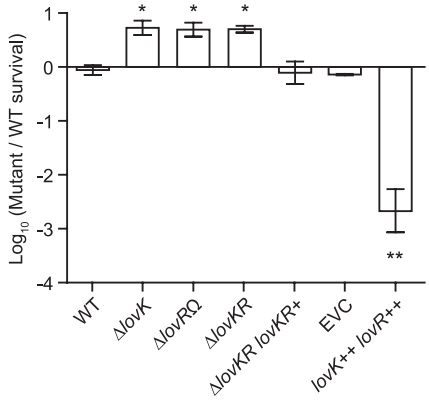

FIG 5 *lovK* and *lovR* function as negative regulators of osmotic stress survival. Cell survival (CFU from stressed/unstressed cultures) was measured after 6 h of exposure to 150 mM sucrose. Data are means ± SEM ($n = 6$; *, one-way ANOVA, $P < 0.001$; Dunnett's posttest for comparison to wild type, $P < 0.01$; **, posttest, $P < 0.001$). EVC, empty vector control.

lon at native expression levels. Moreover, the fact that deletion of either *lovK* or *lovR* results in derepression comparable to that observed when both genes are deleted suggests that *lovK* and *lovR* function together to modulate σ^T-dependent transcription.

LovK and LovR modulate cell survival under stress. The microarray and transcriptional fusion data presented above provide evidence that the LovK-LovR system functions to repress transcription of genes in the σ^T (i.e., general stress) regulon. We next sought to test whether deletion or overexpression of *lovK* and/or *lovR* has functional consequences for cellular survival during osmotic stress. The stress condition tested in this assay was previously demonstrated to elicit a differential stress survival phenotype in *C. crescentus* Δ*sigT*, Δ*phyR*, and Δ*phyK* mutants (2, 17, 25). Deletion of *lovK*, *lovR*, or both results in a reproducible and statistically significant increase in cell survival following stress relative to that in wild-type *C. crescentus* (one-way ANOVA, $P < 0.001$; Dunnett's posttest, $P < 0.01$) (Fig. 5). This enhanced survival phenotype in the Δ*lovK-lovR* strain can be complemented by single-copy integration of the *lovK-lovR* locus, with its native promoter, into the ectopic *vanR* locus on the chromosome (Fig. 5). Overexpression of *lovK-lovR* has the opposite effect on cell survival; cells coordinately overexpressing *lovK* and *lovR* exhibit reduced survival compared to wild-type and empty vector control strains ($P < 0.001$) (Fig. 5).

Increased survival of the *lovK* and *lovR* null strains and decreased survival of the *lovK-lovR* overexpression strain provide a functional correlate with the repressive role for LovK-LovR in transcription of the general stress regulon described above (Table 2; Fig. 3 and 4). However, these data do not rule out mechanisms independent of the general stress pathway by which LovK-LovR could modulate transcription and cell survival during stress. Experiments in which we tested epistasis between the LovK-LovR system and known genes in the general stress pathway are described below.

LovK-LovR-dependent regulation of the general stress system requires *sigT* and *phyR*. We next tested whether the effect that the LovK-LovR system exerts on transcription in the general stress pathway requires the known general stress regulatory genes *sigT* and *phyR*. As expected, we observe that deletion of *sigT* or *phyR* ablated transcription from the general stress reporter construct, P_{sigU}-*lacZ* (Fig. 6). In Δ*sigT* and Δ*phyR* strains, deletion or overexpression of the *lovK* and *lovR* genes had no effect on reporter plasmid transcription in either the presence or absence of stress (Fig. 6A and B). These data provide evidence that the transcriptional effect exerted by the LovK and LovR two-component proteins on the *sigU* promoter requires *sigT* and *phyR*. Moreover, at the level of stress survival, deletion of *lovK* and *lovR* did not enhance survival of a strain that is also missing *sigT* (i.e., a Δ*sigT* Δ*lovK-lovR* triple mutant). Similarly, overexpression of *lovK* and *lovR* did not further reduce the stress survival of a strain lacking *sigT* (Fig. 6C). These data provide evidence that the observed increase in survival in the *lovK-lovR* null strains and decrease in survival in the *lovK-lovR* overexpression strains also require *sigT*.

***lovK* complements the transcriptional and cell survival defects of a *phyK* null mutant.** Further epistasis analysis of transcriptional regulation of the general stress reporter construct (P_{sigU}-*lacZ*) in a Δ*phyK* mutant background provided an unexpected result: overexpression of *lovK* partially complemented a *phyK*-null allele, increasing baseline transcription from P_{sigU}. Moreover, *lovK*-dependent transcription from P_{sigU} was upregulated by osmotic stress (Fig. 7A). This result provides evidence that the LovK sensor histidine kinase not only can substitute for the PhyK general stress sensor kinase but also is regulated by osmotic stress. Individual overexpression of *lovR* did not complement the Δ*phyK* defect in basal or stress-induced transcription from P_{sigU} (Fig. 7A). Importantly, when *lovK* was coordinately overexpressed with *lovR*, complementation of the Δ*phyK* defect was no longer observed (Fig. 7A). This result suggests that LovR may buffer LovK from activating σ^T-dependent transcription in the Δ*phyK* background. To investigate the effect of endogenously expressed *lovR* on the ability of *lovK* to complement the loss of *phyK*, we repeated this experiment in a Δ*phyK* Δ*lovK-lovR* mutant background. In the absence of *lovR* expressed from its native chromosomal locus, complementation of the Δ*phyK* transcriptional defect by *lovK* was more complete; transcription from P_{sigU} approached wild-type levels in the absence of stress in this genetic background (Fig. 7B). We also observed stress-dependent activation of transcription in this strain as above (Fig. 7B). Again, coordinate overexpression of *lovR* with *lovK* abolished complementation of Δ*phyK* with respect to transcription from the general stress

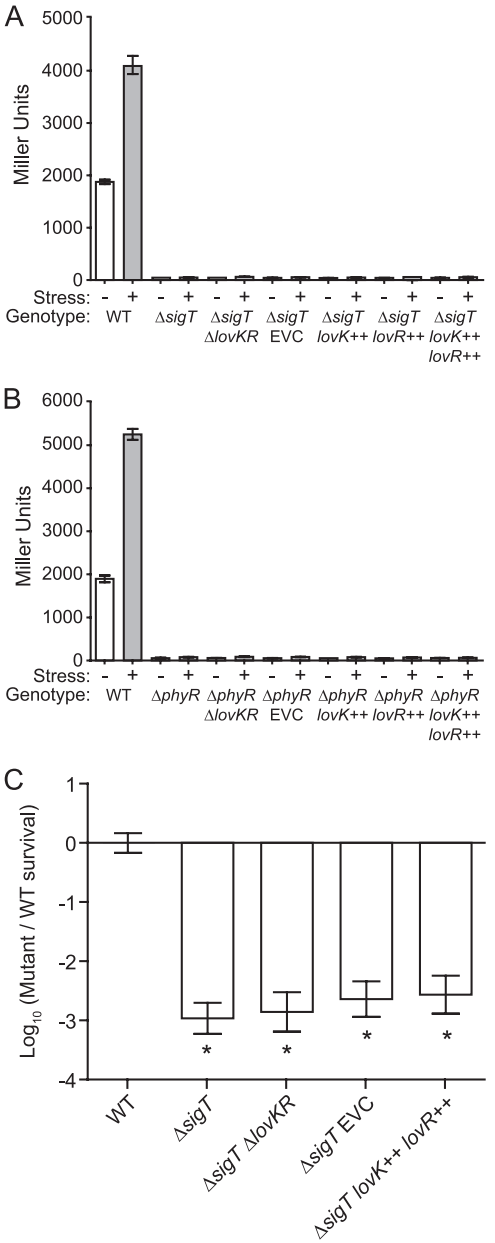

FIG 6 *lovK-lovR*-dependent regulation of P_{sigU}-*lacZ* transcription and osmotic stress survival require the general stress regulators *sigT* and *phyR*. The effect of *lovK-lovR* deletion or overexpression on P_{sigU}-*lacZ* transcription in strains lacking *sigT* (A) or *phyR* (B) was evaluated as for Fig. 3. All strains were grown and assayed in parallel; data are means ± SEM ($n = 3$) for each strain and stress condition. (C) Osmotic stress survival of Δ*sigT* mutant strains in which the chromosomal *lovK-lovR* genes are either deleted (Δ*lovKR*) or coordinately overexpressed (*lovK++lovR++*). EVC, empty vector control. Data are means ± SEM ($n = 3$; *, one-way ANOVA, $P < 0.001$; Dunnett's posttest to wild type, $P < 0.01$; none of the data for mutant strains in panel C are statistically distinct from each other).

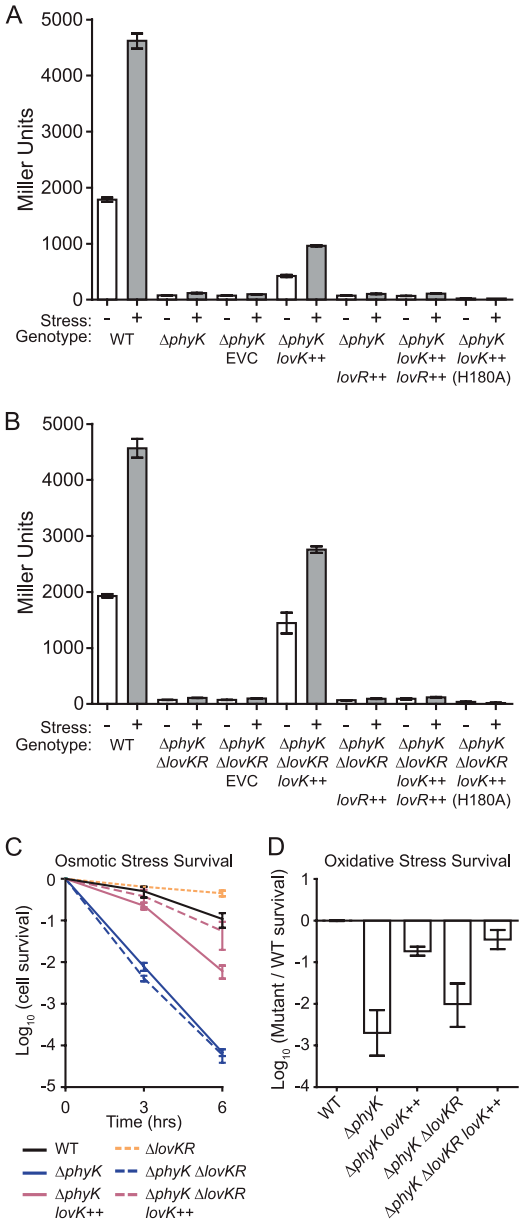

FIG 7 *lovK-lovR* and *phyK* epistasis analysis reveals that *lovK* can complement the transcriptional and survival defects of Δ*phyK*. P_{sigU}-*lacZ* transcription in Δ*phyK* (A) and Δ*phyK* Δ*lovK-lovR* (B) mutant backgrounds in which *lovK*, *lovR*, and *lovK*(*H180A*) are overexpressed (evaluated as for Fig. 3). (C) Cell survival (CFU of stressed/unstressed cultures) was measured at 0, 3, and 6 h after osmotic stress in strains overexpressing *lovK* in Δ*phyK* and Δ*phyK* Δ*lovK-lovR* mutants. Survival of wild-type and Δ*lovK-lovR* strains is shown for comparison. (D) Relative survival following 60 min of exposure to 0.2 mM H_2O_2 in strains overexpressing *lovK* in Δ*phyK* single and Δ*phyK* Δ*lovK-lovR* triple mutant backgrounds. For each panel, three independent cultures were grown and assayed in parallel (data are means ± SEM). EVC, empty vector control.

reporter construct, P_{sigU}-*lacZ* (Fig. 7B). Importantly, the conserved histidine phosphorylation site in LovK (H180) was required to complement the loss of *phyK* (Fig. 7). Finally, deletion of the *lovK-lovR* locus did not affect P_{sigU} transcription in a *phyK*-null background. Together, these data provide evidence for a model in which the LovK sensor kinase can functionally substitute for the PhyK general stress sensor kinase. However, when the preferred phosphotransfer partner of LovK (namely, LovR) is present at high levels, LovK does not functionally substitute for PhyK.

As *lovK* expression complements the defect of Δ*phyK* in transcription from the general stress reporter P_{sigU}-*lacZ*, we next sought to test whether *lovK* can functionally complement *phyK* in two distinct stress survival assays. We subjected wild-type *C. cres-*

centus, a Δ*phyK* mutant, and a Δ*phyK* Δ*lovK-lovR* triple mutant to both osmotic stress (150 mM sucrose) and oxidative stress (0.2 mM H_2O_2). Both the Δ*phyK* single mutant and the Δ*phyK* Δ*lovK-lovR* triple mutant showed significant and statistically equivalent survival defects in high-osmotic-strength medium and in the presence of H_2O_2 (Fig. 7C and D). Expression of *lovK* from a vanillate-inducible promoter partially restored cell survival under both stress conditions in the Δ*phyK* background. Expression of *lovK* in the Δ*phyK* Δ*lovK-lovR* background almost completely restored cell survival to wild-type levels. These survival data support a model in which LovK can function to activate the general stress response system in the absence of PhyK.

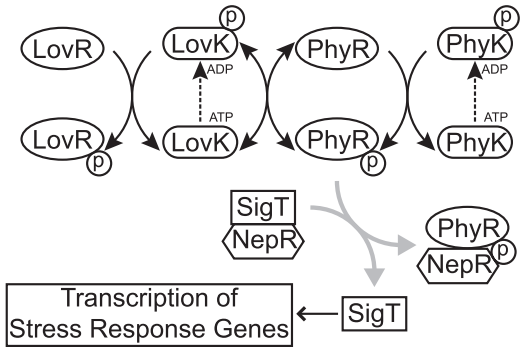

FIG 8 Proposed regulatory network linking the PhyK-PhyR and LovK-LovR two-component systems in control of *C. crescentus* general stress response.

DISCUSSION

Regulation of the general stress response by a cytoplasmic sensor system. The physical and chemical composition of natural environments is complex and in a constant state of flux. While studies of environmental regulation of cell physiology typically focus on one signal or closely related signals, it must be the case that diverse stimuli detected by sets of sensors have an integrated effect on the biology of a cell. This study provides evidence for regulation in which one two-component sensory system (PhyK-PhyR) indirectly activates transcription of a second two-component system (LovK-LovR). The LovK-LovR system functions, in turn, to repress PhyK-PhyR-mediated transcription (Table 2 and Fig. 3 and 4; also, see Table S2 in the supplemental material). The signaling activity of each of these two-component sensory systems can be affected by a range of signals in the environment of the *C. crescentus* cell. Thus, the magnitude of regulatory control between this pair of sensor/signaling systems is likely tuned by environmental conditions.

It is presumed that the transmembrane sensor kinase PhyK monitors the status of the periplasm (25), while LovK is a cytoplasmic protein that can sense the physicochemical environment of the interior of the cell. The sensor domain at the amino terminus of LovK is a photosensory LOV domain (16) that binds an FMN cofactor (33) and can regulate the activity of the HWE-family histidine kinase at the carboxy terminus of the protein in response to visible-light absorption (34). However, the capacity of the LovK photosensor to undergo classical LOV domain photochemistry (i.e., light-dependent cysteinyl-C4a adduct formation) is affected by the redox state of the FMN cofactor; LOV photochemistry and light-regulated kinase activity require that the flavin be in the oxidized state (33). Notably, the midpoint reduction potential (at pH 7) of LovK has been measured at −260 mV (33), which is poised near the cytoplasmic redox potential (20). Thus, the capacity of LovK to function as a bona fide photosensor in the cell may be conditional, requiring the cytosol to be in a certain redox range. Indeed, our attempts to modulate transcription of the general stress pathway using various light stimuli under standard growth conditions have been unsuccessful to date (data not shown). We also note that, although overexpression of *lovK* and *lovR* has been reported to strongly affect the adhesive properties of *C. crescentus* (34), this adhesion phenotype is not genetically linked to any of the mutations we have engineered at the *sigT* locus (data not shown). Thus, we conclude that the regulation of cell adhesion by *lovK* and *lovR* is not a result of transcriptional changes in the general stress regulon.

Cross-regulation? A discussion of possible mechanisms. The molecular mechanism of LovK-LovR-mediated repression of the general stress regulon has yet to be established. While two-component systems are generally insulated from each other (38), there is precedent for cross-regulation in other species (18, 27, 29). LovK has known kinase/ATPase activity (34). However, histidine kinases can also function as phosphatases (19, 23, 31). We envision two possible cross-regulation models for LovK-LovR-dependent repression of the PhyK-PhyR-σ^T pathway: (i) LovK functions as a PhyR phosphatase and a LovR kinase, thereby reducing the cellular concentration of phosphorylated PhyR; (ii) LovR functions as a PhyK phosphoryl acceptor, with LovK functioning as a LovR phosphatase. Each of these models predicts a reduction of the levels of phosphorylated PhyR via phosphoryl flow through LovK and LovR, thereby attenuating σ^T-dependent transcription. The LovK-LovR system could thus be considered a functional analog of the histidine kinase-like PhyP protein of *Sphingomonas* sp. strain FR1, which appears to function as a PhyR phosphatase (22). Genetic epistasis experiments on the two-component regulators in this pathway (Fig. 3, 6, and 7) support model 1, in which LovK and PhyK act as opposing regulators of phospho-PhyR levels (Fig. 8). The logic underlying this conclusion follows.

In a wild-type genetic background, PhyK is the primary PhyR kinase and LovK is the primary LovR kinase. While overexpression of LovK does not attenuate PhyR/σ^T-dependent transcription, increasing the concentration of the primary LovK phosphoacceptor, LovR, partially attenuates σ^T-dependent transcription. We propose that unphosphorylated LovK can function as a PhyR phosphatase and that increasing the cellular concentration of LovR results in a shift of the cellular pool of phospho-LovK (LovK∼P) toward LovK. In this model, coordinate overexpression of LovK and LovR further increases the cellular pool of unphosphorylated LovK, further reducing steady-state levels of PhyR∼P and, thus, attenuating transcription from σ^T-dependent promoters. Our model, in which LovK interacts with PhyR and LovR, is additionally supported by the result that overexpression of LovK partially complemented loss of *phyK* in the presence of an endogenous copy of *lovR* and almost fully complemented the Δ*phyK*-null transcriptional and cell survival defects in the absence of *lovR*. Furthermore, overexpression of *lovR* abolished this complementation.

On the surface, these results are somewhat paradoxical: depending on the genetic background, LovK can function as either a positive or negative regulator of general stress signaling. We argue that these data can be understood in the context of a model in which LovK can function as both a kinase and a phosphatase of

PhyR. In a wild-type background, our data support a model in which LovK, in concert with LovR, reduces the level of PhyR~P. In other words, equilibrium favors the LovK-LovR two-component system as a net PhyR phosphatase. However, in the absence of the primary PhyR kinase (i.e., PhyK), PhyR is predicted to exist almost entirely in its unphosphorylated state. Under these conditions, LovK can partially complement the loss of PhyK presumably by driving the forward kinase reaction to produce PhyR~P. The activity of LovK as an apparent PhyR kinase is accentuated in the absence of the primary LovK phosphoacceptor, LovR. Unexpectedly, in this genetic background, σ^T-dependent transcription is activated by osmotic stress. This result suggests a new sensory role for LOV histidine kinases. However, we cannot exclude the possibility that LovK (via PhyR/σ^T) regulates the expression of other sensory proteins that affect σ^T-dependent transcription.

While these genetic data do not conclusively rule out other potential models, we believe the model presented in Fig. 8 is the most probable regulatory topology underlying LovK-LovR- and PhyK-PhyR-dependent regulation of σ^T-dependent transcription and the general stress response.

Conclusion. We present here a combination of genetic and genomic data that provide evidence for control of the *C. crescentus* general stress response by a pair of two-component signaling systems. *lovK* and *lovR* contain a known ECF σ regulatory motif in their promoters and are upregulated by stress through a mechanism that requires the ECF σ-factor, σ^T. The PhyK-PhyR system functions as a positive regulator, whereas the LovK-LovR system functions as negative regulator of the general stress response. In short, our data provide evidence for a multisensor regulatory system that has the capacity to integrate a range of intra- and extracellular signals to control stress physiology in *C. crescentus*.

Within this regulatory network, the single-domain response regulator, LovR, is independently controlled at the transcriptional level and can modulate whether LovK adds a phosphoryl group to or removes one from PhyR. An analogy to the single-domain response regulator, DivK, which functions to modulate the opposing kinase and phosphatase activities of the PleC and DivJ sensor histidine kinases in *C. crescentus*, can be drawn (31). This role for LovR and DivK as modulators of TCS protein phosphorylation state contrasts with single-domain response regulators such as CpdR and CheY, which function as regulators of proteolysis (1, 21) and flagellar rotation (30, 35), respectively.

Our results raise the question of whether regulation of the general stress pathway by LOV histidine kinases is general in the alphaproteobacteria. In the mammalian pathogen *Brucella abortus*, the LOV histidine kinase LOV-HK is a known virulence factor and has been reported to regulate cell proliferation in macrophages in response to light (41). Whether this phenotype is a result of LOV-HK-dependent regulation of stress signaling in *B. abortus* is unknown. A phylogenetic analysis of LOV domains in bacterial genomes (24) has revealed close homologs of *C. crescentus* LovK in many alphaproteobacterial species, including *Methylobacterium extorquens*, *Rhizobium leguminosarum*, and *Erythrobacter litoralis*. Studies on orthologous general stress signaling genes in these species and others will test the generality of LOV histidine kinases as regulators of stress signaling in this bacterial clade.

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