The ChvG-ChvI and NtrY-NtrX Two-Component Systems Coordinately Regulate Growth of Caulobacter crescentus

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ABSTRACT Two-component signaling systems (TCSs) are comprised of a sensory histidine kinase and a response regulator protein. In response to environmental changes, sensor kinases directly phosphorylate their cognate response regulator to affect gene expression. Bacteria typically express multiple TCSs that are insulated from one another and regulate distinct physiological processes. There are examples of cross-regulation between TCSs, but this phenomenon remains relatively unexplored. We have identified regulatory links between the ChvG-ChvI (ChvGI) and NtrY-NtrX (NtrYX) TCSs, which control important and often overlapping processes in alphaproteobacteria, including maintenance of the cell envelope. Deletion of chvG and chvl in Caulobacter crescentus limited growth in defined medium, and a selection for genetic suppressors of this growth phenotype uncovered interactions among chvGI, ntrYX, and ntrZ, which encodes a previously uncharacterized periplasmic protein. Significant overlap in the experimentally defined ChvI and NtrX transcriptional regulons provided support for the observed genetic connections between ntrYX and chvGI. Moreover, we present evidence that the growth defect of strains lacking chvGI is influenced by the phosphorylation state of NtrX and, to some extent, by levels of the TonB-dependent receptor ChvT. Measurements of NtrX phosphorylation in vivo indicated that NtrZ is an upstream regulator of NtrY and that NtrY primarily functions as an NtrX phosphatase. We propose a model in which NtrZ functions in the periplasm to inhibit NtrY phosphatase activity; regulation of phosphorylated NtrX levels by NtrZ and NtrY provides a mechanism to modulate and balance expression of the NtrX and ChvI regulons under different growth conditions.

IMPORTANCE TCSs enable bacteria to regulate gene expression in response to physicochemical changes in their environment. The ChvGI and NtrYX TCSs regulate diverse pathways associated with pathogenesis, growth, and cell envelope function in many alphaproteobacteria. We used Caulobacter crescentus as a model to investigate regulatory connections between ChvGI and NtrYX. Our work defined the ChvI transcriptional regulon in C. crescentus and revealed a genetic interaction between ChvGI and NtrYX, whereby modulation of NtrYX signaling affects the survival of cells lacking ChvGI. In addition, we identified NtrZ as a periplasmic inhibitor of NtrY phosphatase activity in vivo. Our work establishes C. crescentus as an excellent model to investigate multilevel regulatory connections between ChvGI and NtrYX in alphaproteobacteria.

KEYWORDS Caulobacter crescentus, ChvG, ChvI, ChvT, NtrX, NtrY, cell envelope, histidine kinase, response regulator, two-component regulatory systems
phosphorylated RRs (1). RR phosphorylation generally alters the activity of effector domains that change gene expression. TCSs are modular, and the output of a particular RR may vary between different organisms (2–5). TCSs that regulate host interactions in pathogens and symbionts are often conserved in related free-living organisms and enable responses to similar physiochemical cues present in the environment (2, 6–8).

Although typical TCSs rely on a single HK and RR pair, many systems incorporate additional proteins, such as activators or inhibitors, to form more complex signaling networks (9–17). Historically, most TCSs have been considered to be insular systems, but in many bacteria, cross-regulation between HKS and RRs may integrate multiple environmental cues (9, 11, 14, 18–20). Even when HK and RR pairs are well insulated, TCSs can interact at the transcriptional level (20, 21). For example, one TCS may regulate the expression of other TCS genes, or multiple TCSs may influence transcription of the same downstream gene (22–24).

ChvG-ChvI (ChvGI) and NtrY-NtrX (NtrYX) are conserved alphaproteobacterial TCSs that often regulate similar physiological processes, raising the possibility that they work together in a coordinated fashion (25, 26). The ChvG HK and ChvI RR were originally identified as pleiotropic regulators in the plant pathogen Agrobacterium tumefaciens, affecting virulence, detergent tolerance, and pH sensitivity (8, 27). Subsequent work has linked ChvGI to host interaction, cell motility, acid sensing, and exopolysaccharide production in a variety of Alphaproteobacteria (6, 7, 28–32). In most characterized systems, the periplasmic protein ExoR binds to ChvG and inhibits its kinase activity (15, 16, 33). Acidic pH activates the ChvGI system by triggering rapid proteolysis of ExoR (34). However, not all organisms with ChvGI, including Caulobacter crescentus, encode an ortholog of ExoR. These bacteria must regulate ChvG kinase activity by a different mechanism.

Like ChvGI, NtrYX (consisting of the NtrY HK and NtrX RR) is conserved in many Alphaproteobacteria, including multiple pathogens and symbionts (35–39). Although early studies concluded that NtrYX regulates nitrogen metabolism, recent work suggests that, in certain alphaproteobacteria, it also controls exopolysaccharide biosynthesis, cell motility, and cell envelope composition (25, 35, 39–44). Given that these processes are also regulated by ChvGI, it is conceivable that ChvGI and NtrYX act coordinately. However, no work to date has identified a substantial genetic interaction between these TCSs (25, 26).

C. crescentus, a free-living alphaproteobacterium found in freshwater and soil environments, encodes both the ChvGI and NtrYX systems (45, 46). C. crescentus ChvGI activates transcription of the small regulatory RNA chvR, which posttranscriptionally represses the TonB-dependent receptor gene chvT (6). Examination of reporters of chvR transcription indicated that ChvGI is activated by growth in defined medium, acidic pH, DNA damage, growth at stationary phase, and cell envelope stress (6, 47). However, aside from chvR, genes regulated by ChvGI have not been defined. C. crescentus NtrYX is less well characterized than ChvGI, but a recent study established that NtrX is phosphorylated in stationary phase in defined medium as a result of acidification (48). In addition, NtrX appears to play a core role in regulating C. crescentus physiology, as ntrX is essential for growth in complex medium and ΔntrX cells grow more slowly than wild-type (WT) cells in defined medium (49).

In this study, we initially took a reverse genetic approach to characterize the role of ChvGI in regulating C. crescentus physiology. Deletion of chvG and chvl caused a distinctive growth defect in defined medium. By exploiting this defect, we identified striking genetic interactions between chvG and ntrY, ntrX, and ntrZ (a previously uncharacterized gene). Epistasis analysis provided evidence that unphosphorylated NtrX is detrimental to cells lacking chvG or chvl. We defined the Chvl transcriptional regulon and discovered that it overlaps significantly with genes regulated by NtrX. In addition, we found that NtrZ promotes NtrX phosphorylation in vivo, likely by inhibiting NtrY phosphatase activity. We conclude that ChvGI and NtrYX interact at multiple transcriptional levels, working both in concert and in opposition to regulate C. crescentus growth in defined medium.
RESULTS

Loss of the ChvGl system limits growth in defined medium. To investigate the physiological role of ChvGl in C. crescentus, we generated strains with in-frame deletions of chvG or chvI and examined their growth. The deletion strains grew normally in complex medium (peptone-yeast extract [PYE]), but they displayed a distinctive growth defect in defined medium (M2 minimal salts medium with xylose as carbon source [M2X]) (Fig. 1A and B; see Fig. S1 in the supplemental material). Although overnight cultures of each strain inoculated from PYE agar plates grew to similar densities in M2X, strains lacking chvG or chvI exhibited reduced growth capacity after dilution, only reaching a terminal optical density at 660 nm (OD_{660}) of 0.1 (Fig. 1A). This lower cell density correlated with fewer CFU (Fig. 1B). Ectopic overexpression of chvG (chvG++11) or chvI (chvI++11) from a xylose-inducible promoter (xylose is the sole carbon source in M2X medium) fully rescued growth of ΔchvG or ΔchvI strains, respectively, under these conditions (Fig. 1A and B).

To evaluate if the growth defect was related to the number of cell divisions in defined medium, we resuspended cells in M2X from PYE agar plates and diluted cultures to several low starting densities. ΔchvI cells carrying EV or genetic rescue plasmid (+ +) were suspended in M2X medium and immediately diluted to an OD_{660} of 0.001 or 0.0001. Points represent averages from three biological replicates ± SD. *, P < 0.05; one-way ANOVA followed by Dunnett's posttest comparison to ΔchvI EV; 10⁻⁴ dilution at 30.5 h.

FIG 1  Loss of chvG or chvl limits culture density in defined M2X medium. (A) Growth curves, measured by optical density (OD_{660}) of WT, ΔchvG, and Δchvl strains bearing empty vector (EV) or genetic rescue plasmid (+ +) integrated at the xylose locus. Primary M2X cultures, inoculated from PYE plates, all grow to high density (left). However, upon back dilution to an OD_{660} of 0.025, ΔchvG and ΔchvI EV strains saturate at a significantly lower OD (right). Points represent averages from three biological replicates ± SD. ****, P < 0.0001, one-way ANOVA followed by Dunnett’s posttest comparison to WT EV at 24 h. (B) Growth curves, measured by CFU, corresponding to the cultures in panel A. Points represent averages from three biological replicates ± SD. ***, P < 0.0005; one-way analysis of variance (ANOVA) followed by Dunnett's posttest comparison to WT EV at 24 h. (C) Growth of cultures inoculated from PYE agar plates at different starting densities, measured by CFU. Δchvl cells carrying EV or genetic rescue plasmid (+ +) were suspended in M2X medium and immediately diluted to an OD_{660} of 0.001 or 0.0001. Points represent averages from three biological replicates ± SD. *, P < 0.05; one-way ANOVA followed by Dunnett's posttest comparison to ΔchvI; 10⁻⁴ dilution at 30.5 h.
contribute to growth in M2X primary cultures (Fig. S2B). Moreover, cell density alone did not determine the ability of DchvI cultures to grow in M2X, as denser back dilutions from DchvI overnight cultures did not reach higher viable cell counts (Fig. S2C).

Together, our results suggest that the diminished growth capacity of DchvI or DchvG cells in M2X medium is a function of the time since resuspension from PYE plates and growth phase.

ChvI phosphorylation is critical for growth in defined medium. Given the similarity between the phenotypes displayed by ΔchvI and ΔchvG strains, we predicted that phosphorylation of ChvI might be important for growth in M2X medium. To test this hypothesis, we generated strains harboring chvI alleles encoding changes to the conserved sites of phosphorylation (D52A, D52N, and D52E). Growth of strains carrying the nonphosphorylatable alleles chvI(D52A) and chvI(D52N) was similar to DchvI cells in M2X (Fig. 2A and Fig. S3A). In contrast, chvI(D52E) cultures reached higher densities than ΔchvI cultures, suggesting that ChvI(D52E) is active, albeit not to the same level as phosphorylated ChvI (Fig. 2A and Fig. S3A). Substitutions of the phosphorylatable aspartate with glutamate often act as phosphomimetic mutations and constitutively activate RRs (50–53). Thus, this result supports a critical role for ChvI phosphorylation during growth in M2X medium.

To further examine the importance of ChvI phosphorylation, we overexpressed chvG and chvl alleles from a xylose-inducible promoter in knockout backgrounds. Although overexpression of chvG rescued growth of ΔchvG cells (Fig. 1B), overexpression of chvG(H309A),
a catalytic-histidine mutant, did not, indicating that phosphorylation of ChvG, and by extension, Chvl, is important for growth in M2X medium (Fig. 2B). Overexpression of chvl(D52E) only partially rescued growth of Δchvl and ΔchvG strains in M2X, and these strains grew similarly to the strain encoding chvl(D52E) at the native locus (Fig. 2A and B and Fig. S3B). However, in contrast to native expression of chvl(D52E), overexpression of this allele resulted in cell filamentation in M2X but not PYE, consistent with a cell division defect (Fig. 2C and Fig. S3C). Thus, overexpression of phosphomimetic chvl(D52E) appears to interfere with cell division and, perhaps as a result, only partially rescues growth in M2X medium.

Mutations in ntrY, ntrX, and a gene of unknown function rescue the growth defect of Δchvl and ΔchvG strains. To better understand why ChvGI is critical for growth in defined medium, we employed a selection strategy to isolate second-site mutations that alleviate the growth defect of ΔchvG and Δchvl cells. ΔchvG and Δchvl M2X overnight cultures were back diluted and grown until they reached high density, presumably due to proliferation of suppressor strains (Fig. 3A). We then isolated single colonies with WT-like growth in M2X and used whole-genome sequencing to identify any acquired mutations (Fig. 3B).

In multiple independent strains, we identified nonsynonymous substitutions in the genes encoding the HK NtrY and its cognate RR NtrX (Fig. 3C). The ntrY mutations (L70H and A123V) are located in predicted transmembrane helices, which are involved in transmitting information from periplasmic sensing domains to the kinase domain (Fig. 3C) (54–56). We identified only one ntrX allele, A19V, which lies in helix 1 (α1) of the receiver domain (Fig. 3C). α1 is involved in the interaction interface between the HK dimerization and histidine phosphotransfer (DHp) domain and the RR REC domain (57, 58). We also note that the ntrX(A19V) strain was isolated on PYE plates and appeared to grow normally in M2X, suggesting that this is not a complete loss-of-function allele (49).

In addition to mutations in ntrY and ntrX, we identified three mutants with nonsynonymous substitutions in a gene encoding a putative periplasmic protein of unknown function (CCNA_03863, here referred to as ntrZ) (Fig. 3C). The mutations identified (Y92C and I99N) are both located outside the predicted signal sequence (Fig. 3C). Therefore, mutations in the NtrYX TCS and NtrZ appear to affect the growth of Δchvl and ΔchvG cells in M2X medium.

Overexpression or deletion of ntrX modulates the Δchvl growth phenotype. Several studies have noted similar phenotypes of chvGI and ntrYX mutants (25, 26, 41); however, to our knowledge, none have established a genetic link between these TCSs. To better understand the connections between ChvGI and NtrYX in C. crescentus, we first characterized the genetic relationship between chvl and ntrX. Replacement of ntrX with the ntrX(A19V) allele restored growth of Δchvl cells in M2X (Fig. 4A), ruling out other background mutations as causal for suppression of the Δchvl phenotype. However, overexpression of ntrX(A19V) in the presence of the native ntrX allele did not rescue growth of Δchvl cells (Fig. 4A). This result indicates that the ntrX(A19V) allele is recessive and suggests that the presence of WT NtrX contributes to the growth phenotype of Δchvl cells.

To examine this notion further, we tested the effect of deleting ntrX in the Δchvl strain. ΔntrX cells had a slight growth defect in M2X but were clearly distinct from Δchvl cells (Fig. 4B). Deletion of ntrX in a Δchvl background rescued growth, indicating that the presence of ntrX is indeed detrimental to Δchvl cells (Fig. 4B). Suppression of the Δchvl growth phenotype was not due to a second-site mutation, as restoration of the ntrX locus (Δchvl ΔntrX::ntrX) restored the growth defect in M2X medium (Fig. 4B).

Given that the presence of ntrX limits growth of Δchvl cells in M2X, we next tested whether overexpression of ntrX affects growth of WT cells. Overexpression of ntrX only moderately slowed growth of WT cells. However, overexpression of the nonphosphorylatable ntrX(D53A) allele dramatically impaired the growth of WT cells in M2X, similar to the defect observed in Δchvl cells (Fig. 4C). Overexpression of either ntrX or ntrX (D53A) exacerbated the growth defect of Δchvl cells (Fig. 4C). Together, these results
support a model in which unphosphorylated NtrX limits growth capacity in M2X medium, especially in \( \text{Dchv}\text{I} \) cells. NtrZ is a predicted periplasmic protein that functions upstream of NtrY to regulate levels of phosphorylated NtrX.

We next examined the nature of the \( \text{ntrY} \) and \( \text{ntrZ} \) suppressor mutations. Overexpression of the \( \text{ntrY} \) and \( \text{ntrZ} \) alleles identified in our suppressor strains restored growth of \( \text{ΔchvI} \) cells in M2X (Fig. 5A). In addition, overexpression of WT \( \text{ntrY} \), but not WT \( \text{ntrZ} \), significantly suppressed the \( \text{ΔchvI} \) growth defect (Fig. 5A). We conclude that the \( \text{ntrY} \) and \( \text{ntrZ} \) mutations are dominant, likely gain-of-function, alleles. As deletion of \( \text{ntrX} \) rescued the growth of \( \text{ΔchvI} \) cells, overexpression of \( \text{ntrY} \) or \( \text{ntrZ} \) mutant alleles likely promotes phosphorylation and/or sequestration of NtrX. We attempted, but failed, to construct \( \text{ΔchvI ΔntrY} \) and \( \text{ΔchvI ΔntrZ} \) double-mutant strains, suggesting that deletion of either \( \text{ntrY} \) or \( \text{ntrZ} \) is synthetically

**FIG 3** Mutations in the NtrYX TCS and a gene of unknown function suppress the growth defect of \( \text{ΔchvG} \) and \( \text{ΔchvI} \) strains in M2X medium. (A) Schematic representation of the suppressor selection protocol. Primary overnight cultures in M2X medium were back diluted to an OD\(_{660}\) of 0.025 and grown until cultures grew to high turbidity. Single colonies were isolated for confirmation and sequencing. (B) Growth curves, measured by optical density (OD\(_{660}\)), of WT, \( \text{ΔchvI} \), and suppressor strains isolated from either the \( \text{ΔchvI} \) strain (\( \text{ΔIS}\# \)) or the \( \text{ΔchvG} \) strain (\( \text{ΔGS}\# \)) upon back dilution in M2X medium. Points represent averages from three biological replicates ± SD. ****, \( P<0.0001 \), one-way ANOVA followed by Dunnett’s posttest comparison to \( \text{ΔchvI} \) at 24 h. (C) Whole-genome sequencing results of each suppressor strain. The suppressor strain, parental strain, and identified polymorphism(s) are indicated. Mutations in \( \text{ntrY} \), \( \text{ntrX} \), and \( \text{ntrZ} \) (\( \text{CCNA}_03863 \)) are in bold. Domain structures of NtrY, NtrX, and NtrZ are diagrammed with domains in blue, signal peptides in green, transmembrane helices in yellow, and identified mutations in red.
lethal with chvI deletion. This inability to isolate either double mutant also hinted that NtrY and NtrZ function in the same pathway. To test this possibility, we evaluated the phenotype of ntrY and ntrZ deletions in M2X medium. However, the growth of both single deletions and the ΔntrY ΔntrZ double deletion was indistinguishable from WT, thus preventing epistasis analysis (Fig. 5B).

Phosphorylation of NtrX is likely important for stationary-phase survival (48), and thus, we hypothesized that deletion of the ntrY HK, and perhaps also ntrZ, might lead to a stationary-phase defect. Indeed, we observed significantly lower CFU in both ΔntrY and ΔntrZ cultures than WT cultures after 48 h of growth in M2X (Fig. 5C). Notably, ΔntrY ΔntrZ cultures behaved similarly to the single mutants, suggesting that NtrY and NtrZ indeed function in the same pathway.

To place ntrY and ntrZ relative to one another, we evaluated the ability of ntrY and ntrZ alleles to rescue stationary-phase survival in the deletion strains. Both ΔntrY and ΔntrZ cells were fully rescued by ectopic overexpression of each respective WT allele.
Overexpression of ntrY(L70H), but not ntrY, also fully rescued the phenotype of ΔntrZ cells (Fig. 5D). In contrast, neither WT ntrZ nor ntrZ(I99N) rescued ΔntrY cells (Fig. 5D). These data provide evidence that NtrZ functions upstream of NtrY, potentially as a kinase activator and/or phosphatase inhibitor.

We next examined the phosphorylation state of NtrX directly using Phos-tag gel electrophoresis. To detect NtrX by Western blotting, we constructed strains carrying C-terminally hemagglutinin (HA)-tagged ntrX (ntrX-HA) encoded at the native ntrX locus. Phos-tag analysis of WT lysates at stationary phase revealed distinct bands for phosphorylated (NtrX~P) and unphosphorylated (NtrX) NtrX-HA (Fig. 5E). Deletion of ntrZ ablated NtrX~P, consistent with a role for NtrZ in promoting NtrX phosphorylation (Fig. 5E).

(Fig. 5D). Overexpression of ntrY(L70H), but not ntrY, also fully rescued the phenotype of ΔntrZ cells (Fig. 5D). In contrast, neither WT ntrZ nor ntrZ(I99N) rescued ΔntrY cells (Fig. 5D). These data provide evidence that NtrZ functions upstream of NtrY, potentially as a kinase activator and/or phosphatase inhibitor.

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Surprisingly, ΔntrY cells displayed higher levels of NtrX phosphorylation than WT cells, suggesting that NtrY primarily acts as a phosphatase in vivo and is not required for NtrX phosphorylation. NtrZ indeed acts upstream of NtrY, as deletion of ntrZ in the ΔntrY background did not affect NtrX phosphorylation (Fig. 5E). We conclude that NtrZ promotes NtrX phosphorylation by inhibiting NtrY phosphatase activity.

Our characterization of the ntrY, ntrX, and ntrZ mutants suggested that phosphorylation of NtrX may rescue the growth of ΔchvI or ΔchvG cells. NtrX is phosphorylated under acidic conditions, such as those encountered during the stationary phase in M2G or M2X medium (48). Therefore, we tested whether the pH of M2X affected the growth of Sinorhizobium meliloti cultures. For both WT and ΔchvI strains, primary overnight cultures diluted in M2X at pH 7.0 reached similar CFU at 8 h as those diluted in standard M2X (pH 7.2) (Fig. 1B; Fig. S4). WT cultures were relatively unaffected by growth in M2X at pH 6.0 but had significantly fewer CFU in M2X at pH 5.5 versus pH 7.0 (Fig. S4C). In contrast, ΔchvI cells were markedly less fit in M2X at pH 6.0 than at pH 7.0 and displayed an intermediate growth yield at pH 5.5 relative to M2X at either pH 6.0 or pH 7.0 (Fig. S4C). These results are consistent both with suppression of the ΔchvI growth defect by NtrX phosphorylation and an important role for ChvGI in acid stress responses (6).

ChvI and NtrX regulate transcription of an overlapping set of genes. Although ChvGI is known to regulate the expression of chvR, the complete ChvI transcriptional regulon is not known. To examine the regulatory link between ntrX and chvI in greater detail, we performed a transcriptome deep sequencing (RNA-seq) experiment to comprehensively define ChvI-dependent gene regulation. As ΔchvI cells grow poorly in M2X medium, we exploited overexpression of the phosphomimetic chvI(D52E) allele to assess ChvI-dependent transcription in PYE medium. Excluding the internal chvI control, we identified 162 genes with >1.5-fold change in ΔchvI chvI(D52E) cells compared to ΔchvI EV cells (Fig. 6A and Table S1). Of those, 140 were upregulated and 22 were downregulated, indicating that ChvI primarily serves as a transcriptional activator. Consistent with previous work, chvR was upregulated, while chvT was downregulated by overexpression of chvI(D52E) (6, 47). In addition, expression of both chvG and hprK was enhanced in cells expressing chvI(D52E), pointing to positive autoregulation of the chvIG-hprK operon. We also observed regulation of multiple genes involved in envelope maintenance, metabolism, protein quality control, and transport (Fig. S5A). ChvI-dependent genes included multiple genes encoding proteases/peptidases (CCNA_01341, CCNA_02846, CCNA_01955, CCNA_02721, mmpA, CCNA_02594, CCNA_01121, and CCNA_01202), peptidyl-prolyl and disulfide isomerases (CCNA_02889, CCNA_01654, CCNA_01759, CCNA_01653, CCNA_00378, and CCNA_00379), members of the β-barrel assembly machine (BAM) complex (bamA, bamB, bamD, bamE, and bamF), members of the Tol-Pal complex (tolB, ybgF, and tolQ), and lipopolysaccharide biosynthesis genes (CCNA_01497, CCNA_03454, CCNA_01496, and lpxC). Moreover, nearly 40% of ChvI regulon genes encoded hypothetical proteins or proteins of unknown function (Fig. S5A). MEME analysis of the top 35 upregulated operons identified a putative ChvI binding motif, with GCC direct repeats 11 nucleotides (nt) apart, that closely resembles the recently characterized binding motif of Sinorhizobium meliloti ChvI (Fig. S5B) (5).

As our regulon was defined by overexpression of a phosphomimetic ChvI mutant, we sought to validate our data set under more physiological conditions. We constructed transcriptional β-galactosidase reporters for six selected regulon genes, from nstA (2-fold activation) to CCNA_03987 (128-fold activation). Strains carrying these transcriptional reporters were initially grown in PYE medium, followed by back dilution and 4 h of growth in M2X medium. Consistent with previous reports, the reporter for the chvR promoter (PchvR) was expressed in M2X in a chvI-dependent manner (Fig. 6B) (6, 47). The remaining reporters, apart from PnstA, which was expressed at low levels, also exhibited clear chvI dependence, supporting our RNA-seq results (Fig. 6B). Unlike the other reporters, PchvI was only 2-fold lower in ΔchvI cells than WT cells, indicating that additional factors promote expression of the chvIG-hprK operon.

We next compared our ChvI regulon with a previously published NtrX regulon that was determined using DNA microarrays (49). This experiment measured relative gene
expression between *C. crescentus* WT (strain NA1000) and ΔntrX cells during exponential growth in M2G medium, a condition where NtrX is expected to be largely unphosphorylated (48, 49). Surprisingly, a large fraction of the genes regulated by ChvI were also represented in the genes regulated by NtrX (Fig. 6A). That is, many of the genes upregulated by ChvI also appeared to be upregulated by NtrX (and likewise for downregulated genes, as shown in the heat map).

**FIG 6** The ChvI and NtrX regulons overlap significantly. (A) Heat map of log₂(fold change) for genes in the ChvI regulon (fold change > 1.5, false-discovery rate [FDR] *P* < 0.05) defined by RNA-seq (ΔchvI chvI(D52E) + EV versus ΔchvI EV). Log₂(fold change) is also shown for a microarray data set comparing RNA levels in WT and ΔntrX cells (49); gray cells indicate no data. The expression of upregulated genes (blue) is activated by ChvI or NtrX, whereas the expression of downregulated genes (yellow) is repressed by ChvI or NtrX. The genes most strongly regulated in the ChvI regulon are annotated (expression of those in bold is investigated further in panels B and C). (B) *lacZ* transcriptional reporter activity for a subset of genes in the ChvI regulon in WT and ΔchvI strains in M2X medium (average ± SD, *n* = 3). ****, *P* < 0.0001, one-way ANOVA followed by Šidák’s posttest comparison for indicated pairs. (C) *lacZ* transcriptional reporter activity for the same genes as in panel B in WT and ΔntrX strains in M2X medium (average ± SD, *n* = 4). **, *P* < 0.01; ****, *P* < 0.0001; one-way ANOVA followed by Šidák’s posttest comparison for indicated pairs.
genes). Using cutoffs of 1.5-fold change for the ChvI RNA-seq data and 2.5-fold change for the NtrX microarray data, we established that the ChvI regulon is significantly enriched for NtrX-dependent genes (6.31-fold enrichment, $P = 8.99 	imes 10^{-19}$, hypergeometric test). To confirm this overlap, we evaluated the effect of deleting ntrX on our ChvI regulon reporters. Strains carrying the transcriptional reporters were grown to log phase (OD$_{660}$ of 0.1 to 0.2) in M2X medium before assaying β-galactosidase activity. Four of the six reporters exhibited ntrX dependence, including two genes (CCNA_00889 and chvR) not evaluated in the NtrX microarray experiment (Fig. 6C). We note that transcription from the chvI promoter ($P_{chvI}$) was activated by a variety of cellular conditions (dashed arrow) and phosphorylates Chvl (blue). Phosphorylated Chvl regulates genes involved in envelope maintenance, transport, metabolism, and cell division (blue oval). In addition, Chvl represses expression of chvT. NtrY (purple) is repressed by NtrZ under acidic conditions in defined medium (dashed arrow), reducing dephosphorylation of NtrX (red). Unphosphorylated NtrX regulates much of the Chvl regulon, likely via upregulation of the chvG-hprK operon (gray arrow). In addition, unphosphorylated NtrX upregulates chvT and regulates expression of genes involved in nitrogen metabolism and transport (red oval). Phosphorylated Chvl and unphosphorylated NtrX oppose each other in regulating growth in defined medium, partially via differential regulation of chvT. The transcriptional role of phosphorylated NtrX and source of phosphorylation (question marks) are not yet known.

**FIG 7** Deletion of chvT partially restores growth of ΔchvI cells in M2X medium. (A) Optical density (OD$_{660}$) of WT, ΔchvI, and ΔchvI ΔchvT strains, with empty vector (EV) or chvT overexpression vector (+‡), 24 h after back dilution to an OD$_{660}$ of 0.025 in M2X medium (average ± SD, $n = 4$). ***, $P < 0.0005$; ****, $P < 0.0001$; one-way ANOVA followed by Dunnett’s posttest comparison to ΔchvI EV. (B) Proposed model for the regulatory interactions between ChvGI and NtrYXZ. ChvG (orange) is activated by a variety of cellular conditions (dashed arrow) and phosphorylates Chvl (blue). Phosphorylated Chvl regulates genes involved in envelope maintenance, transport, metabolism, and cell division (blue oval). In addition, Chvl represses expression of chvT. NtrY (purple) is repressed by NtrZ under acidic conditions in defined medium (dashed arrow), reducing dephosphorylation of NtrX (red). Unphosphorylated NtrX regulates much of the Chvl regulon, likely via upregulation of the chvG-hprK operon (gray arrow). In addition, unphosphorylated NtrX upregulates chvT and regulates expression of genes involved in nitrogen metabolism and transport (red oval). Phosphorylated Chvl and unphosphorylated NtrX oppose each other in regulating growth in defined medium, partially via differential regulation of chvT. The transcriptional role of phosphorylated NtrX and source of phosphorylation (question marks) are not yet known.

**DISCUSSION**

The importance of the *C. crescentus* ChvGI system for growth in defined medium. To our knowledge, the only reported physiological phenotype for chvGI mutants in *C. crescentus* is sensitivity to the antibiotic vancomycin (47). However, previous work indicated that ChvGI might be important for growth in defined medium (6).
In fact, *chvG* mutants in other alphaproteobacteria are sensitive to nutritional conditions, although most, with the exception of *Brucella abortus*, exhibit particularly poor growth in complex media (4, 8, 27, 59). Deletion of *chvG* or *chvI* in *C. crescentus* caused a distinctive growth defect in M2X medium. Δ*chvI* cultures do grow in M2X upon inoculation from PYE plates, suggesting that the physiological state of the cell in PYE agar is initially amenable to growth in defined medium (Fig. 1). However, this tolerance is limited by time, with only higher inocula able to reach high cell density (Fig. 1; see Fig. S2 in the supplemental material). Why, then, can primary overnight cultures persist at high density until back dilution in fresh M2X? One possibility is that the low pH of M2X at stationary phase preserves Δ*chvI* and Δ*chvG* cells, potentially by triggering phosphorylation of NtrX (Fig. 7B) (48). This model is supported by the observation that Δ*chvI* cells reach higher CFU when back diluted in M2X at pH 5.5 versus pH 6.0 (Fig. S4C). As ~20% of the NtrX pool is phosphorylated in M2G medium at pH 6.0 versus ~40% at pH 5.5 (by Phos-tag analysis), the pH 6 to 5.5 transition would significantly change the level of unphosphorylated NtrX (48). However, as Δ*chvI* and Δ*chvG* cells also appear to be sensitive to acidic pH, additional factors are likely at play (Fig. S4C). For example, several extracytoplasmic function (ECF) sigma factors are involved in resistance to stationary-phase stress and might play a protective role in Δ*chvI* and Δ*chvG* cells (60–62).

A recent study failed to identify *chvG* or *chvI* as being important for fitness in M2X medium (63). However, in this work, M2X cultures were inoculated from PYE starter cultures at high enough density to reach saturation in 5 doublings. Thus, these experiments likely mimicked our primary overnight cultures, obscuring detection of fitness defects for *chvGI* mutants.

**The Chvl transcriptional regulon in *C. crescentus***. Although ExoR or Chvl transcriptional regulons have been defined in several alphaproteobacteria (4, 5, 26, 64), the *C. crescentus* Chvl regulon was unknown prior to this work. We employed RNA-seq to detect direct and indirect transcriptional targets of Chvl in *C. crescentus* (Fig. 6; Table S1). Our Chvl regulon contained several classes of genes noted in other alphaproteobacteria, including those encoding outer membrane proteins and transporters, metabolic enzymes, lipopolysaccharide biosynthesis enzymes, and stress response proteins (4, 5, 26, 64). We note, in particular, that the *C. crescentus* Chvl regulon contained a large number of genes encoding proteins involved in envelope maintenance, including nearly the entire BAM complex, the envelope integrity protein EipA, members of the Tol-Pal complex, and a variety of chaperones and proteases (Fig. 6 and Table S1) (65–68). The idea that Chvl is involved in envelope integrity is consistent with previous observations that Chvl is activated by envelope stress and confers resistance to antibiotics targeting the cell wall (47). However, ~40% of genes regulated by Chvl are annotated generically or as hypotheticals, and thus, more work will be required to characterize the pathways downstream of the Chvl system. In *A. tumefaciens*, *S. melloti*, and *C. crescentus*, Chvl appears to suppress cell motility and/or chemotaxis (5, 15, 20, 26, 69, 70). However, unlike in *A. tumefaciens* and *S. melloti*, *C. crescentus* Chvl does not regulate any obvious flagellar or chemotaxis genes, suggesting that effects on motility may be due to posttranscriptional regulation and/or alterations in cell cycle progression (5, 26, 71–73).

Overexpression of the phosphomimetic *chvl(D52E)* allele induced cell filamentation in M2X, implicating Chvl in regulating cell division and cytokinesis in particular. Chvl upregulates several genes related to cell division, including *zauP*, members of the Tol-Pal complex, *smc*, and *nstA* (68, 74–77). We note that overexpression of a proteolytically stable mutant form of *nstA* induces cell filamentation, and both *zauP* and the Tol-Pal complex are involved in regulating cytokinesis (74, 77). Perhaps overinduction of these regulon genes, in combination with the cellular state in M2X medium, interferes with proper cell division.

**Genetic interactions between Chvl and NtrYX**. The severe growth defect of Δ*chvG* and Δ*chvI* cells in M2X medium allowed us to uncover the first known genetic interaction between *chvG* and *ntrYX* (Fig. 3). Importantly, deletion of *ntrX* suppressed the growth defect of Δ*chvI* cells, whereas overexpression of nonphosphorylatable *ntrX*
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(D53A) was deleterious for growth in both Δchvl and WT cells (Fig. 4). These results suggest that the activity of unphosphorylated NtrX is particularly detrimental in cells lacking chvl. Although unphosphorylated response regulators are often assumed to be inactive, multiple RRs are known to affect transcription in their unphosphorylated states (78–82).

Thus, we propose that phosphorylated Chvl and unphosphorylated NtrX oppose each other to regulate growth in defined medium (Fig. 7B). We note that a past study examining connections between Chvl and NtrX in S. meliloti did not test whether perturbations in NtrYX signaling might affect Δchvl phenotypes (25). We predict that a similar Chvl-NtrX relationship may be conserved in other alphaproteobacteria.

As gain-of-function mutations in ntrY and ntrZ suppress the growth defect of Δchvl cells, we also propose that phosphorylation of NtrX relieves its detrimental activity, possibly by changing its transcriptional regulon. Although the global transcriptional effects of NtrX phosphorylation have yet to be characterized, in vitro phosphorylation of B. abortus NtrX does induce conformational changes and alters, but does not weaken, binding to the ntrY promoter (83). Interestingly, we were unable to construct Δchvl ΔntrY and Δchvl ΔntrZ strains, suggesting that these gene deletion combinations are synthetically lethal in PYE. As ntrX is also essential in PYE (20), the balance between unphosphorylated and phosphorylated NtrX may be important for growth in both complex and defined media.

To identify downstream genes that mediate the oppositional relationship between Chvl and NtrX, we compared their transcriptional regulons (Fig. 6 and Table S1). The Chvl regulon strongly overlapped with that of NtrX, as 80% of the top 30 Chvl-activated genes are also activated by NtrX (per our >1.5-fold cutoff). In contrast, only 20% of the top 30 NtrX-activated genes exhibit >1.5-fold Chvl dependence (49). Given that chvl, chvG, and hprK are upregulated by NtrX, it may be the case that NtrX simply activates expression of the chvIG-hprK operon, thereby altering transcription of genes in the Chvl regulon (Fig. 7B, gray arrow). However, further work is required to define the mechanism by which NtrX affects the Chvl regulon, as it may also indirectly affect ChvGI signaling or directly regulate expression of regulon genes. The overlap we observed between the Chvl and NtrX regulons may be restricted to C. crescentus and close relatives, as chvG and chvl are not transcriptionally regulated by NtrYX in Rhodobacter sphaeroides (40). However, ChvGI and NtrYX may still be transcriptionally linked in more distantly related alphaproteobacterial species, as ntrX is part of the A. tumefaciens ExOR regulon (26).

The majority of overlapping genes in the Chvl and NtrX regulons cannot account for the detrimental effect of unphosphorylated NtrX on Δchvl cells. Therefore, we focused on eight genes that exhibited opposing transcriptional regulation by Chvl and NtrX (Fig. S6). Given that NtrX largely reinforces the ChvGI TCS, these oppositional effects may reflect direct transcriptional regulation by NtrX or the effects of unique NtrX regulon genes. Only deletion of chvT improved the growth of Δchvl cells, indicating that suppression of chvT RNA levels by Chvl may be important for growth in M2X medium (Fig. 7). chvT is linked to diverse phenotypes in C. crescentus, including survival in stationary phase, sensitivity to cell wall-targeting antibiotics, and sensitivity to bacteriocins (47, 84, 85). However, the molecule(s) transported by ChvT remains undefined. High ChvT levels may contribute to defects in transport in defined medium and/or alter membrane integrity, impacting the viability of cells lacking ChvGI (47). It is clear, though, that altered chvT expression cannot fully explain the growth deficiency of Δchvl cells in M2X medium. This growth defect may result from the collective action of several genes, and therefore, manipulation of individual candidates may not rescue growth in M2X. Moreover, 15 genes in the Chvl regulon are absent from the NtrX microarray data set, raising the possibility that one or more are differentially regulated by Chvl and NtrX (Fig. 6; Table S1). Chvl and NtrX might also interact more indirectly, as each regulates unique subsets of genes that may affect growth in M2X medium.

On the role of NtrZ. NtrYX is associated with a wide range of physiological responses, from nitrogen metabolism to redox sensing and cell envelope maintenance.
Despite these phenotypic observations, little is known about NtrY activity in vivo and the regulation of NtrY via its periplasmic domain. Our work reveals a surprising phosphatase pathway involving the previously uncharacterized protein NtrZ. Phos-tag analysis of NtrX phosphorylation in vivo clearly demonstrates that NtrY is dispensable for NtrX phosphorylation and suggests that NtrY primarily acts as an NtrX phosphatase (Fig. 5). In addition, NtrZ appears to inhibit NtrY phosphatase activity, as deletion of ntrZ abolishes NtrX phosphorylation only when ntrY is present (Fig. 5). Thus, we propose that NtrZ inhibits NtrY phosphatase activity, stabilizing the pool of phosphorylated NtrX (Fig. 7). In the future, we are interested in determining whether NtrZ physically interacts with the NtrY periplasmic domain or affects NtrY activity indirectly. Several known periplasmic or membrane-bound TCS regulators directly interact with HK periplasmic domains (33, 86–88). Although our model does not exclude the possibility that NtrY phosphorylates NtrX under certain conditions, there is clearly another source of NtrX phosphorylation in C. crescentus. An additional HK may phosphorylate NtrX, although the most likely candidate, NtrB, does not phosphorylate NtrX in vitro (49). Alternatively, some metabolic intermediates, such as aspartyl-phosphate or carbamoyl-phosphate, can serve as phosphodonor for RRs in vivo and in vitro (1, 89–92).

Interestingly, we initially placed ntrZ upstream of ntrY by examining the similar stationary-phase survival phenotypes of ΔntrY and ΔntrZ cells (Fig. 5). Given our Phos-tag results, these phenotypes suggest that both phosphorylated and unphosphorylated NtrX play important roles in stationary-phase survival. This idea is consistent with the fact that NtrX phosphorylation increases upon entry into stationary phase but eventually decreases (48).

Fernández et al. reported that NtrX phosphorylation is triggered by acidic pH in defined medium (48). Therefore, we hypothesize that inhibition of NtrY by NtrZ may be enhanced by acidic pH. Thus, NtrZ and ExoR potentially share a regulatory theme in which their activities toward their respective HKs are affected by low pH (16, 33, 34). However, it remains to be seen if, as with ExoR, proteolysis plays a role in controlling NtrZ-dependent regulation of NtrY (16). Given the results of our suppressor selection, it would appear that mutations in NtrZ or in the transmembrane helices of NtrY may bypass regulation by acidic pH. In an alignment of 100 NtrZ homologs, ranging from 41% to 100% sequence identity, the Y92 position is conserved as an aromatic residue (95% Y), while I99 is conserved as an aliphatic hydrophobic residue (51% I, common L and V substitutions), suggesting that these residues are important for NtrZ function. Further work is required to determine how mutation of Y92 or I99 may alter interaction with the NtrY periplasmic domain or affect a different aspect of NtrZ function. An alignment of the noncytoplasmic portion of 250 NtrY sequences, ranging from 37% to 100% identity, reveals that the L70 position is largely conserved as an aliphatic hydrophobic residue (94% L), whereas A123 is conserved as a hydrophobic residue (93% A), albeit with occasional bulky aromatic substitutions. It is unsurprising, then, that the L70H and A123V substitutions appear to affect NtrY function. In fact, several studies have identified transmembrane mutations that alter HK kinase and phosphatase activities (93–95).

Unlike the periplasmic kinase regulator ExoR, which contains Sel1-like repeats typically involved in protein-protein interactions, NtrZ does not contain any conserved domains or motifs (107). Moreover, NtrZ appears restricted to the order Caulobacterales, albeit with a few distant homologs in other alphaproteobacteria. Notably, neither B. abortus, S. mellioti, nor A. tumefaciens contains NtrZ homologs, and conservation of the noncytoplasmic region of NtrY is quite low between C. crescentus and these organisms (24 to 27% identity). However, L70 is largely conserved (L or V), and A123 is entirely conserved between these NtrY homologs. Thus, the L70H and A123V substitutions may have similar effects on NtrY activity in these organisms, and further analyses of these mutants may shed light on a conserved NtrY activity switch. Moreover, it will be
interesting to see if NtrY also primarily acts as a phosphatase and/or is regulated by peri-plasmic effectors in *S. melloti*, *A. tumefaciens*, and *B. abortus*.

**MATERIALS AND METHODS**

**Strains and plasmids.** All plasmids were constructed using standard molecular biology techniques. See Table S2 in the supplemental material for strain, plasmid, and primer information. Plasmids for generating in-frame deletions and allele replacements were generated by cloning homologous upstream and downstream regions into pNPT5138. Transcriptional reporter plasmids were generated by cloning 400 to 500bp upstream of the open reading frame (ORF) into pRlac290. For overexpression strains, ORFs were inserted into pMT585, a plasmid for xyllose-inducible expression that integrates at the *xyl* locus. Plasmids were transformed into *C. crescentus CB15* strain by electroporation or triparental mating. In-frame deletion and allele replacement strains were generated by a double recombination strategy involving sacB counterselection on PYE plates supplemented with 3% sucrose (96). For Δ*ntrX* knockout strains, counterselection was carried out on M2X plus 0.5% sucrose plates due to their growth defect on PYE. Δ*ntrX* and ΔchvI Δ*ntrX* strains were grown only on M2 medium. Construction of ΔchvI Δ*ntrY* and ΔchvI Δ*ntrZ* mutants was attempted using both PYE and M2X counterselection methods. All *C. crescentus* strains were grown at 30°C. For strains carrying pRlac290 plasmids, oxytetracycline was added to 1 μg/ml in liquid and 2 μg/ml in M2X agar or 1 μg/ml in M2X agar. M2X medium contained 6.1 mM Na2HPO4, 3.9 mM KH2PO4, 9.3 mM NH4Cl, 0.25 mM CaCl2, 0.5 mM MgSO4, 1:1,000 100 μg/ml ferrous sulfate chelate solution (Sigma), and 0.15% xyllose. PYE medium contained 0.2% peptone, 0.1% yeast extract, 0.5 mM CaCl2, and 1 mM MgSO4.

**Measurement of growth in M2X medium.** Primary M2X cultures were inoculated from plates to an approximate density of OD660 of 0.02 to 0.10 and grown overnight. Overnight cultures were back diluted to an OD660 of 0.025, and OD660 was recorded at the indicated times. To enumerate CFU, samples were taken at the indicated time points, and 10-fold serial dilutions were plated on PYE agar. For pH experiments, M2X medium was adjusted to the indicated pH using HCl.

For experiments with controlled starting densities, cells were resuspended in M2X medium directly from PYE plates. These resuspensions were then diluted to the indicated OD660 and the titers of the cultures were determined for CFU. In washing experiments, 1 ml resuspended cells were spun down at 8,000 × g for 3 min and resuspended in 1 ml fresh M2X medium once (1×) or twice (2×) before dilution. Plotting and statistical analyses were carried out using Prism (GraphPad).

**Microscopy.** Samples of ΔchvG and ΔchvI cells were taken from overnight M2X or PYE plus 0.15% xylose cultures and imaged with a DMi6000 B (Leica) microscope in phase contrast using a HC PL APO 8,000/0.15 objective. Microscopy. Samples of ΔchvG and ΔchvI cells were taken from overnight M2X or PYE plus 0.15% xylose cultures and imaged with a DMi6000 B (Leica) microscope in phase contrast using a HC PL APO 8,000/0.15 objective. Images were captured using an Orca-R2 C10600 digital camera (Hamamatsu) controlled by Leica Application Suite X (Leica). Images were processed using Fiji (97, 98).

**Phos-tag gel electrophoresis and Western blotting.** Two-milliliter M2X cultures of strains containing *ntrX* or *ntrY* were grown overnight and back diluted to an OD660 of 0.01. Samples were collected after 22 h (0.25 ml - OD660 i.e., volume [ml] = 0.25/OD660 of culture) and frozen at −80°C. Samples were thawed, resuspended in 2.5 × SDS loading buffer (125 mM Tris [pH 6.8], 25% glycerol, 5% SDS, 5 mM diithiothreitol [DTT], and 0.01% bromophenol blue) containing 1.5% benzamide (Sigma), and immediately loaded onto Phos-tag gels.

Phos-tag electrophoresis was performed as described (48) using 8% acrylamide gels copolymerized with 33 μM Phos-tag acrylamide (Nard) and 150 μM ZnCl2. Proteins were transferred to polyvinylidene difluoride (PVDF; Bio-Rad) in a wet-transfer apparatus. Membranes were probed with monoclonal HA-tag antibody (1:2,000 dilution; Invitrogen; 2-2.2.14), incubated with anti-mouse IgG-horseradish peroxidase (IgG-HRP) conjugate (1:5,000 dilution; Invitrogen), and developed with ProSignal Pico spray (Prometheus). Blots were imaged using a Bio-Rad ChemiDoc MP Imager. Bands were quantified using Fiji (97, 98).

**Trancriptome deep sequencing.** Two milliliters of deep culture were inoculated with ΔchvI EV (ΔchvI xylI:pMT585) and ΔchvI chvI (ΔchvI chvI (pMT585-chvI(D52E)) cells and grown overnight. Cultures were diluted to an OD660 of 0.001 in 2 ml fresh PYE and grown for 22.5 h. Cultures were diluted to an OD660 of 0.075 in 5 ml PYE plus 0.15% xylose and grown for 3.5 h before Trizol extraction and RNA isolation, as described previously (101). RNA-seq libraries were prepared using an Illumina TrueSeq stranded RNA kit and sequenced on an Illumina NextSeq 500 instrument at the University of Chicago Functional Genomics Facility. Sequencing data were analyzed using CLC Genomics Workbench 20 (Qiagen) by mapping reads to the *C. crescentus* NA1000 genome (84). Motif searching was carried out using MEME (102). Heatmaps were generated using Java TreeView3, and hypergeometric analysis was performed using phyper in R (103, 104).
**β-Galactosidase assays.** For assays of WT versus ΔchvI strains, cells were grown overnight in 2 ml PYE medium. Then, 500 μl of each culture was centrifuged at 8,000 x g for 3 min and resuspended in 500 μl M2X medium. Resuspended cultures were used to inoculate 2 ml M2X medium at a starting OD660 of 0.075. Cultures were grown for 4 h before assaying β-galactosidase activity. For assays of WT versus ΔntrX strains, cells were grown overnight in 2 ml M2X medium. Overnight cultures were diluted in M2X medium such that they would reach OD660 of 0.1 to 0.2 after 23.5 h of growth. β-Galactosidase assays were performed as previously described using 200 μl of M2X culture plus 100 μl sterile PYE medium as an emulsifier (105). Plotting and statistical analyses were carried out using Prism.

**Data availability.** RNA-seq data are available in the NCBI’s Gene Expression Omnibus (GEO) database (106) under the GSE168965.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**

PDF file, 4.6 MB.

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