Convergence of Alarmone and Cell Cycle Signaling from Trans-Encoded Sensory Domains

Stefano Sanselicio, Patrick H. Viollier

Department of Microbiology and Molecular Medicine, Institute of Genetics and Genomics in Geneva (iGE3), Faculty of Medicine, University of Geneva, Geneva, Switzerland

ABSTRACT  Despite the myriad of different sensory domains encoded in bacterial genomes, only a few are known to control the cell cycle. Here, suppressor genetics was used to unveil the regulatory interplay between the PAS (Per-Arnt-Sim) domain protein MopJ and the uncharacterized GAF (cyclic GMP-phosphodiesterase–adenyllyl cyclase–FhlA) domain protein PtsP, which resembles an alternative component of the phosphoenolpyruvate (PEP) transferase system. Both of these systems indirectly target the Caulobacter crescentus cell cycle master regulator CtrA, but in different ways. While MopJ acts on CtrA via the cell cycle kinases DivJ and DivL, which control the removal of CtrA at the G1-S transition, our data show that PtsP signals through the conserved alarmone (p)ppGpp, which prevents CtrA cycling under nutritional stress and in stationary phase. We found that PtsP interacts genetically and physically with the (p)ppGpp synthase/hydrolase SpoT and that it modulates several promoters that are directly activated by the cell cycle transcriptional regulator GcrA. Thus, parallel systems integrate nutritional and systemic signals within the cell cycle transcriptional network, converging on the essential alphaproteobacterial regulator CtrA while also affecting global cell cycle transcription in other ways.

IMPORTANCE  Many alphaproteobacteria divide asymmetrically, and their cell cycle progression is carefully regulated. How these bacteria control the cell cycle in response to nutrient limitation is not well understood. Here, we identify a multicomponent signaling pathway that acts on the cell cycle when nutrients become scarce in stationary phase. We show that efficient accumulation of the master cell cycle regulator CtrA in stationary-phase Caulobacter crescentus cells requires the previously identified stationary-phase/cell cycle regulator MopJ as well as the phosphoenolpyruvate protein phosphotransferase PtsP, which acts via the conserved (p)ppGpp synthase SpoT. We identify cell cycle-regulated promoters that are affected by this pathway, providing an explanation of how (p)ppGpp-signaling might couple starvation to control cell cycle progression in Caulobacter spp. and likely other Alphaproteobacteria. This pathway has the potential to integrate carbon fluctuation into cell cycle control, since in phosphotransferase systems it is the glycolytic product phosphoenolpyruvate (PEP) rather than ATP that is used as the phosphor donor for phosphorylation.
FIG 1 MopJ and PtsP are pleiotropic regulators that control motility and cell cycle progression in Caulobacter crescentus. (A) Model showing the C. crescentus cell cycle and the relevant cell cycle transcriptional regulators CtrA and GcrA, as well as the recently described PAS domain protein MopJ (23). The thin black vertical line represents the flagellar filament (composed of FljK, FljM, and other flagellins), before it rotates (wavy line). The thick vertical black line represents the stalk, and the white oval represents the chromosome, whose replication is initiated at the C. crescentus origin of replication (Cori). The thin slanted black lines represent the polar pili (composed of the PilA pilin). The expression of MopJ and CtrA is transcriptionally activated by GcrA (blue arrows), while CtrA activates expression of the methylase CcrM, the flagellin FljM, and the pilin PilA. Expression of the flagellin FljK by CtrA is indirect (7). Shown underneath is a model of the (p)ppGpp-dependent signaling pathways in stationary-phase C. crescentus cells described in the text. Dashed arrows indicate connections that are poorly defined. (B, top) Motility assay on swarm (0.3%) agar for WT, mopJ::himar, ∆mopJ, and ∆ptsP single mutants, the ∆mopJ ∆ptsP double mutant, and two

(Continued)
on a conserved histidine (His) in an ATP-dependent manner (11). However, phosphorylation of CtrA underlies a multicomponent His-Asp (HA) relay, regulated in time and space (12) to restrict CtrA activity and its presence during the cell cycle. CtrA is abundant in G1 phase, degraded at the G1-S transition, resynthesized in S-phase after transcriptional activation from its promoter by the conserved regulator GcrA (13, 14) (Fig. 1A), and subsequently phosphorylated by the HA relay acting on CtrA. In the absence of CpdR, CtrA protein levels no longer oscillate during the cell cycle (15, 16).

The conserved alarmone (p)ppGpp (guanosine 3’,5’-bispyrophosphate) is induced under different starvation conditions, and in stationary phase it also interferes with CtrA oscillations through an unknown mechanism (17–21). Although conditions of nitrogen or carbon starvation are known to result in the induction of (p)ppGpp via the synthase/hydrolase SpoT in C. crescentus (17–20), it is unclear how nutritional changes are perceived and relayed to SpoT to keep cells idling in the (motile) G1 phase. Interestingly, a nutritional downshift has been used for the enrichment of nitrogen or carbon starvation are known to result in the induc-

RESULTS

Mutations in the GAF domain of PtsP suppress the motility de-
fect of \( \Delta \text{mopJ} \) cells. Prolonged incubation of \( \Delta \text{mopJ} \) colonies on swarm agar gives rise to highly motile flares growing out from the poorly motile \( \Delta \text{mopJ} \) background (23). Whole-genome sequenc-
ing of two such \( \Delta \text{mopJ} \) motility suppressors (Fig. 1B) revealed a single missense mutation (S104P or Q153P) in the GAF domain-
encoding region of PtsP (CCNA_00892) (Fig. 1C) in each strain. PtsP resembles E1 regulatory components of the phosphoenolpy-
ruvate (PEP)-dependent transport system (PTS) that typically use PEP rather than ATP as the phospho donor to phosphorylate cli-
ent proteins such as the Hpr phospho-carrier protein (25).

We investigated the role of PtsP in motility by constructing an in-frame deletion in \( \text{ptsP} \) (\( \Delta \text{ptsP} \)) in wild-type (WT; NA1000) cells, and we observed a reduction in motility on swarm agar (Fig. 1B) that was restored by complementation with a plasmid carrying \( \text{ptsP} \) (pMT335-\( \text{ptsP} \)) (Fig. 1B). Flow cytometry (Fig. 2A and B) and differential interference contrast (DIC) microscopy (Fig. 2C and D) additionally revealed that the \( \Delta \text{ptsP} \) mutation reduced the number of G1-phase cells in the exponential and sta-
tionary phases and caused a mild perturbation in cytokinesis, akin to that observed with the \( \Delta \text{mopJ} \) strain (Fig. 1B and 2A to D) (23). The \( \Delta \text{ptsP} \) mutation accentuated the defects of the \( \Delta \text{mopJ} \) strain (Fig. 1B and 2A to D), indicating that MopJ and PtsP control similar functions.

Since the \( \Delta \text{ptsP} \) mutation impaired swarming motility, while the \( \text{ptsP} \) (S104P) and \( \text{ptsP} \) (Q153P) constructs appeared to enhance it by way of a replacement of a polar residue with a secondary structure-breaking proline, we reasoned that the GAF\(^{\text{S104P}}\) and GAF\(^{\text{Q153P}}\) mutations might confer a gain-of-function mutation to PtsP. To test this idea, we deleted the GAF-encoding residues (residues 33 to 159) of \( \text{ptsP} \) from WT and \( \Delta \text{mopJ} \) cells to determine if the GAF domain simply acts as an autoinhibitory domain. Using swarming motility as readout (Fig. 1D), we observed that the resulting \( \text{ptsP} \Delta \text{GAF} \) (S104P) and \( \text{ptsP} \Delta \text{GAF} \) (Q153P) double mutant exhibited a slight increase in motility compared to their parental strains on swarm agar. We also observed that the growth rates of the \( \Delta \text{mopJ} \) \( \text{ptsP} \Delta \text{GAF} \) double mutant and the \( \text{ptsP} \Delta \text{GAF} \) single mutant were diminished compared to the WT, akin to the \( \Delta \text{mopJ} \) \( \text{ptsP} \) (S104P) and \( \Delta \text{mopJ} \) \( \text{ptsP} \) (Q153P) strains (Fig. 1E), supporting the notion that the \( \Delta \text{GAF} \) mutation can relieve auto-
inhibition or at least partially phenocopy the point mutations. In

Figure Legend Continued

spontaneously isolated \( \Delta \text{mopJ} \) motility suppressors, \( \Delta \text{mopJ} \) \( \text{ptsP} \) (S104P) and \( \Delta \text{mopJ} \) \( \text{ptsP} \) (Q153P). (Bottom) Complementation of the \( \Delta \text{ptsP} \) motility defect with \( \text{ptsP} \) (pMT335-\( \text{ptsP} \)) [pMT335-\( \text{ptsP} \)], but not with empty \( \text{ptsP} \) (pMT335 [pMT335]) also restored WT (NA1000) cell motility. (C) Domain organization of PtsP from the N to C terminus, indicating the total length in amino acids (aa) of the protein. Asterisks indicate the position of the suppressive mutation in the PtsP GAF domain. (D) Motility assay on soft (0.3%) agar with WT, \( \Delta \text{mopJ}, \Delta \text{ptsP}, \Delta \text{mopJ} \) \( \text{ptsP} \) (S104P), \( \text{ptsP} \Delta \text{GAF} \), and \( \Delta \text{mopJ} \) \( \text{ptsP} \) \( \Delta \text{GAF} \) strains. (E) The \( \text{ptsP} \) (S104P) or \( \text{ptsP} \) (Q153P) suppressor mutations in \( \Delta \text{mopJ} \) (top) and the deletion of the GAF domain of \( \text{ptsP} \) in the WT or in the \( \Delta \text{mopJ} \) background (bottom) increased the doubling time of cells. Growth curves are shown for the WT, \( \Delta \text{mopJ} \) \( \text{ptsP} \) (S104P), \( \Delta \text{mopJ} \) \( \text{ptsP} \) (Q153P), \( \text{ptsP} \Delta \text{GAF} \), and \( \Delta \text{mopJ} \) \( \text{ptsP} \) \( \Delta \text{GAF} \) cells in PYE. Error bars in the graph indicate standard deviations. (F) Immunoblot showing the steady-state levels of \( \text{PtsP}, \text{CtrA}, \text{GcrA}, \) and \( \text{CcrM} \) during the cell cycle of WT cells (top) or \( \Delta \text{mopJ} \) \( \text{ptsP} \) (S104P) cells (bottom). The time (in minutes) after synchronization is indicated above the blots. (G) Fluorescence and DIC images show the localization pattern of \( \text{PtsP-GFP} \) (C-terminal fusion of \( \text{P} \) to GFP) expressed under the control of \( \text{P} \) [xylose inducible] at the xylI locus in WT cells.
contrast, the growth rates of the ΔmopJ and ΔptsP single mutants were similar to that of the WT (see Fig. S1 in the supplemental material).

PtsP affects CtrA accumulation in stationary phase and during the cell cycle. To explore the possibility that the reduced growth rate of the ΔmopJ ΔptsP(S104P) mutant (the mutant exhibiting the lowest growth rate) stems from a deregulated cell cycle, we conducted immunoblotting experiments using antibodies against CtrA, GcrA, and the DNA methyltransferase CcrM, whose gene is directly regulated by CtrA (Fig. 1A), in synchronized cells. We observed that the cycling of CtrA and CcrM was altered in the ΔmopJ ΔptsP(S104P) strain versus the WT, while the appearance of GcrA seemed not affected or only mildly affected (Fig. 1F). Difficulties in obtaining a stable ΔmopJ ΔptsP(S104P) strain prevented us from exploring if this mutation in isolation also affects the cell cycle.

The altered cycling of CtrA and CcrM in ΔmopJ ptsP(S104P) cells prompted us to assay the CtrA-activated pPpilA-lacZ, pPsciP-lacZ, and pPfljK-lacZ promoter-probe plasmids (note that PfljK is indirectly activated by CtrA, while the others are directly activated) in exponential-phase (Fig. 3A) and stationary-phase (Fig. 3B) ptsP and mopJ single and double mutant cells. We observed a commensurate reduction in promoter activity in the ΔmopJ ΔptsP double mutant compared to the ΔptsP and ΔmopJ single mutants, with the strongest effect occurring in stationary phase (Fig. 3B). In contrast, in the ΔmopJ ptsP(S104P) and ΔmopJ ptsP(Q153P) suppressor mutants, there was a strong upregulation of LacZ activity relative to the WT (Fig. 3A and B). Immunoblotting using polyclonal antibodies against FljK, SciP, and PilA confirmed these transcriptional trends of the ΔmopJ ΔptsP(S104P) and ΔmopJ ΔptsP(Q153P) suppressor mutants (Fig. 3C and D). (Note that the PilA protein is absent from stationary-phase WT cells for reasons that are currently unknown, but it likely operates at the post-transcriptional level [compare Fig. 3B and D]).

We also observed a strong reduction in CtrA steady-state levels in stationary-phase ΔptsP cells, similar to the response of ΔmopJ cells observed previously (Fig. 3E) (23). This effect was not apparent in exponential-phase cells (Fig. 3E), and only a weak effect was seen during the transition from exponential to stationary phase (see Fig. S2A in the supplemental material). In contrast, CtrA-M2, a version of CtrA that is no longer degraded by the ClpXP protease because the C-terminal proteolytic signal has been masked (10),

FIG 2 MopJ and PtsP promote the accumulation of G1-phase cells. (A and B) FACS analysis of ΔmopJ and ΔptsP mutant strains and the ΔmopJ ΔptsP double mutant strain showed a reduction in G1 phase. Genome content (FL1-A channel) and cell size (FSC-A channel) were analyzed by FACS during exponential (A) and stationary (B) phases in M2G. (C and D) ΔmopJ and ΔptsP single mutants and the ΔmopJ ΔptsP double mutant showed filamentation. DIC images of WT, ΔmopJ and ΔptsP single mutants, and the ΔmopJ ΔptsP double mutant during exponential (C) and stationary (D) growth phases in M2G.
accumulates to near-wild-type steady-state levels in stationary-phase ΔmopJ or ΔptsP cells (Fig. 3E). CtrA accumulation is similarly restored when the CdpR proteolytic regulator of CtrA is inactivated (15, 16) (see Fig. S2B), indicating that MopJ and PtsP (indirectly) protect CtrA from degradation in stationary phase. In contrast, the steady-state levels of CtrA in stationary ΔmopJ ptsP(S104P) and ΔmopJ ptsP(S153P) cells were near (or exceeded) WT levels (Fig. 3E), showing that the ptsP suppressor mutations act positively on CtrA abundance, at least in the context of a ΔmopJ mutation. In support of the idea that ptsP mutations additionally affect ctrA promoter activity, LacZ measurements (β-galactosidase assays) of strains harboring the P_{ctrA}-lacZ reporter plasmid revealed a strong reduction in stationary ΔmopJ and ΔmopJ ptsP cells, but near-wild-type activity in ΔmopJ ptsP(S104P), ΔmopJ ptsP(P153P), and ΔmopJ ptsPΔGAF mutants (Fig. 3F).

We conclude that MopJ and PtsP influence CtrA at the post-transcriptional level, while PtsP additionally promotes ctrA transcription. GcrA and CtrA both positively and directly regulate transcription of the ctrA gene via the P1 and P2 promoter, respectively (13, 14, 26). Thus, our finding that CtrA abundance, but not P_{ctrA}-lacZ activity, is reduced in stationary-phase ΔmopJ cells (Fig. 3E and F) implies that P_{ctrA} Activity can be sustained in a (largely) CtrA-independent manner in stationary phase, perhaps by GcrA or a related pathway (this is explored further below [Fig. 4G; see also Fig. S3C in the supplemental material]).

PtsP signals via SpoT. To further dissect the PtsP signaling pathway genetically, we isolated a motility suppressor of the ΔmopJ mutant and found by genome sequencing an in-frame deletion encoding residues 493 to 514 of the C-terminal regulatory domain of the (p)ppGpp synthase/hydrolase SpoT (27) in this strain (ΔptsP spoTΔ222) (Fig. 4A and B). The spoTΔ222 mutation also improved the motility of the ΔptsP mopJ::himar1 double mutant (Fig. 4B; see also Fig. S3A in the supplemental material), although to a lesser extent, possibly because of a contribution of MopJ to motility. Consistent with the notion that the spoTΔ222 allele is a gain-of-function mutation that causes an ectopic increase in (p)ppGpp levels, induction of (p)ppGpp from the heterologous (p)ppGpp constitutively active synthase RelA* (which lacks the C-terminal regulatory domain) of Escherichia coli (17, 21) is sufficient to improve motility of ΔptsP cells on swarm agar (Fig. 4C), thus acting analogous to the spoTΔ222 mutation. In contrast, the ΔspoT deletion phenocopies the motility of the ΔptsP mutant and the motility of the ΔspoT mopJ::himar1 mutant strain resembles that of the ΔptsP mopJ::himar1 strain (Fig. 4B).

As for the ΔptsP strain, CtrA abundance and P_{ctrA}-lacZ activity were strongly reduced in stationary-phase ΔspoT cells (Fig. 3E and F). CtrA levels were restored in stationary-phase ΔptsP spoTΔ222 double mutant cells (Fig. 3E), and P_{ctrA}-lacZ activity in exponential- or stationary-phase ΔptsP spoTΔ222 cells was elevated relative to the WT (Fig. 3F). Moreover, experiments using CtrA-dependent promoter probe plasmids revealed that transcriptional activity in ΔptsP spoTΔ222 double mutant cells was higher than in the WT (Fig. 4F), unlike the ΔptsP single mutant (Fig. 3B). Lastly, pulldown experiments using epitope-tagged variants of SpoT or PtsP (Fig. 4D and E; see also Fig. S3B in the supplemental material) revealed that both proteins interact directly or indirectly.

**PtsP and SpoT act on GcrA target promoters.** The difference in motility between the ΔptsP spoTΔ222 double mutant and the ΔptsP spoTΔ222 mopJ::himar1 triple mutant strains (Fig. 4F) raised the possibility that MopJ is regulated by the PtsP pathway. Indeed, we previously showed that expression of a transcriptional fusion of the mopJ promoter to the lacZ reporter gene (P_{mopJ}-lacZ) is regulated by (p)ppGpp; artificial induction of (p)ppGpp during exponential growth augmented P_{mopJ}-lacZ activity, while it was diminished in stationary-phase ΔspoT cells (23). As shown in Fig. 4G, P_{mopJ}-lacZ is also downregulated by the ΔptsP deletion to the same extent as by the ΔspoT mutation. Conversely, P_{mopJ}-lacZ is restored in exponential-phase ΔptsP spoTΔ222 cells, even exceeding the values for WT cells (Fig. 4G). These results mirrored those obtained with the P_{ctrA}-lacZ reporter plasmid, and since mopJ and ctrA are both targets of GcrA, we hypothesized that PtsP/SpoT signaling may affect other GcrA target promoters. In support of this idea, we found that the activity of P_{npq-lacZ}, a transcriptional reporter of the GcrA target promoter P_{npq} directing expression of the TipF flagellar regulator/cyclic-di-GMP receptor protein (14, 28), showed a PtsP/SpoT-dependent response similar to that with P_{ctrA}-lacZ and P_{mopJ}-lacZ (see Fig. S3C in the supplemental material).

**DISCUSSION**

Two concerted pathways involving the PAS domain protein MopJ (23) and the GAF domain protein PtsP are now known to promote the accumulation of the conserved cell cycle regulator CtrA in stationary phase and when _C. crescentus_ cycles during exponential growth. While we previously established that MopJ acts on the components that regulate CtrA phosphorylation and stability (23), our work here revealed that PtsP signals through the (p)ppGpp synthase/hydrolase SpoT. Induction of (p)ppGpp during starvation and ectopically in nutrient-rich medium (19, 21) enhances CtrA levels while reducing DnaA synthesis and/or stability, ultimately slowing growth and cell cycle progression and inducing a G1-phase arrest (19, 21, 29, 30).

Although the effects of (p)ppGpp on CtrA and DnaA abundance are reported to occur at the post-transcriptional level, we additionally report evidence of a transcriptional induction (directly or indirectly) of GcrA target promoters based on population-based measurements. While a specific and direct

---

**FIG 3** PtsP regulates CtrA synthesis in stationary phase. (A and B) Promoter-probe assays of transcriptional reporters carrying a flbM, sciP, pilA, or flJK promoter fused to a promoterless lacZ gene in WT, ΔmopJ or ΔptsP single mutants, the ΔmopJ ΔptsP double mutant, and suppressor mutants ΔmopJ ptsP(S104P) and ΔmopJ ptsP(Q153P) in exponential (exp.) (A) and stationary (stat.) (B) phases. The graphs show lacZ-encoded β-galactosidase activities, measured in Miller units. Error bars indicate standard deviations (SD). (C and D) Immunoblot showing the steady-state levels of the major flagellin FljK, the SciP negative regulator, and the PilA structural subunit of the pilus filament in WT, ΔmopJ and ΔptsP single mutants, the ΔmopJ ΔptsP double mutant, and suppressor mutants ΔmopJ ptsP(S104P) and ΔmopJ ptsP(Q153P) in exponential (C) and stationary (D) phases. The steady-state levels of the MreB actin are shown as a loading control. (E) Immunoblot showing the steady-state levels of CtrA (or CtrA-M2), PtsP (or PtsPΔGAF) and MreB (loading control) in various mutants in exponential and stationary phases. (F) Promoter-probe assays of transcriptional reporters carrying the _ctrA_ promoter fused to a promoterless lacZ gene in the WT and various mutants in exponential (left) and stationary (right) growth phases. The graphs show lacZ-encoded β-galactosidase activities measured relative to the WT. Error bars show the SD.
FIG 4  Genetic and physical interactions between PtsP and the (p)ppGpp synthase SpoT. (A) Domain organization of Caulobacter SpoT. The asterisk marks the position of the suppressor mutation. The hydrolase and synthase domains are also indicated, along with two conserved regulatory domains in the C-terminal part of SpoT. (B) Motility assay on a swarm agar plate of WT, ΔptsP and ΔspoT single mutants, ΔmopJ ΔptsP, ΔptsP mopJ::himar1, and ΔspoT mopJ::himar1 double mutants, the spontaneous motility suppressor of the ΔptsP mutant, ΔptsP spoT(Δ22), and the ΔptsP spoT(Δ22) mopJ::himar1 triple mutant. (C) Swarm agar assay with WT and ΔmopJ and ΔptsP single mutants upon expression of the constitutive active form of E. coli RelA fused to the FLAG (M2) tag (RelA = -M2) in the presence of xylose. The controls harboring the inactivated form of RelA = (RelA = -E335Q-M2) and the empty vector are also shown. The arrowhead points to the increase in motility in ΔptsP cells upon (p)ppGpp production by RelA = induction. (D) Identification of SpoT by tandem mass spectrometry (MS/MS) on a silver stained gel following tandem affinity purification (TAP) from extracts of WT cells expressing PtsP-TAP from pMT335 under the control of the P_van promoter. (E) Coimmunoprecipitation (Co-IP) of PtsP with green fluorescent protein (GFP)-tagged SpoT from a GFP-TRAP affinity matrix (ChromoTek GmbH, Planegg-Martinsried, Germany). Precipitated samples were probed for the presence of PtsP by immunoblotting using antibodies against PtsP. Cell lysates used as input are also shown. (F) Promoter-probe assays of transcriptional reporters carrying the fljM, sciP, pilA, or fljK promoter fused to a promoterless lacZ gene in WT and ΔptsP spoT(Δ22) cells in stationary phase. Error bars show the standard deviations (SD). (G) Promoter-probe assays of transcriptional reporter carrying the mopJ promoter fused to a promoterless lacZ gene in WT, ΔptsP and ΔspoT single mutants, ΔptsP spoT(Δ22), ΔmopJ ptsP(S104P), ΔmopJ ptsP(Q133P) suppressor mutants, and ptsPΔGAF and ΔmopJ ptsPΔGAF mutants cells in stationary (top) and exponential (bottom) phases. Error bars show SD.
mechanism may underlie GcrA-dependent promoter control, it is possible that the effect of (p)pGpp is indirect, perhaps due to an extension of the cell cycle stage when GcrA is active (Fig. 1A). In a complementary study, González and Collier (31) recently reported that loss-of-function mutations in ptsP partially mitigate the cell division defect of cells lacking the CcmR DNA methyltransferase (32, 33). In the absence of CcmR, GcrA targets, including the promoters of the cell division genes ftsZ and its regulator mipZ, are poorly active (14, 32), because GcrA is no longer efficiently recruited (14, 33). Our finding that PtsP and SpoT affect GcrA target promoter activity is consistent with the result that ptsP suppressor mutations augment mipZ and ftsZ expression, and thus ptsP mutations surface as suppressor mutations that enhance growth of CcmR-deficient cells (31). While the abundance and localization of PtsP do not appear to change during the cell cycle (Fig. 1F and G), PtsP is upregulated in stationary phase (Fig. 3E; see also Fig. S2C in the supplemental material). The N-terminal GAF domain seems to fulfill a critical sensory role for PtsP, because gain-of-function mutations in the GAF domain emerged here as motility suppressors of the ΔmopJ mutant, and as suppressors of CcmR-deficient cells in the study by González and Collier. Remarkably, mutations in mopJ (CCNA_00999; not identified as mopJ [31]), divL (encoding a key component of the HA relay for CtrA that is regulated by MopJ [23]), and/or ctra itself can co-occur with ptsP mutations, reinforcing the genetic relationship between the PtsP and MopJ signaling pathways detailed here. Moreover, our observations that the activity of the mopJ promoter (PmopJ) increased upon induction of (p)pGpp and that PmopJ is also a direct GcrA target reveal an additional layer of complexity in the intricate interplay of these two signaling pathways that affect the cell cycle and motility.

Under natural conditions, (p)pGpp is induced during carbon, ammonium, or iron exhaustion in Caulobacter spp. (18), but it is also present in reduced amounts during growth in rich (peptone-yeast extract [PYE]) medium. Unlike for E. coli, amino acid starvation is not sufficient to induce (p)pGpp in Caulobacter spp. or in several other alphaproteobacteria (27), but SpoT is required for recovery from fatty acid starvation in C. crescentus (34). The mechanism underlying SpoT activation for lipid starvation in E. coli involves an interaction of the C-terminal regulatory domain of SpoT with the acyl carrier protein (35), an essential factor for fatty acid synthesis. How SpoT is activated by other starvation conditions is less clear, but our findings raise the intriguing possibility that PtsP couples (p)pGpp production by SpoT with carbon starvation (or other nutrient limitation in stationary phase), for example, through fluctuations in the glycolytic intermediate PEP, the phosphorphan of PtsP (36). As glutamine inhibits phosphorylation of Sinorhizobium meliloti PtsP in vitro (37), PtsP (and thus SpoT) signaling may be regulated in additional ways, for example, via the PtsN (EII) component of the alternative PTS system (PTS*)

MATERIALS AND METHODS

Growth conditions. Caulobacter crescentus NA1000 and derivatives were cultivated at 30°C in PYE rich medium or in M2 minimal salts plus 0.2% glucose (M2G) supplemented by 0.4% liquid PYE (39). Escherichia coli S17-1 (40) and EC100D cells (Epicientre Technologies, Madison, WI) were cultivated at 37°C in Luria broth (LB) rich medium. Agar (1.5%) was added into M2G or PYE plates, and motility was assayed on PYE plates containing 0.3% agar. Antibiotic concentrations used for C. crescentus included kanamycin (solid, 20 μg/ml; liquid, 5 μg/ml), tetracycline (1 μg/ml), spectinomycin (liquid, 25 μg/ml), spectinomycin-streptomycin (solid, 30 and 5 μg/ml, respectively), gentamicin (1 μg/ml), and nalidixic acid (20 μg/ml). When needed, β-xylene or sucrose was added at 0.3% final concentration, glucose at 0.2% final concentration, and vanillate at 500 or 50 μM final concentration. For the experiments in stationary phase in PYE, cultures with an optical density at 600 nm (OD600) of >1.4 were used, with the exception of those with motility suppressors: NA1000 ΔmopJ ptsPΔ104 and with an OD600 of ~1.1 and NA1000 ΔmopJ ptsPΔ213 and with an OD600 of ~1.7 were used. Swarmer cell isolation, electroporation, biparental mating, and bacteriophage φC30-mediated generalized transductions were performed as described in reference 39.

Motility suppressors of ΔmopJ and ΔptsP mutant cells. Spontaneous mutations that suppress the motility defect of the ΔmopJ mutation appeared as “flares” that emanated from nonmotile colonies after approximately 3 days of incubation. Two isolates were subjected to whole-genome sequencing, and mutations in the ptsP gene (ptsPΔ104 and ptsPΔ213) were found. In the first one, the serine codon (TCG) at position 104 in ptsP was changed to one encoding proline (CCG). In the second, the glutamate codon (CAG) at position 153 in ptsP was changed to one encoding proline (CCG). Spontaneous mutations that suppressed the motility defect of the ΔptsP mutant appeared as “flares” that emanated from the nonmotile colony after approximately 3 days of incubation. Two isolates were subjected to whole-genome sequencing, and a mutation in the spoT gene (spoT312) was found in one isolate, with residues 493 to 514 of the SpoT-coding sequence deleted.

Tandem affinity purification. The tandem affinity purification procedure was based on that described previously in reference 41. Briefly, when the culture (1 liter) reached an OD660 of 0.4 to 0.6 in the presence of 50 μM vanillate, cells were harvested by centrifugation at 6,000 × g for 10 min. The pellet was then washed in 50 ml of buffer I (50 mM sodium phosphate [pH 7.4], 50 mM NaCl, 1 mM EDTA) and lysed for 15 min at room temperature in 10 ml of buffer II (buffer I plus 0.5% n-dodecyl-β-D-maltoside, 10 mM MgCl2, two protease inhibitor tablets [for 50 ml of buffer I; Complete EDTA-free; Roche], 1× Ready-Lyse lysosome [Epicentre], 500 U of DNase I [Roche]). Cellular debris was removed by centrifugation at 7,000 × g for 20 min at 4°C. The supernatant was incubated for 2 h at 4°C with IgG-Sepharose beads (GE Healthcare Biosciences) that had been washed once with IPP150 buffer (10 mM Tris-HCl [pH 8], 150 mM NaCl, 0.1% NP-40). After incubation, the beads were washed at 4°C three times with 10 ml of IPP150 buffer and once with 10 ml of tobacco etch virus (TEV) protease cleavage buffer (10 mM Tris-HCl [pH 8], 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 1 mM dithiothreitol). The beads were then incubated overnight at 4°C with 1 ml of TEV solution (TEV cleavage buffer with 100 U of TEV protease per milliliter [Promega]) to release the tagged complex. CaCl2 (3 μM) was then added to the solution. The sample with 3 ml of calmodulin-binding buffer (10 mM β-mercaptoethanol, 10 mM Tris-HCl [pH 8], 150 mM NaCl, 1 mM magnesium acetate, 1 mM imidazole, 2 mM CaCl2, 0.1% NP-40). After incubation, the beads were washed at 4°C with calmodulin beads (GE Healthcare Biosciences) that previously had been washed once with calmodulin-binding buffer. After incubation, the beads were washed three times with 10 ml of calmodulin-binding buffer and eluted 5 times with 200 μl IPP150 calmodulin elution buffer (calmodulin-binding buffer with 2 mM EGTA instead of CaCl2). The eluates were then concentrated using Amicon Ultra-4 spin columns (Ambion).

Flow cytometry. Fluorescence-activated cell sorting (FACS) was performed as described previously (33). Cells in exponential growth phase (OD600 0.3 to 0.6) or in stationary phase (diluted to obtain an OD600 of 0.3 to 0.6), cultivated in M2G, were fixed in ice-cold 70% ethanol solution. Fixed cells were resuspended in FACS staining buffer (pH 7.2; 10 mM Tris-HCl, 1 mM EDTA, 50 mM Na-citrate, 0.01% Triton X-100) and then
treated with RNase A (Roche) at 0.1 mg/ml for 30 min at room temperature. Cells were stained in FACS staining buffer containing 0.5 μM of SYTOX green nucleic acid stain solution (Invitrogen) and then analyzed using a BD Accuri C6 flow cytometer instrument (BD Biosciences, San Jose, CA, United States). Flow cytometry data were acquired and analyzed using the CFLOW Plus v1.0.264.15 software (Accuri Cytometers Inc.). A total of 20,000 cells were analyzed from each biological sample. The forward scatter (FSC-A) and green fluorescence (FL1-A) parameters were used to estimate cell sizes and cell chromosome contents, respectively. Reported experimental values represent the averages of 3 independent experiments. The relative chromosome number was directly estimated from the FL1-A value of NA1000 cells treated with 20 μg/ml rifampin for 3 h at 30°C, as described previously (33). Rifampin treatment of cells blocks the initiation of chromosomal replication but allows ongoing rounds of replication to finish.

Cell generation time determinations. Cell growth in PYE or M2G medium was in an incubator at 30°C under agitation (190 rpm) and monitored at OD660. Generation time values were extracted from the curves by using the Doubling Time Application. Values represent the averages of 3 independent clones.

Bacterial strains, plasmids, and oligonucleotides, as well as methods for immunoblotting, coimmunoprecipitation, microscopy, and β-galactosidase assays are described in the supplemental material.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01415-15/-/DCSupplemental.

REFERENCES
1. Guttenplan SB, Kearns DB. 2013. Regulation of flagellar motility during biofilm formation. FEMS Microbiol Rev 37:849–871. http://dx.doi.org/10.1111/1574-6976.12018.
2. Skerker JM, Laub MT. 2004. Cell-cycle progression and the generation of asymmetry in Caulobacter crescentus. Nat Rev Microbiol 2:325–337. http://dx.doi.org/10.1038/nrmicro864.
3. Quon KG, Marczynski GT, Shapiro L. 1996. Cell cycle control by an essential bacterial two-component signal transduction protein. Cell 84:83–93. http://dx.doi.org/10.1016/S0092-8674(00)00995-2.
4. Sommer JM, Newton A. 1991. Pseudoreversion analysis indicates a direct role of cell division genes in polar morphogenesis and differentiation in Caulobacter crescentus. Genetics 129:623–630.
5. Laub MT, McAdams HH, Feldblyum T, Fraser CM, Shapiro L. 2000. Global analysis of the genetic network controlling a bacterial cell cycle. Science 290:2144–2148. http://dx.doi.org/10.1126/science.290.5499.2144.
6. Fiebig A, Herrou J, Fumeaux C, Radhakrishnan SK, Viollier PH, Crosson S. 2014. A cell cycle and nutritional checkpoint controlling bacterial surface adhesion. PLoS Genet 10:e1004101. http://dx.doi.org/10.1371/journal.pgen.1004101.
7. Fumeaux C, Radhakrishnan SK, Ardisson S, Therazal L, Frandi A, Martins D, Nesper J, Abel S, Jenal U, Viollier PH. 2014. Cell cycle transition from S-phase to G1 in Caulobacter is mediated by ancestral virulence regulators. Nat Commun 5:4081. http://dx.doi.org/10.1038/ncomms5081.
8. Quon KC, Yang B, Domian II, Shapiro L, Marczynski GT. 1998. Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin. Proc Natl Acad Sci U S A 95:120–125. http://dx.doi.org/10.1073/pnas.95.11.120.
9. Bastedo DP, Marczynski GT. 2009. CtrA response regulator binding to the Caulobacter chromosome replication origin is required during nutrient- and antibiotic stress as well as during cell cycle progression. Mol Microbiol 72:139–154. http://dx.doi.org/10.1111/j.1365-2958.2009.06630.x.
10. Bastedo DP, Quon KC, Shapiro L. 1997. Cell type-specific phosphorylation and proteolysis of a transcriptional regulator controls the G1-to-S transition in a bacterial cell cycle. Cell 90:415–424. http://dx.doi.org/10.1016/S0092-8674(00)80502-4.
11. West AH, Stock AM. 2001. Histidine kinases and response regulator proteins in two-component signaling systems. Trends Biochem Sci 26:369–376. http://dx.doi.org/10.1016/S0968-0004(01)01852-7.
12. Tsokos CG, Laub MT. 2012. Polarity and cell fate asymmetry in Caulobacter crescentus. Curr Opin Microbiol 15:74–87. http://dx.doi.org/10.1016/j.mib.2012.10.011.
13. Holtzendorff J, Hung D, Brende P, Reisenauer A, Viollier PH, McAdams HH, Shapiro L. 2004. Oscillating global regulators control the genetic circuit driving a bacterial cell cycle. Science 304:983–987. http://dx.doi.org/10.1126/science.1095191.
14. Fieravanti A, Fumeaux C, Mohapatra SS, Bompad C, Brilli M, Frandi A, Castric V, Villaret V, Viollier PH, Biondi EG. 2013. DNA binding of the cell cycle transcriptional regulator GcrA depends on N6-adenosine methylation in Caulobacter crescentus and other Alphaproteobacteria. PLoS Genet 9:e1003541. http://dx.doi.org/10.1371/journal.pgen.1003541.
15. Iniesta AA, McGrath PT, Reisenauer A, McAdams HH, Shapiro L. 2006. A phospho-signaling pathway controls the localization and activity of a protease complex critical for bacterial cell cycle progression. Proc Natl Acad Sci U S A 103:10935–10940. http://dx.doi.org/10.1073/pnas.0604554103.
16. Biondi EG, Reisinger SJ, Skerker JM, Arif M, Perchuk BS, Ryan KR, Laub MT. 2006. Regulation of the bacterial cell cycle by an integrated genetic circuit. Nature 444:899–904. http://dx.doi.org/10.1038/ nature05321.
17. Potrykus K, Cashel M. 2008. (p)ppGpp: still magical? Annu Rev Microbiol 62:35–51. http://dx.doi.org/10.1146/annurev.micro.081807.162603.
18. Boutte CC, Crosson S. 2011. The complex logic of stringent response regulation in Caulobacter crescentus: starvation signalling in an oligotrophic environment. Mol Microbiol 80:695–714. http://dx.doi.org/10.1111/j.1365-2958.2011.07602.x.
19. Boutte CC, Henry JT, Crosson S. 2012. (p)ppGpp and polyphosphate modulate cell cycle progression in Caulobacter crescentus. J Bacteriol 194:28–35. http://dx.doi.org/10.1128/JB.00593-11.
20. Lesley JA, Shapiro L. 2008. Spot regulates DNA stability and initiation of DNA replication in carbon-starved Caulobacter crescentus. J Bacteriol 190:6867–6880. http://dx.doi.org/10.1128/JB.00700-08.
21. Gonzalez D, Collier J. 2014. Effects of (p)ppGpp on the progression of the cell cycle of Caulobacter crescentus. J Bacteriol 196:2514–2525. http://dx.doi.org/10.1128/JB.01575-14.
22. De Nisco NJ, Abo RF, Wu GM, Penterman J, Walker GC. 2014. Global analysis of cell cycle gene expression of the legume symbiont Sinorhizobium meliloti. Proc Natl Acad Sci U S A 111:3217–3224. http://dx.doi.org/10.1073/pnas.1400241111.
23. Sanselisco S, Berge M, Therazal L, Radhakrishnan SK, Viollier PH. 2015. Topological control of the Caulobacter cell cycle circuitry by a polarized single-domain PAS protein. Nat Commun 6:7005. http://dx.doi.org/10.1038/ncomms8005.
24. Crosson S, Hossain MT, Crosson S. 2014. Global-binding PAS domains in a genomic, cellular, and structural context. Annu Rev Microbiol 68:261–286. http://dx.doi.org/10.1146/annurev-micro-121809-151631.
25. Tchieu JH, Norris V, Edwards JS, Saier MH, Jr. 2001. The complete phosphotransferase system in Escherichia coli. J Mol Microbiol Biotechnol 3:329–346.
26. Domian II, Reisenauer A, Shapiro L. 1999. Feedback control of a master bacterial cell-cycle regulator. Proc Natl Acad Sci U S A 96:6648–6653. http://dx.doi.org/10.1073/pnas.96.12.6648.
27. Boutte CC, Crosson S. 2015. Bacterial lifestyle changes stringent response activation. Trends Microbiol 23:174–180. http://dx.doi.org/10.1016/j.tim.2013.01.002.
28. Davis NJ, Cohen Y, Sanselisco S, Fumeaux C, Ozaki S, Luciano J, Guerrero-Ferreira RC, Wright ER, Jenal U, Viollier PH. 2013. De- and repolarization mechanisms of flagellar morphogenesis during a bacterial.
cell cycle. Genes Dev 27:2049–2062. http://dx.doi.org/10.1101/gad.222679.113.

29. Gorbatyuk B, Marczynski GT. 2005. Regulated degradation of chromosome replication proteins DnaA and CtrA in Caulobacter crescentus. Mol Microbiol 55:1233–1245. http://dx.doi.org/10.1111/j.1365-2958.2004.04459.x.

30. Leslie DJ, Heinen C, Schramm FD, Thüring M, Aakre CD, Murray SM, Laub MT, Jonas K. 2015. Nutritional control of DNA replication initiation through the proteolysis and regulated translation of DnaA. PLoS Genet 11:e1005342. http://dx.doi.org/10.1371/journal.pgen.1005342.

31. Gonzalez D, Collier J. 2015. Genomic adaptations to the loss of a conserved bacterial DNA methyltransferase. mBio 6:e00952-15. http://dx.doi.org/10.1128/mBio.00952-15.

32. Gonzalez D, Collier J. 2013. DNA methylation by CcrM activates the transcription of two genes required for the division of Caulobacter crescentus. Mol Microbiol 88:203–218. http://dx.doi.org/10.1111/mmi.12180.

33. Murray SM, Panis G, Fumeaux C, Viollier PH, Howard M. 2013. Computational and genetic reduction of a cell cycle to its simplest, primordial components. PLoS Biol 11:e1001749. http://dx.doi.org/10.1371/journal.pbio.1001749.

34. Stott KV, Wood SM, Blair JA, Nguyen BT, Herrera A, Mora YGP, Cuajungco MP, Murray SR. 2015. (p)ppGpp modulates cell size and the initiation of DNA replication in Caulobacter crescentus in response to a block in lipid biosynthesis. Microbiology 161:553–564 http://dx.doi.org/10.1099/mic.0.000052.

35. Battesti A, Bouveret E. 2006. Acyl carrier protein/SpoT interaction, the switch linking SpoT-dependent stress response to fatty acid metabolism. Mol Microbiol 62:1048–1063. http://dx.doi.org/10.1111/j.1365-2958.2006.05442.x.

36. Pfluger K, de Lorenzo V. 2008. Evidence of in vivo cross talk between the nitrogen-related and fructose-related branches of the carbohydrate phosphotransferase system of Pseudomonas putida. J Bacteriol 190:3374–3380. http://dx.doi.org/10.1128/JB.02002-07.

37. Goodwin RA, Gage DJ. 2014. Biochemical characterization of a nitrogen-type phosphotransferase system reveals that enzyme EII(Ntr) integrates carbon and nitrogen signaling in Sinorhizobium meliloti. J Bacteriol 196:1901–1907. http://dx.doi.org/10.1128/JB.01489-14.

38. Karstens K, Zschiedrich CP, Bowien B, Stulke J, Gorke B. 2014. Phosphotransferase protein EIIANtr interacts with SpoT, a key enzyme of the stringent response, in Ralstonia eutropha H16. Microbiology 160:711–722. http://dx.doi.org/10.1099/mic.0.075226-0.

39. Ely B. 1991. Genetics of Caulobacter crescentus. Methods Enzymol 204:372–384.

40. Simon R, Prieger U, Pühler A. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Nat Biotechnol 1:784–790. http://dx.doi.org/10.1038/nbt1183-784.

41. Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Séraphin B. 1999. A generic protein purification method for protein complex characterization and proteome exploration. Nat Biotechnol 17:1030–1032. http://dx.doi.org/10.1038/13732.