signals impinge upon cell cycle progression. Extensively studied (10), little is known about how environmental resources are allocated in a nutrient-dependent manner. Indeed, a population of cell types have different roles with respect to the nutrient environment, one might predict that differentiation of the swarmer cell is more sensitive to nutrient limitation than division of the stalked cell and that this underlies nutrient-dependent swarmer accumulation.

A complex series of molecular regulatory events govern the swarmer-to-stalked transition (Fig. 1B). The final two steps of this developmental transition are initiation of chromosome replication and growth of a stalk. The origin-binding response regulator CtrA initially represses replication initiation. CtrA is both deactivated by dephosphorylation and proteolyzed at the swarmer-to-stalked transition (11, 39), and the concentration of the replication initiation factor DnaA peaks in this same period (18), promoting chromosome replication (6). The two-component receiver protein DivK is central in the regulation of these events; its phosphorylation state determines cell fate. Briefly, the swarmer cell determinant PleC localizes to the flagellar pole and functions as a phosphatase of DivK (32, 34, 51). In its unphosphorylated state, DivK stabilizes CtrA thereby inhibiting replication initiation (3, 24). The stalked-cell determinant DivJ replaces PleC at the flagellar/nascent stalked pole during the swarmer-to-stalked transition and is activated as a kinase of DivK (32, 40). Phosphorylated DivK (DivK-P) represses a polar signaling complex (47), ultimately promoting the deactivation and proteolysis of CtrA (3) and replication. DivK-P and PleC also activate the PleD regulator (34), which cues stalk development (35).

We have identified two signaling molecules, ppGpp and inorganic polyphosphate (polyP), that are involved in inhibiting the swarmer-to-stalked transition upon exhaustion of glucose from the growth medium. ppGpp (guanosine 3’,5’-bispyrophosphate)
is the effector of the stringent response, whereby orderly growth inhibition promotes survival in starvation. In the stringent response, transcription is globally reprogrammed for adaptation to starvation via the activity of ppGpp on RNA polymerase (46). ppGpp also inhibits DNA replication via its activity on replication factors (16, 30). In *C. crescentus*, ppGpp is synthesized by the enzyme SpoT (30). polyP consists of linear chains of phosphate residues, which are synthesized by polyphosphate kinase (Ppk1) (1). In addition to storing phosphorus, polyP helps bacteria adapt to changing nutrient availability by regenerating ATP or GTP (25), and activating the Lon protease (28, 42). polyP levels increase in regulating sigma factors, and activating the Lon protease (28, 42), in response to nutrient deprivation. Specifically, we present evidence that ppGpp and polyP inhibit several critical regulatory events of the swarmer-to-stalked transition, including CtrA phosphorylation (29), DivJ localization, replication initiation, and stalk development (group of ovals) or phosphorylation (yellow circles). Perpendicular lines represent signal inhibition. Black lines indicate signaling events that occur during normal cell cycle progression in nutrient-replete medium; orange lines indicate signaling events that occur during nutrient limitation. Signaling events may be direct or indirect.

**MATERIALS AND METHODS**

**Culture conditions and growth measurements.** NA1000 strains were grown in M2G minimal medium (13) or peptone-yeast extract (PYE) complex medium at 30°C. Glucose exhaustion experiments were done on cultures grown in M2G to an optical density at 660 nm (OD$_{660}$) of <0.3. Cells were switched to M2G with 0.02% glucose (M2G/1/10) and then diluted to an OD$_{660}$ of 0.13. Experiments with the origin-labeled strains were done in PYE with 0.2% glucose, and tetR-yellow fluorescent protein (YFP) was induced for 1 h with 0.15% xylose. *ppk1* overexpression experiments were done in PYE supplemented with 0.3% xylose for 1 h before growth analysis. Kanamycin was used for plasmid selection in *C. crescentus* at 5 μg/ml in liquid media and 25 μg/ml on solid media. *Escherichia coli* strains TOP10 and Mach1 (Invitrogen, Carlsbad, CA) were used for cloning. *E. coli* strains were grown in Terrific broth supplemented with 50 μg/ml kanamycin at 30°C.

*C. crescentus* NA1000 cultures were serially passaged so that they were maintained in log phase (OD$_{660}$ ≤ 0.3) for at least 36 h before experiments were conducted. Density measurements for individual cultures were taken regularly up to an OD$_{660}$ of 0.3, and growth rates were determined by fitting the data to the exponential growth equation $y(t) = y_0 e^{kt}$, where $t$ is time, $y$ is cell density, and $k$ is growth rate. Error in doubling time was calculated from the distribution of mean cell population doubling times among independent biological replicates.

For analysis of cell population growth rate data, the doubling times of Δ*spoT* and Δ*ppk1* in-frame null mutants and point mutants were compared to that of four independent paired cultures of wild-type strain NA1000; the mean doubling time of the wild type was normalized to 1. Δ*spoT*: Δ*cenT* and Δ*ppk1*: Δ*ppk1* complemented strains were grown in PYE plus 0.3% xylose; their doubling times were compared to those of empty vector control Δ*spoT*:EV and Δ*ppk1*:EV strains, which were normalized to those of Δ*spoT* and Δ*ppk1* strains, respectively. Growth rate measurements for four independent cultures were carried out for each strain.

**Strain construction.** All experimental strains (Table 1) were derived from wild-type *C. crescentus* strain NA1000 (15). The Δ*ppk1* strain was made by amplifying ~500 bases upstream and downstream of *ppk1* (gene CC1710), ligating into pNPT5138, and following a double-recombination gene replacement protocol described previously (17). The *ppk1* deletion primers are as follows: up forward, 5'-GGATCCCTCCC TGTGGGAAAGG-3'; up reverse, 5'-AAGCTTAGGCGCTGTTCCAC ATTC-3'; down forward, 5'-GAATTCCGGCTCTTTAGCCTATTC C-3'; down reverse, 5'-GGATCCATGACCAATCCACGCTGT-3'. The Δ*ppk1* strain was made in an analogous fashion using the following primers: up forward, 5'-GGATCCGAGCGTATTGAGCAGC TA-3'; up reverse, 5'-AAGCTTGTCTGCGGGTTGCTGCCAT TCT-3'; down forward, 5'-GAATTCCTCCC TGTGGGAAAGG-3'; down reverse, 5'-GGATCCGACAGCATCCCTGGACTCA-3'. The Δ*spoT*:ori-yfp and Δ*ppk1*:ori-yfp strains were made by the same deletion method in the NA1000 CCO006::tetO sylx::tetR-yfp (also known as NA1000 ori-yfp) background (50). The boundaries of the pNPT5138 *spoT* deletion plasmid have been described previously (4). Strains NA1000 Δ*ppk1*:ori-yfp, NA1000 Δ*spoT*:divJ-1-yfp, and NA1000 Δ*ppk1*:ori-yfp were made by mating *E. coli* strain S17-1/pBCS18T-termDivJ-mYFP (29) with the respective recipient strains. The NA1000 CFP-paraB strain (45) was a gift from the lab of Lucy Shapiro. The Δ*spoT*: CFP-paraB, Δ*ppk1*: CFP-paraB, and Δ*spoT*:Δ*ppk1* CFP-paraB strains were made by deleting *spoT*, *ppk1*, or both, respectively, in the cyan fluorescent protein (CFP)-paraB background. The *spoT*:Y323A, *ppk1*:H460A, and hemagglutinin (HA)-tagged strains were all made by PCR amplification of regions around the mutation with gene stitching mutagenesis (21), ligation into pNPT5138, and use of the double-recombination gene replacement protocol described previously (17).

**Microscopy and image analysis.** Light microscopy was conducted with a Leica CTR5000 microscope, and images were acquired with a Hamamatsu ORCA-ER camera. Fixed cells were immobilized on 1% agarose pads before imaging. Cells were fixed at room temperature (RT) for 8 min by mixing 500 μl of culture with 100 μl of 16% paraformaldehyde, 0.0001% glutaraldehyde, and 20 μl 1 M NaPO$_4$, pH 7.4, then washed and resuspended in phosphate-buffered saline (PBS). For each strain and time point, 400 to 1,500 cells from each of three independent biological replicates were imaged and counted, and the final values are the means for the replicates. Origin foci were counted manually. DivJ-YFP puncta were counted in ImageJ from threshold binary phase and fluorescent images.

For transmission electron microscopy for polyP granules, log phase
**TABLE 1 Strains and plasmids**

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Cells were switched from M2G to M2G, then collected after 5 h. The cells were fixed as described above, washed with water, spotted onto Formvar-carbon-coated 400 mesh copper grids, air dried, and visualized without staining. For transmission electron microscopy for stalk analysis, cells were prepared as described previously (3); briefly, cells were harvested, fixed, stained with 1% uranyl acetate, spotted onto Formvar-carbon-coated 400 mesh copper grids, and air dried. Stalks were imaged at $16,800$ magnification for polyP analysis and at $1,890$ magnification for stalk analysis and at carbon-coated 400 mesh copper grids, and air dried. Grids were imaged without staining. For transmission electron microscopy for stalk analysis, Formvar-carbon-coated 400 mesh copper grids, air dried, and visualized

**RESULTS AND DISCUSSION**

ppGpp and polyP lengthen division times in complex medium. ppGpp and polyP are signaling molecules involved in adjusting growth rates during starvation, nutrient shifts, and stress. Given the result that ppGpp regulates replication initiation in *C. crescentus* (30) and the known role of polyP in nutrient-dependent control of bacterial physiology (9), we sought to test the role of these two molecules in the regulation of *C. crescentus* growth and development. Preliminary growth experiments with deletion strains (Table 1) in peptone-yeast extract (PYE) complex medium yielded a surprising result: both ΔΔspoT and ΔΔppk1 strains grow faster than the wild-type NA1000 strain (Fig. 2A). The fast-growth phenotype can be complemented by integration of a single copy of spoT or ppk1, respectively, at an ectopic locus (Fig. 2A).

To confirm that this phenotype is due to the known catalytic activities of these two enzymes, we built strains with single point mutations in the ppGpp synthetase domain of SpoT (Y323A) (23) or the polyP synthesis site in Ppk1 (H460A) (26). These point mutant proteins were stable (see Fig. S1C in the supplemental material) and phenocopied the full gene deletions (Fig. 2A), indicating that the fast-growth phenotypes are due to the loss of ppGpp and polyP. We confirmed by thin-layer chromatography (TLC) that the spoT(Y323A) strain failed to produce ppGpp in glucose starvation (Fig. 2B). Furthermore, we visualized unstained *C. crescentus* cells from cultures that had exhausted their sole carbon source by transmission electron microscopy: wild-type cells and cells bearing an HA-tagged ppk1 produce electron-dense intracellular granules that are known to be phosphorus rich (8) (Fig. 2C). In contrast, cells lacking ppk1 or possessing a single amino acid substitution in the predicted Ppk1 active site produce no prominent electron-dense granules when grown under identical conditions. In *E. coli*, mutants defective in ppGpp synthesis also exhibit a defect in polyP accumulation (27). However, the *C. crescentus* ΔΔspoT null mutant produces granules indistinguishable from the wild type under these conditions, even though the transcription of ppk1 is strongly regulated by spoT under abrupt starvation conditions (4). From these data, we conclude that polyP synthesis and the accompanying slowing of division time require a catalytically active ppk1, but not spoT, under these tested conditions.

Since loss of ppk1 increases growth rate in complex medium, we sought to determine if ppk1 overexpression is sufficient to slow growth under similar conditions. We integrated a single copy of either ppk1 or an empty vector under the control of a xylose-
inducible promoter at the chromosomal xylX locus (33). Cells expressing ppk1 in PYE complex medium supplemented with xylose showed no significant difference in growth rate relative to the empty-vector control (Fig. 2D). Since the C. crescentus genome encodes a putative exopolyphosphatase (encoded by ppx1, which is just downstream of ppk1), we reasoned that an intact Ppx1 may be responsible for breaking down excess polyP. We therefore overexpressed ppk1 in a Δppx1 background. Relative to that of the empty-vector control, the growth rate of ppk1-overexpressing cells in this genetic background was significantly slower. We repeated these growth experiments in strains lacking spoT, obtaining nearly identical growth rate changes compared to strains with intact spoT. Thus, ppk1 overexpression is sufficient to slow growth in complex medium in the absence of ppx1, and spoT is not required for this phenotype. Unlike Ppk1, which possesses one active site, SpoT contains both ppGpp synthase and hydrolase domains. We do not currently understand how these two activities are differentially regulated, but we do know that the rates of ppGpp synthesis differ between swarmer and stalked cells (see Fig. 5). Given this complexity, we were uncertain how we would interpret the results of a spoT overexpression experiment and have therefore not assessed the sufficiency of ppGpp in modulating growth rate and cell differentiation at this time.

The known roles of polyP involve promoting growth in the face of shifting nutrient conditions (28). Here, we present novel evidence that polyP can also function to “brake” bacterial growth under nutrient-replete conditions. Our data provide evidence that ppGpp also functions to slow growth not only in starvation but also in nutrient-rich conditions.

To more fully understand the observed changes in growth rate, we sought to test whether the swarmer and stalked stages of the division cycle were equally shortened in the ΔspoT and Δppk1 strains or whether one phase was differentially shortened. We used strains expressing tetR-YFP with tetO arrays near the origin...
of replication (50) to distinguish swarmer and stalked cells by replication status. Cells growing in PYE medium were imaged by phase-contrast and fluorescence microscopy, and the fraction of cells with one origin focus (swarmers) was calculated (Fig. 2E). The ΔspoT and Δppk1 strains have a smaller fraction of cells with a single origin than the wild-type strain (P < 0.05). This finding suggests that the small amounts of ppGpp and polyP produced in log phase C. crescentus cells in complex medium are sufficient to prolong the swarmer stage, delaying either replication initiation or segregation.

ΔspoT cells fail to properly halt growth under glucose exhaustion. As ppGpp and polyP levels are increased during starvation, we reasoned that the preferential inhibition of the swarmer-to-stalked transition might be accentuated under a starvation condition and that these molecules may be required for swarmer accumulation. Although glucose limitation was previously reported to have no effect on swarmer accumulation (14), we thought this negative result might be a function of the glucose concentration used (2.2 mM). To test the effects of lower concentrations of glucose on swarmer accumulation, we conducted a glucose exhaustion assay in which cultures growing in M2G were switched to M2G1/10 (1.1 mM glucose). In minimal defined medium, wild-type, Δppk1, and ΔspoT cells initially grow at the same rate (see Fig. S2 in the supplemental material). We do not know why growth rate differences between these strains are observed in complex (Fig. 2A) but not minimal medium, but we speculate that growth rate-limiting steps vary in different medium conditions.Soon after cultures are switched to M2G1/10, they cease growth as glucose is exhausted (Fig. 3A). As observed in other species (53), the ΔspoT strain is impaired in its ability to restrain growth upon nutrient deprivation and attains a higher culture density (Fig. 3A); this greater density is partially due to an increased cell size, which is also observed in other species (52). Inability to restrain growth is presumably detrimental, as the ΔspoT strain has reduced viability after carbon starvation (30).

spoT and ppk1 inhibit DivJ localization in glucose exhaustion. One of the early events in the swarmer-to-stalked transition is the phosphorylation of DivK, which indirectly signals initiation of this cell type switch. DivK is phosphorylated by DivJ, which first must be localized to the nascent stalked pole and activated (40, 41). Localization of DivJ to the pole is thus an important marker of the swarmer-to-stalked transition. To test how glucose exhaustion affects swarmer accumulation with respect to DivJ localization, we built strains with DivJ-YFP fusions in the wild-type, ΔspoT, and Δppk1 backgrounds, grew the cells to log phase in M2G defined medium, switched the cells to M2G1/10, and determined the proportions of cells with (stalked) and without (swarmers) a DivJ-YFP focus before and after glucose exhaustion. All strains in nutrient-replete M2G medium had ~25% of cells without a DivJ focus, indicating that ~25% of cells in these populations are swarmers (Fig. 3B). After glucose exhaustion the proportion of cells without a DivJ focus nearly doubled in wild-type cells, while remaining near 25% in the ΔspoT and Δppk1 strains (Fig. 3B). These data provide evidence that the activities of spoT and ppk1 are important for inhibiting localization, and presumably activation, of DivJ during glucose exhaustion. Because DivJ localization is thought to be required for activity (40, 41), we speculate that in wild-type glucose-exhausted cells in which DivJ fails to localize, DivK is not phosphorylated and the developmental transitions dependent upon DivK—P do not proceed. We note that many of the DivJ foci in Δppk1 cells after glucose exhaustion are dimmer than the foci in the other strains. In addition, we observed that after prolonged glucose starvation—several hours past the time of the data presented here—all strains exhibited many cells with two bright DivJ foci.

spoT and ppk1 inhibit replication initiation in glucose exhaustion. The swarmer-to-stalked transition involves multiple signaling events and morphological changes, one of which is initiation of chromosome replication. We therefore studied the effects of glucose exhaustion on swarmer accumulation with respect to replication initiation. ParB is a protein that binds at oriC and is involved in chromosome partitioning: strains with a CFP-ParB fusion exhibit fluorescent foci at each oriC in the cell (45) and can be used to quantify oriC within a cell (5). We used this fluorescent protein fusion in the wild-type, ΔspoT, and Δppk1 backgrounds to assay the proportion of cells with a single ParB focus after glucose exhaustion. We collected samples from cultures growing in M2G and cultures 5 h after the switch to M2G1/10. In M2G medium,
motivated by our observed stalk development phenotype, we investigated protein levels of PodJ, a protein known to govern polar organelle development. Though levels of this protein do not appear to depend on spoT or ppk1, we note a genetically distinct mechanism of starvation-dependent cell cycle regulation in the control of PodJ stability. PodJ, which exists as long and short forms across the cell cycle, is a localization factor for the swarmer cell determinant PleC (22, 49). In wild-type swarmer cells grown under nutrient-replete conditions, the short form of PodJ is degraded while the long form begins to accumulate at the swarmer-to-stalked transition. However, during glucose exhaustion the short form of PodJ is stabilized and the long form accumulates to lower levels. This effect is independent of both spoT and ppk1 (see Fig. S3 in the supplemental material) and provides evidence for additional nutrient-dependent cell cycle effectors beyond ppGpp and polyP.

SpoT and ppk1 affect the stability of the replication inhibitor CtrA. Replication initiation is reciprocally controlled by DnaA and CtrA. CtrA binds and occludes oriC to prevent initiation during the swarmer stage and is subsequently deactivated and proteolyzed to allow replication during the swarmer-to-stalked transition (11, 39). In starvation, CtrA is stabilized in swarmer cells (19). DnaA initiation replication by binding oriC, separating the strands, and recruiting other replication factors (18). DnaA levels peak during the swarmer-to-stalked transition in replete medium (19). However, in carbon starvation ppGpp activates the premature proteolysis of DnaA to prevent replication initiation (30). We sought to test whether polyP also impacts DnaA stability and whether ppGpp and polyP affect the stability of the replication inhibitor CtrA.

To measure CtrA and DnaA protein levels during the swarmer-to-stalked transition, we isolated swarmer cells from wild-type, spoT, and ppk1 cultures growing in replete M2G or at the inflection point of glucose exhaustion (Fig. 3A). We then resuspended the swarmer cells in M2G or spent medium from a wild-type culture at the glucose exhaustion inflection point, respectively, and harvested samples for Western blots at various times after resuspension. The results of these experiments show that, in replete medium, CtrA and DnaA levels in the spoT and ppk1 strains are similar to those in the wild-type strain (Fig. S1A; see Fig. S1A in the supplemental material). During glucose exhaustion, DnaA is proteolyzed in the wild-type strain and stabilized in the spoT strain, as reported previously for abrupt starvation (30). DnaA levels in the ppk1 strain are similar to wild-type levels (see Fig. S1A in the supplemental material). Consistent with previous observations (19), CtrA in wild-type swarmer cells is stabilized during glucose exhaustion. However, CtrA in spoT and ppk1 swarmer cells is improperly proteolyzed under the same conditions (Fig. 4). These data indicate that ppGpp and polyP are either directly or indirectly involved in inhibiting CtrA proteolysis during glucose deprivation, which likely contributes to their inhibitory effect on replication initiation (Fig. 2E and 3C). We note that cyclic di-GMP has been previously shown to modulate CtrA proteolysis (12) and stalk development (35) as well; thus, both of these processes are reciprocally regulated by two guanine nucleotide second messengers: c-di-GMP, which promotes stalked-cell development, and ppGpp, which inhibits it.

We note that CtrA proteolysis is part of the DivK-P-dependent developmental program: therefore, the effects of
ppGpp and polyP on CtrA may be downstream of the effect of these molecules on DivJ localization.

Swarmer cells produce ppGpp at a higher rate than stalked cells upon starvation. As ppGpp signaling is specifically important for inhibiting the development of swarmer cells, we predicted that swarmers and stalked cells might differentially accumulate ppGpp in starvation. To test whether differential rates of ppGpp synthesis occur between cell types, we incubated cells with KH$_2$PO$_4$ to label nucleotides and separated the swarmers and stalked cells, which were then glucose starved. ppGpp levels were monitored by TLC. In this experiment the “stalked” category includes early stalked to late predivisional cells. The data show that swarmer cells consistently accumulate ppGpp at a faster rate and to a greater extent than stalked cells upon glucose starvation (Fig. 5). A higher rate of ppGpp synthesis in swarmer cells relative to stalked cells provides an additional layer of regulation that may contribute to preferential inhibition of the swarmer-to-stalked transition upon nutrient limitation.

Concluding remarks. In this work we demonstrate that exhaustion of glucose from the growth medium inhibits the developmental transition from swarmer to stalked cell type and that this phenomenon is regulated by the activity of two starvation-associated molecules: ppGpp and polyP. ppGpp signaling activates the proteolysis of DnaA (30), inhibits the localization of DivJ, and inhibits the proteolysis of CtrA. polyP production inhibits the localization of DivJ and the proteolysis of CtrA. Both ppGpp and polyP function to preferentially block the transition from swarmer to stalked-cell type relative to subsequent transitions of the cell cycle. It may be that ppGpp and polyP inhibit some factor upstream of DivJ localization, leading to inhibition of CtrA proteolysis through the canonical DivK–P pathway (Fig. 1B). Future studies will be aimed at deciphering the detailed molecular mechanism by which ppGpp and polyP exert these effects.

This work establishes novel signaling functions for the starvation effectors ppGpp and polyP and shows that they can modulate cell cycle progression by inhibiting signaling events that occur only at the swarmer-to-stalked transition.

ACKNOWLEDGMENTS

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