Phosphotransferase-dependent accumulation of (p)ppGpp in response to glutamine deprivation in Caulobacter crescentus

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The alarmone (p)ppGpp is commonly used by bacteria to quickly respond to nutrient starvation. Although (p)ppGpp synthetases such as SpoT have been extensively studied, little is known about the molecular mechanisms stimulating alarmone synthesis upon starvation. Here, we describe an essential role of the nitrogen-related phosphotransferase system (PTS¹⁰) in controlling (p)ppGpp accumulation in Caulobacter crescentus. We show that cells sense nitrogen starvation by way of detecting glutamine deprivation using the first enzyme (EΙ patients) of PTS¹⁰. Decreasing intracellular glutamine concentration triggers phosphorylation of EΙ patients and its downstream components HPr and EIIA¹⁰. Once phosphorylated, both HPr~P and EIIA¹⁰~P stimulate (p)ppGpp accumulation by modulating SpoT activities. This burst of second messenger primarily impacts the non-replicative phase of the cell cycle by extending the G1 phase. This work highlights a new role for bacterial PTS systems in stimulating (p)ppGpp accumulation in response to metabolic cues and in controlling cell cycle progression and cell growth.
To face the environmental changes, organisms have developed complex regulatory mechanisms that integrate stimuli and stresses. Once activated, these signalling pathways modulate essential cellular processes such as DNA replication, cell division or cell growth. For example, upon nutrient starvation, yeast cells access to a specific quiescent state that enhances stress resistance and survival. Bacteria also select many strategies to survive in challenging environments. One of the most studied bacterial adaptations to harsh conditions is certainly the formation of endospores in *Bacillus subtilis*, which requires asymmetric cell division and differentiation of the prespore. Other bacteria take advantage of their asymmetric cell division to adapt to starvation conditions. It is the case of the aquatic α-proteobacterium *Caulobacter crescentus* that divides asymmetrically to give birth to two different daughter cells: a chemotactically active motile swarmer cell and a sessile stalked cell. Whereas the stalked cell grows and reinitiates DNA replication immediately at birth to ultimately divide again, the newborn swarmer cell enters first into a pre-replicative (G1) phase (Fig. 1a). In nutrient-replete conditions, the G1/swarmer cell differentiates into a stalked cell (swarmer-to-stalked transition) and concomitantly initiates chromosome replication (G1-to-S transition). Upon nitrogen starvation, *C. crescentus* extends its swarmer phase to promote spreading and colonization of new environments. Asymmetric cell division might be a strategy commonly used by α-proteobacteria to generate daughter cells with different cell fates.

Nutritional stresses are also known to be associated with the accumulation of an alarmone, the guanosine tetra- and penta-phosphate commonly called (p)ppGpp. Burst of intracellular (p)ppGpp alarmone allows cells to quickly adapt to a nutrient stress by affecting important cellular processes such as transcription, translation or DNA replication (reviewed in refs 8 and 9). For example, (p)ppGpp interferes with cell cycle steps by the direct binding of the alarmone to the DNA primase DnaG, which stops DNA replication in *B. subtilis*. As a consequence of its pivotal role in stress adaptation, (p)ppGpp became crucial for virulence of several bacterial pathogens, long-term persistence, competence and biofilm formation. In *Escherichia coli*, (p)ppGpp level is regulated by two proteins, RelA and SpoT. RelA is a monofunctional (p)ppGpp synthetase stimulated by amino acids starvation, in contrast to SpoT, which is a bifunctional synthetase-hydrolase enzyme responding to a wide range of nutritional stresses such as carbon, phosphate or fatty acid starvation. *C. crescentus* possesses a single RelA/SpoT homologue that was named SpoT because of its bifunctional activity. Previous studies showed that (p)ppGpp can modulate cell cycle progression in *C. crescentus* by delaying simultaneously the swarmer-to-stalked differentiation and the G1-to-S transition. Nitrogen or carbon starvation was described to trigger accumulation of (p)ppGpp but the regulatory networks sensing these stresses and activating SpoT remain uncovered. Furthermore, interacting partners of SpoT are largely unknown even if SpoTCc was shown to co-fractionate with the 70S ribosomal subunit.

Ammonium (NH$_4^+$) is the preferred inorganic nitrogen source for most of living cells. There are only two reactions that efficiently assimilate NH$_4^+$ (Fig. 1b). The first one is catalysed by the NADP-dependent assimilative glutamate dehydrogenase. The other one is mediated by the ATP-dependent glutamine synthetase (GlnA). There is no NADP-dependent glutamate dehydrogenase encoded in the genome of *C. crescentus*, suggesting that the assimilation of inorganic nitrogen is strictly dependent on the glutamine synthetase (GlnA) activity. In most bacteria, nitrogen metabolism is tightly regulated by a well-characterized pathway, which involves the universal nitrogen sensor GlnD (Fig. 1b, reviewed in refs 17 and 18). When *E. coli* is grown in nitrogen-deplete (−N) conditions, the PII uridylyltransferase GlnK catalyses the transfer of uridine monophosphate (UMP) groups to PII regulatory proteins, GlnB and GlnK. GlnK~UMP no longer inhibits the ammonia channelAmtB, and GlnB~UMP stimulates deadenylylation of GlnA by the adenylyltransferase GlnE, and thereby promotes glutamine synthetase activity (Fig. 1b). In nitrogen-replete (+N) conditions, GlnB inhibits the transcription of *glnA*, by stimulating dephosphorylation of the transcriptional activator NtrC, and promotes the addition of the adenine monophosphate groups by GlnD to GlnA, which slows down the glutamine synthetase activity (Fig. 1b).

In this work, we unravel the regulatory network that stimulates (p)ppGpp accumulation in *C. crescentus* in response to nitrogen starvation. In particular, we uncover the essential role of the nitrogen-related phosphoenolpyruvate (PEP) phosphotransferase system (PTS) in transducing glutamine deprivation signal to (p)ppGpp accumulation, which in turn controls the cell cycle progression. The cell cycle control described here constitutes a

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**Figure 1 | The *C. crescentus* swarmer cell lifetime is extended upon nitrogen starvation.** (a) Asymmetric cell division of *C. crescentus* gives birth to a non-replicative swarmer cell that goes through G1 phase before replicating and a replicative stalked cell that directly enters into S phase. Upon nitrogen starvation (−N), swarmer cells extend their G1 phase. (b) In *E. coli*, ammonium can be assimilated either by the NADP-dependent glutamate dehydrogenase (GdhA) to generate glutamate (Glu) from α-ketoglutarate (α-KG) or by the glutamine synthetase (GlnA) to produce glutamine (Gln) from Glu, this latter being recycled by the glutamate synthase (GOGAT). GlnA is regulated at different levels by the GlnD/GlnB/GlnE and NtrBC pathways, and GlnD senses intracellular pool of Gln.
new PTS$_{Ntr}$-dependent regulatory role, illustrating the diversity of the cellular processes regulated by PTS systems.

**Results**

Glutamine deprivation signals nitrogen starvation. Previous studies showed that nitrogen starvation extends the swarmer cell lifetime in *C. crescentus*°. By following DNA content and cell cycle-regulated proteins (the flagellin and the stalked-associated protein StpX) in synchronous or asynchronous population of *Caulobacter* cells, we confirmed the specific extension of the G1/swarmer phase in response to nitrogen starvation (Supplementary Fig. 1a–e). By contrast, stalked cells can complete DNA replication once initiated, despite the absence of a nitrogen source (Supplementary Fig. 1f).

To understand how nitrogen starvation affects the differentiation of G1/swarmer cells, we focused our work on proteins involved in nitrogen assimilation and metabolism. First, we created an in-frame deletion of the predicted gene coding for the general sensor for nitrogen availability, glnD (CCNA_00013). In contrast to wild-type cells, ΔglnD cells were unable to use ammonium as a nitrogen source. Indeed, the ΔglnD mutant did not grow and accumulated G1/swarmer cells when ammonium was used as the sole nitrogen source (Supplementary Fig. 2a,b). As expected, the G1 block and the growth were rescued in the presence of glutamine (Supplementary Fig. 2c). Indeed, as for glnD mutants in *E. coli*°, *C. crescentus* ΔglnD is auxotrophic for glutamine. Thus this result shows that G1/swarmer cells accumulation in ΔglnD is a consequence of its inability to use ammonium as a nitrogen source. Interestingly, the ΔglnD mutant strain cultivated in a complex peptone yeast extract (PYE) medium accumulated G1/swarmer cells (Fig. 2c,d). As a consequence, ΔglnD cells also exhibited (i) a slower growth than the wild-type strain and (ii) a bigger motility halo than the wild-type, despite the growth defect (Fig. 2a,b). Indeed, the overrepresentation of the G1/swarmer cells in a ΔglnD population increases the overall motility and the doubling time of the strain. Thus, our results indicate that G1/swarmer lifetime is extended in the absence of glnD (Fig. 2a–d). Again, addition of glutamine suppressed all these phenotypes (Fig. 2d), suggesting that defects of ΔglnD are a consequence of glutamine availability in the complex PYE medium. Indeed, PYE is mainly composed of yeast extract, in which glutamine is the less-abundant amino acid (°0.2%, see the ‘Methods’ section).

In *E. coli*, glutamine auxotrophy displayed by glnD mutant strains comes from the under-expression and lower activity of the glutamine synthetase GlnA. In the absence of GlnD, the PII protein GlnB is not uridylylated, and thereby constitutively stimulates (i) the dephosphorylation of transcriptional activator NtrC~P by NtrB, which subsequently inhibits the NtrC~P-dependent expression of glnA, and (ii) the adenylylation (+adenine monophosphate) of GlnA by the adenylytransferase GlnE, thereby inhibiting the glutamine synthetase activity (Fig. 1b). *C. crescentus* encodes three PII protein homologues (glnB CCNA_02406, glnK CCNA_01400 and glnC CCNA_00555), one adenylytransferase homologue (glnE CCNA_02839), three GlnA homologues (glnA CCNA_02407, glnA2 CCNA_03230 and glnA3 CCNA_03240) and two NtrC homologues (ntrC CCNA_01815 and ntrX CCNA_01817). Single in-frame deletions of each of these genes were created and tested for growth, motility and G1 accumulation in complex PYE medium. We found that only ΔglnB, ΔntrC and ΔglnA recapitulated ΔglnD phenotypes, and that all these phenotypes could be suppressed by adding glutamine to the medium (Fig. 2d). However, it is noteworthy that the motility defect in ΔntrC was not completely rescued by

![Figure 2](https://example.com/image2.png)  
**Figure 2 | The C. crescentus G1/swarmer cell lifetime is dictated by intracellular glutamine concentration.** (a–d) Extension of the G1/swarmer lifetime in a ΔglnD strain can be compensated by addition of glutamine. Motility (a), growth (b) and DNA content (c) of wild-type (WT; RH50) and ΔglnD (RH577) grown in complex media without (PYE) or with glutamine (PYEQ) media. (d) G1/swarmer lifetime is extended in glutamine auxotrophic mutants. Doubling time, motility and G1 proportion were measured in WT (RH50), ΔglnD (RH577), ΔglnB (RH771), ΔglnA (RH772), ΔglnE (RH874), ΔglnD ΔglnE (RH875) and ΔntrC (RH1458) grown in complex media without (PYE) or with glutamine (PYEQ), and normalized to the WT (100%). All these phenotypes can be rescued by addition of glutamine (PYEQ). Error bars = s.d.; n = 3.
addition of glutamine, suggesting that NtrC also controls motility independently of G1/swarmer cell accumulation (Fig. 2d). Moreover, deleting glnE alleviated the ΔglnD defects, supporting the fact that inactivation (adenylation) of GlnA by GlnE is the causative effect of the glutamine auxotrophy detected in ΔglnD cells (Fig. 2d). Unexpectedly, neither GlnA2 nor GlnA3 seems to indicate as *.

To fish out key actors participating to the G1/swarmer extension in response to glutamine deprivation, we isolated spontaneous mutations that increase motility of the wild-type strain on PYE swarm agar plates supplemented with glutamine. Whole-genome sequencing of one candidate revealed a unique mutation (D81G) in the hydrolase domain of SpoT, the protein synthesizing (p)ppGpp (Fig. 3a and Supplementary Fig. 4a). It has been shown that mutations abolishing, at least partially, the hydrolase activity of SpoT without altering its synthetase activity, were all found in the hydrolase domain. Interestingly, the conserved aspartate, corresponding to the D81 in C. crescentus SpoT, was found to be required for the hydrolase activity of SpoT in Streptococcus dysgalactiae, which suggests that the D81G mutation may reduce hydrolase activity of SpoT in C. crescentus as well. The spoTΔDoug strain had a growth delay in PYE and accumulated G1/swarmer cells in exponential phase of growth (Fig. 3b; Supplementary Fig. 5a–c and Supplementary Movie 1), even when glutamine was added to the medium (Supplementary Figs 5a–c and 6), confirming that the spoTΔDoug mutant is insensitive to glutamine. By contrast, in-frame deletion of spoT (ΔspoT) displayed a motility defect on swarm agar and contained less G1/swarmer cells, in comparison with the wild-type (Fig. 3b; Supplementary Fig. 5b,c and Supplementary Movie 1). Most importantly, introducing ΔspoT in ΔglnD cells completely

Figure 3 | SpoT and EINtr modulate (p)ppGpp accumulation on nitrogen starvation. (a) Mutations isolated in SpoT and EINtr that modulate G1/swarmer lifetime. Black arrowheads indicate the mutations (spoTΔDoug and ptsPΔL83Q) that increase G1/swarmer lifetime. White arrowheads indicate the positions of 3 himar transposon insertions in the ptsP locus out of the 16 that suppressed spoTΔDoug phenotypes. The exact positions of the remaining 13 transposon insertions into the ptsP locus were not determined. (b) SpoT and EINtr control G1/swarmer lifetime. Doubling time, motility and G1 proportion were measured in wild-type (WT, RH50), ΔglnD (RH577), ΔspoT (RH755), ΔglnD ΔspoT (RH756), spoTΔDoug (RH752), ΔptsP (RH758), ΔptsP spoTΔDoug (RH727) ΔglnD ΔptsP (RH940), ptsPΔL83Q (RH748) and ΔspoT ptsPΔL83Q (RH728) grown in complex media (PYE) and normalized to the WT (100%). Error bars = s.d.; n = 3. (c,d) Glutamine auxotrophy leads to (p)ppGpp accumulation. Intracellular levels of (p)ppGpp detected by TLC after nucleotides extraction of WT (RH50), ΔglnD (RH577), ΔspoT (RH755), ΔglnD ΔspoT (RH756), spoTΔDoug (RH752), ΔptsP (RH758) and ΔptsP spoTΔDoug (RH727) grown in +N or −N conditions. Error bars = s.d.; n = 3. Statistically significant differences by Student’s t-test in comparison with the WT are indicated as *P < 0.05% and **P < 0.01% (n = 3).
suppressed the accumulation of G1/swarmer cells (Fig. 3b and Supplementary Fig. 5b,c). This result highlights the role of (p)ppGpp produced by SpoT in response to glutamine deprivation to control the cell cycle. We thus checked the (p)ppGpp production in −N or +N conditions. In agreement with previous studies, we found that (p)ppGpp concentration increases on glutamine deprivation, that is, in the wild-type strain grown without nitrogen source (−N) or in ΔglnD cultivated with (+N) or without (−N) NH₄Cl (Fig. 3c,d; Supplementary Figs 1g and 5d). As already mentioned in previous works, we also observed a low amount of (p)ppGpp produced by wild-type cells in stress conditions (Fig. 3c and Supplementary Fig. 5d). This (p)ppGpp steady-state level was slightly higher in the spoTD₈₁G strain in +N conditions (Fig. 3c and Supplementary Fig. 5d), which would explain the phenotypes displayed by spoTD₈₁G that is slower growth, bigger motility halo and G1 accumulation (Fig. 3b), even in the presence of glutamine (Supplementary Figs 5a–c and 6). Nevertheless, spoTD₈₁G accumulated similar levels of (p)ppGpp than wild-type cells upon nitrogen starvation (−N), suggesting that SpoTD₈₁G is still sensitive to nitrogen starvation (Fig. 3d). In contrast, the disappearance of (p)ppGpp accumulated upon nitrogen starvation is slower in spoT₈₁G cells than in wild-type cells (Supplementary Fig. 5e). These results support that D₈₁G mutation mainly affects the hydrolyase activity of SpoT. It’s noteworthy that an artificial increase of (p)ppGpp levels in non-stressed conditions (Fig. 3c and Supplementary Fig. 5d). This (p)ppGpp steady-state level might determine the time spent by swarmer cells in G1 phase. Altogether, our findings indicate that glutamine deprivation increases (p)ppGpp level, which in turn, will extend the lifetime of G1/swarmer cells.

**PTS₅ⁿʳ** promotes (p)ppGpp accumulation on nitrogen starvation. To identify factors that participate to the activation of SpoT in response to nitrogen starvation, we selected for transposon insertions that improve growth of spoTD₈₁G cells on complex medium (PYE). Indeed, the accumulation of (p)ppGpp in spoTD₈₁G cells decreases the growth rate on PYE medium (Fig. 3b and Supplementary Fig. 5a). We identified multiple transposon insertions (34 out of 50 clones) into spoTD₈₁G itself. The remaining 16 clones harboured a transposon insertion into the ptsP gene (CCNA_000892), coding for a nitrogen-related PEP-phosphotransferase (PTS) protein homologue, called PtsP or EI_Ntr in Enterobacteria (Fig. 3a). Canonical PTS systems are composed of several components that form a phosphorylation cascade initiated by autophosphorylation of the first protein called EI, using PEP as phosphoryl donor (reviewed in ref. 21). The phosphoryl group is then transferred from EI~P to HPr and then to EIIA proteins. When the PTS system is used to take up sugars, the phosphoryl group is ultimately transferred from EIIA~P to transported carbohydrates by using specific permeases (EIIB and EICC components). In many other cases, PTS systems are dedicated to regulatory functions implying that PTS components (EI, HPr or EIIA) phosphorylate or interact with regulatory target proteins. Nitrogen-related PTS (PTS₅ⁿʳ) systems are so far considered as unusual PTS systems that respond to nitrogen availability, but their regulatory roles in bacterial physiology remain poorly understood (reviewed in ref. 21).

An in-frame deletion of ptsP in the parental spoTD₈₁G strain suppressed the spoTD₈₁G phenotypes, confirming the genetic interaction between ptsP and spoT₈₁G (Fig. 3b). In addition, ΔptsP phenocopied ΔspoT in terms of motility, G1/swarmer cells accumulation (Fig. 3b; Supplementary Figs 5a–c and 7a–c), and capability to suppress G1/swarmer cells accumulation of ΔglnD cells (compare ΔglnD ΔptsP to ΔglnD ΔspoT in Fig. 3b; Supplementary Figs 5a–c and 7a–c). Interestingly, we isolated another candidate than spoTD₈₁G in the gain-of-motility screen, which harboured a mutation (L83Q) in the GAF domain of El_Ntr (ptsL₈₁QG Fig. 3a). As ptsL₈₁QG phenocopies spoTD₈₁G (Fig. 3b; Supplementary Figs 5a–c and 7a–c), ΔptsP suppresses spoTD₈₁G defects and ΔspoT suppresses ptsL₈₁QG defects (Fig. 3b), we wondered whether ptsP (EI₅ⁿʳ) is upstream or downstream of SpoT. To test that, we measured the (p)ppGpp levels in a spoTD₈₁G ΔptsP background. As shown in Fig. 3d, no (p)ppGpp accumulation was detected in spoTD₈₁G ΔptsP cells starved for nitrogen (−N). However, spoTD₈₁G ΔptsP cells still produced a low amount of (p)ppGpp, whether a nitrogen source was added to the medium or not (Fig. 3c). This constitutive low levels of (p)ppGpp produced by spoTD₈₁G ΔptsP cells is very close to the (p)ppGpp level detected in non-starved wild-type cells (Fig. 3c). Interestingly, there is a systematic correlation between the amount of (p)ppGpp produced by the cells and the time spent by these cells in G1 phase. Indeed, ΔptsP or ΔspoT swarmer cells do not produce detectable levels of (p)ppGpp (Fig. 3c) and have shortened G1 phase (Fig. 3b), whereas spoTD₈₁G ΔptsP and wild-type cells have similar levels of (p)ppGpp and G1 lifetime (Fig. 3c,b). Altogether, these results support the role played by (p)ppGpp in determining the G1 lifetime, and show that El₅ⁿʳ regulates (p)ppGpp levels by controlling SpoT.

Glutamine inhibits El₅ⁿʳ autophosphorylation. To understand how glutamine deprivation is transduced to SpoT, we first looked at the autophosphorylation level of El₅ⁿʳ. Indeed, as described above, accumulation of G1/swarmer cells (Fig. 3b) observed in a ptsL₈₁QG background, are not compensated by supplying an exogenous source of glutamine (Supplementary Fig. 6). Moreover, it has been recently shown, in the closely related α-proteobacterium Sinorhizobium meliloti, that binding of glutamine to the conserved N-terminal GAF domain of El₅ⁿʳ inhibits its autophosphorylation. To check whether the phosphorylation of El₅ⁿʳ is also sensitive to glutamine in C. crescentus, we performed in vitro autophosphorylation assays with a purified fraction containing El₅ⁿʳ using [⁴⁰P]PEP as a phosphoryl donor, in the presence or absence of glutamine. We found that autophosphorylation of El₅ⁿʳ was strongly reduced by glutamine (Fig. 4a and Supplementary Fig. 8a). In contrast, autophosphorylation of the El₅ⁿʳ₈₁Q mutant form was not modulated by the presence of glutamine (Fig. 4a), suggesting that the mutation L₈₁Q prevents glutamine binding to the highly conserved region of the El₅ⁿʳ GAF domain (Supplementary Fig. 4b).

Phosphorylated PTS₅ⁿʳ proteins trigger (p)ppGpp accumulation. To unrav el how El₅ⁿʳ controls SpoT activity, we first searched for components that could participate to PTS₅ⁿʳ phosphorylase (Fig. 5a). Besides ptsP (EI₅ⁿʳ), we found a unique HPr homologue (ptsH, CCNA_00241) and another nitrogen-related PTS₅ⁿʳ component, EI₅ⁿʳ (ptsN, CCNA_03710). We created single in-frame deletions of the two genes (ΔptsH and ΔptsN) and found that the proportion of G1/swarmer cells in ΔptsH (without HPr) or ΔptsN (without EI₅ⁿʳ) strain was reduced in comparison with the wild-type strain in complex medium (Fig. 5b), a phenotype already described for ΔptsP (without EI₅ⁿʳ) and for ΔspoT (Fig. 5b). Interestingly, strains expressing a non-phosphorylatable version of EI₅ⁿʳ (EI₅ⁿʳ₈₁₆₆A) accumulated G1 cells as much
as the loss-of-function mutant (ΔptsN), that is, less than the wild-type strain (Fig. 5b), suggesting that the PTS Ntr pathway is slightly phosphorylated in complex medium PYE and that the last component of the PTS Ntr phosphorelay, EIIA Ntr, controls Spol activity.

To validate the conservation of PTS Ntr phosphorelay and the inhibitory effect of glutamate on this cascade, we checked the phosphorylation level of EIIA Ntr in +N or −N conditions.

To this purpose, we performed in vivo phosphorylation assays on WT and Δpts (without EIIA Ntr) strains expressing a xylose-inducible tagged version of EIIA Ntr (PxyIX::3FLAG-ptsN; Fig. 4b,c and Supplementary Fig. 8b,c). In agreement with our previous data, we found that the phosphorylation of EIIA Ntr is enhanced in the absence of nitrogen sources (−N) in comparison with the +N conditions (Fig. 4b,c and Supplementary Fig. 8b,c). In addition, we showed that EIIA Ntr is required in vivo for EIIA Ntr phosphorylation, since phosphorylated EIIA Ntr was undetectable in Δpts cells starved for nitrogen (Fig. 4b,c and Supplementary Fig. 8b,c).

In addition, we measured the (p)ppGpp levels in the single PTS Ntr mutants first in +N conditions. Consistent with the G1 accumulation in PYE, we found that PTS Ntr mutant strains (ΔptsP, ΔptsH, ΔptsN or ptsN H66A) produced significantly lower amount of (p)ppGpp than the wild-type strain in the presence of a nitrogen source (Fig. 5c). Moreover, EIIA Ntr H66A partially abrogated the cell cycle and developmental defects of ptsPL83Q (EI Ntr ΔE35) supporting the fact that overphosphorylation of EIIA Ntr in ptsP L83Q cells is partially responsible for (p)ppGpp accumulation and subsequent G1-to-S transition delay (Fig. 5b). On the contrary, a strain expressing a phosphomimetic mutant of EIIA Ntr (EIIA Ntr H66A) had increased proportion of G1 cells independently of the presence of EIIA Ntr (Fig. 5b). Altogether, these data suggest that the phosphorylated form of EIIA Ntr (EIIA Ntr~P) controls Spol activity.

However, (p)ppGpp measurements in −N conditions showed that Spol is also controlled in an EIIA Ntr~P-independent way. Indeed, in contrast to cells devoid of EIIA Ntr (ΔptsP) or HPr (ΔptsH), which did not accumulate (p)ppGpp upon nitrogen starvation (Fig. 5d), the absence of EIIA Ntr ~P in ΔptsN or ptsN H66A cells did not abolish (p)ppGpp accumulation upon nitrogen starvation (Fig. 5d), showing that Spol is still sensitive to nitrogen availability in the absence of EIIA Ntr ~P. On the basis of these results, we propose a model in which HPr ~P controls the intracellular levels of (p)ppGpp by at least two ways, in an EIIA Ntr ~P-dependent way but also independently of EIIA Ntr ~P.

Phosphorylated EIIA Ntr directly interacts with Spol. Since most of the regulatory functions of PTS components are mediated by protein–protein interactions, we checked whether HPr and EIIA Ntr were able to interact with Spol by performing bacterial two-hybrid (BTH) assays. To test this, T18 or T25 domains of Bordetella pertussis adenylate cyclase23 were fused to coding regions of HPr (ptsH) and EIIA Ntr (ptsN, ptsN H66A, and ptsN H66A) and Spol (spoT and spoT D184C). We found that both the wild-type EIIA Ntr (ptsN) and the phosphomimetic mutant of EIIA Ntr (EIIA Ntr H66A) were able to interact with Spol versions (Fig. 6a,b and Supplementary Fig. 9a), while the non-phosphorylatable mutant EIIA Ntr H66A was not (Fig. 6a,b and Supplementary Fig. 9a). Both T18-EIIA Ntr H66A and T25-EIIA Ntr H66A can, respectively, interact with T25-HPr and T18-HPr (Fig. 6c and Supplementary Fig. 9a), showing that EIIA Ntr H66A is functional in the BTH assays. Altogether, these findings suggest that EIIA Ntr is phosphorylated in vivo in E. coli. Indeed, there are two PTS systems in E. coli, a canonical one composed of EI (ptsI), HPr (ptsH) and EIIA (ptsM), as well as a nitrogen-related one composed of EIIA Ntr (ptsN), Npr (npr) and EIIA Ntr (ptsN), and both pathways can cross-talk to some extent24 (Supplementary Fig. 9b). To test whether Caulobacter EIIA Ntr is phosphorylated in vivo in E. coli, strains deleted for npr (Npr) or for both ptsP (EI Ntr) and ptsI (EI) genes were created. As illustrated on Fig. 6a and Supplementary Fig. 9c, the interaction between EIIA Ntr and Spol was abolished in the Δnpr strain while EIIA Ntr H66A retained the ability to interact with Spol. Likewise,
EIINtr, HPr and EIINtr control G1/swarmer lifetime. G1 proportion was measured in wild-type (WT; RH50), ptsNH66E, in the presence (MG1655 ΔptsP (RH1755), ΔptsP (RH1758), ΔptsPΔΔptsP (RH1748), ΔptsN (RH1819), ptsNΔ66E (RH2019), ptsNΔ66E (RH2017), ΔptsP ptsNΔ66E (RH2018), ΔptsP ptsNΔ66A (RH2016) grown in complex media (PYE) and normalized to the WT (100%). Error bars are indicated as *.

The red colour indicates positive interactions. (a) Schematic representation of the PTSNtr pathway in C. crescentus. (b) The PTSNtr components EIINtr, HPr and EIINtr control G1/swarmer lifetime. G1 proportion was measured in wild-type (WT; RH50), ΔspoT (RH1755), ΔptsP (RH1758), ptsPΔΔptsP (RH1748), ΔptsN (RH1819), ptsNΔ66E (RH2019), ptsNΔ66E (RH2017), ΔptsP ptsNΔ66E (RH2018), ΔptsP ptsNΔ66A (RH2016) grown in complex media (PYE) and normalized to the WT (100%). Error bars are indicated as *.

The red colour indicates positive interactions. (a) In contrast to EIINtrΔ66A, EIINtrΔΔP and EIINtrΔΔE directly interact with SpoT. MG1655 cyaA::frt (RH785) or MG1655 cyaA::frt Δnpr (RH2122) strains coexpressing T18- fused to ptsN, ptsNΔ66A, ptsNΔ66E or ZIP with T25- fused to spoT, spoTΔN or ZIP were spotted on MacConkey agar base plates supplemented with 1% maltose and 1 mM IPTG. Plates were incubated for 3–4 days at 30°C. The red colour indicates positive interactions. (b) The absence of E. coli EI and EIINtr proteins abolishes interaction between SpoT and EIINtr but not with EIINtrΔΔE. β-galactosidase assays were performed on strains coexpressing T25-SpoT and T18-EIINtr, T18-EIINtrΔΔP or T18-EIINtrΔΔE in the presence (MG1655 cyaA::frt (RH785)) or absence (MG1655 cyaA::frt ΔptsP ΔptsI (RH2124)) of EI and EIINtr proteins. Error bars = s.d.; n = 3. (c) In contrast to HPrΔΔE, HPrΔP directly interacts with EIINtrΔΔE. MG1655 cyaA::frt (RH785) strain coexpressing T18- fused to ptsN, ptsNΔ66A, ptsNΔ66E or ZIP with T25- fused to hpr, hprΔΔE, or ZIP were spotted on MacConkey agar base plates supplemented with 1% maltose and 1 mM IPTG. Plates were incubated for 3–4 days at 30°C. The red colour indicates positive interactions.

The phosphorylated form of EIINtr interacts with SpoT in BTH assays. (a) In contrast to EIINtrΔ66A, EIINtrΔΔP and EIINtrΔΔE directly interact with SpoT. MG1655 cyaA::frt (RH785) or MG1655 cyaA::frt Δnpr (RH2122) strains coexpressing T18- fused to ptsN, ptsNΔ66A, ptsNΔ66E or ZIP with T25- fused to spoT, spoTΔN or ZIP were spotted on MacConkey agar base plates supplemented with 1% maltose and 1 mM IPTG. Plates were incubated for 3–4 days at 30°C. The red colour indicates positive interactions. (b) The absence of E. coli EI and EIINtr proteins abolishes interaction between SpoT and EIINtr but not with EIINtrΔΔE. β-galactosidase assays were performed on strains coexpressing T25-SpoT and T18-EIINtr, T18-EIINtrΔΔP or T18-EIINtrΔΔE in the presence (MG1655 cyaA::frt (RH785)) or absence (MG1655 cyaA::frt ΔptsP ΔptsI (RH2124)) of EI and EIINtr proteins. Error bars = s.d.; n = 3. (c) In contrast to HPrΔΔE, HPrΔP directly interacts with EIINtrΔΔE. MG1655 cyaA::frt (RH785) strain coexpressing T18- fused to ptsN, ptsNΔ66A, ptsNΔ66E or ZIP with T25- fused to hpr, hprΔΔE, or ZIP were spotted on MacConkey agar base plates supplemented with 1% maltose and 1 mM IPTG. Plates were incubated for 3–4 days at 30°C. The red colour indicates positive interactions.

no more β-galactosidase activity was detected in a ΔptsP ΔptsI background (without E. coli EI proteins) expressing T25-SpoT and T18-EIINtr, while the interaction between SpoT and EIINtrΔΔE remained unchanged in this background (Fig. 6b). Finally, the expression of Caulobacter ptsH (HPr) from the inducible pBAD promoter (pBAD33-ptsHc) in a Δnpr
background restored the interaction between EIIA\textsuperscript{Ntr} and SpoT only when arabinose was added to the medium (Supplementary Fig. 9c), indicating that Caulobacter HPr and EIIA\textsuperscript{Ntr} proteins can be phosphorylated by E. coli PTS systems, and that only the phosphorylated form of EIIA\textsuperscript{Ntr} interacts with SpoT. In contrast to EIIA\textsuperscript{Ntr}, no interaction was detected between HPr (or HPr\textsubscript{H18A}) and SpoT (or SpoT\textsubscript{D81G}) on MacConkey maltose agar plates (Supplementary Fig. 9d). The fact that HPr interacts with EIIA\textsuperscript{Ntr} (Fig. 6c and Supplementary Fig. 9a) shows that HPr is functional in the BTH assays.

Altogether, these BTH data strongly suggest that (i) HPr and EIIA\textsuperscript{Ntr} are both phosphorylated in E. coli and (ii) EIIA\textsuperscript{Ntr} \textasciitilde P is the only form of EIIA\textsuperscript{Ntr} able to interact with SpoT, thereby supporting a model in which SpoT activity is controlled directly by EIIA\textsuperscript{Ntr} \textasciitilde P, and indirectly by HPr \textasciitilde P (Fig. 8).

**Phosphorylated EIIA\textsuperscript{Ntr} inhibits hydrolase activity of SpoT.** Interestingly, the deletion of ptsN (EIIA\textsuperscript{Ntr}) did not abolish the G1 accumulation of spoT\textsubscript{D81G} cells in contrast to ΔptsP (EIINtr) or ΔptsH (HPr; Fig. 7a). The fact that SpoT\textsubscript{D81G}, which harbours a reduced hydrolase activity (Supplementary Fig. 5c), is insensitive to the presence of EIIA\textsuperscript{Ntr} \textasciitilde P suggests that EIIA\textsuperscript{Ntr} \textasciitilde P might inhibit the hydrolase activity of SpoT rather than stimulating its synthetase activity. This could explain why ΔptsN or ptsN\textsubscript{H66A} are still able to accumulate high levels of (p)pGpp on nitrogen starvation, while ΔptsP or ΔptsH cannot (Fig. 5d). To validate our hypothesis, we engineered Caulobacter strains in which the only (p)pGpp synthetase activity was supplied by the unrelated E. coli RelA protein, and we measured the endogenous hydrolase activity of SpoT in different genetic backgrounds. To this end, we first abolished the synthetase activity of SpoT in several backgrounds (spoT\textsubscript{D81G}, ptsN\textsubscript{H66A}, ptsN\textsubscript{H66E} and ΔptsP), by replacing the tyrosine 323 of SpoT by an alanine (SpoTY323A; ref. 14). As expected, all these strains displayed a G1 accumulation similar to a ΔspoT strain in PYE complex medium (Fig. 7c). In a second time, we inserted a truncated version of E. coli RelA (p)Gpp synthetase at the xylose locus, leading to an artificial (p)pGpp accumulation in Caulobacter upon addition of xylose (PxyI\textsubscript{X::relA-FLAG}; ref. 16). Since the hydrolase domain of RelA is inactive, the only (p)pGpp hydrolase activity in these strains was carried out by the Caulobacter SpoT protein, while the only (p)pGpp

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**Figure 7 | EIIA\textsuperscript{Ntr} \textasciitilde P inhibits the hydrolase activity of SpoT.** (a) spoT\textsubscript{D81G} is insensitive to the absence of EIINtr. G1 proportion was measured in wild-type (WT; RH50), spoT\textsubscript{D81G} (RH1752), spoT\textsubscript{D81G} ΔptsP (RH1727), spoT\textsubscript{D81G} ΔptsH (RH2013), spoT\textsubscript{D81G} ΔptsN (RH1999), spoT\textsubscript{D81G} ΔptsN ΔptsP (RH2014) and spoT\textsubscript{D81G} ΔptsN ΔptsH (RH2015) grown in complex media (PYE) and normalized to the WT (100%). Error bars = s.d.; n = 3. (b) The hydrolase activity of SpoT is required for growth on an artificial exogenous production of (p)pGpp. Growth of spoT\textsubscript{Y323A}, spoT\textsubscript{D81G} Y323A, ptsN\textsubscript{H66A}spoT\textsubscript{Y323A}, ptsN\textsubscript{H66E}spoT\textsubscript{Y323A} and ΔptsP spoT\textsubscript{Y323A}, expressing a truncated version of E. coli RelA from the inducible xylI\textsubscript{X::relA-FLAG} promoter (PxyI\textsubscript{X::relA-FLAG}) on PYE medium supplemented with 0.1% of xylose. (c) Reduction of SpoT hydrolase activity led to a G1 extension on an artificial exogenous production of (p)pGpp. Flow cytometry analysis to determine DNA content in asynchronous population of WT, spoT\textsubscript{Y323A}, spoT\textsubscript{D81G} Y323A, ptsN\textsubscript{H66A}spoT\textsubscript{Y323A}, ptsN\textsubscript{H66E}spoT\textsubscript{Y323A} and ΔptsP spoT\textsubscript{Y323A} with (dark grey bars) or without (light grey bars) PxyI\textsubscript{X::relA-FLAG} in PYE medium supplemented with 0.1% of xylose. The data were normalized to the WT without PxyI\textsubscript{X::relA-FLAG} (100%). Error bars = s.d.; n = 3. (d) The hydrolase activity of SpoT is required to degrade (p)pGpp in \textasciitilde + N condition. Intracellular levels of (p)pGpp detected by TLC after nucleotides extraction of spoT\textsubscript{Y323A}, spoT\textsubscript{D81G} Y323A, ptsN\textsubscript{H66A}spoT\textsubscript{Y323A} and ΔptsP spoT\textsubscript{Y323A} containing PxyI\textsubscript{X::relA-FLAG} in \textasciitilde + N or \textasciitilde – N medium supplemented with 0.1% xylose.
Adaptation to starvation conditions requires sophisticated regulatory mechanisms that sense an external stimulus and translate it into an internal molecular response. In this report, we uncovered how Caulobacter copes with nitrogen starvation by triggering (p)pGpp accumulation (Fig. 8), which in turn will control the cell cycle and development by extending the G1/swarmer phase. Increasing the time spent in the non-replicative (G1), motile phase reflects the adaptation of Caulobacter cells to their natural environment, that is, freshwater in which nutrients can rapidly be limiting. Interestingly, G1 arrest also occurs during the intracellular trafficking of Brucella abortus, and on nitrogen and carbon starvation in Sinorhizobium meliloti. In addition, the G1 block encountered by S. meliloti cells starved for nitrogen and carbon is also dependent on (p)pGpp. Therefore, (p)pGpp-dependent mechanisms delaying DNA replication initiation could be a common feature used by z-proteobacteria in response to harsh conditions such as infection or starvation.

As previously suggested in the literature, our data indicate that stalked cells are able to complete replication upon nitrogen starvation, supporting that only swarmer cells are responsive to nitrogen depletion. Indeed, even if the speed of chromosome duplication is slowed down in nitrogen-starved conditions, the stalked cell seems to be unable to stop ongoing DNA replication. Intracellular glutamine concentration is known to vary in bacteria, up to 10-fold depending on nitrogen availability. As a consequence, monitoring intracellular glutamine concentration is an efficient strategy to evaluate nitrogen availability, and subsequently adjust nitrogen assimilation. In E. coli, the uridylyltransferase GlnD is known to directly sense the intracellular glutamine pool, and according to it, to modify uridylylation level of regulatory PII proteins (GlnB and GlnK), which in turn will adapt nitrogen metabolism. For instance, in the absence of glutamine, GlnD will increase ammonium transport, as well as the expression and activity of the glutamine synthetase. Intriguingly, three GlnA paralogs are encoded into the genome of Caulobacter crescentus, suggesting a functional redundancy, and the presence of multiple glutamine synthetase is conserved in several z-Proteobacteria. Even though we showed that only the glutamine synthetase encoded by glhA is necessary for assimilating ammonium in complex and minimal media, we do not exclude that the other paralogs (GlnA1 and GlnA3) also participate in ammonium assimilation under specific growth conditions.

Discussion

Adaptation to starvation conditions requires sophisticated regulatory mechanisms that sense an external stimulus and...
cycle thanks to (p)pGpp alarmone. It would be interesting to check if the PTS$^{\text{Ntr}}$ system is used by other z-proteobacteria to relay nitrogen starvation (glutamine deprivation) to (p)pGpp production and subsequent G1 arrest.

Only a few mechanisms triggering (p)pGpp accumulation in nutrient-limiting conditions have so far been deciphered at the molecular level. When *E. coli* cells are starved for amino acids, the (p)pGpp synthetase RelA is directly activated by ribosomes whose A site is occupied by an uncharged tRNA$^{32}$, whereas the bifunctional (p)pGpp synthetase/hydrolase SpoT is regulated by an acyl carrier protein in response to fatty acid starvation$^{33,34}$. In this report, we discovered a new molecular mechanism stimulating (p)pGpp accumulation in response to nutrient starvation. This mechanism involves the PTS$^{\text{Ntr}}$ system as an important metabolic sensor that translates a glutamine deprivation signal into a (p)pGpp accumulation signal. Our data suggest that EIIA$^{\text{Ntr}}$-$^{\text{P}}$ directly reduces the hydrolase activity of SpoT, while HPr-$^{\text{P}}$ indirectly activates (p)pGpp production upon nitrogen starvation (Fig. 8). Historically, the PTS system was discovered as a phosphorylation cascade involved in the regulation of sugar uptake and carbon catabolite repression$^{32,35}$. Afterwards, a second phosphotransferase system (PTS$^{\text{Ntr}}$) was proposed to be connected to nitrogen metabolism but this connection remained poorly described$^{31}$. The direct inhibition of EN$^{\text{Ntr}}$ autophosphorylation by glutamine observed in *E. coli* and *S. mellii*o$^{22,26}$, as well as now in *C. crescentus* (Fig. 4), reinforces the idea that nitrogen constitutes a signal for PTS$^{\text{Ntr}}$ systems. The fact that the GAF domain, highly conserved in all EN$^{\text{Ntr}}$ proteins (Supplementary Fig. 4b), is required for binding glutamine suggests that the glutamine-dependent control of EN$^{\text{Ntr}}$ phosphorylation might be a common feature in PTS$^{\text{Ntr}}$ system.

In contrast to its EIIA paralog, the EIIA$^{\text{Ntr}}$ component is not associated with permeases, but rather carries out regulatory functions, by interacting with its target(s). For example, the unphosphorylated form of EIIA$^{\text{Ntr}}$ inhibits pyruvate dehydrogenase activity in *Pseudomonas putida* by interacting with the EI subunit$^{37}$. Our work constitutes so far the first example of regulatory functions mediated by the phosphorylated form of EIIA$^{\text{Ntr}}$ (EIIA$^{\text{Ntr}}$$^{\text{P}}$). Indeed, our results support the conclusion that only the phosphorylated form of EIIA$^{\text{Ntr}}$ interacts with SpoT to inhibit its hydrolase activity. This is further supported by the fact that EN$^{\text{Ntr}}$ and SpoT are found in the same protein complex during stationary phase$^{38}$ Interestingly, a direct interaction between the non-phosphorylated form of EIIA$^{\text{Ntr}}$ and SpoT has recently been found in the β-proteobacterium *Ralstonia eutropha* by BTH but no function was assigned for this connection$^{39}$. This differential interaction between phosphorylated or non-phosphorylated form EIIA$^{\text{Ntr}}$ and SpoT illustrates the evolutionary plasticity of PTS$^{\text{Ntr}}$ components with their targets. Besides EIIA$^{\text{Ntr}}$$^{\text{P}}$, we know that phosphorylated HPr also controls (p)pGpp accumulation on nitrogen starvation, but how this regulation works at the molecular level remains an open question. HPr-$^{\text{P}}$ could interact with an unknown factor (X in Fig. 8), which in turn could modulate the abundance of SpoT or activate its synthetase activity, to subsequently increase the global (p)pGpp pool. Although we have now uncovered the pathway that stimulates (p)pGpp accumulation in response to nitrogen starvation, understanding how (p)pGpp affects the G1-to-S transition at the molecular level will be a challenge for future work.

Methods
Bacterial strains and growth conditions. Oligonucleotides, strains and plasmids used in this study are listed in Supplementary Tables 1, 2 and 3, altogether with construction details provided in the Supplementary Methods. *E. coli* Top10 was used for cloning purpose, and grown aerobically in Luria–Bertani (LB) broth (Sigma)$^{40}$. Electrocompetent cells were used for transformation of *E. coli*.
resuspended in PSG-labelling (−N) supplemented with KH2PO4, xylose (0.1%) and with (+N) or without (−N) glucose (9.3 mM).

**BTH assays.** BTH assays were performed as described previously in refs 23 and 42. Briefly, 2 μl of MG1655 cyaA::frt (RH758), MG1655 cyaA::frt ΔepsI (RH1222), MG1655 cyaA::frt ΔPips (RH1224) strains expressing T18 and T25 fusions were spotted on MacConkey Agar Base plates supplemented with ampicillin, kanamycin, and IPTG (1 mM) and incubated for 3–4 days at 30 °C. All proteins were fused to T25 at their N-terminal extremity (pT25) or to the T18 at their N-terminus (pT18(X) and pT18) extremity. BTH assays in both directions (T25-X with T18-Y or T18-Y with T25-X) gave similar results. The β-galactosidase assays were performed as described in ref. 46. Briefly, 50 μl E. coli BTH strains cultivated overnight at 30 °C in LB medium supplemented with kanamycin, ampicillin, and IPTG (1 mM) were resuspended in 800 μl of Z buffer (60 mM NaH2PO4, 40 mM Na2HPO4, 10 mM KCl, 1 mM MgSO4) and lysed with chloroform. After the addition of 200 μl ONPG (4 mg ml−1), reactions were incubated at 30 °C until colour turned yellowish. Reactions were then stopped by the addition of 500 μl of 1 M Na2CO3, and absorbance at 420 nm was measured. Miller units are defined as (OD420 nm x t)/([OD420 nm x v]), where, [OD420 nm] is the absorbance of the template-free β-galactosidase assay, and t and v are the time of the reaction (min) and the volume of cultures used in the assays (ml). All the experiments were performed with three biological replicates and Miller units of the T25-X T18-ZIP combination were used as a blank and subtracted.

**Immunoblot analysis.** Immunoblot analyses were performed as described in ref. 46, with the following primary antibodies: α-Flaggin (1:5,000; ref. 47), α-FLAG (1:5,000; Stratagene), α-Mreb (1:5,000; ref. 46) and secondary antibodies: α-LTC (1:1000) or (p)ppGpp: Still Magical? Annu. Rev. Microbiol. 62, 35–51 (2008).

**Preparation of fractions containing EIN<sup>frt</sup> or EIN<sup>frtL83Q</sup>.** Fractions containing EIN<sup>frt</sup> or EIN<sup>frtL83Q</sup> proteins were purified from NA1000 and NA1000 pXMCS2 (RH748) strains, respectively. C. crescentus strains were grown in 150 ml PYE liquid media (OD<sub>600</sub> ~0.7), collected by centrifugation for 15 min at 6,000 g, 4 °C, washed with 4 ml ice-cold phosphate buffered-saline containing 0.05% Triton X-100, complete EDTA-free anti-protease, 20 mg ml<sup>−1</sup> lysozyme, 10 U ml<sup>−1</sup> DNase I. Cells were first lysed by sonication, then zirconium beads were added and cells were disrupted by Fastprep for 20 s. Lysates were pelleted at 15,000g at 4 °C. Samples were subjected to electrophoresis in a 12% SDS-polyacrylamide gel. SDS-polyacrylamide gels were then dried and imaged on a MP Phosphor system (Packard) and analysed with Cyclone Phosphor Imager (PerkinElmer). Band intensities were quantified with ImageJ software by using the WT (−Xyl, −N) as the background.

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