(p)ppGpp modulates cell size and the initiation of DNA replication in *Caulobacter crescentus* in response to a block in lipid biosynthesis

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Stress conditions, such as a block in fatty acid synthesis, signal bacterial cells to exit the cell cycle. *Caulobacter crescentus* FabH is a cell-cycle-regulated β-ketoacyl-acyl carrier protein synthase that initiates lipid biosynthesis and is essential for growth in rich media. To explore how *C. crescentus* responds to a block in lipid biosynthesis, we created a FabH-depletion strain. We found that FabH depletion blocks lipid biosynthesis in rich media and causes a cell cycle arrest that requires the alarmone (p)ppGpp for adaptation. Notably, basal levels of (p)ppGpp coordinate both a reduction in cell volume and a block in the over-initiation of DNA replication in response to FabH depletion. The gene *ctrA* encodes a master transcription factor that directly regulates 95 cell-cycle-controlled genes while also functioning to inhibit the initiation of DNA replication. Here, we demonstrate that *ctrA* transcription is (p)ppGpp-dependent during fatty acid starvation. CtrA fails to accumulate when FabH is depleted in the absence of (p)ppGpp due to a substantial reduction in *ctrA* transcription. The (p)ppGpp-dependent maintenance of *ctrA* transcription during fatty acid starvation initiated from only one of the two *ctrA* promoters. In the absence of (p)ppGpp, the majority of FabH-depleted cells enter a viable but non-culturable state, with multiple chromosomes, and are unable to recover from the miscoordination of cell cycle events. Thus, basal levels of (p)ppGpp facilitate *C. crescentus'* re-entry into the cell cycle after termination of fatty acid starvation.

INTRODUCTION

The *Caulobacter crescentus* cell cycle is controlled by four core oscillating master regulators: DnaA, GcrA, CtrA and CcrM (Collier et al., 2007). Each contributes to the genetic circuitry that ensures cell cycle events occur at the correct time and place. When a stress condition activates a cell cycle checkpoint, *C. crescentus* exits the cell cycle and inhibits cell division (Britos et al., 2011). This study focuses on how *C. crescentus* adapts to a block in lipid biosynthesis induced by depletion of FabH. In nature, bacteria may experience fatty acid starvation in the presence of microbially produced antibiotics that block fatty acid synthesis, such as cerulenin or thiolactomycin (Zhang et al., 2006).

The FabH β-ketoacyl-acyl carrier protein synthase condenses malonyl-acyl carrier protein with acetyl-CoA, forming β-ketoacyl-acyl carrier protein, which is a key intermediate in fatty acid biosynthesis. Although shown not to be essential in *E. coli* except in cells unable to produce (p)ppGpp (Yao et al., 2012), *fabH* is an essential gene in *C. crescentus* (Christen et al., 2011). Moreover, *Escherichia coli fabH* mutants were found to have a 70% reduction in cell volume compared with WT, suggesting that cell size is regulated by the rate of fatty acid biosynthesis (Yao et al., 2012). Early studies found that blocking fatty acid biosynthesis caused *C. crescentus* to lose motility (O’Neill & Bender, 1989) and the capacity to replicate DNA while arresting the cell cycle at the stalked or predivisional cell stage (Contreras et al., 1979). Furthermore,
a previous study (Dawson et al., 1981) found that cell size was reduced in starved marine Vibrio cultures after transition from rich media to a salt solution. In E. coli, the alarmone (p)pGpp mediated a reduction in cell size, and a change in shape from rod to coccoid, in response to isoleucine (p)ppGpp mediated a reduction in cell size, and a change in shape from rod to coccoid, in response to isoleucine starvation (Traxler et al., 2008). When (p)pGpp was artificially induced in C. crescentus, cell size was reduced but crescent morphology was maintained (Gonzalez & Collier, 2014).

In addition to modulating cell size, (p)pGpp was found to inhibit the transcription of fabH and other lipid biosynthetic genes in E. coli (My et al., 2013; Podkorytov & Larson, 1996). The transcription of a group of lipid biosynthetic genes (including plsX, which is in the same operon as fabH) was also inhibited in carbon-starved C. crescentus (Boutte & Crosson, 2011), suggesting a conserved regulatory pathway. In general, (p)pGpp helps cells shift their metabolism from anabolic to a catabolic focus, where cells attempt to re-assimilate key metabolites into central metabolism (Boutte & Crosson, 2011; Traxler et al., 2008).

In C. crescentus, (p)pGpp is produced by the RelA/SpoT homologue (Rsh)-family member SpoT during starvation conditions (Dalebroux & Swanson, 2012; Potrykus & Cashel, 2008). Unlike E. coli, SpoT is the sole Rsh and (p)pGpp synthetase/hydrolase of C. crescentus, as this organism does not have a RelA homologue (Boutte & Crosson, 2011; Boutte et al., 2012; Lesley & Shapiro, 2008). In C. crescentus, SpoT mediates a block in DNA replication in response to carbon starvation. Notably, the DnaA and CtrA transcription factors have antagonistic functions in regulating the initiation of DNA replication, whereby DnaA initiates replication and CtrA blocks it (Collier, 2012). To initiate DNA replication in C. crescentus, CtrA must be destroyed by proteolysis for DnaA to function. When C. crescentus faces carbon starvation, SpoT responds by mediating DnaA proteolysis (Lesley & Shapiro, 2008) and, in contrast, protecting CtrA from proteolysis (Boutte et al., 2012). Together, the simultaneous proteolysis of DnaA and stabilization of CtrA prevent the initiation of DNA replication under nutrient-limiting conditions. Interestingly, a recent report showed that the Lon protease degrades DnaA to prevent DNA replication under adverse growth conditions (Jonas et al., 2013). Also, when (p)pGpp levels are artificially induced in rich media, DnaA synthesis is reduced (Gonzalez & Collier, 2014).

C. crescentus ctrA is notably transcribed from two temporally regulated promoters, P1 and P2 (Domin et al., 1999; Schredl et al., 2012). CtrA is destroyed by the ClpXP protease during the G1-to-S transition, allowing DNA replication initiation (Iniesta et al., 2006; McGrath et al., 2006). Then, CtrA returns to help limit DNA replication initiation to only once per cell cycle. During the S-phase of the cell cycle, GcrA activates the transcription of ctrA P1 (Holtzendorff et al., 2004) after this locus becomes hemimethylated during DNA replication (ReisenaUer & Shapiro, 2002). Once CtrA accumulates, it inhibits transcription from P1 and activates its own transcription from P2 (Domin et al., 1999). This promoter switch has been proposed to finely tune CtrA accumulation – with low levels during the S-phase and high levels during the G2- and G1-phases of the cell cycle (Domin et al., 1999).

In response to carbon starvation, (p)pGpp programmes the cell to upregulate catabolic genes and downregulate anabolic genes (Boutte & Crosson, 2011). Specifically, 379 genes were upregulated and 382 genes were downregulated threefold, including genes unrelated to metabolism (Boutte & Crosson, 2011). Furthermore, the same authors subsequently demonstrated that (p)pGpp slows the swarmer-to-stalked cell transition (Boutte et al., 2012). As a result, SpoT mutants have a faster doubling time than wild-type C. crescentus. Conversely, expression of a truncated E. coli relA gene in C. crescentus increases (p)pGpp levels, slowing down the swarmer-to-stalked cell transition (Gonzalez & Collier, 2014).

Although much is known about how C. crescentus responds to carbon starvation, little is known about how it responds to other types of starvation, including fatty acid starvation. Here, we demonstrate that (p)pGpp prevents most cells that have been starved for fatty acids from entering a viable but non-culturable (VBNC) state. Adaptive responses mediated by (p)pGpp in response to a block in lipid biosynthesis include a reduction of cell volume and transcriptional activation of ctrA. When FabH is depleted in the absence of SpoT, cell volume is maintained and CtrA does not accumulate, resulting in multiple chromosomes in individual cells. Although alive, most of these cells cannot form colonies due to a miscoordination of (p)pGpp-regulated cell cycle events.

**METHODS**

**Bacterial strains, media, plasmids, and primers.** Strains and plasmids are described in Table S1 (available in the online Supplementary Material). Primer sequences are shown in Table S2. Details on the construction of strains and plasmids can be found in the online Supplementary Material. Primer sequences are shown in Table S2. Details on the construction of strains and plasmids can be found in the online Supplementary Material. C. crescentus cultures were grown at 28 °C on agar plates or in 1.5 cm diameter glass or plastic culture tubes containing either peptone yeast extract (PYE) or minimal (M2G) medium (Ely, 1991) supplemented with 0.5 mM vanillate, kanamycin (broth, 5 µg ml⁻¹; agar, 25 µg ml⁻¹), spectinomycin (broth, 25 µg ml⁻¹; agar, 50 µg ml⁻¹), nalidixic acid (agar only, 3 µg ml⁻¹) and ampicillin (agar only, 3 µg ml⁻¹). To isolate C. crescentus swarmer cells for synchronized culture, swarmer and stalked cells were separated (from exponentially growing cultures in M2G broth) onto a 125 r.p.m. rotating platform at 28 °C. E. coli strains were grown in or on LB medium (Miller, 1972) containing ampicillin (broth, 50 µg ml⁻¹; agar, 50 µg ml⁻¹), kanamycin (broth, 15 µg ml⁻¹; agar, 50 µg ml⁻¹), and nalidixic acid (agar only, 18 µg ml⁻¹). To isolate C. crescentus swarmer cells for synchronized culture, swarmer and stalked cells (from exponentially growing cultures in M2G broth) were separated using Ludox AS-40 colloidal silica (Sigma-Aldrich) density gradients (Evinger & Agabian, 1977).

**Measurement of transcription using Miller assays.** The Miller units (β-galactosidase activities) of promoter-lacZ constructs were measured (Miller, 1972) from cultures in PYE broth supplemented with the appropriate antibiotic after 24 h of FabH repletion or depletion. OD₆₆₀ was noted and LacZ activity (420 nm) was...
measured with a Beckman Coulter DU 640 UV–visible spectrophotometer (path length, 1 cm). The dnaA-, gcrA- or ctra–lacZ reporters were expressed from plasmid pKlacZ290 and the ccrM–lacZ reporter was integrated into the chromosome at the native ccrM locus (Table S1). Significance (P<0.05) from triplicate or quadruplicate experiments was determined using paired or unpaired Student t-tests.

**Immunoblot.** Immunoblot samples were taken from cultures normalized based on OD₆₆₀. To resolve proteins in cell lysates, 8 (for DnaA), 12 (for CcrM) and 15 % SDS-PAGE (for GcrA, CtrA and FabH) were used (Sambrook et al., 1989). FabH antisera (Josman) was isolated from rabbits inoculated with purified His-FabH. Proteins were transferred to Immobilon PVDF membranes (Millipore). Donkey anti-rabbit conjugated to horseradish peroxidase (Jackson ImmunoResearch) was used to detect polyclonal antibodies reacting with DnaA, GcrA, CtrA, CcrM (gifts from Lucy Shapiro, Stanford University) or FabH. The following antisera were diluted: anti-FabH 1:4000, anti-DnaA 1:20,000, anti-GcrA 1:4000, anti-CtrA 1:20,000 and anti-CcrM 1:5000. The blots were viewed with Western-Lightning Plus-ECL chemiluminescent reagent (PerkinElmer) using HyBlot CL autoradiography films (Denville Scientific). Photoshop (Adobe) was used to optimize the brightness and contrast of scanned films.

**Depletion of FabH.** Single colonies were inoculated into 3 ml PYE–vanillate broth and grown overnight, as described above. When required, overnight PYE–vanillate cultures were subcultured into M2G–vanillate broth. All exponential-phase cultures were then washed three times, in PYE or M2G broth to remove vanillate, and M2G–vanillate broth. All exponential-phase cultures were then normalized to a starting OD₆₆₀ of 0.100, and 3 ml of the OD₆₆₀ 0.100 PYE culture was used to inoculate 3 ml PYE medium supplemented with vanillate. After 4–7 h of incubation at 28 °C on a shaking platform, PYE cultures without vanillate were diluted back to OD₆₆₀ 0.100. After 24 h of incubation, these cultures reached an OD₆₆₀ between 0.1 and 0.6.

Exponentially growing M2G–vanillate cultures were normalized to OD₆₆₀ 0.5 and then were washed as described above. The final cell pellet was resuspended in 200 µl M2G. Five microlitres of the suspension was used to inoculate 3.3 ml M2G–vanillate cultures, 13.7 µl SM1131 was added to 3.3 ml M2G, and 20 µl SM1309 or SM1455 was added to 3.3 ml M2G broth. After 17 h of incubation, these cultures reached an OD₆₆₀ between 0.1 and 0.6.

**Growth curves.** Single colonies of FabH-depletion strains were inoculated into 3 ml of PYE supplemented with vanillate and were incubated overnight in a 28 °C incubator with 125 r.p.m. rotational movement. If the cultures passed into stationary phase during the overnight incubation, they were diluted so the turbidity could double before starting the growth curve. The cells were then washed as described above and were normalized to OD₆₆₀ 0.065. Cultures were incubated as described above.

**VBNC assay.** To determine the percentage of cells in a VBNC state, FabH-depletion strains were cultured as described above. After 24 h, all cultures were normalized to OD₆₆₀ 0.150, and 1 × 10⁻⁵ [for cultures lacking both FabH and (p)ppGpp] and 1 × 10⁻⁶ [for all other cultures] final dilutions were plated onto PYE–vanillate agar. They were incubated at 28 °C for 2 days for c.f.u. determination. Simultaneously, live/dead staining was performed (see details below) for immediate viewing under a fluorescent microscope while aliquots of unstained cultures were fixed in a solution of 30 mM sodium phosphate buffer (pH 7.5) and 2.5 % formaldehyde for direct counts. Direct counts were determined using a Petroff–Hauser counting chamber and an upright light microscope using phase-contrast. Predivisional cells were counted as one cell as they will lead to 1 c.f.u. on an agar plate. Since the direct counts, c.f.u. determination and live/dead staining were performed on the same cultures, the percentage of VBNC cells could be determined. First, the number of live cells was calculated by multiplying the percentage of live cells (staining) by the total number of cells (direct counts). Then, to determine the percentage of live cells that formed colonies, the number of c.f.u. was divided by the number of live cells. Finally, the percentage of live cells that did not form colonies represents the percentage of cells in a VBNC state.

**Microscopy.** Strains were grown as described above. Two to five microlitres of cultures were viewed on 1 % agarose slides. Cell morphology was observed with a Zeiss Axiosimager M1 microscope with a Hamamatsu OCRA-ER digital camera using differential interference contrast (DIC) settings.DIC was used in combination with fluorescence filters depending on the dyes or markers. Zeiss AxioVision software was used to measure cell length and width, and these measurements were used to calculate cell volume and surface area as previously described (Yao et al., 2012).

**Live/dead staining.** The cells were grown as described above. At 24 h, 1.5 ml culture was centrifuged for 2 min at 7430 g. The supernatant was removed and 100 µl 0.85 % NaCl was added to the pellet and then was split into two equal volumes. For one set, 1 ml 0.85 % NaCl was added. For the second set, 1 ml 70 % 2-propanol was added (negative control). The cells were centrifuged, the supernatants were removed, and the cell pellets were resuspended in 1 ml 0.85 % NaCl. Then 5 µl resuspended cells were added to 5 µl pre-mixed SYTO 9 and propidium iodide dyes (from a LIVE/DEAD BacLight Viability kit; Molecular Probes). After incubating the samples for 15 min in the dark, propidium iodide was viewed with a Zeiss Axiosimager M1 microscope using a DAPI filter (70 ms exposure), and SYTO 9 (40 ms exposure) was viewed with a GFP filter. Predivisional cells were only counted as two cells if a clear septum was visible with fluorescence microscopy. This counting procedure was used since some compartmentalized late predivisional cells had mixed staining where one cell compartment stained with SYTO 9 and the other with propidium iodide.

**RNA isolation and purification.** RNA was isolated using a phenol/ chloroform extraction from 50 ml cultures of C. crescentus in M2G supplemented with vanillate as necessary. RNA was precipitated and subsequently cleaned using the RNasy kit (Qiagen). RNA was eluted using 1:20 TE buffer (pH 7.4) to RNA-free water. RNA concentration was measured using a Nanodrop spectrophotometer. The RNA (3 µg) was treated with DNase, and cleaned using RNA Clean & Concentrate (Zymo Research). RNA quality was checked by measuring the A₂₆₀/A₂₈₀ ratio and running an RNA sample on a 0.8 % agarose gel (Argueta et al., 2006).

**Preparation of cDNA.** cDNA was produced using SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocol, with 1.5 µg RNA. The RACEextrAR primer (Schredl et al., 2012) (Table S2) was added to the mixture for 5'-RACE analyses. Random hexamers and the ctra Rev primer (Tan et al., 2010) (Table S2) were used for cDNA synthesis for real-time quantitative PCR (qPCR) analyses. For 5'-RACE applications, the RNA template was removed using alkaline hydrolysis. The cDNA was concentrated using DNA Clean & Concentrate (Zymo Research).

**qPCR of ctra.** Real-time qPCR was performed as previously described (Cuajungco et al., 2012), with minor modifications appropriate for our experiments. The amplification reactions were performed in a Bio-Rad CFX96 machine using SensiMix SYBR Green Master Mix (Bioline) with the following thermocycling conditions: 5 min at 95 °C followed by 40 cycles of 30 s at 95 °C, 45 s at 55 °C, 30 s at 72 °C. The primer sets (Table S2) for ctra (ctrA Fwd and ctra

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Flavolipid and fatty acid biosynthesis (Kim et al. 2014) and CC2677 (normalization control) (CC2677 Fwd and CC2677 Rev) genes were obtained from a recent publication (Tan et al., 2010). The qPCR efficiency (E) and correlation coefficient (R²) were: E = 91 (±1)% and R² = 0.99 for ctrA (n = 3); and E = 94 (±5)% and R² = 0.99 for CC2677 (n = 3).

5′-RACE of ctrA. DT88 adapters (Argueta et al., 2006) (Table S2) were ligated (overnight at 18 °C) to the single-stranded cDNA template using RNA ligase (Promega). After ligation, PCR was performed using the primers DT89 and 5′ RACE ctrA Rev (Schrödl et al., 2012) (Table S2). A 1:50 dilution of the resulting PCR was used as the template for a second PCR using DT89 (Argueta et al., 2006) and 5′ RACE ctrA Nested Rev (Schrödl et al., 2012). The resulting product was separated on a 2.5% agarose gel at 80 V and visualized with ethidium bromide. The bands were cut from the gel, extracted and sequenced.

Flow cytometry. Samples for flow cytometry were isolated from exponentially growing cells treated with rifampicin for 3 h as previously described (Winzler & Shapiro, 1995) except that DyeCycle Orange (Invitrogen) was used to stain the DNA as in a recent publication (Lesley & Shapiro, 2008). The data were collected using a FACStar Plus machine (Becton Dickson) and analysed using FlowJo software (Tree Star). Flow cytometry results were obtained from triplicate or quadruplicate independent cultures.

Online Supplementary material. The online Supplementary Material includes methods, four tables (including Table S3, which describes the media used in (p)ppGpp assays), references and seven figures.

RESULTS

FabH is a cell-cycle-regulated protein

Published microarray data (McGrath et al., 2007) suggested that fabH (CC1369) is a cell-cycle-regulated gene, with significantly lower transcript levels in swarmer cells than stalked or predivisional cells. To independently confirm these data, a transcriptional reporter was created. fabH is the second gene of a two-gene operon that also contains plsX; the transcriptional pattern of the two genes is similar during the C. crescentus cell cycle (McGrath et al., 2007), suggesting that both genes are normally expressed from one promoter. PlsX has been proposed to integrate phospholipid and fatty acid biosynthesis (Kim et al., 2009). As shown in Fig. 1(a), PplsX/fabH-lacZ transcription is significantly lower (P < 0.05) in swarmer cells than stalked or predivisional cells. Our data confirm published microarray data (McGrath et al., 2007), which directly measured mRNA levels in terms of relative changes in fabH transcript levels during the cell cycle. To see if FabH protein levels follow fabH transcript abundance, anti-FabH antibodies were generated in rabbits and FabH protein accumulation was monitored during the cell cycle by immunoblot. The specificity of the FabH antibody was confirmed using a FabH-depletion strain (Fig. S1). As shown in Fig. 1(b), FabH accumulated in stalked and early predivisional cells, with lower FabH levels in swarmer cells. Thus, FabH accumulation peaked (Fig. 1b) when maximum fatty acid biosynthesis was required during the S-phase of the cell cycle (O’Neill & Bender, 1987).

A block in FabH-mediated lipid biosynthesis obstructs cell growth

C. crescentus FabH-depletion strains in WT or spoT mutant genetic backgrounds were constructed with the sole copy of fabH under control of the vanillate promoter (Thanbichler et al., 2007) at the vanillate locus. We were unable to knockout fabH in a WT genetic background, confirming previously published results showing that fabH is required for growth on PYE (rich) medium (Christen et al., 2011). FabH was depleted after 3 h of growth in the absence of vanillate (Fig. S1), indicating that FabH is an unstable protein. In PYE medium, cell growth slowed significantly after 7 h of FabH depletion (with turbidity increasing 1.6-fold in FabH-depleted cultures compared with 167.5-fold in FabH-replete cultures between 7 and 22 h) and completely arrested by 22 h, independent of the presence or absence of SpoT (Fig. 2a).

(p)ppGpp facilitates C. crescentus’s re-entry into the cell cycle after termination of fatty acid starvation

When FabH was depleted for 24 h in the presence of SpoT, 96 ± 9% of cells could recover from the block in lipid biosynthesis and form colonies on medium containing vanillate, the inducer of fabH transcription in our mutants (Fig. 1b). In contrast, only 21 ± 8% of cells depleted of FabH for 24 h in the absence of SpoT yielded colonies on PYE medium containing vanillate. Similar results (15 ± 1% of cells yielded colonies) were found in a SpoT Y323A genetic background, where SpoT was catalytically inactive (Boutte et al., 2012), indicating that (p)ppGpp is required for this phenotype.

To determine if the non-growing FabH-depleted cells represented a bacteriostatic or a bactericidal state, live/dead staining was used. Of cells expressing FabH, 93 ± 4% (2878 total) were alive, compared with 80 ± 6% (1861) of cells depleted of FabH (Fig. 2b). Similar percentages were observed in cells lacking SpoT, where 97 ± 1% of (2271) FabH-expressing cells were alive compared with 78 ± 9% of (769) FabH-depleted cells. In a SpoT Y323A genetic background, 82 ± 8% of (940) FabH-replete cells were alive compared with 75 ± 3% of (1085) FabH-depleted cells (Fig. 2b). Although 78 ± 9% of (769) ΔspoT FabH-depleted cells were alive (Fig. 2b, c), and (as described below) physiologically active (capable of synthesizing both proteins, as [13]S)methionine pulse-labelled CtrA; Fig. S6), 77% of live cells were VBNc. Similar results were obtained in SpoT Y323A FabH-depleted cells, where 80% of live cells were in a VBNc state. Thus, (p)ppGpp prevents the majority of cells in culture from entering a VBNC state after depletion of FabH in C. crescentus. This is similar to E. coli, where fabH or spoT can be deleted individually but colonies lacking both fabH and spoT cannot be obtained (Yao et al., 2012).

Based on the known biochemical function of FabH, we asked if phospholipid biosynthesis was blocked in these strains. Indeed, a 26-fold reduction in relative phospholipid
biosynthesis was observed in cells depleted of FabH compared with cells expressing FabH (Fig. S2).

A reduction in cell volume is mediated by (p)ppGpp in response to a block in lipid biosynthesis

It was previously reported (Yao et al., 2012) that E. coli fabH mutants have 70% less volume than WT cells, and a recent publication (Gonzalez & Collier, 2014) showed that expression of a truncated E. coli RelA in non-starved C. crescentus reduced cell size by half. Here, microscopy showed that FabH depletion in WT C. crescentus reduced cell volume to 51% of the volume of FabH-expressing cells when grown in PYE broth (Fig. 2c, d). This reduction in cell volume increased the surface-area-to-volume ratio to 161% of that of FabH-replete SpoT+ cells (Fig. S3). Similar changes were observed in M2G broth (not shown). In contrast, when FabH was depleted in SpoT mutants, cell volume was not reduced (Fig. 2c, d). Cell size was normalized to the FabH-replete condition for each strain representing 100% since the ΔspoT and SpoT Y323A strains were larger than WT. We attribute the cell size phenotype to (p)ppGpp synthesis because the SpoT Y323A point mutant, which cannot synthesize (p)ppGpp (Boutte et al., 2012), behaved in a similar manner to ΔspoT when FabH was depleted (Fig. 2d). Thus, (p)ppGpp modulates a reduction in cell size, which is presumably advantageous under starvation conditions (Yao et al., 2012).

(p)ppGpp is required for CtrA accumulation when lipid biosynthesis is blocked

To measure the abundance of the core cell cycle master regulators under conditions of FabH depletion, cell lysates were probed for master regulator protein levels by immunoblot assays. As shown in Fig. 3(a), DnaA, GcrA and CcrM accumulation was drastically reduced when lipid biosynthesis was blocked. However, CtrA accumulated only when (p)ppGpp was produced (Fig. 3a), as confirmed with immunoblot experiments in the SpoT Y323A genetic background (Fig. 4a). Interestingly, a transient increase in CtrA accumulation was observed after 3–9 (spoT+) or 4.5–6 h (ΔspoT) of FabH depletion (Fig. S1). CtrA accumulation was also maintained in carbon-starved (Boutte et al., 2012; Britos et al., 2011) or stationary phase C. crescentus cultures (Gorbatyuk & Marczynski, 2005).

Transcription of the cell cycle master regulators is altered when FabH is depleted in WT or ΔspoT strains

We asked if the changes in master regulator protein accumulation, in response to FabH depletion, resulted
Fig. 2. (a) FabH depletion blocks cell growth. The cells were washed and suspended in either PYE or PYE–vanillate broth. All cultures were diluted at 7 and 22 h (arrows). Error bars indicate SD from triplicate independent samples. (b) (p)ppGpp is required to prevent the majority of FabH-depleted cells from entering a VBNC state. Dilutions from cultures normalized to OD_{660} 0.150 were plated after 24 h of incubation onto PYE–vanillate agar for c.f.u. determination, fixed for direct counts of cell number, and stained to determine the percentage of live cells. The numbers of cells or colonies are indicated on the left y-axis whereas the percentages are represented on the right y-axis. Error bars indicate SD from triplicate or quadruplicate trials. (c) FabH depletion induces a bacteriostatic state. BacLight LIVE/DEAD staining (Invitrogen) reveals live cells (green) and dead cells (red). 2-Propanol was used as a negative control. Although 24 h of FabH depletion (PYE) in ΔspoT strains blocked the
recovery of c.f.u., the majority cells were alive and in a VBNC state. Images are representative of six or seven independent experiments. DIC, Differential interference contrast. (d) (p)ppGpp mediates a reduction in cell volume in response to FabH depletion (PYE). Each strain was normalized to FabH-replete cell volume represented as 100 % and the relative percentage change in cell volume under FabH-depletion conditions at 24 h is shown. FabH-depleted spoT+ cells had half the volume of FabH-replete spoT+ cells. In contrast, no reduction in volume was observed in FabH-depleted cells that could not produce (p)ppGpp. Error bars indicate SD from measurements from 225–424 cells per condition. Van, Vanillate.

from altered transcription. As shown in Fig. 3(b), the transcription of dnaA and ctrA increased (by 150 % for dnaA and by 70 % for ctrA) in a SpoT-dependent manner when FabH was depleted. In contrast, ccrM transcription decreased by 50 % in a FabH-dependent manner. The transcription of gcrA only decreased by 27 % in the absence of both FabH and SpoT. Thus, high levels of ccrM transcription are FabH-dependent and the transcription of dnaA

![Fig. 3](http://mic.sgmjournals.org)
Fig. 4. (a) (p)pGpp-dependent transcription of ctrA in FabH-depleted cells (M2G). Relative ctrA mRNA levels were normalized to FabH-replete cells in a SpoT+ genetic background as 100%. Error bars indicate SD from triplicate independent experiments from cultures grown for 17 h in M2G broth. Below, a representative immunoblot shows CtrA protein accumulation in the same strains grown for 17 h in M2G broth. (b) 5'-RACE on ctrA revealed (p)pGpp-dependent ctrA P2 transcription during fatty acid starvation. Grey boxes mark where P1 RACE products should appear whereas black boxes indicate P2 RACE products. Transcription from ctrA P1 is GcrA-dependent (Holtzendorff et al., 2004) and ctrA P2 is (p)pGpp-dependent under fatty acid starvation. This gel is representative of triplicate independent experiments from cultures grown for 17 h in M2G broth. (c) In the absence of CtrA, multiple chromosomes accumulate in individual FabH-depleted SpoT mutant cells. The number of cells containing one, two or three chromosomes in rifampicin-treated unsynchronized cultures is shown. FL2-H::DyeCycle Orange indicates peak emission values. Each chromosome peaks at 200 FL-H::DyeCycle Orange in PYE broth and at 250 FL-H::DyeCycle Orange in PYE–vanillate broth. Graphs are representative of flow cytometry analysis from triplicate or quadruplicate independent cultures grown for 24 h in PYE broth with or without vanillate.
and *ctrA* is SpoT-dependent when cells are starved for fatty acids. These changes did not result from the presence or absence of the *fabH* inducer, as we showed that vanillate did not alter the transcription of these cell cycle master regulatory genes (Fig. S4). Post-transcriptional regulation may explain the lack of DnaA and GcrA protein accumulation in FabH-depleted cultures, despite high levels of *dnaA* and *gcrA* transcription. We observed a SpoT-dependent 70% increase in *ctrA* transcription under FabH-depletion conditions and a 38% decrease in the absence of SpoT. This clearly implicates SpoT in the transcriptional regulation of *ctrA* during fatty acid starvation.

**ctrA transcription is (p)ppGpp-dependent in the absence of FabH**

We used real-time qPCR experiments to more accurately measure *ctrA* transcript levels in the absence of FabH. Although the transcriptional reporter experiments were performed in rich PYE medium, we could not find a reference gene that was consistent in all conditions in PYE medium. To circumvent this problem, RNA was harvested from FabH mutants grown in M2G minimal medium. We found that 17 h of FabH depletion in M2G broth was phenotypically similar to 24 h of FabH depletion in PYE broth (as shown with regard to CtrA accumulation in Fig. 4a); thus, all M2G broth experiments were performed 17 h post FabH depletion. The CC2677 reference gene was stable under the various growth conditions and in our genetic backgrounds in M2G medium. As shown in Fig. 4(a), in minimal medium, CtrA only accumulated in FabH-depleted strains that produce (p)ppGpp. Thus CtrA is regulated in a similar manner by (p)ppGpp in both rich and minimal media. Although no major changes in *ctrA* transcript levels were observed in cells expressing FabH in a WT or ΔspoT background, a substantial reduction in *ctrA* mRNA [to 29% (ΔspoT) or 33% (SpoT Y323A) of FabH-replete SpoT 

**Individual FabH-depleted ΔspoT cells accumulated multiple chromosomes**

Flow cytometry analysis was used to measure the DNA content of rifampicin-treated cells because high levels of CtrA are required to block the over-initiation of DNA replication (Schredl et al., 2012), and FabH-depleted ΔspoT cells lack CtrA accumulation. Cells expressing FabH or SpoT had either one or two chromosomes (Fig. 4c). In contrast, cells lacking both FabH and SpoT accumulated two or three chromosomes per cell, suggesting dysregulation of DNA replication. The percentages of cells with different numbers of chromosomes determined by flow cytometry analysis and the visualization of MipZ-eYFP foci are shown in Table S4. MipZ-eYFP binds near the origin of replication (Thanbichler & Shapiro, 2006) and therefore provides a reliable measurement for the number of initiation events for DNA replication per cell. Notably, these results indicate that, although DnaA accumulation was low under FabH-depletion conditions (Fig. 3a), the DnaA molecules present are active enough to over-initiate DNA replication in the absence of CtrA (Fig. 4c).

**Basal levels of (p)ppGpp orchestrate the response to a block in lipid biosynthesis**

In an attempt to determine if (p)ppGpp levels increase during fatty acid starvation, cultures were labelled with [32P]orthophosphate, nucleotides were extracted, and these nucleotides were separated using TLC. Although we were able to visualize abundant (p)ppGpp production in response to carbon starvation, no increase in (p)ppGpp production was observed when lipid biosynthesis was blocked genetically (FabH deletion; Fig. S7a) or pharmacologically [with cerulenin, which blocks FabF, FabB and FabH activity (Price et al., 2001); Fig. S7b]. Thus, notably lower levels of (p)ppGpp are produced under fatty acid starvation than under carbon starvation conditions.

**DISCUSSION**

This study demonstrates that a block in FabH-mediated lipid biosynthesis obstructs cell growth. We propose a

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**5′-RACE was used to determine if (p)ppGpp contributed to the expression of the known *ctrA* transcripts, P1, P2 

**DISCUSSION**

This study demonstrates that a block in FabH-mediated lipid biosynthesis obstructs cell growth. We propose a
model where basal levels of (p)ppGpp prevent cells from entering a dormant, or VBNC, state, which can be induced by fatty acid starvation. This model is also supported by experimental work in *E. coli*, where cells lacking FabH or SpoT were viable but colonies lacking FabH and SpoT could not be obtained (Yao et al., 2012). In response to fatty acid starvation, we found that (p)ppGpp production leads to decreased cell size, via an unknown mechanism, and to the maintenance of CtrA accumulation. CtrA then prevents the over-initiation of DNA replication. As a result of these adaptive responses, cells may re-enter the cell cycle once lipid biosynthesis resumes. In contrast, in the absence of (p)ppGpp, cells maintain their size and over-initiate DNA replication. Consequently, most cells cannot restore growth.

**Cell size and DNA replication during fatty acid starvation**

A reduction in cell size is presumably adaptive, resulting in a larger surface-area-to-volume ratio (which could help the cell in bringing in additional nutrients; Fig. S3) and a smaller volume of cytoplasm (which would require fewer protein molecules to achieve a given concentration; Fig. 2d). Bacterial cell size is carefully regulated (Campos et al., 2014), with large size associated with rapid growth (Weart et al., 2007). For example, *E. coli* coordinates the initiation of DNA replication with cell size – cells will not initiate DNA replication until they reach an appropriate size (Hill et al., 2012). In fatty-acid-starved *C. crescentus*, blocking the initiation of DNA replication is critical since cell division in the absence of new lipids for the synthesis of membrane would likely be a futile, and deadly, endeavour. Many cell cycle events depend on DNA replication. By blocking the initiation of DNA replication, this cell cycle cascade can be paused, allowing cells to re-enter the cell cycle when conditions improve.

**There are multiple levels of control of cell cycle regulators in the absence of lipid biosynthesis**

Interestingly, although DnaA accumulation is low in FabH-depleted ΔspoT cells, DnaA activity appears to be high, perhaps due to a lack of regulatory-inactivation-of-DnaA activity, which has been well documented in *C. crescentus* (Collier & Shapiro, 2009; Fernandez-Fernandez et al., 2011, 2013). Although (p)ppGpp has been reported to inhibit dnaA transcription in *E. coli* (Chiaramello & Zyskind, 1990; Zyskind & Smith, 1992), we observed a SpoT-dependent increase in dnaA transcription in *C. crescentus* starved for fatty acids (Fig. 3b). However, this was notably correlated with decreased DnaA protein accumulation (Fig. 3a). This suggests that either dnaA mRNAs are poorly translated or DnaA is degraded, possibly by the Lon protease (Jonas et al., 2013). Transcription of gcrA was also high (Fig. 3b) despite a lack of GcrA accumulation (Fig. 3a). Perhaps SpoT binding of *C. crescentus* ribosomes (Boutte & Crosson, 2011) inhibits translation, as found in *E. coli* (Srivatsan & Wang, 2008). It was recently reported (Boutte & Crosson, 2011) that transcription of the 16S rRNA gene *C. crescentus* decreased (in a SpoT-independent manner) in response to starvation of carbon or glutamate but not phenylalanine. However, while no decrease in *ctrA* translation initiation was observed in our strains (Fig. S5), it is possible that translation could be inhibited after its initiation. Interestingly, it was recently reported (Gonzalez & Collier, 2014) that artificially increasing (p)ppGpp levels in non-starved *C. crescentus* cultures reduced DnaA synthesis independently of proteolysis.

Another notable result was that the (p)ppGpp-dependent maintenance of *ctrA* transcription was carried out only by the *ctrA* P2 promoter. We hypothesize that P1 does not fire because GcrA does not accumulate in FabH-depleted cells (Fig. 3a). Presumably, once CtrA accumulates prior to nutrient limitation, it can activate its own transcription from P2, which exhibited abundant transcripts in our 5'-RACE reactions from (p)ppGpp-producing cultures. Additionally, the discrepancy between measurements of *ctrA* mRNA levels in FabH-depleted ΔspoT strains compared with FabH-replete strains (62% with the transcriptional reporters in PYE and 29% with qPCR in M2G media) could have resulted from media differences or from the lack of degradation of the LacZ reporter.

A surprising result was that *ccrM* transcription and CcrM protein accumulation were quite low in FabH-depleted SpoT+ cells despite the accumulation of CtrA protein. Our previously published results (Schredl et al., 2012) suggested that the CtrA-mediated activation of *ccrM* transcription is quite robust. However, the data here suggest another level of regulation for *ccrM* transcription. Here, we found that high levels of *ccrM* transcription were dependent on FabH, or active lipid biosynthesis, but not CtrA or (p)ppGpp levels (Fig. 3b). These results are in contrast to the regulation of *C. crescentus* *ccrM* mRNA levels during carbon starvation, in which *ccrM* transcription was repressed in a SpoT-dependent manner (Boutte & Crosson, 2011). These differences could result from a fatty-acid-starvation-induced transcriptional repressor that renders (p)ppGpp-mediated repression unnecessary. Alternatively, high levels of (p)ppGpp could be required for the repression of *ccrM* transcription. We think these alternatives are more likely than low CtrA-P levels in cells depleted of FabH, as CtrA-P has been shown to be required to block the initiation of DNA replication (Chen et al., 2011) and our flow cytometry and MipZ-eYFP microscopy results clearly indicate that the presence of CtrA in our FabH-depleted strain blocked the over-initiation of DNA replication.

**Fatty acid starvation leads to a distinctive response compared with carbon starvation**

During carbon starvation in *C. crescentus*, (p)ppGpp accumulation led to significantly reduced *ccrM* transcription, whereas no changes were noted in *dnaA*, *gcrA* or *ctrA* transcription (Boutte & Crosson, 2011). Our cell cycle master regulator gene transcription levels suggest that *C. crescentus* uses (p)ppGpp to respond quite differently to starvation for
carbon (Boutte et al., 2012) compared with fatty acids. Notably, the former study (Boutte & Crosson, 2011) focused on the transcriptional response to starvation within minutes of its onset whereas this study focused on the response hours post-onset. Yet, (p)ppGpp accumulated after 2 h of carbon starvation (Fig. S7) but not after 2 h of cerulenin treatment (Fig. S7b). Thus, we highlight the differences between the two types of starvation. First, C. crescentus notably increases (p)ppGpp levels in response to carbon (Boutte et al., 2012; Lesley & Shapiro, 2008), but not fatty acid, starvation. Second, (p)ppGpp directs distinct genetic programmes in response to the starvation stimuli. For example, ccrM is repressed in a SpoT-dependent manner under carbon starvation but in a SpoT-independent manner in response to fatty acid starvation. We also observed a SpoT-dependent increase in dnaA and ctrA transcription when lipid biosynthesis was blocked. Changes in transcription of these cell cycle regulators were not observed during carbon starvation (Boutte & Crosson, 2011).

In contrast to E. coli, which produces increased levels of (p)ppGpp in response to fatty acid starvation (Gong et al., 2002; Seyfzadeh et al., 1993), C. crescentus does not noticeably increase (p)ppGpp levels, as analysed by TLC of [32P]orthophosphate-labelled nucleotides. In E. coli, SpoT-mediated (p)ppGpp synthesis presumably arises from non-acylated acyl carrier protein binding to and inducing SpoT synthase activity during fatty acid starvation (Battesti & Bouveret, 2006; Potrykus & Cashel, 2008). Although we did not observe increased (p)ppGpp accumulation under genetically or chemically induced fatty acid starvation conditions, use of the SpoT Y323A point mutant (Boutte et al., 2012) demonstrated that (p)ppGpp indeed modulates both cell size and the initiation of DNA replication in response to fatty acid starvation. Thus, in C. crescentus, basal levels of (p)ppGpp mediate critical responses to a block in de novo lipid biosynthesis.

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