ppGpp-Mediated Synthesis of Factors Controlling DNA Replication and Cell Size in Response to a Block in Lipid Biosynthesis in *Caulobacter crescentus*

A thesis submitted in partial fulfillment of the requirements

For the degree of Master of Science in Biology

By

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May 2014
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ACKNOWLEDGEMENTS

First and foremost I would like to thank my thesis advisor, Sean Murray. It was he who first encouraged me to pursue my Master’s degree in his laboratory at California State University Northridge (CSUN). Had it not been for his constant encouragement and dedication to his students, this thesis may have never come to be. He has always been there to encourage and support me; from providing me with opportunities to learn new techniques to agreeing to let me present my research in various locations, he always had my best interest in mind. He values my scientific opinion, is always willing to discuss new ideas, and works with me to design experiments to test my hypotheses. Thank you, Sean, for everything you have done to help me.

Next, I would like to thank my thesis committee members, Michael Summers and David Bermudes. For taking the time to critically read my thesis, always being there to answer my questions, and helping shape this project into what it has become you have my sincere appreciation.

I would like to thank the CSUN Interdisciplinary Research Institute for the Sciences for their support of this work. I would not have been able to devote as much time on the research as I did over the past two years without their financial support. The level of this work shows just how much their support has helped.

I would also like to thank the following current and former members of my lab: Kristina Stott, Yannet Perez, Anabel Herrera, Bao Nguyen, and Ram Wolman. They supported me throughout the development and completion of my project. Not only do I value their time and help throughout the past few years, but also I value their friendship.

Last, but certainly not least, I would like to thank my family and friends. They have always been and continue to be willing to listen to me ramble, complain, and talk excitedly about my research. They have continually encouraged me to pursue my dreams, whatever they may be, and have never been anything but supportive of my choices.

Thank you all for everything you have done to make this possible.
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ABSTRACT

ppGpp-Mediated Synthesis of Factors Controlling DNA Replication and Cell Size in Response to a Block in Lipid Biosynthesis in *Caulobacter crescentus*

By

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Master of Science in Biology

How organisms survive starvation is poorly understood at the cellular level. This study uses the model organism *Caulobacter crescentus*, which evolved to survive long periods of starvation, to address this important question. To determine how the cell cycle circuitry integrates with lipid biosynthesis, we created a FabH-depletion strain to induce fatty acid starvation. In response to starvation various bacterial species produce a molecule known as (p)ppGpp, an intracellular signaling molecule, which is linked to their survival until nutrients become available. (p)ppGpp facilitates the expression of certain genes whose protein products are required to survive starvation. We discovered that this regulon includes CtrA, a cell cycle regulatory protein, which blocks the initiation of DNA replication, and PhaC, an enzyme that polymerizes hydroxybutyrate monomers, forming polyhydroxybutyrate (PHB). CtrA, which normally blocks the initiation of DNA replication, fails to accumulate when FabH is depleted in the absence of (p)ppGpp due to a dramatic decrease in *ctrA* transcription. FabH depleted cells lacking the ability to produce (p)ppGpp contain multiple chromosomes and, although viable after 24 hours of FabH depletion, are unable to produce colonies. Using fluorescent microscopy we were able to show that PHB, a carbon storage molecule, is produced within the cell in a CtrA-
and (p)ppGpp-dependent manner within a localized region of the cell and modulates cell size in response to nutrient availability.

Here we present a novel model for the survival of fatty acid starvation in *Caulobacter crescentus*. (p)ppGpp is responsible for the prevention of the over-initiation of DNA replication via CtrA. Subsequently, CtrA is responsible for the production of PHB that provides not only carbon for the survival of starvation but a mechanism through which cells decrease size and thus nutrient requirements. Thus, my model reveals a three-component genetic pathway that explains the critical changes bacteria make in response to starvation.
INTRODUCTION

Starvation in Bacteria

Bacteria rarely experience the exponential growth seen in the laboratory environment. Within the natural environment, bacteria are often found under nutrient limiting conditions. Thus, bacteria have evolved systems to survive within their environmental niche. Starvation can be caused by environmental cues such as a decrease in nutrient availability or by an antibiotic that creates a block in a biosynthetic pathway; both cause the bacterium to initiate various strategies that allow them to exit the cell cycle until conditions are permissive for growth. These strategies can include: the ability to differentiate into different cell types allowing for motility through the assembly of a flagellum, production of a vegetative cell, or protection gained by the formation of a spore.

In general, the starvation response can be induced by limitation of nutrients such as glucose, ammonium, phosphate, iron, amino acids, or lipids (Potrykus et al., 2010; Boutte and Crosson, 2011). Oligotrophic environments and competition for nutrients require that bacteria be able to quickly adapt to changing conditions. Thus, how an organism responds to these external stressors can be greatly influenced by the environment to which it is adapted. Growth in starvation conditions can cause physiological and morphological changes such as decreased cell size (Dawson et al., 1981), decreased antibiotic sensitivity (Nguyen et al., 2011), and activation of the stringent response (Atkinson et al., 2011).
Stringent response

One of the ways that bacteria are known to respond to starvation is through the activation of the stringent response via the synthesis of the small signaling molecule (p)ppGpp. This small molecule helps them survive the process until nutrients become available by coordinating the activation of genes necessary to both enter and exit a bacteriostatic, or non-replicating, state. Most bacteria have the ability to synthesize (p)ppGpp; however, some obligate intracellular pathogens and bacterial symbionts seem to lack the ability to produce this intracellular signal. This is likely due to their persistent growth in an invariable environmental niche (Mittenhuber, 2001).

Synthesis of (p)ppGpp is performed by RelA, which converts GTP and ATP into (p)ppGpp by transferring the pyrophosphate from ATP onto GTP. Subsequently, SpoT converts ppGpp to GDP and pyrophosphate. Although many organisms possess both RelA and SpoT, it is possible to have only the ability to either synthesize or degrade (p)ppGpp or have a RelA/SpoT homologue (RSH) enzyme capable of both synthesis and degradation (Atkinson et al., 2011). The organism used in this study, *Caulobacter crescentus*, has only one RSH enzyme known as SpoT (Figure 1) that, in addition to the enzymatic domains, also has two regulatory domains.

The TGS domain is characteristic of threonine-tRNA synthase, GTPase, and SpoT indicating that it is likely to bind a nucleotide. Additionally, it has been shown that the acyl carrier protein (ACP) of organisms such as *E. coli* have the ability to bind the TGS domain and activate the synthesis of (p)ppGpp in response to fatty acid starvation (Dalebroux et al., 2010). The ACT domain is characteristic of aspartate kinase, chorismate mutase and TyrA and functions as a ligand binding domain; however,
Figure 1. Schematic representation of SpoT and potential ACP interaction. *C. crescentus* SpoT is a RelA/SpoT homologue (RSH) enzyme possessing the ability to both synthesize and hydrolyze (p)ppGpp (Dalebroux et al., 2011).
unlike the TGS domain, the majority of ACT domains are known to interact with amino acids and are generally referred to as the regulatory domain in amino acid metabolism (Grant, 2006).

(p)ppGpp is able to bind RNA polymerase (RNAP) and modulate RNAP activity (Figure 2) downregulating the transcription of genes required for translation and upregulating genes involved in the stress response. (p)ppGpp signaling allows pathogenic organisms to sense the changing environments and upregulate virulence genes (Dalebroux et al., 2010). Furthermore, recent in vivo and in vitro experiments show that pppGpp is less potent than ppGpp with respect to regulation of growth rate, RNA/DNA ratios, and transcriptional regulation in E. coli (Mechold et al., 2013).

A mutation in the C. crescentus SpoT enzyme that changes tyrosine, the 323rd amino acid, to alanine (Y323A) renders the enzyme unable to synthesize (p)ppGpp (Figure 3). Strains harboring this mutation can then be used to prove the necessity of (p)ppGpp rather than the presence or absence of SpoT as the cause for physiological and morphological changes seen (Boutte et al., 2012). C. crescentus is able to synthesize detectable levels of (p)ppGpp as a result of glucose and ammonium starvation, but not amino acid or phosphate starvation. Different mechanisms sense glucose and ammonium starvation; the ACT domain is required for high levels of (p)ppGpp to be produced in response to carbon starvation whereas the TGS domain is required for the synthesis of (p)ppGpp in response to ammonium starvation (Boutte and Crosson, 2011).

Microarray analysis revealed that in response to glucose starvation, 379 C. crescentus genes were upregulated at least threefold and 382 genes were downregulated at least threefold. Of those genes that were upregulated, 120 were catabolic genes.
Figure 2. ppGpp modulates RNAP activity. ppGpp is able to bind RNA polymerase (RNAP) and modulate the transcription of genes in response to starvation.
Figure 3. A mutation in the active site of SpoT removes (p)ppGpp synthesis. Thin layer chromatography during carbon starvation of wild type (WT), spoT(Y323A), and ΔspoT shows that of those strains only WT is able to produce (p)ppGpp in response to carbon starvation (Boutte and Crosson, 2012).
Interestingly, genes related to the metabolism of the nutrient granules polyhydroxybutyrate and polyphosphate were also upregulated. 166 of the identified genes that were downregulated were anabolic genes and genes involved in cell division and DNA replication (Boutte and Crosson, 2011).

In addition, the *C. crescentus* cell cycle is regulated by (p)ppGpp; *ccrM*, a DNA methylase, is also downregulated during carbon starvation. Secondly, SpoT mediates the proteolysis of DnaA (Lesley and Shapiro, 2008) and protects CtrA from proteolysis in response to carbon starvation (Boutte et al., 2012). Together, the simultaneous proteolysis of DnaA and stabilization of CtrA prevent the initiation of DNA replication under nutrient-limiting conditions. In a subsequent study, it was determined that ppGpp slows the swarmer-to-stalked cell transition (Boutte et al., 2012). SpoT mutants have a faster doubling time than wild type *Caulobacter*. Swarmer and stalked cells respond differently to carbon starvation with higher levels of (p)ppGpp in the swarmer cell (Figure 4).

**SigT during carbon starvation**

SigT is an alternative sigma factor belonging to the extra-cytoplasmic function (ECF) sigma factor family (Britos et al., 2011). During carbon starvation, SigT activates genes involved in signal transduction and gene regulation, and transport and metabolism. Many of the genes activated by SigT peak at the swarmer to stalked cell transition indicating its role in the cell cycle. Interestingly, SigT has been shown to have a role in the degradation of CtrA during carbon starvation. When starved of carbon in the presence of SigT, CtrA is degraded to 26±7%. However, when SigT is deleted from the genome, carbon starvation does not trigger the degradation of CtrA, and CtrA is maintained at
Figure 4. Differential synthesis of ppGpp in swarmer and stalked cells. A synchronized population of C. crescentus was split. One group of swarmer cells were immediately placed under carbon starvation and ppGpp accumulation was observed. The second group was allowed to progress to the stalked phase before being placed under carbon starvation. ppGpp levels were measured using thin layer chromatography and the relative intensity was graphed (Boutte et al., 2012).
57±18%. Upon screening for SigT-regulated proteins that cause this change in CtrA accumulation it was determined that the loss of the sigma factor SigU, histidine kinase HK4, or response regulator PhyR, restored the CtrA levels seen in the SigT mutant.

**Caulobacter crescentus**

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 differentiation process to ensure that cell cycle events occur at the correct time and place. When a stress activates a cell cycle checkpoint, *C. crescentus* will exit the cell cycle. This study focuses on the molecular mechanisms that mediate exit from the cell cycle in response to a block in lipid biosynthesis that can be induced by the depletion of FabH.

The FabH β-ketoacyl-acyl carrier protein synthase condenses malonyl-ACP with acetyl-CoA, forming β-ketoacyl-acyl carrier protein, which is a key intermediate in fatty acid biosynthesis. Although recently shown to not be essential in *E. coli* (Yao et al., 2012) except in a spoT mutant genetic background, *fabH* is an essential gene in *Caulobacter* (Christen et al., 2011). *E. coli* fabH mutants were found to have a 70% reduction in cell volume compared to wild type, suggesting that cell size is regulated by the rate of fatty acid biosynthesis (Yao et al., 2012). When fatty acid biosynthesis was blocked in *C. crescentus*, the cell cycle arrested at the stalked/predivisional stage (Contreras et al., 1979). Furthermore, cells lost motility (O’Neill and Bender, 1989) and the capacity to replicate DNA (Contreras et al., 1979). These early papers critically linked lipid biosynthesis with bacterial cell cycle progression. Here, we integrate this process using molecular genetic techniques that were not available 30 years ago.
Polyphosphate

Polyphosphate (polyP) consists of linear chains of phosphate residues that, in *C. crescentus*, are synthesized by Ppk1. In addition to acting as a phosphorous storage molecule, polyP is known to help bacteria adapt to environmental changes by acting as a phosphate reservoir for the regeneration of ATP and GTP (Ishge et al., 2002). Loss of polyP synthesis in *C. crescentus*, similar to the loss of ppGpp synthesis, results in a faster growth rate (Boutte et al., 2012).

*E. coli* mutants lacking the ability to synthesize (p)ppGpp also show a defect in the ability to synthesize polyP; however, this is not the case in *C. crescentus*. Although (p)ppGpp regulates *ppk1* transcription (Boutte and Crosson, 2011), loss of (p)ppGpp synthesis does not affect the ability of *C. crescentus* to produce polyP with granules appearing similar to wild type (Boutte et al., 2012). Additionally, the loss of either (p)ppGpp or polyP synthesis results in an increase in the initiation of DNA replication under glucose exhaustion (Boutte et al., 2012).

Furthermore, loss of the ability to synthesize polyP results in decreased stationary phase survival; after 24 hours of stationary phase in minimal media, no colony forming units (CFU) are a produced in strains lacking polyP. Overexpression of *ppk1* from an inducible promoter not only complements the mutation, but also gives cells better survival in stationary phase when compared to wild type (Henry and Crosson, 2013). Localization of polyphosphate is dynamic and cell cycle regulated; cells contain a single central polyP granule and as the cell cycle progresses the cell elongates and a second granule forms in the developing daughter cell (Figure 5; Henry and Crosson, 2013). This localization ensures that polyP granules are present in both the swarmer and
Figure 5. Polyphosphate granules are cell cycle regulated. A synchrony of wild-type *C. crescentus* was monitored for polyphosphate during the cell cycle. The relative percentage of cells with either one or two foci at each time point was recorded (Henry and Crosson, 2013).
stalked cell. The localization of *ppk1* is also dynamic, however, it localizes independent of the polyP granules and can quickly relocalize between the original and newly forming polyP foci. PolyP localization was found to be dependent upon chromosome segregation; mitomycin C and hydroxyurea were used to halt DNA replication in swarmer cells. Allowing cells to elongate decreased their ability to produce a second polyP granule and blocked their capacity to properly segregate polyP granules between the mother and daughter cells (Henry and Crosson, 2013).

**Polyhydroxybutyrate**

Polyhydroxybutyrate (PHB), a polymer comprised of R-3-hydroxybutyrate monomers (Figure 6), is an important bacterial polymer that has applications in a wide variety of fields. Diverse assortments of bacteria are able to synthesize PHB, which forms insoluble granules within the cell that primarily function as an energy storage compound (Anderson & Dawes, 1990). This is similar in function to the storage of excess energy in mammals within fat cells. A few of the notable uses for PHB include the production of biodegradable plastics, biomedical implants, and the use of chiral hydroxybutyrate monomers as starting materials for the production of enantiomerically pure chemicals and pharmaceuticals (Anderson & Dawes, 1990). In recent years, PHB research has seen an exponential increase in the rate of publication indicating that the study of PHB is a rapidly growing field (Figure 7).
Figure 6. Chemical Structure of polyhydroxybutyrate (PHB). (Reusch, 2013)
Figure 7. PHB related PubMed publications by year
Maurice Lemoigne, a biologist at the Pasteur Institute, first discovered PHB in granular inclusion bodies within the cytoplasm of *Bacillus megaterium* in the mid 1920s (Lemoigne, 1923). However, it wasn’t until the late 1980s that the bacterial PHB synthase, now known as either PhaC or PhbC, was characterized (Schubert *et al.*, 1988). PHB accumulation in *C. crescentus* was measured to be approximately 18% of the cell dry weight when grown in the presence of glucose (Qi and Rehm, 2001). Gas chromatography-mass spectroscopy and gel permeation chromatography of the purified polymer indicated that it was comprised solely of 3-hydroxybutyrate.

Remarkably, both prokaryotes and eukaryotes possess the ability to synthesize R-3-hydroxybutyrate (R-3HB), a metabolite of fatty acid biosynthesis (Figure 8), in response to starvation. Better known as ketone bodies, R-3-HB is one of the factors produced by the liver during starvation. When starvation lasts a period of several days the brain is able to switch from glucose to a mixture of glucose and ketone bodies to fulfill energy requirements (Guyton and Hall, 2000).

In addition to its function as a carbon storage molecule (Figure 9A), PHB has also been implicated in the genetic competence of bacteria and alterations in protein folding. Medium chain length PHB was identified in the membranes of species unable to produce PHB granules (Reusch, 2013). This form of PHB typically consists of 100-200 polymerized 3-HB monomers that form a complex with inorganic polyphosphate and are hypothesized to play a role in the transport of cations and DNA across the membrane (Figure 9B). Short chain conjugated PHB (cPHB) consists of ≤ 10 polymerized 3-HB monomers that are covalently bound to proteins located within the cytoplasm or cellular membrane such as OmpA in *E. coli* (Figure 9C). cPHB has been found in all
Figure 8. Synthesis of R-3-hydroxybutyrate in eukaryotes (left) and prokaryotes (right). In both eukaryotes and prokaryotes, acetoacetyl-CoA is formed by condensation of two molecules of acetyl-CoA. In prokaryotes, NADPH reduces acetoacetyl-CoA to form R-3-hydroxybutyryl-CoA, which is then polymerized to PHB. In eukaryotes, a third acetyl-CoA condenses with acetoacetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA), which decomposes to form acetoacetate and acetate. NADH is then able to reduce acetoacetate to R-3-hydroxybutyrate (Reusch, 2013).
Figure 9. Classes of poly-(R)-3-hydroxybutrate. (A) Storage PHB. Ultrathin section of an *Azotobacter chroococcum* cell showing granules of PHB surrounded by a membrane (X 56,200) (Nuti et al., 1972); (B) Medium-chain length PHB. Sketch of PHB molecule surrounding and noncovalently associated with a molecule of calcium polyphosphate in the bilayer; (C) Short-chain PHB covalently attached (cPHB) to protein (Reusch, 2013).
compartments of both eukaryotes and prokaryotes. Due to the ease of synthesis, requiring only acetate and reducing potential, along with the ubiquitous nature of medium and short chain PHB, it is theorized that PHB is among the first biological polymers to have been synthesized (Huang and Reusch, 1996).

As of 2013 the polyhydroxyalkanoate synthases can be arranged into four classes, numbered I though IV. Class I synthases are characterized by their activity towards three to five carbon hydroxyacyl-CoA monomers. Class II synthases are characterized by their activity towards six to fourteen carbon hydroxyacyl-CoA monomers. Synthases of Class I and II are comprised of a single subunit; however, the class III and IV synthases are composed of two non-identical subunits. Therefore, it is interesting to note that class III synthases are able to act upon hydroxyacyl-CoA monomers utilized by both class I and class II. Finally, there is the class IV synthase that has only recently been characterized in Bacillus sp. This synthase is only able to act upon the shorter hydroxyacyl-CoA monomers similar to the class I synthase (Jendrossek, 2009). Interestingly, Caulobacter crescentus possesses a class I synthase which is the largest PHB synthase known with a length of 673 amino acids (2019 nucleotides) and a molecular mass of 73.6 kDa (Qi and Rehm, 2001). Previous bioinformatics analyses have shown that all PHB synthases share a conserved cysteine residue where the growing PHB chain can be attached via a covalent bond. Comparison between PHB synthase genes and esterases suggests that PHB synthases contain an \( \alpha/\beta \) hydrolase fold (Jendrossek, 2009). Through the use of multiple sequence alignments it was determined that the N-terminus of the PHB synthase is subject to variation between species (Qi and Rehm, 2001).
Recent localization studies have revealed that *C. crescentus* PhaZ, a PHB depolymerase that binds to PHB storage granules, has a dynamic localization pattern while the cell is metabolically active (Parry et al., 2014). After the addition of the 2,4-dinitrophenol (DNP), a chemical that rapidly depletes cells of ATP and GTP by disengaging oxidative phosphorylation, cells lose metabolic activity and the dynamic localization of proteins within the cell is lost (Parry et al., 2014). PhaZ labeled with mCherry and visualized in both a metabolically active and DNP treated state showed that PhaZ-mCherry formed fluorescent foci (1–2 per cell) that moved inside active (untreated) *C. crescentus* cells that is consistent with binding to PHB granules. Metabolic depletion by DNP treatment dramatically reduced motion (Figure 10; Parry et al., 2014).

PhaC is known to be associated with the growing PHB chain (Qi and Rehm, 2001), however, in addition to PhaC many other proteins known as phasins or PHB granule associated proteins (PGAPs) are known to co-localize with PHB granules (Wahl et al., 2012). In *Ralstonia eutropha* a protein known as PhaM has been identified that was able to bind both a phasin on the surface of PHB granules and DNA. This interaction was able to direct the localization of PHB and coordinate PHB segregation with DNA replication (Wahl et al., 2012). Overexpression of PhaM resulted in multiple small PHB granules that were associated with the chromosome whereas deletion of PhaM resulted in large PHB granules with impaired segregation of PHB to daughter cells (Wahl et al., 2012).

A phasin (PhaP) is a protein that accumulates during PHB biosynthesis and promotes PHB accumulation. The PHB regulatory protein (PhaR) negatively regulates PhaP production preventing PhaP from accumulating in cells that are not producing PHB.
Figure 10. Movement of PhaZ in metabolically active and inactive *C. crescentus*. Means square displacement (MSD), measured as the square of the distance between an object’s current position and its original position over time. This confirms the loss of PhaZ mobility in metabolically inactive (DNP treated) *C. crescentus* (Parry et al., 2014).
(York et al., 2002). Deletion of \( \text{phaR} \) caused a decrease in PHB yields and a double mutant lacking both \( \text{phaR} \) and \( \text{phaP} \) exhibited a greater loss in PHB production in \textit{Ralstonia eutropha}. The regulation of PHB accumulation is quite complex; it is proposed that PhaR works as a negative regulator by possessing two domains: one DNA binding domain that binds the promoter of PhaP and a second that binds PHB. When there are increasing levels of PHB present within the cell, PhaR is titrated away from the promoter activating the transcription of \( \text{phaP} \). When PhaR is bound to the PHB granule, and the PHB granule is no longer increasing in size, newly synthesized PhaR can no longer bind the PHB granule, allowing it to then bind the promoter of \( \text{phaP} \) and prevent its transcription (York et al., 2002).

**This study**

How do bacteria respond to and survive fatty acid starvation? To address this important question, we have chosen to use the model organism \textit{Caulobacter crescentus}, a fresh-water bacterium that has evolved to survive long periods of starvation, to determine the effect of fatty acid starvation and characterize its response. Fatty acid starvation is generally induced by antibiotics such as cerulenin that block enzymes in the metabolic pathway responsible for the synthesis of lipids that comprise the bacterial cell membrane. Furthermore, we have taken steps to determine how the bacterial cell cycle circuitry integrates with lipid biosynthesis by searching for the genetic cause of the physiological changes we have observed such as the ability to exit and reenter the cell cycle and decrease cell size in response to starvation.
In this study, we demonstrate that (p)ppGpp is required for transitioning from a bacteriostatic state to vegetative growth. Adaptive responses mediated by (p)ppGpp in response to a block in lipid biosynthesis include a reduction of cell volume and transcriptional activation of \textit{ctrA}. When FabH is depleted in the absence of SpoT, normal cell volume is maintained and CtrA does not accumulate, resulting in multiple chromosomes in individual cells. Although alive, these cells cannot form colony-forming units as a result of a miscoordination of cell cycle events regulated by SpoT (Stott, 2012). In this study I show that CtrA regulated by SpoT’s synthesis of (p)ppGpp and not another unknown function.

In our research, we have found that when \textit{C. crescentus} cells are starved for fatty acids, PHB granules (that are visible when using a glowing (fluorescent) PHB dye viewed under a microscope) accumulate in a (p)ppGpp-dependent manner during fatty acid starvation. Fatty acids are needed to generate new cell membrane during bacterial cell division, allowing the cell to grow in size before dividing. By using a fluorescent dye to visualize PHB granules, we were able to demonstrate that the growth media also has a visible effect on PHB granule production. When grown in nutrient-rich media, \textit{C. crescentus} produces PHB granules only after key nutrients have been depleted due to the growth of the organism. However, when grown in nutrient-limited media, PHB granules are always present. We have generated a \textit{C. crescentus} strain in which the \textit{phaC} gene has been removed to observe the effects of its loss in PHB granule production on bacterial growth and physical shape or size. We found that this mutant strain exhibited not only a defect in growth rate, but in the regulation of cell size. Therefore, PHB granules may help to modulate cell size and growth rate relative to the availability of nutrients.
MATERIALS AND METHODS

**Bacterial strains, media, and plasmids**

Strains and plasmids are listed in Table 1. *Caulobacter crescentus* cultures were grown in either peptone yeast extract (PYE) or minimal media supplemented with glucose (M2G) (Ely, 1991) on a 125 rpm shaking platform at 28°C. *C. crescentus* strains were supplemented with vanillate (0.5 mM), kanamycin (5 µg mL\(^{-1}\) in liquid or 20 µg mL\(^{-1}\) on solid media), or tetracycline (1 µg mL\(^{-1}\) in liquid or 2 µg mL\(^{-1}\) on solid media) as indicated. *Escherichia coli* cultures were grown using Luria-Bertani (LB) media at 37°C (Miller, 1972). Cultures were supplemented with kanamycin (30 µg mL\(^{-1}\) in liquid or 50 µg mL\(^{-1}\) on solid media), or tetracycline (12 µg mL\(^{-1}\) in liquid or 12.5 µg mL\(^{-1}\) on solid media) as indicated.

**Construction of the phaC knockout strain SM1276**

To create a non-polar deletion in *phaC*, the upstream (US) and downstream (DS) flanking regions of *phaC* were amplified using polymerase chain reaction (PCR). A third PCR was performed using the US and DS products as template to produce a PCR product that could be digested and inserted into the suicide vector pNTPS138. The resulting plasmid was sequenced and electroporated into NA1000. Sucrose selection and PCR using the \(\DeltaphaCUSFwd\) and \(\DeltaphaCDSRv\) primers were used to identify colonies in which *phaC* had been removed.
### Table 1. Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype and phenotype</th>
<th>Source</th>
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<tr>
<td><strong>Caulobacter crescentus</strong></td>
<td></td>
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<tr>
<td>NA1000</td>
<td>Wild Type</td>
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<td>SM689</td>
<td>ΔcpxR::Ω (strepRspecR)</td>
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<td>SM961</td>
<td>psIS14 with PyrL::creA(D51E)Δ3Ω (chlRstrepRspecR)(high copy vector)</td>
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<td>SM968</td>
<td><em>divJ</em>::Tn5 (kanR)</td>
<td>Wheeler and Shapiro, 1999</td>
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<td><em>pleC</em>::Tn5 (kanR)</td>
<td>Wheeler and Shapiro, 1999</td>
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<td>SM1131</td>
<td>PvanA::fabHΔfabH (specR)</td>
<td>Kristina Stott and Sean Murray</td>
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<td>SM1247</td>
<td>ΔspoT</td>
<td>Lesley et al., 2006</td>
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<td>SM1276</td>
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<td>Boutte et al., 2012</td>
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</tr>
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<td>S17</td>
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<td>Simon et al., 1983</td>
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<td>DH10B</td>
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<td>Thanhbichler et al 2007</td>
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<td>SM1127</td>
<td>Top 10 pMT517 (PvanA::fabH) (specR)</td>
<td>Kristina Stott and Sean Murray</td>
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<td>SM1068</td>
<td>TOP10 pNTPS138::fabH-KO</td>
<td>Kristina Stott and Sean Murray</td>
</tr>
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<td>Primer Name</td>
<td>Sequence</td>
<td>Source</td>
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<td>QPCR CC2677 RV</td>
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<td>5’RACEctrA RV</td>
<td>CCG CAG GGT GCG CAG AAC ATC GAT</td>
<td>Schredl et al., 2012</td>
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<td>5’RACEctrA nested RV</td>
<td>GCA GGA TAA GAT CGT AGT GTG AGA T</td>
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<td>DT88</td>
<td>GAA GAG AAG GTG GAA ATG GCG TTT TGG</td>
<td>Argueta et al., 2006</td>
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<td>DT89</td>
<td>CGC CAT TTC CAC CTT CTC TTC</td>
<td>Argueta et al., 2006</td>
</tr>
</tbody>
</table>
construct was conjugated into the resulting strain. Strains containing the FabH depletion construct were identified using sucrose selection and verified using PCR (Stott, 2012).

**Construction of phaC transcriptional and translational reporter strains**

The promoter of phaC (PphaC) was PCR amplified using phaC-(Pr/TNL) FWD and RV primers as needed. The PCR product was digested and ligated into pRKLacZ290 or pJC326C. Then, PphaC/pRKLacZ290 or pJC326C was transformed into chemically competent TOP10 E. coli. The plasmid was isolated and the sequence was confirmed, the plasmid was electroporated into S-17 E. coli allowing for conjugation into C. crescentus.

**Depletion of FabH**

Single colonies were grown overnight in 3 mL of media containing vanillate (PYE or M2G) as previously described. Cultures were then centrifuged at 6500 rpm for 2 min and the pellets were then resuspended in media lacking vanillate. This was repeated three times. Each culture was then split and added to media with or without vanillate. After 24 hours in PYE or 17 hours in M2G tests were performed. The 24 and 17 hour time points were chosen because, although unable to form CFU, we were previously able to use the LIVE/DEAD BacLight (Molecular Probes) to stain and confirm C. crescentus was still alive at this time point (Stott, 2012 for PYE data; this study for M2G, data not shown).
Growth curves and viability assay

Individual colonies were grown overnight in 3 mL cultures as previously described (Methods, bacterial strains, media, and plasmids) according to the conditions of the strain being tested. Cultures were diluted to an OD$_{660}$ of 0.3 and allowed to double before proceeding. Cultures were then diluted to an OD$_{660}$ of 0.1. The OD$_{660}$ was monitored and recorded every 1.5 hours (PYE) or 2 hours (M2G). At the same time dilutions were plated to measure the number of colony forming units (CFU) mL$^{-1}$.

Microscopy

Strains were grown as previously described. For staining with Nile Blue A (NB) (Ostle and Holt, 1981) 2µL of NB was added to 48µL of live cells and allowed to incubate in the dark for 5 minutes before visualization. For visualization 4-5 µL were placed on 1% agarose slides and visualized using a Zeiss AxioVision microscope with a Hamamatsu ORCA-ER digital camera using differential interference contrast (DIC) and/or the appropriate fluorescent filter set.

Immunoblot

1 mL immunoblot samples were taken from cultures and centrifuged at 8,000 rpm for 2 minutes. After centrifugation, the supernatant was removed and the pellets were frozen on dry ice. Using SDS buffer the samples were normalized based on resuspending 1 mL of pelleted 0.35 OD$_{660}$ cells in 0.1 mL of 2x SDS Buffer. FabH and CtrA were resolved using 15% SDS-PAGE. Proteins were transferred to PVDF membranes and polyclonal rabbit antibodies for CtrA (1:20,000) or FabH (1:4,000) were detected with
donkey anti-rabbit antibodies conjugated to horseradish peroxidase. Western Lightning®
Plus-ECL Enhanced Chemiluminescence Substrate (Perkin-Elmer, Inc.) was used to
expose HyBlot CL autoradiography films (Denville Scientific, Inc.) for visualization.

**Synchrony**

Differential centrifugation using Ludox AS-40 colloidal silica (Sigma Aldrich,
Inc.) allowed for the separation of the swarmer cells from the stalked cells (Evinger and
Agabian, 1977). Swarmer cells were removed using a Pasteur pipette and washed to
remove residual Ludox. Isolated swarmer cells were then inoculated into M2G and
allowed to progress through the cell cycle. Samples to be used for either immunoblot or
microscopy were taken every twenty minutes starting at time zero.

**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 RNeasy Kit (Qiagen). RNA was eluted using a 1:20
TE buffer (pH 7.4) to DEPC solution. RNA concentration was measured using a
nanodrop spectrophotometer. 3 µg of RNA was treated with DNase, and cleaned using
RNA Clean & Concentrate -5 (Zymo Research). RNA quality was checked by measuring
the 260/280 ratios 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 manufacturers protocol using 1.5µg of RNA. The RACEctrARV primer was added to the mixture for 5’-RACE analyses. Random hexamers and the QPCRctrARV primer were used for cDNA to be used in qPCR analyses. For 5’-RACE applications the RNA template was removed using alkaline hydrolysis. cDNA was concentrated using DNA Clean & Concentrate (Zymo Research).

5’-RACE of ctrA

DT88 adapters were ligated to the single stranded cDNA template using RNA ligase (Promega). Ligation took place overnight in a water bath at 18ºC. After ligation, PCR was performed using the primers DT89 and RACEctrARV. A 1:50 dilution of the resulting PCR was used as the template for a second PCR using DT89 and RACEctrAnestedRV. The resulting product was separated on a 2.5% agarose gel at 80V and visualized. Resulting bands were cut from the gel, extracted, and sequenced.

qPCR of ctrA

qPCR was performed as previously described (Cuajungco et al., 2012) with minor modifications to suit our experiments. The amplification reactions were done in a Bio-Rad CFX96 machine using SensiMix SYBR Green Master Mix (Bioline) with the following thermocycling conditions: 5 min 95ºC followed by 40 cycles (30s at 95ºC, 45s at 55ºC, 30s at 72ºC). The primer sets for ctrA and CC2677 (normalization control) genes were obtained from a recent publication (Tan et al., 2010).
RESULTS

Transcription and protein accumulation of CtrA is (p)ppGpp-dependent during fatty acid starvation

To determine if the decrease in *ctrA* transcription and CtrA accumulation previously seen in the ΔspoT genetic background was a result of the loss of SpoT or the loss of the synthesis of (p)ppGpp by SpoT we constructed a *FabH*-depletion mutation in a strain that had the SpoT(Y323A) mutation. The SpoT(Y323A) mutation inactivates the synthetase domain of SpoT while retaining the SpoT protein structure.

A polyclonal antibody raised against CtrA (gift from Lucy Shapiro, Stanford University) was used to assay CtrA accumulation in a split culture subjected to FabH repletion or depletion in the absence of ppGpp. Immunoblots of lysates from the newly constructed strain were compared to the strains possessing the FabH-depletion in a wild-type or ΔspoT genetic background (Stott, 2012). CtrA protein accumulation during FabH depletion in the SpoT Y323A point mutant mirrored that observed in the ΔspoT genetic background indicating that the loss of CtrA accumulation was a result of the loss of (p)ppGpp synthesis and not another unknown function of SpoT (Figure 11).

To determine if the decrease in CtrA accumulation in the ΔspoT and SpoT(Y323A) strains after FabH-depletion was due to a decrease in transcription qPCR was used to quantify the mRNA levels of *ctrA*. Transcription of *ctrA* decreased approximately 70% in strains lacking the ability to synthesize (p)ppGpp indicating that transcription of *ctrA* is maintained during fatty acid starvation in a (p)ppGpp-dependent manner (Figure 11). Although 30% of *ctrA* transcripts remained in FabH-depleted cells lacking (p)ppGpp, no CtrA protein accumulated. We expect that ClpXP-mediated
Figure 11. Transcription and protein accumulation of CtrA is (p)ppGpp dependent

ctrA mRNA levels in minimal media were measured using qPCR and normalized using
the internal standard CC2677. Values shown above are relative to the average ctrA
mRNA levels observed in the +FabH +SpoT condition. Western blot samples from the
same strains and growth conditions are shown below the qPCR data. The data are
averages from triplicate experiments. Y-error bars indicate standard deviation.
proteolysis completely proteolizes the CtrA molecules synthesized as Sean Murray confirmed that there was no change in CtrA half-life (unpublished results). CtrA remains an unstable protein (Domian et al., 1997; Schredl et al. 2012).

To determine which promoter of *ctrA* was maintained by (p)ppGpp during fatty acid starvation, I used 5’-RACE to map the transcriptional start sites of *ctrA* during fatty acid starvation as compared to the transcriptional start sites during exponential growth (Figure 12). It was determined that *ctrA* transcription in strains starved for fatty acids (depleted of FabH) required (p)ppGpp to maintain transcription from the previously described *ctrA* P2 promoter (Figure 12; Domian et al., 1999). Furthermore, transcription from *ctrA* P1 was FabH-dependent, as no P1 transcripts were observed under FabH-depletion conditions.

**Regulation of bacterial cell size in response to fatty acid starvation**

Previous research indicated that bacterial cells possess the ability to decrease cell size in response to starvation (Dawson et al, 1981); however, it appeared as though our SpoT mutants (∆spoT and SpoT Y323A) lacked the ability to properly respond to fatty acid starvation by reducing cell volume.

To determine if the SpoT mutants were indeed unable to decrease cell size in response to fatty acid starvation we measured the length and width of each cell, calculating the average surface area and volume of each strain during either exponential growth or fatty acid starvation. Our calculations determined that strains lacking the ability to produce (p)ppGpp were unable to decrease cell size in response to fatty acid starvation (Figure 13). The ability to decrease cell size is an adaptive response allowing
Figure 12. Transcription of *ctrA* from the P2 promoter is (p)ppGpp-dependent. 5’-RACE revealed that the *ctrA* P1 promoter is FabH-dependent and is lost after 17 hours of FabH-depletion in minimal media. Transcription from the P2 promoter is (p)ppGpp-dependent during fatty acid starvation. The data is representative of triplicate experiments.
Figure 13. Cell length changes in a PHB-dependent manner. *C. crescentus* was grown in M2G. Cultures were split and half were depleted of FabH to induce fatty acid starvation. Cells that were able to produce PHB were able to decrease cell size. Those that were unable to produce PHB (No PHB or No ppGpp) were unable to decrease cell size in response to starvation. Dots indicate the mean size and the vertical lines reflect the range of the data. 50% of the measured cell lengths fall within the box. Over 200 cells were measured for each condition. Students’ T-test revealed that the only significant change in size in response to starvation occurred in the strain that was able to produce both ppGpp and PHB.
the cell to decrease nutrient requirements and increase the density of low abundance cytoplasmic factors.

Previously, Kristina Stott was looking at the localization of a green fluorescent protein (GFP) tagged protein (Stott, 2012). It appeared as though the protein was mislocalized during fatty acid starvation, however, the fluorescent foci were later determined to be polyhydroxybutyrate granules (Figure 14).

*C. crescentus* PhaC polyhydroxybutyrate synthase is essential for normal growth

Due to the presence of PHB granules during fatty acid starvation we set out to determine the phenotype of cells unable to produce PHB. A non-polar *phaC* knockout (*ΔphaC*) of *C. crescentus*, which rendered strain unable to produce PHB granules, was created. The *ΔphaC* mutation was verified using both PCR amplification of the gene locus and staining for PHB with Nile Blue A.

To determine if PHB production altered the growth of *C. crescentus*, cell biomass was measured using a UV-Vis Spectrophotometer at 660nm in either PYE or M2G media every 1.5 or 2 hours respectively. Samples of the cultures were diluted and plated onto PYE agar plates at each time point to allow for the counting of colony forming units (CFU). The *ΔphaC* strains exhibited a growth defect in PYE and M2G as well as a decreased ability to form CFU when compared to wild type (Figure 15).
Figure 14. GFP fluorescent PHB foci are produced in a SpoT-dependent manner. Cells were grown in PYE and fatty acid starvation (±FA) was induced via FabH depletion or the antibiotic cerulenin. Arrows indicate GFP fluorescent PHB granules. (Kristina Stott and Sean Murray, unpublished data). These results are representative of triplicate experiments.
Figure 15. *C. crescentus* PhaC is essential for normal growth. Optical Density at 660 nm and CFU/mL of wild type and ΔphaC grown in (A) M2G or (B) PYE.
**phaC transcription is mediated by (p)ppGpp**

To determine if the loss of PHB production in strains unable to synthetize (p)ppGpp was due to changes in the transcription of phaC, transcriptional (DNA → RNA) and translational (RNA → protein) reporter constructs were created and moved into wild-type *C. crescentus* and the SpoT mutant backgrounds. Using cells growing in exponential and stationary phase both in M2G and PYE it was determined that a.) Low levels of phaC transcription occur during exponential growth in both wild type and strains lacking (p)ppGpp and b.) Increases in phaC transcription during stationary phase occurred only in the wild type strain (Figure 16). The ratio of transcripts that were subsequently translated remained fairly stable between strains (data not shown). This correlates with Nile Blue A staining where PHB is always present in cells grown in M2G and only present when cell density is high while grown in PYE (Figure 17). Thus, PHB are only present when nutrient availability is low due to either growth in minimal media supplemented with glucose or depletion of a nutrient source due to stationary phase in rich media.

*C. crescentus* PHB granules dynamically localize during the cell cycle

*C. crescentus* exhibits a unique cell cycle in which the bacterium can exist in two forms. A synchronized culture, in which all cells begin as swarmer (motile) cells become stalked (non-motile) cells and ultimately divide together, was visualized under the fluorescent microscope with Nile Blue A (Figure 18). It was determined that PHB granules localize either centrally in the swarmer cell, at the cell pole prior to forming a
Figure 16. Transcription of \textit{phaC} is ppGpp-dependent. Miller assays were conducted on four independent colonies during exponential growth and stationary phase (24 hours) in either (A) PYE or (B) M2G. Translation index remained constant between strains for each condition (data not shown). Bars represent Miller Units and y-error bars indicate standard deviation from quadruplicate trials.
Figure 17. PHB production is media and growth phase dependent. *C. crescentus* was grown in M2G and PYE. PHB granules (detected with Nile Blue A) are always present when grown in M2G. PHB formation in PYE is dependent upon OD.
Figure 18. PHB granules are cell cycle regulated. This graph represents data from two independent synchrony experiments. A minimum of 200 cells was counted at each time point.
stalked cell, the returns to the center of the dividing cell giving rise to one PHB granule in each cell upon division (Figure 19).

To quantify this cell cycle localization, the number of bacterial cells with either one or two PHB foci was counted at each time point. These results mirror the results seen previously for polyP localization in *C. crescentus* (Henry and Crosson, 2013). These results imply that the segregation of PHB granules between the swarmer and stalked cell may be tied to a similar mechanism as polyP or to polyP localization.

PHB accumulation is altered by the stability of CtrA

To determine the regulator of PHB production in *C. crescentus* a mutant screen was conducted to search for mutants (Figure 20) with altered PHB accumulation or localization. Increased stability or expression of CtrA leads to an increase in the abundance of PHB within the cell (Figure 21). When CtrA stability is decreased due to the loss of the PleC approximately 60% of the bacterial cells loss the ability to produce PHB. However, when CtrA stability is increased due to the ectopic expression of a stable CtrA mutant (CtrAD51E) more than 50% of the cells contained three or more PHB foci (Figure 21). Other mutants (DivJ, CpdR, and CtrAΔ3Ω) that increase the stability and accumulation of CtrA also increased the number of PHB foci per cell relative to wild type (Figure 21).

Bioinformatic analyses and data mining revealed that CC_0509 has homology with the negative regulator of PhaP production (PhaR). Microarray data suggests that it is cell cycle regulated similar to other CtrA regulated proteins (Figure 22;
Figure 19. Microscopy of PHB during the cell cycle. Wild type *Caulobacter crescentus* was synchronized and PHB localization was visualized using Nile blue A. These results are representative of two independent synchrony experiments. Above the microscopy data is a schematic representation of the relative localization of PHB foci during the cell cycle. The localization of PHB at the pole during the swarmer to stalked cell transition is similar to that of proteins involved in CtrA stability (CtrA, ClpXP, CpdR, and DivJ; Figure 20)
Figure 20. Genes and mutations that alter the stability of CtrA. Deletion of PleC results in decreased CtrA stability (increased proteolysis). Deletion of DivJ or CpdR results in increased CtrA stability (decreased proteolysis). CtrAΔ3Ω has the last three amino acids removed. These are needed to target CtrA for proteolysis. CtrAD51EΔ3Ω. The D51E causes the CtrA to mimic the active (phosphorylated) form while also removing the tag required for proteolysis (Δ3Ω).
Figure 21. PHB production is altered by the stability of CtrA. A minimum of 300 of cells was counted in each genetic background. The number of PHB granules within each cell were counted and characterized as 0, 1, 2, or 3+ PHB foci. The relative abundance of each category is shown above.
Figure 22. Cell cycle transcription of CC_0509. Microarray analysis of CC_0509 shows that CC_0509 has a profile similar to that of CtrA regulated genes; high in the swarmer cell (T= 0min), decreased in the stalked cell (T= 12-48 min), gradually increasing as the cell prepares for division (T= 60min +). Transcription at each time point is relative to non-synchronized wild type transcription (McGrath et al., 2007). Below the microarray graph is a CtrA Western blot showing the accumulation of CtrA during the cell cycle.
McGrath et al., 2007) and chromatin immunoprecipitation data shows that it has a CtrA binding domain in its promoter region (Laub et al., 2002).

**PhaC protein analysis**

The amino acid sequence of a protein offers many insights into the possible structure and functions of a given peptide. To gain a better understanding into the potential structure and function of *C. crescentus*’ *phaC*, I identified motifs within the *phaC* sequence and then generated structural models through comparison with known protein structures. By looking at the sequence alignment generated using the amino acid sequences we can see that the N-terminal domain of *C. crescentus* does not align well with many of the other genera. As the largest known PHA synthase, it is possible that the *C. crescentus* N-terminal fragment may contain regulatory domains not seen in the sequences of other organisms.

The amino acid sequence of *C. crescentus*’ PhaC was input into the MyHits motif scanner (Pagni et al., 2007). This tool allows you to search multiple motif databases including PROSITE and Pfam among others. After submitting a sequence, the program then generates a single output listing the results from all of the databases. On the next page is a schematic of *C. crescentus*’ PhaC annotated with the various motif matches (Figure 23). It is interesting to note that, within the *C. crescentus* PhaC, the N-terminal region defined by homology with other organisms begins at approximately the 179th amino acid. Between the 2nd and 179th amino acid there are 47 alanine residues (Figure 24). These alanine rich regions are common in transcriptional repressor domains and are commonly found in repressor proteins (Pagni et al., 2007).
Figure 23. MyHits Motif Scan Output.

[1]: A CK2 phosphorylation site is a site phosphorylated by casein kinase II, a serine/threonine kinase whose activity is independent of cyclic nucleotides.

[2]: A leucine zipper consists of leucine residues spaced approximately 7 amino acids apart. Leucine zippers can function as either the DNA binding domain of transcription factors or in forming protein-protein interactions.

[3]: Though not commonly found in bacterial species, *C. crescentus* does, in fact, possess the myristoyl transferase needed for myristoylation, a co-translational lipid modification of amino acid residues, to occur.

[4]: Protein Kinase C phosphorylation site.

[5]: Integrin binding domain that can potentially play a role in bacterial aggregation and biofilm formation.

**PhaC_N**: The PhaC N-terminal region, as determined by analysis of multiple PhaC genes.

**Alanine-Rich Region**: 47 alanine residues are located between amino acids 2 and 179 of *C. crescentus phaC* (see Figure 24).
Figure 24. Location and sequence of alanine-rich region within PhaC.
To determine the evolutionary history of PhaC, an alignment was constructed using nucleotide sequences for *phaC* from 21 different bacterial genomes (Table 3). Nucleotide (Appendix 1) and amino acid (Appendix 2) alignments were used to check that homologous domains were aligned. Subsequently, using MrBayes (a downloadable freeware) a Bayesian analysis was conducted. For this analysis I chose to run 3 million generations and generated a tree by eliminating the first 25% of the calculations. The output was converted into an .nwk file and subsequently opened using MEGA5 for visualization.

After 3 million generations and eliminating the first 25% of data to account for oscillation, a tree was visualized in MEGA . This tree followed the evolutionary path that the Proteobacteria are believed to have followed. Alphaproteobacteria gave rise to the common ancestor of Beta- and Gammaproteobacteria that then split to form the two groups. The outliers of my tree are known to inhabit environments similar to that of the Proteobacteria (soil and aquatic environments) (Figure 25).

The structure of the alpha/beta hydrolase domain of PhaC remains highly conserved between species. However, the structural prediction servers often do not know what to do with the N-terminus. The confidence with which they predict the N-terminal region remains low, but the presence of several motifs within this region indicates that this region may aid in the control of the enzymatic activity or localization. The alanine-rich region is likely to play a role in DNA binding as it is commonly found in repressor proteins. The RGD motif, though not thoroughly studied in bacteria, has been implicated in the formation of biofilms and cell-cell interaction (Pagni et al., 2007). This is interesting to note because *C. crescentus* is a biofilm forming bacterial species.
Table 3. Accession numbers of organisms used for Bayesian analysis of *phaC*.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Locus</th>
<th>Size (bp)</th>
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<th>Source</th>
</tr>
</thead>
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<td>1983</td>
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<tr>
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<td>1266</td>
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</table>
Figure 25. Bayesian tree with posterior probabilities. The classes of Proteobacteria along with Cyanobacteria and Actinobacteria are labeled. All unlabeled sequences are Alphaproteobacteria. This tree highlights the evolution and transfer of the phaC gene.
DISCUSSION

Bacteria living in unpredictable environments found it evolutionarily necessary to be able to synthesize the small molecule (p)ppGpp. Thus, the stringent response is well conserved in bacterial species. The synthesis of this molecule, and the cytoplasmic concentration of it, acts as a rheostat to bind RNA polymerase and appropriately modulate transcription in response to different forms starvation. Among the physiological effects seen during starvation are blocks in DNA replication and decreases in cell size.

In this thesis we set out to characterize the molecular mechanisms behind these changes as a result of fatty acid starvation in *Caulobacter crescentus*. Consequently, I have been able integrate polyhydroxybutyrate production into our previous model for the survival of fatty acid starvation (Figure 26). Kristina Stott’s model identified SpoT as a regulator of CtrA production during fatty acid starvation that prevented the over-initiation of DNA replication (Stott, 2012). However, I have enhanced her model by determining that the regulator of CtrA during fatty acid starvation is (p)ppGpp, the small molecule synthesized by SpoT. The (p)ppGpp-dependent maintenance of *ctrA* transcription was carried out by the *ctrA* P2 promoter. When *FabH* is depleted in the absence of (p)ppGpp *C. crescentus* is neither able to reduce cell size nor produce PHB granules. The loss of the PHB synthase (PhaC) renders cells unable to reduce cell size in response to fatty acid starvation indicating that the reason cells lacking (p)ppGpp are unable to reduce cell size is due to the loss of PHB production. A cell cycle regulator of PHB production was discovered; increases in CtrA stability caused a relative increase in the number of PHB granules present within a cell. Alternatively, decreases in CtrA stability resulted in a relative decrease in the number of PHB granules present within a cell.
Figure 26. Proposed model for the survival of fatty acid starvation.
Based on our results, I have generated a model for regulating DNA replication and cell size when cells are starved for fatty acids. According to this model fatty acid starvation in a normal cell triggers the stringent response resulting in low levels of (p)ppGpp (Jimmy Blair and Sean Murray, unpublished data), which is able to increase CtrA accumulation and PHB production. (p)ppGpp-dependent \textit{ctrA} transcription is carried out by \textit{ctrA} P2, which maintains levels of CtrA sufficient to prevent the over-initiation of DNA replication by DnaA. Additionally, (p)ppGpp-dependent PHB accumulation allows the cell to store carbon and reduce cell size in response to fatty acid starvation. Starvation-dependent cell size reduction is advantageous in that it increases the relative abundance of cytoplasmic factors and increases the surface area to volume ratio.

Research in \textit{Ralstonia eutropha} showed that PHB production was regulated by the stringent response. They were able to show that a loss in the ability to synthesize (p)ppGpp resulted in a loss in the ability to produce PHB granules. They were also able to show that this loss was likely due to the decreased production of cofactors and a decrease in PhaB activity (York et al., 2012). Our research confirmed that the stringent response regulates PHB production in \textit{C. crescentus}; both of our SpoT mutants (\textit{ΔspoT} and SpoT(Y323A)) were unable to produce polyhydroxybutyrate (PHB) in response to fatty acid starvation (Figure 14). These PHB granules were able to fluoresce in the GFP wavelength; it is possible that some previously published bacterial mutants that were interpreted as having mislocalized GFP-fusion proteins were simply PHB granules. This led us to construct a \textit{ΔphaC} strain of \textit{C. crescentus} that was unable synthesize PHB. The loss of the ability to synthesize PHB led to a dramatic decrease in the growth rate (Figure
as well as a decrease in the ability to form colony-forming units. Transcriptional (Figure 16) and translational (not shown) reporters for *phaC* showed that *phaC* transcription was maintained at a basal level during the cell cycle but that stationary phase induced a (p)ppGpp-dependent increase in *phaC* transcription. The translation index of *phaC* did not differ between conditions indicating that a change in the production of *phaC* was likely due to transcriptional control. Further transcriptional studies could help us to determine changes in *phaC* transcription as a result of fatty acid starvation or if a different PHB synthetic enzyme was responsible for the loss of PHB production in *C. crescentus*. *In vitro* assays using lysates and intermediates to determine the rate of synthesis by each of the enzymes in the PhaABC PHB synthetic pathway would be informative, as it would determine the rate-limiting step that prevents PHB production in SpoT mutants.

Previous research indicated that PhaR acted as a regulator of PHB production in *Ralstonia eutropha* (York et al., 2002). *C. crescentus* has a PhaR homolog known as CC_0509 (PhaR\textsubscript{CC}). Chromatin immunoprecipitation was able to show that PhaR\textsubscript{CC} has a CtrA binding domain in its promoter region, and microarray data shows that it is cell cycle regulated in a pattern similar to other CtrA regulated genes (Figure 22; Laub et al., 2002; McGrath et al., 2007). Our research shows that increasing CtrA stability (and presumably abundance) positively increases PHB accumulation within the bacterial cell (Figure 21). We hypothesize that further studies will confirm that increases in CtrA stability will result in increased transcription of *phaR\textsubscript{CC}*. Regulation of PHB production by PhaR has many levels. If *C. crescentus* follows the model seen in other organisms, decreases in PhaR accumulation should lead to increases in PHB production via increased
synthesis of CC_2159 (phaP homolog) or a currently unidentified phasin; this is due to decreased binding (repression) of the phaP promoter by PhaR. Additionally, it is believed that PhaR also affects PHB accumulation using a PhaP-independent pathway. We hypothesize that the loss of PhaR will result in a dramatic decrease in PHB production. Loss of PhaP would cause a greater decrease in PHB production than the loss of PhaR, and the loss of both PhaR and PhaP would cause the greatest decrease in PHB accumulation (York et al., 2002). These changes in phaR and phaP transcription could be confirmed using transcriptional reporters placed in the various CtrA stability mutants or by placing a phaP transcriptional reporter plasmid in phaR deletion strain. Through the manipulation of PhaR expression, PHB accumulation can be modulated within the cell; increases in PhaR may decrease PHB accumulation and decreases in PhaR would increase PHB accumulation (York et al., 2002). Notably, reduced PhaR synthesis has a different effect on PHB accumulation than deletion of PhaR; whereas reduced PhaR synthesis increases PHB accumulation, deletion of phaR actually decreases it (York et al., 2002). Protein-protein interaction screens using PhaR as the bait could be used to determine the interacting phasin ultimately responsible for coordinating PHB production.

Microscopy revealed that the localization of PHB was both dynamic and cell cycle regulated. PHB granules localize to specific locations within the cell and segregate evenly upon division. The question then becomes, what factors are controlling the cell cycle localization of PHB? Research in C. crescentus has shown that another nutrient granule, polyphosphate (polyP), is controlled by DNA replication and chromosome segregation (Henry and Crosson, 2013). Blocks in DNA replication or chromosome segregation prevent the formation and segregation of new polyP granules indicating that
their synthesis is tied to DNA replication. The N-terminus of *C. crescentus* PhaC is the longest of all known PHB synthetases. Construction of an N-terminal deletion could help determine whether this region functions in the localization of PHB in *C. crescentus*. Further testing could be done to determine if the localization of PHB is dependent upon DNA replication, chromosome segregation, or polyP localization.

We saw that when *C. crescentus* lost the ability to synthesize PHB via the deletion of *phaC* we saw a reduction in growth rate. Interestingly, we observed no change in the accumulation of any of the master regulators between wild-type and ΔphaC. This indicates that the cells do not grow poorly due to a lack of any of the core master regulator proteins (Data not shown). Accordingly, loss in the ability of strains to produce (p)ppGpp (and thus PHB) resulted in a decrease in the ability to form CFU after depletion of FabH. Further testing could be used to reveal if it is the loss of PHB production in the strains unable to synthesize (p)ppGpp that results in the loss of viability. If the ΔphaC strain after FabH-depletion has decreased ability to form CFU in response to fatty acid starvation, FACS could be used to distinguish if it results from the strains inability to prevent the overinitiation of DNA replication or production of PHB. It is possible that the decrease in growth rate may be due to a build up of PHB intermediates synthesized by PhaA or PhaB and blocked by the loss of PhaC. Therefore, I believe that a mutation in PhaA would be a better indicator of the loss of PHB production on growth rate.

We still have yet to elucidate the mechanism for fatty acid starvation to signal the stringent response in *C. crescentus*. In *Escherichia coli* it is believed that SpoT interacts with the acyl-carrier protein (ACP). Under fatty acid starvation, the fatty acid biosynthetic complex disassociates freeing ACP to bind the TGS domain of SpoT.
(Dalebroux et al., 2010). To determine if this interaction occurs in *C. crescentus*, protein-protein interaction can be assayed using the bacterial two-hybrid system. Using SpoT mutants lacking the various domains the location of the interaction could be further explained.

We know that low levels of (p)ppGpp are able to affect these changes in *C. crescentus* in response to fatty acid starvation. It would be interesting to use deep sequencing to determine how low levels of (p)ppGpp (Jimmy Blair and Sean Murray, unpublished results) differentially alter the expression of genes in response to fatty acid starvation as compared to high levels of (p)ppGpp induced by carbon starvation (Boutte and Crosson, 2011).

Due in part to the wide range of biotechnology applications that PHB granules possess and its current implication in cell growth, the characterization of PHB production has many benefits to both the biotechnology industry and microbiologists alike. *C. crescentus* is known to be well adapted to low nutrient environments; one such adaptation being the production of PHB. *Caulobacter's* relatively simple genetics provides an ideal system to analyze the function and regulation of its single PHB synthase (PhaC). I have identified a mechanism in which, when starved for fatty acids, *Caulobacter* cells produce PHB granules in a ppGpp-dependent manner. Significantly, the use of PHB in the biotechnology industry remains low due to its cost prohibitive production. Through further study and characterization of this pathway we can work to make the use of PHB a financially viable alternative. Finally, we have added an additional level suggesting how some bacteria may be able to use PHB to reduce cell size in response to starvation.
REFERENCES


Stott KV. (2012) *Caulobacter crescentus* SpotT is essential for preventing the over-initiation of DNA replication during fatty acid starvation. CSUN Master's Thesis.


APPENDICES

Appendix 1. Alignment of phaC