GcrA PROTEOLYSIS IN *CAULOBACTER CRESCENTUS* SWARMER CELLS IS

ClpP-DEPENDENT

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By

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Signature page</td>
<td>ii</td>
</tr>
<tr>
<td>Acknowledgment</td>
<td>iii</td>
</tr>
<tr>
<td>Abstract</td>
<td>ix</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Cell cycle of <em>Caulobacter crescentus</em></td>
<td>1</td>
</tr>
<tr>
<td>Master regulators of <em>Caulobacter crescentus</em></td>
<td>7</td>
</tr>
<tr>
<td>GcrA master regulator</td>
<td>8</td>
</tr>
<tr>
<td>CtrA master regulator</td>
<td>17</td>
</tr>
<tr>
<td>DnaA master regulator</td>
<td>21</td>
</tr>
<tr>
<td>CcrM master regulator</td>
<td>25</td>
</tr>
<tr>
<td>Importance of proteolysis in regulation of <em>Caulobacter’s</em> cell cycle</td>
<td>27</td>
</tr>
<tr>
<td>The process of proteolysis in bacteria</td>
<td>30</td>
</tr>
<tr>
<td>McpA and the ClpXP protease in <em>Caulobacter crescentus</em></td>
<td>33</td>
</tr>
<tr>
<td>Material and Methods</td>
<td>37</td>
</tr>
<tr>
<td>Bacterial strains, media, and plasmids</td>
<td>37</td>
</tr>
<tr>
<td>Construction of <em>clpX</em>Ω construct</td>
<td>37</td>
</tr>
<tr>
<td>Mini-synchrony</td>
<td>39</td>
</tr>
<tr>
<td>GcrA half-life</td>
<td>39</td>
</tr>
<tr>
<td>Construction of ClpX depletion strain</td>
<td>40</td>
</tr>
<tr>
<td>PCR</td>
<td>41</td>
</tr>
<tr>
<td>Agarose gel electrophoresis and Gel extraction</td>
<td>42</td>
</tr>
<tr>
<td>Miniprep</td>
<td>43</td>
</tr>
<tr>
<td>Digestion and ligation</td>
<td>44</td>
</tr>
</tbody>
</table>

iv
Transformation 46
DNA sequencing 47
Electroporation 47
Growth curve 48
Microscopy 48
Western blot 48
UV mutagenesis 49

Results 51

Testing for GcrA accumulation in various protease mutants 51
GcrA degradation is ClpP-dependent 53
Construction of ClpX depletion strain 55

CFU/ml/OD of Caulobacter crescentus decreases when ClpX is depleted 60
Morphology and DNA content of Caulobacter cells with depleted ClpX 60
ClpX depletion does not affect GcrA levels but increases CtrA levels 65
Screen for a clpX bypass suppressor 67

Discussion 71

Overview of results 71
Possible explanations for GcrA degradation 72
More speculation on GcrA degradation 74

References 78
LIST OF TABLES

Material and Methods

Table 1. Bacterial strains 38
LIST OF FIGURES

Introduction

Figure 1: Centrifugation of *Caulobacter crescentus* 2
Figure 2: The cell cycle of *Caulobacter crescentus* 4
Figure 3: *Caulobacter* cell cycle regulation 6
Figure 4: The four oscillating master regulators in *Caulobacter crescentus* 9
Figure 5: gcr*A* is essential for rapid growth in *Caulobacter crescentus* 10
Figure 6: GcrA accumulation and proteolysis in swarmer cells and stalked cells of *Caulobacter crescentus* 11
Figure 7: The gcr*A* promoter sequence 13
Figure 8: GcrA is negatively regulated by the CtrA response regulator 14
Figure 9: Association of GcrA and CtrA with promoter DNA in *vivo* 16
Figure 10: ΔgrcA cells are viable but have lower growth rate and morphological defects 18
Figure 11: Localization of ClpXP, CpdR, RcdA, and CtrA are cell cycle regulated 20
Figure 12: Proteolysis of DnaA is ClpP-dependent 22
Figure 13: Increased proteolysis of DnaA upon nitrogen starvation 24
Figure 14: ClpXP degradation model 29
Figure 15: Proteolysis of McpA is ClpX-dependent 34
Figure 16: Proteolysis of McpA is ClpP-dependent 35

Results

Figure 1: Absence of GcrA protein in swarmer cells of various protease mutants 52
Figure 2: GcrA degradation is ClpP-dependent 54

Figure 3: Construction of ClpX depletion strain 57-59

Figure 4: Cell viability of *Caulobacter crescentus* decreases when ClpX is depleted in PYEG media 61

Figure 5: Inducible xylose promoter 62

Figure 6: Morphology and DNA content of *Caulobacter crescentus* with depleted ClpX 63-64

Figure 7: When ClpX is depleted, CtrA levels increase while GcrA levels remain unchanged 66

Figure 8: Screen for clpX bypass suppressor 68

Figure 9: UV mutagenesis 69

Discussion

Figure 1: Possibilities for GcrA degradation 73

Figure 2: Adaptor protein can increase substrate degradation by ClpXP 77
ABSTRACT

THE PROTEOLYSIS OF THE CELL CYCLE MASTER REGULATOR GCR A IN CAULOBACTER CRESCENTUS SWARMER CELLS IS CLP-P-DEPENDENT

By

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Masters of Science in Biology

GcrA is responsible for the regulation of bacterial genes involved in motility, polar development, cell wall biogenesis, amino acid metabolism and transport, chromosome replication, repair, and recombination. Moreover, regulated proteolysis of proteins is a critical component of the bacterial cell cycle. The GcrA master regulator in Caulobacter crescentus accumulates in stalked cells but not in swarmer cells (although it is transcribed and translated in both cell types) because of swarmer-cell-specific proteolysis (Collier et al., 2006). We know that degradation of GcrA in swarmer cells is ClpP-dependent because when ClpP was depleted in swarmer cells, GcrA became more stable (Sean Murray, unpublished results). ClpP, with the help of chaperones, degrades regulatory and structural proteins to ensure the localization of polar organelles at the correct cell pole during correct time of the cell cycle. ClpP has two known chaperones, ClpA and ClpX. In this research we show that GcrA degradation in swarmer cells is ClpA independent. To test if ClpX is the chaperone, a ClpX depletion strain was created since clpX is an essential gene, but no change in GcrA accumulation was observed when ClpX was depleted. However, ClpX may have a higher affinity for GcrA than for other proteins that accumulate to toxic levels. A bypass suppressor screen was performed to see if a ClpX knockout could be viable. Such a strain would allow us to conclusively test if
GerA degradation is ClpX dependent. Unfortunately, no ClpX bypass suppressor was obtained.
INTRODUCTION

*Caulobacter crescentus* is a nonpathogenic gram-negative bacterium that is found in fresh water. *Caulobacter crescentus* is a good model organism for studying the bacterial cell cycle. *Caulobacter* species are located in diverse environments including contaminated water (MacRae *et al*., 1991), sediment environments like gold mines (Inagaki *et al*., 2003), and moderately high nutrient environments like wastewater (Mannisto *et al*., 1999; North *et al*., 2004; Curtis & Brun, 2010). Moreover, *Caulobacter* is a good model organism for studying the regulation and differentiation of the bacterial cell cycle because it can easily be used for studying genetic, biochemical, and cell biological analysis. It encodes ~3,700 predicted genes whose expression can be examined by DNA microarrays (Ausmees & Jacobs-Wagner, 2003).

The cell cycle of *Caulobacter crescentus* is about 2.5 hours in minimal media (Curtis & Brun, 2010). During each cell cycle, *Caulobacter crescentus* divides asymmetrically and forms two different progeny cells: a motile swarmer cell and a nonmotile stalked cell. The swarmer cell uses its flagellum for swimming and looking for a good source of nutrients. The stalked cell has a sticky substance at the end of its stalk called holdfast that is used to attach near a food source (Collier & Shapiro, 2007). Separation of these two types of cells can be easily achieved since swarmer cells are denser and will sink near the bottom of test tube and stalked cells will float near the top of the test tube after density centrifugation as shown in Figure 1 (Courtesy of Sean Murray).

**Cell cycle of Caulobacter crescentus**
Figure 1: Centrifugation of *Caulobacter crescentus*. Density centrifugation allows the mixed culture of *Caulobacter crescentus* to separate with swarmer cells near the bottom and stalked cells near the top of a test tube (Courtesy of Sean Murray).
*Caulobacter crescentus* has an intriguing cell cycle similar to the G1, S, and G2 phases of eukaryotes as shown in Figure 2. In a nutrient poor environment, stalked cells remain attached to a surface, however, the swarmer cells can use their flagella to swim away and find another source of nutrients. When a swarmer cell finds a nutrient-rich environment, it loses its flagellum and forms a stalk at the same pole previously occupied by the flagellum, thus becoming a stalked cell. Differentiation of swarmer cells into stalked cells is similar to the G1-S phase transition of eukaryotes. Then the stalked cell attaches to a surface near the nutrient rich environment and starts dividing. During S-G2 phase, the growing stalked cell forms a new flagellum and a new chemosensory protein (McpA) at the pole opposite the stalked pole (Gorbatyuk & Marczynski, 2005). During the predivisional stage, a swarmer compartment begins to bud off a stalked cell, eventually forming a cell with both swarmer and stalked compartments. At this point, the late predivisional cell forms a septum and divides (G2 phase). The stalked progeny will continue dividing if there are enough resources around. The swarmer progeny has a flagellum and can swim away to find a nutrient-rich environment if there are not enough resources nearby to differentiate into a stalked cell (Curtis & Brun, 2010). This dimorphic life cycle allows for the bacteria to live in competitive environments.

It is suggested that *Caulobacter crescentus* has two different developmental programs. One is a cyclic developmental program where the motherstalked cell undergoes cell division and the re-enters the cell cycle to its predivisional stage. The other one is when the swarmer cell undergoes a noncyclic developmental program in which it synthesizes holdfast, loses its flagellum and pili, and grows a stalk instead as shown in Figure 2 (Curtis & Brun, 2010). Swarmer cells cannot initiate DNA replication
Figure 2: The cell cycle of *Caulobacter crescentus*. During non-cyclic development or G1 phase, a swarmer cell differentiates into a stalked cell. During the cyclic development, the stalked cell starts DNA replication in S phase and forms a predivisional cell including swarmer and stalked compartments in G2 phase. This Figure is from (Curtis and Brun, 2010).
or cell division. In contrast, the stalked cell is able to replicate its chromosome and begin cell division (Curtis & Brun, 2010; Degen & Newton, 1972). Swarmer cells and stalked cells have different proteins due to the expression or repression of genes at different time points of the cell cycle and the proteolysis of proteins that are not needed (Jacobs-Wagner, 2004). Master regulator proteins of *Caulobacter crescentus* regulate the transcription of these genes. *Caulobacter crescentus* has four master regulator proteins: CtrA, GcrA, DnaA, and CcrM that control cell cycle regulated genes (Collier & Shapiro, 2007). For instance, CtrA regulates genes responsible for flagella and pili biogenesis, holdfast synthesis, DNA methylation, and chemotaxis. As it shown in Figure 3, flagella and pili biogenesis, chromosome segregation, DNA methylation, and cytokinesis occur at different stages of the cell cycle.

Moreover, degradation and synthesis of some proteins in *Caulobacter crescentus* characterize the dimorphic cell cycle. For instance, the McpA protein is synthesized and localized at the flagellar pole of predivisional cells and then is degraded in a non-motile stalked cell (Gorbatyuk & Marczynski, 2005; Tsai & Alley, 2001). The response regulator, CtrA, also shows the dimorphic synthesis and proteolysis (Gorbatyuk & Marczynski, 2005; Domian *et al.*, 1997). CtrA is present in swarmer cells and inhibits chromosome replication by binding five DNA sites on the replication origin (Gorbatyuk & Marczynski, 2005). The proteolysis of CtrA happens during the transition of swarmer to stalked cells just prior to chromosome replication. CtrA is later synthesized in the predivisional cell and is degraded in stalked progeny during asymmetric division (Gorbatyuk & Marczynski, 2005). Besides CtrA, three other master regulators play a key role in regulation of the cell cycle.
Figure 3: *Caulobacter* cell cycle regulation. During different stages of *Caulobacter* cell cycle, many genes are expressed or repressed. These genes allow the cell cycle to proceed forward by regulating different genetic modules in a temporal pattern. The figure is from (Jacobs-Wagner, 2004).
Master regulators of *Caulobacter crescentus*

As it was mentioned before, *Caulobacter crescentus* has four master regulators, DnaA, CtraA, GcrA, and CcrM. These four master regulators are responsible for the expression or repression of numbers of genes at different stages of the cell cycle. DnaA is responsible for the initiation of DNA replication at the G1 to S phase transition. It reaches maximum accumulation during the first 20-24 minutes of the cell cycle (Collier et al., 2007). Moreover, DnaA activates the transcription of the *gcrA* gene during the transition from swarmer to stalked cells (Collier et al., 2007). GcrA in stalked cells (accumulating at 40-80 minutes of the cell cycle) controls and activates genes in the stalked cells, notably those that are essential for DNA replication elongation. After DNA replication is initiated, GcrA inhibits *dnaA* transcription (Holtzendorff et al., 2004) in stalked cells, but activates the next master regulator, *ctrA*. One of the functions of CtrA is to inhibit the initiation of DNA replication by competing with DnaA for binding to the *Caulobacter* chromosomal origin of replication (*Cori*). Therefore, CtrA and DnaA act as antagonists in DNA replication in *Caulobacter crescentus*. CtrA is present in predivisional cells (80-120 minutes) and in the swarmer compartment of the late-divisional cell. Additionally, CtrA activates *ccrM* during the late divisional stage (100-120 minutes). The CcrM master regulator controls transcription indirectly by methylating the chromosome. Full methylation of the chromosome by CcrM activates *dnaA* transcription in late predivisional cells and represses the transcription of *ctrA* from its first promoter, *ctrA* P1, and its own expression from *ccrM* promoter (Collier et al., 2007). Essentially, DnaA initiates DNA replication, GcrA controls the synthesis of proteins that elongate DNA replication, CtrA inhibits the initiation of DNA replication and activates genes involved
in cell polarity and cell division, and CcrM methylates the DNA (Li et al., 2009). These processes allow for the chromosome to replicate once, and only once, during the cell cycle. These oscillating master regulators are presented in Figure 4.

**GcrA master regulator**

GcrA controls and regulates many genes in stalked cells of Caulobacter crescentus (Holtzendorff et al., 2004). GcrA was thought to be essential in Caulobacter crescentus and to test if that GcrA was an essential protein the coding sequence of the gcrA was placed under the control of a xylose-inducible promoter at the xylX locus of the chromosome. Then, most of the wild-type gcrA coding region at its native locus was substituted with a spectinomycin/streptomycin resistance cassette to construct the strain LS3707. The strain was grown in xylose containing media and the cells were washed to remove the xylose before shifting the PYE (Peptone Yeast Extract) media from xylose to glucose so that they could inhibit transcription from the xylose-inducible promoter. Western blot analysis showed that GcrA was depleted when medium was changed to PYEG (Peptone Yeast Extract Glucose) after two hours. This also indicated that GcrA is an essential protein for rapid growth because the cells lost viability in PYEG as shown in Figure 5. To test whether GcrA accumulation occurred in the stalked cells, Western blots were performed demonstrating that GcrA accumulates in stalked, but not swarmer cells. Next, the half-life of GcrA was measured in an isolated population of swarmer and stalked cells. The result showed that the half-life of GcrA in swarmer cells was ~ 10 minutes and the half-life of GcrA in stalked cells was ~ 44 minutes (Collier et al., 2006) (Figure 6). This indicates why GcrA accumulates in the stalked cells. Its proteolysis is cell cycle regulated since GcrA is specifically degraded in swarmer cells (Figure 6).
Figure 4: The four oscillating master regulators in *Caulobacter crescentus*. DnaA initiates DNA replication during the swarmer to stalked transition and turns on the next master regulator, *gcrA*. GcrA then activates the genes responsible for the elongation of DNA replication and represses transcription from *dnaA*, but turns on the next master regulator, *ctrA*. CtrA inhibits DNA replication during the predivisional stage, represses the expression of *gcrA*, and activates the next master regulator, *ccrM*. CcrM controls the transcription indirectly by fully methylating the chromosome, which causes activation of *dnaA* and repression of *ctrA* and its own transcription. This Figure is from (http://www.unil.ch/dmf/page59925_en.html).
Figure 5: gcrA is essential for rapid growth in Caulobacter crescentus. When Caulobacter cells ($\Delta$gcrA pxyL::gcrA) were placed in xylose containing media, the presence of GcrA was detected by Western blot and the cells were viable, however, when the media was shifted from PYE xylose to PYE glucose, GcrA was not detected and the cells lost their viability. This Figure is from (Holtzendorff et al., 2004).
Figure 6: GcrA accumulation and proteolysis in swarmer cells and stalked cells of *Caulobacter crescentus*. Cells were isolated from a synchronized NA1000 (wild-type), and LS3707 (ΔgcrA pxylX::gcrA) culture. Western blot analysis of GcrA shows that GcrA is accumulated after 40 minutes in the stalked cell. The gray area in the stalked and predivisional cells shows the accumulation of GcrA in wild type (A). The half-life of GcrA in swarmer cells is ~10 min and in the stalked cells is ~44 min (B). This Figure is from (Collier *et al.*, 2006).
To test whether CtrA is responsible for the repression of the gcrA gene, gcrA promoter activity was measured in the ctrA401ts mutant at the restrictive temperature when ctrA was non-functional. For the gcrA promoter construct, a DNA fragment from -507 to +92 of the gcrA transcript initiation site (Holtzendorff et al., 2004) was used which contained a CtrA-binding site, a putative DnaA box, and two putative DNA methylation sites (Figure 7). To measure transcription from the gcrA promoter (-507 to +92), the lacZ gene was fused to promoter of gcrA on the plasmid pRKlacZ290, and then it was introduced into the ctrA401 temperature sensitive mutant and wild type. The ctrA401 temperature sensitive mutant has an allele of ctrA that is functional at 28°C but non-functional at 37°C. The ctrA401 mutant that carried the lacZ gene under the control of the gcrA promoter showed higher gcrA expression at 37°C when ctrA was non-functional but lower gcrA expression at 28°C when CtrA was functional (Figure 8A). This indicates that gcrA is negatively regulated by CtrA. Moreover, Western blot analysis of GcrA indicates that GcrA protein levels increase in the ctrA401ts and cckAts strains when the temperature was shifted from 28°C to 37°C after two to four hours. However, the GcrA levels in wild type increased only ~10% when the temperature was shifted from 28°C to 37°C after four hours (Figure 8B). This indicates that both the CtrA response regulator and CckA histidine kinase (that phosphorylates CtrA) (Jacobs et al., 1999) affect the repression of gcrA as shown in Figure 8 (Holtzendorff et al., 2004).

Furthermore, to observe if GcrA interacts with promoter DNA in vivo, a chromatin immunoprecipitation test was performed (Holtzendorff et al., 2004). In this assay, the ctrA and podJ promoter regions were immunoprecipitated with antibodies
Figure 7: The *gcrA* promoter sequence. The *gcrA* promoter contains a CtrA-binding site (-35 to -10) (underlined), a putative DnaA box (underlined with red line), and two putative DNA methylation sites indicated by boxes. This Figure is from (Collier *et al*., 2006).
Figure 8: GcrA is negatively regulated by the CtrA response regulator. The
\textit{ctrA401ts} mutant shows higher \textit{gcrA} expression at 37°C when \textit{ctrA} is non-functional and
lower \textit{gcrA} expression at 28°C (A). Western blot analysis shows that GcrA protein levels
increase after the temperature for \textit{ctrA401ts} and \textit{cckAts} mutants was shifted to 37°C (B).
This Figure is from (Holtzendorff \textit{et al.}, 2004).
against GcrA or CtrA. They observed a great change in both ctrA and podJ transcript levels after GcrA depletion. Evidently, GcrA regulates both the ctrA and podJ genes (Holtzendorff et al., 2004). CtrA also regulates the expression of ctrA and podJ genes. Additionally, as shown in Figure 9, GcrA binds to the promoter of dnaA, but CtrA does not bind to the promoter of dnaA. In support of this experiment, microarray analysis also showed that dnaA RNA levels changed when GcrA was depleted but not in the ctrA401ts strain at the restrictive temperature (Jacobs et al., 2003). Therefore, GcrA binds to promoter DNA (ctrA, podJ, and dnaA promoter regions), or a different protein binds to these promoters and physically interacts with GcrA (Holtzendorff et al., 2004).

Recent studies showed that GcrA works together with CcrM in the transcription regulation of the ctrA promoter and other promoters (Fioravanti et al., 2013). GcrA binds m6A-marked DNA and associates with RNA polymerase to facilitate transcription initiation at methylated promoters. This transcriptional regulatory mechanism by CcrM and GcrA is conserved in the Alphaproteobacteria. GcrA interacts with RNA polymerase by binding to a component of its core complex. The N-terminal domain of GcrA binds DNA at specific chromosomal sites and affects the transcription of cell cycle regulated genes. It was observed that a significant number of GcrA-bound sequences were associated with CcrM methylation sites. Furthermore, methylation appears to enhance GcrA binding to these target sequences. The binding of GcrA to both DNA strands increases when both strands are fully methylated compared to methylation of only one strand. GcrA target promoters may be regulated in a methylation-dependent manner in vivo. Moreover, GcrA is a transcription factor that had enhanced binding to ctrA promoter and other promoters through the methylation activity of CcrM (Fioravanti et al., 2013).
Figure 9: Association of GcrA and CtrA with promoter DNA in vivo. GcrA binds to the promoters of *ctrA*, *podJ*, and *dnaA*. It positively regulates the expression of *ctrA*, *podJ*, and negatively regulates the expression of *dnaA*. However, CtrA binds only to the *ctrA* and *podJ* promoters. It positively regulates *ctrA* expression and negatively regulates *podJ* synthesis. Lane 1: chromosomal DNA, Lane 2: anti-GcrA immunoprecipitated DNA, Lane 3: anti-CtrA immunoprecipitated DNA. This Figure is from (Holtzendorff *et al.*, 2004).
Another recent study on GcrA in *Caulobacter crescentus* indicated that GcrA is not an essential protein. Cells with ΔgcrA were shown to be viable, but they had slow rate of growth and were elongated because of a deficiency in GcrA as shown in Figure 10. Similarly, the methyltransferase CcrM was not found to be an essential protein. Deletion of either GcrA or CcrM caused defects in cell division; however simultaneous deletion of both GcrA and CcrM suppressed the defect in the cell division. Since both *gcrA* and *ccrM* appear to be co-inherited, they are not present alone but rather they are present or absent together. This implies that *gcrA* and *ccrM* form an independent unessential genetic component that controls transcription of genes during the cell cycle (Murray *et al*., 2013).

**CtrA master regulator**

CtrA is an essential protein that regulates chromosome replication, polar organelle development, and cell division (Collier & Shapiro, 2007). CtrA is present in swarmer cells but not in stalked cells because as swarmer cells differentiate to stalked cells, CtrA is degraded by the ClpXP protease. *ctrA* gene has two promoters, P1 and P2. The *ctrA* P1 promoter becomes inactive when it is fully methylated and becomes active after DNA replication when it is hemi-methylated. The *ctrA* P1 promoter is turned on by GcrA and when a low amount of CtrA accumulates, the *ctrA* P1 promoter is turned off by CtrA. Then CtrA activates its own *ctrA* P2 promoter in late predivisional cells to continue *ctrA* gene transcription. Production of CtrA, and its activation, regulates the expression of many cell cycle regulated genes. The response regulator CtrA becomes active when it receives a phosphate group from the CckA histidine kinase (Curtis & Burn, 2010). Phosphorylated CtrA is present in swarmer cells but it becomes degraded during the transition of a swarmer to a stalked cell. As mentioned before, CtrA is degraded by the
Figure 10: \( \Delta gcrA \) cells are viable but have lower growth rate and morphological defects. \( \Delta gcrA \) cells grown in PYE or M2G were viable but exhibited elongated cell morphology. They had a long lag phase and a slow doubling time (Murray et al., 2013).
ATP-dependent protease called ClpXP. However, to degrade CtrA, the ClpXP protease must localize to the stalked pole of the cell. Localization of the ClpXP protease occurs by an un-phosphorylated response regulator CpdR. CpdR localizes to the stalked pole and causes the co-localization of ClpXP, CtrA, and RcdA to the same pole (Iniesta, et al., 2006). RcdA is a localization factor that helps the protease in degrading CtrA at the correct time of the cell cycle (McGrath, et al., 2006). PopA, a protein that is needed for Caulobacter cell cycle progression and pole morphogenesis, interacts with RcdA and recruit CtrA to the stalked pole for degradation (Abel et al., 2011). Co-localization of ClpXP, CpdR, RcdA, PopA, and CtrA allows ClpXP to degrade CtrA in stalked cells and in the stalked compartment of late pre-divisional cells (Figure 11). CckA, a histidine kinase, localizes to both poles during the early predivisional stage (Jacobs et al., 1999) and phosphorylates both CtrA and CpdR (Collier & Shapiro, 2007). When CckA phosphorylates CpdR and CtrA, it prevents ClpXP localization and CtrA degradation in swarmer cells and in the swarmer compartment of late predivisional cells. As a result, ClpXP and RcdA do not localize to the cell pole and CtrA accumulates and blocks DNA replication (Collier & Shapiro, 2007). However, when CpdR is unphosphorylated, it binds to ClpXP and localizes to the stalked pole where it degrades CtrA, thus allowing for DNA replication to be initiated (Iniesta & Shapiro, 2008) (Figure 11). CckA phosphorylates both CtrA and CpdR indirectly through action of ChpT that encodes a histidine phophotransferase and transfers phosphate between CtrA and CckA. It was involved in two pathways, CckA-ChpT-CtrA and CckA-ChpT-CpdR that each regulate a different level of control for CtrA. In the early predivisional cells when CckA is localized at the cell poles, phosphorylated ChpT transfers the phosphate to CtrA and
Figure 11: Localization of ClpXP, CpdR, RcdA, and CtrA are cell cycle regulated.

During the swarmer to stalked cell transition CpdR localizes to the stalked pole and helps ClpXP, RcdA, and CtrA localize to the same pole where ClpXP degrades CtrA. In the predivisional cells, CckA localizes to both poles and phosphorylates CtrA and CpdR. CpdR~P prevents the localization of ClpXP and RcdA to the pole in the swarmer compartment of predivisional cells and CtrA is not degraded by ClpXP. This Figure is from (Iniesta et al., 2006).
CpdR to phosphorylate them, however, phosphorylated DivK in the stalked compartment of the late predivisional cells and stalked progeny delocalizes CckA to prevent the phosphorylation of both CtrA and CpdR. Dephosphorylated CpdR localizes CtrA to the stalked pole of the cells to become degraded by ClpXP (Biondi et al., 2006; Wheeler & Shapiro, 1999). CtrA inhibits gcrA expression and activates ctrA P2 transcription. CtrA also activates divK expression, which inhibits the CckA-ChpT pathway, leading to increased phosphorylation of CtrA and CpdR (Biondi et al., 2006).

**DnaA master regulator**

DnaA is also cell cycle regulated and it must get degraded after it initiates DNA replication during swarmer to stalked cell transition. In pursuit of finding the protease that degrades DnaA, Gorbatyuk and Marczynski observed that a ClpP-dependent protease degrades DnaA. However, they assumed that ClpP needs an ATP-dependent chaperone such as ClpX or ClpA to degrade DnaA (Gorbatyuk & Marczynski, 2005) as ClpXP degrades CtrA (Jenal & Fuchs, 1998). Gorbatyuk and Marczynski suggested that there could be a chaperone that introduces DnaA to ClpP, but they found out that ClpA was not involved in DnaA proteolysis. Depletion of ClpX did also not alter DnaA proteolysis. Therefore, they suggested that either a different chaperone presents DnaA to ClpP, or DnaA degradation uses the remaining ClpX activity more efficiently than CtrA degradation (Gorbatyuk & Marczynski, 2005). A summary of DnaA proteolysis is shown in Figure 12. When *Caulobacter crescentus* faces carbon or nitrogen starvation, it induces the proteolysis of DnaA and since DnaA initiates chromosome replication, the proteolysis of DnaA prevents the cell from inappropriately replicating its chromosome (Gorbatyuk & Marczynski, 2005). Upon nitrogen starvation, the transition from swarmer to stalk cell
Figure 12: Proteolysis of DnaA is ClpP-dependent. The proteolysis of DnaA increases upon nitrogen starvation. The degradation of DnaA is ClpP-dependent; however the chaperone presenting DnaA to ClpP is not yet known.
differentiation is blocked (Chiaverotti et al., 1981). To see if selective starvation can
affect the proteolysis of CtrA and DnaA, wild-type NA1000 swarmer cells were grown in
M2G media with or without ammonium (Gorbatyuk & Marczynski, 2005). Then, every
15 minutes, samples were collected for Western blot analysis with DnaA and CtrA
antiserum. The results showed that in the presence of nitrogen, CtrA accumulated in
swarmer cells, degraded in transition from swarmer to stalked cells, and was
resynthesized in predivisional cells (Figure 13A). Moreover, DnaA was present during
the cell cycle. However, upon nitrogen starvation DnaA was rapidly degraded during the
transition from swarmer to stalked cells, and it was completely cleared after 90 minutes,
but CtrA was able to persist even after 150 minutes of nitrogen starvation (Figure 13B).
They conducted the same experiment in the stalked cells and monitored the CtrA and
DnaA protein levels. The results showed that stalked cells developed asymmetrically, but
did not divide. Additionally, the CtrA protein was retained in the stalked cells upon
nitrogen starvation, but DnaA protein was absent (Figure 13C). Therefore, these
experiments showed that both swarmer and stalked cells maintained CtrA, but they
accelerated the degradation of DnaA. Since starvation induces proteolysis of DnaA (that
causes the initiation of DNA replication) and maintains CtrA (that blocks the initiation of
DNA replication), the Caulobacter cells do not use their energy for growth and
development, but recycle the amino acids and use them as resources. This physiological
mechanism allows the Caulobacter to survive in limited nutrient environments. However
when nutrients are available, DnaA synthesis increases and becomes quickly replenished
to allow the rapid continuation of chromosome replication of the Caulobacter cells
(Gorbatyuk & Marczynski, 2005).
Figure 13: Increased proteolysis of DnaA upon nitrogen starvation. Western blot analysis of wild type *Caulobacter* grown in M2G with ammonium (+N) media and continued through complete cell cycle showed the presence of DnaA throughout the cell cycle and absence of CtrA in stalked cells (A). Western blot analysis of wild type *Caulobacter* grown in M2G without ammonium (-N) showed increased degradation of DnaA, but not of CtrA (B). The same experiment in the stalked cells showed proteolysis of DnaA and retaining of CtrA (C). This Figure is from (Gorbatyuk & Marczynski, 2005).
CcrM master regulator

CcrM is an adenine DNA methyltransferase that is important for the rapid growth of Caulobacter crescentus cultures (Gonzalez & Collier et al., 2013). CcrM is controlled by cell cycle regulated transcription in the predivisional cell and degradation before cell division to ensure that DNA methylation occurs only in the predivisional cell. The Lon protease is required for the degradation of CcrM but its absence doesn’t affect cell-cycle-regulated transcription of the ccrM gene. Moreover, the Lon protease is not an essential protease for viability in Caulobacter cells, but it affects the normal development of the cell cycle. The Lon protease is present throughout the cell cycle but the presence of CcrM protein only in predivisional cells suggests that rate of the transcription of ccrM gene is greater than the rate of degradation; hence, it causes accumulation of CcrM. Thus, CcrM levels during the cell cycle are regulated by different rates of synthesis and constitutive degradation by the Lon protease (Wright et al., 1996).

Further studies on CcrM indicate that lack of the CcrM enzyme causes reduced accumulation of FtsZ and MipZ proteins that lead to a strong defect in cell division. FtsZ and MipZ are two cell division proteins involved in the cell division of Caulobacter crescentus. Caulobacter cells lacking CcrM became elongated in rich medium and their viability decreased within some hours (Stephens et al., 1996; Gonzalez & Collier et al., 2013) but CcrM-depleted cells grown in minimal medium were viable (Gonzalez & Collier et al., 2013). Low levels of FtsZ were discovered to be the main cause of such a strong cell division defect in ΔccrM cells when cultured in rich medium. Therefore, they concluded that ccrM gene is not essential for viability of Caulobacter cells in minimal medium. Since CcrM promotes the accumulation of FtsZ and MipZ proteins and ftsZ and
mipZ mRNAs, it was reasonable to observe less FtsZ and MipZ in ΔccrM cells by Western blot analysis compared to wild type cells. They initially thought that CcrM, through the actions of DnaA and CtrA, controls the ftsZ and mipZ promoters indirectly. However, it was observed that CcrM was able to control ftsZ promoter without DnaA and CtrA binding sites. This suggested that ftsZ promoter has other motifs that are necessary for the control of ftsZ transcription by CcrM. The conserved CGACTC motif in the ftsZ and mipZ promoter regions and DNA methylation by CcrM are necessary for their efficient transcription. The target motifs of the DNA methyltransferase, CcrM, are GANTC that become hemi-methylated when they are replicating and become fully methylated at the onset of cell division. The initiation of chromosome replication or DNA mismatch repair doesn’t need DNA methylation by CcrM. However, methylation of GANTC is required for the transcription of many genes that are essential for DNA metabolism, cell division, and cell cycle progression (Gonzalez & Collier et al., 2013).

CcrM methylation is important not only for the regulation of dnaA and ctrA (Reisenauer & Shapiro, 2002; Collier et al., 2007), but also for ftsZ and mipZ expression (Gonzalez & Collier et al., 2013). CcrM methylates the chromosome at GANTC sites in the dnaA, ctrA P1, ftsZ and mipZ promoters. There are 4,515 GANTC sites that are recognized by CcrM that are methylated on adenine nucleotide; however, 27 GANTC sites are not methylated at all. During cell cycle progression GANTC site transitions from full to hemi-methylation allowing for promoter activation or inactivation (Kozdon, et al., 2013). Full methylation of the chromosome is essential for the activity of FtsZ and MipZ proteins in normal cell division. When the CGACTC motif in ftsZ and mipZ promoters is unmethylated, there is low activity or gene expression; however when it is
hemimethylated, there is intermediate activity, and when it is fully methylated, there is
highest activity (Gonzalez & Collie, 2013).

**Importance of proteolysis in regulation of *Caulobacter’s* cell cycle**

Bacteria regulate protein levels by degrading and preventing the inappropriate
accumulation of proteins and removing proteins from the cell when they are no longer
needed. Proteolysis is vital for regulation of the cell cycle in *Caulobacter crescentus*. As
we know there is regulation at the level of transcription, translation, protein activity,
localization, and protein stability (Iniesta *et al.*, 2006). For example, transcriptional
regulation occurs in many genes in response to stressful conditions in the environment.
The study of proteolysis allows us to understand processes such as producing amino
acids, reprocessing proteins, and maintaining cell energy since the degradation process
can allow the cell to recycle amino acids (Goldberg & John 1976). In bacteria there are
four families of energy dependent proteases that degrade different proteins. The four
families of energy dependent proteases are ClpAP/XP, ClpYQ, Lon, and FtsH. These
families of proteases are also found in eukaryotic cells. Their proteolytic domain is
controlled by ATPase domains (subunits) placed in the cytoplasm (Gottesman, 2003 &
Lupas *et al.*, 1997).

The first family of energy dependent proteases includes the ClpAP and ClpXP
proteases. ClpAP protease is composed of ClpA (the ATPase domain) and ClpP (the
proteolytic domain) (Levchenko *et al.*, 1997). In 1993 Gottesman found out that another
ATPase subunit, ClpX associates with ClpP, forming the ClpXP protease (Gottesman *et
al.*, 1993). ClpP is known to be conserved in various organisms from bacteria to humans
is about 51 kDa and its proteolytic domain has two seven-member rings (Kessel et al., 1995; Wang et al., 1997 & Gottesman, 2003) that can line up with 14 proteolytic active sites. Generally, ClpP has a multi-subunit serine peptidase where the proteolytic active site resides within a barrel-shaped structure (Wang et al., 1997).

Moreover, ClpX is a hexameric AAA+ enzyme that identifies substrates, works with ATP, and translocates denatured polypeptides into the proteolytic compartment of ClpP for degradation (Keiler et al., 1996). The ClpA and ClpX ATPases act as chaperones and can unfold substrate without degrading it. The regulatory subunits ClpX or ClpA present the unfolded substrate to ClpP for degradation (Tsai & Alley, 2001) as shown in Figure 14. Additionally, in many bacteria, clpA is co-transcribed with clpS (Dougan et al., 2002). ClpS stimulates degradation of aggregated malate dehydrogenase and inhibits the degradation of ssrA-tagged proteins in E. coli by a ClpA-dependent protease. Since ClpS interacts with the N terminus of ClpA, elimination of the N terminus prevents the ability of ClpS to inhibit ClpAP degradation of substrates in vitro (Guo et al., 2002a & Zeth et al., 2002). ClpB, another Clp ATPase, does not associate with proteolytic domain, but acts as chaperone in degrading proteins with DnaJ, DnaK, and GrpE (Motohashi et al., 1999; Zolkiewski, 1999).

The second family of energy dependent proteases includes ClpYQ. The ATPase domain of ClpY is similar to the ATPase domain of ClpX and works with the proteolytic domain, ClpQ, that has a threonine active site (Kessel et al., 1996; Missiakas et al., 1996; RohRwild et al., 1996). The third family of proteases includes the Lon protease, a cytoplasmic serine protease that is considered to be the main quality control protease because mutations in lon decrease the accumulation of abnormal proteins in E. coli
**Figure 14: ClpXP degradation model.** The ClpX chaperone recognizes the substrate, unfolds, and it presents it to ClpP using ATP hydrolysis. The ClpP peptidase then degrades the unfolded protein. This figure is taken from (http://www.bio.umass.edu/mcb/faculty/Chien.html).
(Fukui et al., 2002 & Gottesman, 1996). The Lon protease unfolds the proteins with ATP hydrolysis and translocates the unfolded substrates into the proteolytic domain. Moreover, the Lon protease can degrade a 72-amino acid substrate (Van Melderen et al., 1996 & Gottesman, 2003). Specific proteolytic events in Caulobacter crescentus play a controlling role in cell cycle progression (Alley et al., 1993; Domain et al., 1997; Jenal & Shapiro, 1996; Kelly et al., 1998). For example, the CcrM DNA methyltransferase can be synthesized only in Caulobacter crescentus late predivisional cells, where it methylates the newly replicated chromosome. DNA methylation activity is restricted to late predivisional cells to prevent early methylation of the newly synthesized chromosome. This limitation is achieved by rapid degradation of CcrM through the Caulobacter crescentus Lon protease (Wright et al., 1996).

Finally, the FtsH protease is considered to be an ATP-dependent cytoplasmic zinc metalloprotease that has two transmembrane domains (Tomoyasu et al., 1995). The ATPase domain of FtsH is similar to other hexameric ring AAA proteins and possibly the proteolytic domain has similar multimers and chambers as the other ATP-dependent proteases (Krzywda et al., 2002 & Niwa et al., 2002).

The process of proteolysis in bacteria

The process of proteolysis begins when a substrate binds to the ATPase domain of the protease. Then after ATP hydrolysis the substrate gets unfolded and translocated from the ATPase domain to the proteolytic active sites of the protease domain. However, binding of substrate does not require ATP, but translocation needs ATP hydrolysis and is independent of degradation (Gottesman, 2003 & Lupas et al., 1997). There are some recognition motifs for proteolysis on the N or C terminus of substrates, which are called
intrinsic protease recognition signals (Gottesman, 1996). The sequences near the N or C terminus of substrates are required for adequate degradation by many proteases. The recognition motif of the substrate signals the protease to destroy it at the correct time. For example, in *E. coli*, the amino acids between 1 and 15 (recognition motifs) of the RepA protein signal RepA for degradation by ClpAP (Hoskins *et al.*, 2000a) and another protein in *E. coli*, MuA, is recognized by ClpXP through the signal of the last four amino acids of MuA (Levchenko *et al.*, 1995). Moreover, the recognition of degradation signals allows for the protease ATPase domain to bind to the substrate and unfold it. Since bacteria don’t have ubiquitin, which directs the proteins to the eukaryotes proteasome for degradation, they have these degradation signals or tagging systems for degradation. In *Caulobacter*, there are tags such as the ssrA tag that includes eleven amino acids added by tmRNA or ssrA to the C terminus of polypeptides to stimulate its degradation (Keiler *et al.*, 1996). Generally, ClpXP is responsible for the degradation of ssrA tagged proteins. However, there are some modulators or adaptors that affect the protease’s substrate selection. For example, the SspB adaptor protein stimulates ClpXP to degrade ssrA tagged proteins in *E. coli* (Levchenko, *et al.*, 2000). This adaptor protein, SspB, binds to the ssrA tag and ClpX to increase the binding affinity of the tagged protein to ClpXP. The ssrA tag marks the failed translation products to help ClpXP to recognize the 11-residue ssrA tag directly or by the bounded adaptor protein such as SspB, which helps deliver the substrates to ClpXP (Wah *et al.*, 2002; Chowdhury *et al.*, 2009; Zhou *et al.*, 2001). SspB tethers ssrA tagged proteins to ClpXP to increase the effectiveness of recognition and degradation of those proteins (Wah, *et al.*, 2003).
In *Caulobacter crescentus*, proteolysis plays an important role in biological functions including chromosomal replication, the generation of asymmetry, cell division, and motility (Tsai & Alley, 2001; Alley *et al*., 1991; Jenal & Fuchs, 1998; Jenal & Shapiro, 1996; Guardokus, *et al*., 1996; Quon, *et al*., 1998 & Wright, *et al*., 1996). For example, McpA, the receptor for the chemotaxis response, was shown to undergo time specific proteolysis (Tsai & Alley, 2001 & Le Moual & Koshland, 1996). Synthesis of McpA only occurs in predivisional cells (Tsai & Alley, 2001 & Alley, *et al*., 1991) and McpA localizes to the flagellated cell pole of the swarmer compartment (Tsai & Alley, 2001 & Alley, *et al*., 1992). The C terminus of McpA is necessary for its proteolysis (Tsai & Alley, 2001 & Alley, *et al*., 1993). The five amino acids at the C terminus of McpA in *E. coli* are required for binding the chemoreceptor methyltransferase (CheR) and the chemoreceptor methylesterase (CheB) (Tsai & Alley, 2001; Barnakov, *et al*., 1999; Djordjevic, 1997 & Okumura, *et al*., 1998). Since the McpA chemoreceptor of *Caulobacter crescentus* has similar amino acids to C terminus of McpA in *E. coli* at its C terminus, which are methylated (Tsai & Alley, 2001 & Alley, *et al*., 1991), it is possible that these amino acids bind to CheR and CheB, but it is not clear if these residues are participating in proteolysis (Tsai & Alley, 2001). As previously stated, the ClpXP protease is needed for the degradation of the response regulator CtrA, which is a part of the OmpR family of DNA binding proteins (Jenal & Fuchs, 1998). As we know, ClpX is able to recognize substrates such as McpA and CtrA by their proteolytic signals (Tsai & Alley, 2001 & Levchenko, *et al*., 1997). There are two proteolytic signals in the CtrA response regulator, with one at the N-terminus of CtrA (the first 56 residues) and the other near the C-terminus of CtrA (last 15 amino acid residues) (Ryan, *et al*., 2002).
Therefore, ClpXP was hypothesized to degrade McpA since the C terminus of the McpA is contributed to its proteolysis just like CtrA, specifically the AAL amino acids of McpA (Tsai & Alley, 2001).

**McpA and the ClpXP protease in Caulobacter crescentus**

Since the *clpX* gene is essential in *Caulobacter crescentus* (Jenal & Fuchs, 1998), investigators tried to test whether ClpXP is the protease required for degrading McpA. For that study, they used a *clpX* conditional strain in which *clpX* is expressed under the control of the xylose inducible promoter *Pxylx*, and upon shifting the medium from xylose to glucose, the expression of *clpX* is suppressed. They conducted a series of xylose depletion experiments by growing the *clpX* conditional strain for different times in the absence of xylose. As shown in Figure 15, the degradation of McpA happened in a cell-cycle-dependent manner. After 3 hours of xylose depletion, the degradation of McpA decreased and after 6 hours of xylose depletion, McpA was not degraded anymore. This experiment demonstrated that ClpX is required for the degradation of McpA. To investigate whether ClpP is also involved in degradation of McpA, they conducted the same experiment for ClpP. In this study, they also used a *clpP* conditional strain under the control of the xylose promoter *Pxylx*. As shown in Figure 16, from time 0 to 9 hours of xylose depletion, which is equal to four generations, McpA was degraded by ClpP. However, after 12 hours of ClpP depletion, McpA degradation was inhibited (Figure 16). This indicates that the ClpXP protease is responsible for the degradation of McpA, but ClpP has a weak effect on McpA compared to ClpX; this may be due to the effect of other proteases participating in the proteolysis process in the absence of ClpP (Tsai & Alley, 2001). As a result, these experiments indicate that ClpX acts as a chaperone for
Figure 15: Proteolysis of McpA is ClpX-dependent. ClpX was depleted and the cells were allowed to progress through the cell cycle. McpA cell cycle Western blot analysis for 0, 3, and 6 hours of ClpX depletion suggests that McpA is not degraded in the absence of ClpX (A-C). Optical density measurement at 600 nm for 0, 3, 6, 9, and 12 hours of ClpX depletion indicates that ClpX is the chaperone needed for proteolysis of McpA. This Figure is from (Tsai & Alley, 2001).
Figure 16: Proteolysis of McpA is ClpP-dependent. ClpP was depleted and the cells were allowed to progress through the cell cycle. McpA cell cycle Western blot analysis and growth curve at 600 nm optical density for 0, 3, 6, 9, and 12 hours of ClpP depletion indicate that McpA degradation is ClpP-dependent. This Figure is from (Tsai & Alley, 2001).
Similar to the process showing that McpA was degraded by ClpXP protease, we set to discover if the GcrA master regulator was also degraded by ClpXP or ClpAP. As mentioned before, the GcrA master regulator is present in stalked cells, early predivisional cells, and the stalked compartment of late divisional cells, but is degraded in swarmer cells. Previous research indicated that the proteolysis of GcrA is ClpP-dependent (S. Murray, unpublished data). Since it is known that ClpP has two chaperones, ClpA and ClpX, my research is to find out which of these chaperones helps ClpP to degrade Caulobacter’s GcrA protein.
MATERIALS AND METHODS

Bacterial strains, media, and plasmids

In this research study, all the strains of *Caulobacter crescentus* were grown in PYE (Peptone Yeast Extract) or M2G (Ely, 1991) media with variations of 1.5% agar, 5 µg/ml kanamycin in liquid media, 20 µg/ml kanamycin in PYE plates, 1 µg/ml oxytetracycline in liquid media, and 2 µg/ml oxytetracycline in PYE plates, 0.2% PYE glucose, and 0.3% PYE xylose media (Johnson & Ely, 1977). All the PYE plates of *Caulobacter crescentus* were incubated at 28°C, and the liquid cultures were placed on a shaking platform rotating at 125rpm in a 28°C incubator. However, *E. coli* cultures were incubated in a 37°C incubator for 24 hours. All the strains of *E. coli* were grown in LB (Luria Bertani) media with variations of 30 µg/ml of kanamycin in liquid LB and 50µg/ml of kanamycin in LB plates. All the strains and plasmids used in this project are shown in Table 1.

Construction of *clpX* Ω construct

A *clpX* Ω construct was created by PCR amplification, gel extraction, digestion of upstream and downstream region of the *clpX* gene, PCR purification and ligation of those fragments into the pNPTS138 (kanamycin resistant, sucrose sensitive) plasmid. The pNPTS138 plasmid is 5361 base pairs and is a Litmus 38-derived vector with *oriT*, *nptI*, *sacB*+ genes (with AT rich regions) created by Dickon Alley (Anacor Pharmaceuticals, Inc.). An omega cassette (2000 bp) with streptomycin/spectinomycin resistance (*smr'/spc'*) gene from pBOR (pHP45Ω ligated between two *EcoRI* fragments) was digested with *EcoRI*, gel extracted and ligated into the pNPTS138 plasmid between downstream and upstream regions of *clpX* gene. The omega cassette replaced most of the
### Table 1. Bacterial strains

<table>
<thead>
<tr>
<th>C. crescentus</th>
<th>Genotype/Phenotype</th>
<th>Derivation or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>NA1000 (CB15N)</td>
<td>Wild type</td>
<td>Gift from Lucy Shapiro</td>
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<tr>
<td>UJ945</td>
<td>ftsH::Ωstrep/spec (strep/spec&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Fischer et al 2002</td>
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<td>UJ199</td>
<td>P&lt;sub&gt;xylX&lt;/sub&gt;:clpP clpP::Ωstrep/spec (tet&lt;sup&gt;R&lt;/sup&gt;, strep/spec&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Jenal and Fuchs, 1998</td>
</tr>
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<td>UJ200</td>
<td>P&lt;sub&gt;xylX&lt;/sub&gt;:clpX clpX::Ωstrep/spec (tet&lt;sup&gt;R&lt;/sup&gt;, strep/spec&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Jenal and Fuchs, 1998</td>
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<td>ΔclpS</td>
<td>Gift from Patrick Viollier</td>
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<td>UJ5838</td>
<td>clpA::Ωspec (strep/spec&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Grunenfelder et al, 2004</td>
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<td>SG500</td>
<td>clpB::Ωspec (strep/spec&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>Simao et al 2000</td>
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<td>SM999</td>
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<td>SM1019</td>
<td>P&lt;sub&gt;xylX&lt;/sub&gt;:clpX clpX::Ωstrep/spec</td>
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<td>DH10B</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; mcrA Δ(mrr-hsdRMS-mcrBC) Φ80 lacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara leu) 7697 galU galK rpsL nupG λ&lt;sup&gt;−&lt;/sup&gt;</td>
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<td>TOP10</td>
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<td>LS1914</td>
<td>DH10B pNPTS138 (kan&lt;sup&gt;R&lt;/sup&gt;)</td>
<td>C. Mohr, R. Roberts, and L. Shapiro</td>
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<td>S17-1</td>
<td>F&lt;sup&gt;−&lt;/sup&gt;, lambda(-), thi, pro, recA, restriction (-), modification (+), RP4 derivative integrated into the chromosome with Tet::Mu, Km::Tn7,</td>
<td>(Simon et al., 1983)</td>
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<td>This study</td>
</tr>
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clpX gene and we left 12 codons at the start and 18 codons at the end. The resulting construct was transformed into chemically-competent TOP10 cells (Life Technologies, Inc.) and then transferred to *E.coli* S17 by electroporation.

**Mini-synchrony**

A published mini-synchrony protocol, as described by (Jeng-Wen Tsai and M. R. K. Alley, 2001) was followed. Briefly, a 15 ml culture (*Caulobacter crescentus*) in log phase with plenty of swarmers cells present was centrifuged at 6,000 rpm for 10 minutes at 4°C. The supernatant was removed and the pellet was resuspended in 1 ml of cold M2, transferred to a 2 ml eppendorf tube to rinse off residual media. The eppendorf tube was centrifuged for 3 minutes at 13,000 rpm. Then the supernatant was removed, and the pellet in eppendorf tube was placed on ice immediately. Then the entire cell pellet was resuspended in 900 μl of 1X M2 salts. 900 μl of percoll was added to allow cell separations to be completed in minutes using centrifugal forces, spinning for 20 minutes at 11,000 rpm, 4°C. The swarmer cells were washed two times in 1.5 ml of cold M2 and centrifuged at 7000-8000 rpm for 3 minutes. The pellet was fresh-frozen on dry ice and then stored in freezer.

**GcrA half-life**

To examine the effect of Clp depletion or expression of GcrA stability, Sean Murray measured the half-life of the GcrA using pulse-chase experiment. The swarmer cells of UJ199 (pxtyX::clpP  clpP::Ω) in M2G were labeled with a radioactive precursor [35S]-labeled methionine (10μCi/ml) for 5 minutes and chased for growing amounts of time. At the beginning of chase phase, a nonradioactive methionine was added to the culture to prevent additional incorporation of the radioisotope into GcrA. At different times during
the chase phase, samples of the cells were lysed and immunoprecipitated with antibody against GcrA. The samples were run on a 15% SDS-PAGE gel, dried, and radioisotope was detected using a phosphoimager. Then NIH ImageJ was used to quantify the bands. The half-life of the GcrA is the time it takes for the concentration of the radiolabeled GcrA to get reduced by 50% relative to the level at the beginning of the chase.

**Construction of a ClpX depletion strain**

Strain UJ200 (Jenal & Fuchs, 1998) containing the clpX gene under the xylose inducible promoter was used to construct a depletion strain, PxylX::clpX clpX::Ω.

**Transduction**

PxylX::clpX from UJ200 strain was transferred to *Caulobacter* NA1000 by transduction with phage ϕcr30 (LS178) lysate. 200 µl of wild type *Caulobacter* cells was mixed with 20 µl of bacterial phage ϕcr30 (LS178) and incubated at 28°C for two hours. After incubation, we selected for tetracycline resistance by plating the cells on PYE-tet and then we streaked them isolated colonies.

**Preparation of transducing lysate (Phage ϕcr30)**

We added ~10^6 phage to 0.5 ml of a stationary phase *Caulobacter* culture and left it at room temperature for 15 minutes for phage to attach to cells. Then 4 ml of melted 0.4% PYE agar was added to infected cells and mixture was poured on to a PYE plate. The plates were incubated overnight at 28°C. The next day, we scraped off the top agar, placed it in a 50 ml centrifuge tube and washed the plate with 5 ml PYE and added it to a 15 ml Falcon tube. 100 µl of chloroform was added to kill the residual bacteria (vortexed for 30 seconds every 3 minutes for a total of 12 minutes), and then the tube was centrifuged for 10 minutes at 5,750 rpm. A Stratagene Strat linker 1800 UV DNA-RNA
crosslinker (auto cross-link function) was used to inactivate the viral genome. The phage lysate was refrigerated at 4°C.

**Conjugation**

Through conjugation, pNPTS138 containing the clpX::Ω gene was inserted into PxylX::clpX *Caulobacter* (SM1074). 300 µl of S17 *E. coli* and 1000 µl of *Caulobacter* cells were transferred to a 0.45 µm Millipore PVDF filter on top of wire mesh on a glass stopper connected to a vacuum. Afterward, the filter was washed with 3 ml PYE to remove any salt residue from LB broth that can inhibit *Caulobacter* growth. The filter was removed and placed on a PYE plate to be incubated overnight at room temperature. After incubation, the filter was resuspended in 1 ml PYE broth and different volumes of the resuspended cells were plated on PYE plates. To select for PxylX::clpX clpX::Ω/pNPTS138 as the product of our conjugation, they were placed in PYE broth and plated onto PYE xylose plates containing 3% sucrose. Sucrose resistant colonies were selected for kanamycin sensitivity and spectinomycin resistance to confirm the recombination that contained clpX::Ω without the kanamycin resistance marker (*nptl*) from the plasmid. The resulting construct was inoculated in PYE broth with glucose or xylose to observe no growth in glucose and obtain samples for Western blot and microscopy.

**PCR**

To amplify the downstream and upstream regions of the *clpX* gene from genomic DNA by Polymerase Chain Reaction (PCR), the following primers were used:

Primer 1:
ClpXLko-Fwd: 5’-AAAAAGCTTACTTCAGGAAAGCATTGAAGCTGTCCTTCA-3’

Primer 2:

ClpXLko-Rv: 5’-AAAGAATTCCGTTTTCGTGTCGCCGCTCGCGGCTTT-3’

Primer 3:

ClpXRko-Fwd: 5’-AAAAAAAGAATTCATCTATGCCGAGAAAAAGGGT-3’

Primer 4:

ClpXRko-Rv: 5’-AAAAAAAACTTAAGACCATGTGCAGGAAACACAAACGATA-3’

For DNA amplification (500 bp), the temperature and duration of template denaturation was 94° C for 30 seconds. The temperature and duration for primer annealing to DNA was 65° C for 30 seconds and for primer extension was 72° C for 4 minutes. There were 35 cycles on the thermocycler. The upstream clpX PCR product was run on the gel, cut, and digested with restrictive enzymes EcoR1 and Hind III. The downstream clpX PCR product was cloned into pZero Blunt TOPO (Life Technologies, Inc.). The digested products were purified and then cloned into a plasmid (pNPTS138), and the omega, spectinomycin cassette, was inserted between the two fragments.

**Agarose gel electrophoresis and Gel extraction**
50 ml of 0.8% agarose gel with TAE buffer (Tris/acetate/EDTA) including 3 µl of a 10 mg/ml stock ethidium bromide was run at 120 volts for 40 minutes. Then the gel was observed under UV light with an Alpha Inotech FlourChem digital imaging system to confirm the correct size of the bands, 500 bp for ClpX-DS and 500 bp for ClpX-US. The correct sized bands were cut and extracted by a Qiagen™ gel extraction kit. Then the extracted PCR products were purified by QIAquick® PCR purification kit to obtain the DNA of interest.

**Miniprep**

A Fermentas GeneJET plasma miniprep kit (Fermentas® miniprep kit) was used before digestion. Pelleted cells from 1 ml of overnight *E. coli* cultures were resuspended in 250 µl of the Resuspension Solution and transferred to microcentrifuge tubes. 250 µl of the Lysis Solution was added and mixed carefully by inverting the tubes 6 times to have viscous and slightly clear solution. 350 µl of the Neutralization Solution was added and mixed immediately by inverting the tube 6 times. We centrifuged the tubes for 5 min to pellet the cell debris and chromosomal DNA at 10,000 rpm. Then, the supernatant was transferred to the supplied GeneJET™ spin columns and centrifuged for 1 min. The flow-through was discarded and the columns were placed back into the same collection tube with 500 µl of the Wash Solution. The columns were centrifuged for 60 seconds and flow-through was discarded. The washing procedure was repeated again with an additional 1-minute centrifugation to remove residual Wash Solution. The GeneJET™ spin columns were transferred into a new microcentrifuge tubes with 50 µl of pre-warmed water added to the center of GeneJET™ spin column membrane to elute the plasmid DNA (pBOR, pNPTS138). The columns were incubated for 2 min at room temperature
and centrifuged for 2 minutes. The columns were discarded and the purified plasmid DNA was stored at -20°C.

**Digestion and ligation**

The *clpX*-US PCR was digested with restriction enzymes (Hind III and EcoR1) to make compatible ends. The pNPTS138 plasmid was also digested with restriction enzymes, Hind III and EcoR1 to be prepared for ligation. pNPTS138 plasmid was then ligated to *clpX*-US and the ligated product of *clpX*-US/pNPTS138 was digested with AflII (since AflII can only cut the circular DNA) and then EcoR1 before it was ligated to *clpX*-DS. *clpX*-US/ *clpX*-DS /pNPTS138 was digested with EcoR1 and ligated to a digested Ω cassette (from SM 432). A Fermentas ® Rapid DNA Ligation Kit was used for ligations. A 3:1 (insert to vector) molar ratio was used for ligations. The following digestion and ligation protocols were employed for each step:

**Cloning *clpX*-DS PCR into pZero Blunt TOPO**

10 µl *clpX*-DS PCR  
3 µl salt solution  
4 µl water  
3 µl pCR II-Blunt-TOPO  
Final volume: 20 µl  
5 minutes incubation at room temperature

**Digestion of *clpX*-DS/pZero Blunt TOPO**

20 µl mini-prep of *clpX*-DS/pZero Blunt TOPO  
2 µl AflII  
10 µl NEB buffer2  
10 µl 10XBSA  
56 µl water  
2 hours incubation at 37° C  
2 µl EcoR1 (NEB)  
2 hours incubation at 37° C  
Total: 100 µl

**Digestion of ClpX-US PCR**

10 µl *clpX*-US PCR
2 μl 10 X Fermentas Fast Digest Buffer
1 μl Hind III
1 μl EcoRI
6 μl water
Total: 20 μl
2 hours incubation at 37°C

**Digestion of pNPTS138**
20 μl mini-prep of plasmid
10 μl 10X Fermentas Fast Digest Buffer
2 μl Hind III
2 μl SAP
64 μl water
2 μl EcoRI
Total: 100 μl
2 hours incubation at 37°C

**Digestion of clpX-US/pNPTS138**
20 μl mini-prep of clpX-US/pNPTS138
10 μl NEB buffer2
10 μl 10XBSA
2 μl AflIII
56 μl water
2 hours incubation at 37°C
2 μl EcoRI (NEB)
2 hours incubation at 37°C
Total: 100 μl

**Digestion of pBOR containing Ω cassette**
20 μl mini-prep of plasmid
10 μl 10X Fast Digest Buffer
68 μl water
2 μl EcoRI
Total: 100 μl
4 hours incubation at 37°C

**Digestion of pNPTS138/ ClpX-US-ClpX-DS**
10 μl mini-prep of pNPTS138/ ClpX-US-ClpX-DS
2 μl 10 X Fast Digest Buffer
2 μl EcoRI
6 μl water
Total: 20 μl
2 hours incubation at 37°C

**Control ligation**
4 μl Fermentas Rapid Ligation Buffer  
1 μl T4 DNA ligase  
1 μl vector  
14 μl water  
Total: 20 μl  
2 hours incubation at room temperature

**Ligation of clpX-US to pNPTS138**  
4 μl Fermentas Rapid Ligation Buffer  
1 μl T4 DNA ligase  
1 μl HindIII-EcoRI digested vector (pNPTS138)  
1 μl HindIII-EcoRI digested insert (clpX-US)  
13 μl water  
Total: 20 μl  
2 hours incubation at room temperature

**Ligation of clpX-DS to clpX-US/pNPTS138**  
4 μl Fermentas Rapid Ligation Buffer  
1 μl T4 DNA ligase  
1 μl EcoRI-AflIII digested vector (clpX-US/pNPTS138)  
3 μl EcoRI-AflIII digested insert (clpX-DS)  
11 μl water  
Total: 20 μl  
2 hours incubation at room temperature

**Ligation of Ω cassette to clpX-US/ clpX-DS /pNPTS138**  
4 μl Fermentas Rapid Ligation Buffer  
1 μl T4 DNA ligase  
1 μl EcoRI-digested vector (clpX-US/clpX-DS/pNTPS138)  
5 μl EcoRI-digested insert (Ω cassette)  
9 μl water  
Total: 20 μl  
2 hours incubation at room temperature

**Transformation**

To confirm that we had a plasmid (clpX-US Ω clpX-DS /pNPTS138 or the intermediates produced in cloning) of the correct size (500 bp, 1000 bp, or 3000 bp), ligated products was transformed into chemically competent cells (One Shot® TOP10 E. coli from Invitrogen™). 5 μl of ligated products with a frozen pipette tip was transferred to the
competent cells and incubated on ice for five minutes. The cells were moved to a water bath at 42°C for 30 seconds and moved back to be incubated on ice for two minutes. They were rescued with SOC broth and incubated in a 37°C shaking incubator for one hour before they were plated on LB-kanamycin or LB-streptomycin-spectinomycin plates.

**DNA sequencing**

The plasmid with the insert (ClpX-US Ω ClpX-DS /pNPTS138) was sent to Sequetech™ (Menlo Park, CA) for DNA sequencing. We used BLASTn against *Caulobacter NA1000*, assession number NC 011916 in Genbank.

**Electroporation**

5 µl of plasmid minipreps (*clpX-US Ω clpX-DS /pNPTS138*) were added to 40 µl electrocompetent cells of S17 *E. coli* (LS178) and incubated at room temperature for 5 minutes before transferring them to prechilled 2 mm electroporation cuvettes. The negative control cuvette contained only 40 µl electrocompetent cells. For the DNA-only control, 5 µl of plasmid was added to 50 µl of deionized water and incubated for five minutes at room temperature before transferring the content into pre-chilled cuvette. All the pipette tips and test tubes used in this experiment were kept on ice. The cuvettes were placed into the sample chamber of the electroporator before applying the pulse. The setting of the electroporation was 2.0 kV, 400Ω, 25 µFD. Immediately after the cells were shocked, 900 µl of ice-cold 2X PYE was added to them and mixed gently using a micropipette. The suspensions were transferred to sterile test tubes and incubated for 4 hours at 28°C with moderate shaking. The aliquots (20 µl and 200 µl) were then plated on LB kanamycin plates.
**Growth curve**

To observe the growth curve of *Caulobacter* with our constructed ClpX depletion strain in PYE xylose or glucose media, we inoculated a single colony from PYE xylose plates into 3 ml PYE xylose broth media. The single colonies were grown overnight in a 28°C incubator. After they became turbid, all of the cultures were washed 3 times in 3 ml PYE to remove xylose and were diluted to 0.05 OD$_{600}$ in 15 mm diameter glass tubes in PYE xylose broth and PYE glucose broth. We measured the optical density with an Amersham Novaspec III spectrophotometer every 2 hours for total of 12 hours and each time we took 10µl samples for dilution ($10^{-5}$) and plating (100 µl), 50µl samples for microscopy, and 1 ml samples for Western blot. The plates were incubated at 28°C for two days; we counted the number of colonies on each plate and calculated the colony forming units (CFU) per ml.

**Microscopy**

To compare any morphological defects in *Caulobacter* cells grown in PYE glucose with those grown in PYE xylose, we used the 50 µl samples that we took during growth curve experiment for microscopy. 2.5% formaldehyde and a 30 mM sodium phosphate buffer (pH 7.5) was used to fix the cells and placed on 1% agarose pad on a slide to be viewed with differential interference contrast (DIC) by using a Zeiss Axio Vision microscope with a Hamamatsu OCRA-ER digital camera. We also treated 9.5 µl of the cells with 0.5µl of 2 µg/ml DAPI stain to visualize the DNA content inside the cells.

**Western blot**

To observe the amount of CtrA, GcrA, and ClpX protein in ClpX depletion strain, Western blot analysis was used. The samples that were obtained from the growth curve in
PYE glucose and PYE xylose media were centrifuged to form pellets. The pellets were placed on dry ice for 15 minutes and were then resuspended in SDS buffer and boiled for five minutes to break open the cells. SDS buffer was used to normalize the samples based off 1.5 ml of 0.350 O.D$_{660}$ samples being pelleted and resuspended in 150 µl of 2X SDS buffer. CtrA and GcrA proteins were resolved on 15% SDS-PAGE gels and ClpX proteins were resolved on 10% SDS-PAGE gels. To check the size of our proteins, we used PageRuler™ plus prestained protein ladder (Fermentas, Inc.) as our standard protein ladder. After the proteins of interest migrated halfway on the SDS-polyacrylamide gel (~40 min at 100 V), they were transferred to PDVF membrane (Immobilon from Millipore) at 100 V for 1-2 hours at 4° C. The PDVF membrane was immersed in a solution of 5% nonfat dry milk and TTBS for one hour to block non-specific antibody binding. Then PDVF membrane was incubated with primary antibody (1 µl for CtrA, 5 µl for GcrA, and 5 µl for ClpX) in 20 ml of TTBS 2% milk for 1 hour at room temperature. The PDVF membrane was then washed with TTBS, and incubated with 2 µl of secondary antibody (donkey anti-rabbit conjugated to horseradish peroxidase) (Jackson Research Labs, Inc) in 20 ml of TTBS 2% milk for an hour in room temperature. To produce light from the secondary antibody that is bound to the primary antibody and our specific protein, PVDF membrane was washed in TTBS and treated for 1 minute with a solution of 2 ml ECL Plus Reagent 1 (PerkinElmer, Inc.), 2 ml ECL Plus Reagent 2, and 4 ml deionized water. To capture the light from the secondary antibody, we exposed the membrane covered in the saran wrap to X-ray film (Denville Scientific). The relative intensity of Western blot bands was determined using ImageJ (NIH, Bethesda, MD).

**UV mutagenesis**
The merodiploid strain after the conjugation of S17 *E. coli* containing the clpX::Ω/pNPTS138 plasmid with wild type *Caulobacter* was used for UV mutagenesis. 500 μl (0.18 O.D in exponential phase) of PYE culture in petri plates without the cover were exposed to UV light at different micro Joules exposures (100, 200, 300, and 400 MJ). *Caulobacter* cells were irradiated in the dish with the Stratalinker 2400 with intensity mentioned above. 300 μl of UV exposed cultures were added to 3 ml PYE broth and incubated in a 28°C incubator. Then the exponential cells were spread on selective media (spectinomycin-streptomycin-sucrose) to select for sacB mutants or resolved merodiploids. The growing colonies were patched on PYE-kanamycin and PYE plates to screen for sacB mutants versus deleted plasmid (clpX:Ω or clpX wild type).
RESULTS

Testing for GcrA accumulation in various protease mutants

As previously described in the introduction, GcrA does not accumulate in swarmer cells because it is degraded by a protease or proteases. To find out what type of protease is responsible for the degradation of GcrA, we observed GcrA accumulation in a number of protease mutants. From previous studies, we know there are four families of energy dependent proteases in bacteria including ClpAP/XP, ClpYQ, Lon, and FtsH. Therefore, we intended to find out which of these proteases is responsible for the degradation of GcrA in swarmer cells.

To examine whether GcrA accumulates in swarmer cells in the absence of those proteases, Western blot analysis was performed with anti-GcrA antibodies using cell lysates from the swarmer cells of wild type and various protease and ATPase chaperone mutants. In this experiment, cell lysates from the swarmer cells of wild type and the protease mutants along with mixed wild type cultures (containing both swarmer and stalked cells) were subjected to anti-GcrA Western blot analysis. As it can be observed in Figure 1, the results show that GcrA accumulates in mixed population of Caulobacter cells but not in swarmer cells of wild type, various protease mutants, and ATPase chaperone mutants. If there was GcrA accumulation in one of these mutants, we would able to see the GcrA band and this would suggest that the protease or the chaperone is responsible for GcrA degradation. However, the result indicates that none of these protease mutants (ftsH and lon) or chaperone mutants (clpA, clpS, and clpB) have altered GcrA proteolysis. Moreover, there was no GcrA band in the swarmer cells of wild type, where GcrA does not normally accumulate because of the unknown
Figure 1: Absence of GcrA protein in swarmer cells of various protease mutants.

This Western blot indicates that GcrA accumulates in the unsynchronized wild type (NA1000), but not in the swarmer cells of wild type, clpA, clpS, clpB, ftsH, or lon mutants. This suggests that there is another protease or proteases responsible for degrading GcrA in the swarmer cells (Sean Murray, unpublished results).
protease/proteases that is/are responsible for degrading GcrA. However, since GcrA normally accumulates in the stalked cells, we were able to see a GcrA band in the mixed population of wild type cells. This experiment showed that there should be a different protease or proteases responsible for degrading GcrA in the swarmer cells of *Caulobacter crescentus*.

**GcrA degradation is ClpP-dependent**

Since there is another protease responsible for GcrA degradation, we hypothesized that GcrA degradation could be ClpP-dependent. Previous studies indicated that the ClpP protease is responsible for the degradation of DnaA and CtrA. ClpP degrades CtrA with help of the ClpX chaperone; however, the chaperone that presents DnaA to ClpP is still unknown. ClpP proteolytic domain usually works with either ClpX or ClpA chaperones. To find out whether GcrA is degraded in the swarmer cells by the ClpP protease, which is an essential protein in *Caulobacter crescentus*, anti-GcrA and anti-ClpP Western blot experiments were performed. We used UJ199 strain (Jenal & Fuchs, 1998) (that had clpP gene under control of xylose-inducible promoter (Pxylx::clpP clpP::Ω). ClpP was depleted by growth in glucose-containing media for 12 hours. Western blots of cell extracts from a synchronized UJ199 culture using GcrA antibody suggests that GcrA accumulates in swarmer cells when ClpP is depleted, however, GcrA does not accumulate when ClpP is expressed from xylose-inducible promoter in xylose-containing media (Figure 2A). Moreover, Western blot analysis of UJ199 swarmer cells using ClpP antibody indicates the absence of ClpP when it is depleted and presence of ClpP when it is expressed (Figure 2A).

Further experiments were done to show that GcrA degradation is ClpP-dependent. Since
Figure 2: GcrA degradation is ClpP-dependent. (A) Western blot analysis of UJ199 swarmer cells with anti-GcrA and anti-ClpP suggest that GcrA is degraded in the presence of ClpP. These blots are representative of triplicate results. (B) GcrA half-life indicates that GcrA is less stable when ClpP is present and that GcrA is more stable when ClpP is depleted. These results were obtained from triplicate experiments. (Sean Murray, unpublished results).
we obtained positive results from Western blot analysis, Sean Murray measured GcrA half-life in exponentially growing cultures in minimal media to determine GcrA stability. As it is shown in Figure 2B, when ClpP was present, GcrA half-life was ~28 minutes and when ClpP was depleted, GcrA half-life was ~76 minutes. This shows that GcrA is less stable in the presence of ClpP. The result from Western blots and GcrA half-life experiments indicate that GcrA degradation in swarmer cells is indeed ClpP-dependent.

**Construction of ClpX depletion strain**

Now that we know GcrA degradation is ClpP-dependent, we needed to test which chaperone helps ClpP to degrade GcrA. Previous Western blots showed that ClpA was not the chaperone involved in GcrA degradation (Figure 1). Then we hypothesized that ClpX is the chaperone that helps ClpP degrade GcrA. To test if ClpX is the chaperone that presents GcrA to the ClpP protease, we constructed a ClpX depletion strain.

Using PCR, we amplified the regions upstream (US) and downstream (DS) of *clpX*. In order to insert the *clpX*-US and *clpX*-DS into plasmid pNPTS138, we performed sequential cloning; digesting *clpX*-US and plasmid pNPTS138 with restriction enzymes (Hind III and EcoRI) to prepare the ends of the insert and the vector for directional cloning. Then *clpX*-US was ligated into plasmid pNPTS138 to create pNPTS138 plasmid with *clpX*-US. *clpX*-DS was cloned into plasmid pBTOPO (pZero Blunt TOPO). Plasmid pBTOPO with *clpX*-DS and pNPTS138 plasmid with *clpX*-US were then digested with EcoRI and AflIII and ligated to result in pNPTS138 plasmid with *clpX*-US and *clpX*-DS. To insert the spectinomycin and streptomycin omega cassette (Ω), plasmid pBOR and pNPTS138/*clpX*-US-*clpX*-DS were digested with EcoRI and ligated so that the omega cassette would be inserted between *clpX*-US and *clpX*-DS in the pNPTS138 plasmid. We
then transformed the ligation into *E. coli* TOP10 chemically competent cells, isolated and sequenced the recombinant vector, and performed electroporation to move the constructed plasmid into S17 *E. coli*. Since *clpX* is an essential gene and *Caulobacter* is not viable without it, we needed to construct *clpX* mutant with a functional copy of *clpX* under control of the xylose-inducible promoter (Pxyl). Therefore, we transduced Pxyl::clpX from the UJ200 (SM1074) (Jenal & Fuchs, 1998) into wild type *Caulobacter*. We then transferred the *clpXΩ/PNPTS138* from S17 *E. coli* to Pxyl::clpX *Caulobacter* via conjugation to create the merodiploid strain of Pxyl::clpX+ clpX+ clpX::Ω. To allow for recombination that would delete pNPTS138 from the Pxyl::clpX+ clpX+ clpX::Ω merodiploid strain, it was grown in PYE broth media and then plated on PYE-xylose containing 3% sucrose for counter-selection against sacB gene (with AT rich regions) on the pNPTS138 vector. Plasmid pNPTS138 carries two selection markers, nptI selecting for kanamycin resistance, and sacB selecting for sucrose sensitivity. Sucrose-resistant colonies were screened for kanamycin resistance to test for the loss of the plasmid. Sucrose-resistant and kanamycin resistant colonies didn’t lose the plasmid but obtained sucrose resistance through sacB mutation. Sucrose-resistant colonies that were sensitive to kanamycin were tested for spectinomycin resistance. Kanamycin and spectinomycin sensitive colonies had a wild-type *clpX*. A mutational strain that had a disrupted chromosomal *clpX* (*clpX::Ω*) was sensitive to kanamycin, but resistant to spectinomycin. In this manner we were able to select for the depletion strain, Pxyl::clpX+ clpX::Ω. The process of constructing ClpX depletion strains is shown in Figure 3.
1. Recombination

- **DS Recombinant**
- **US Recombinant**

Merodiploid strain
Figure 3: Construction of ClpX depletion strain. Downstream and upstream regions of clpX gene were amplified and through a series of digestions and ligations, pNPTS138/clpX Ω plasmid was constructed and transferred into S17 E.coli. Pxylx::clpX was transduced from UJ200 to wild type Caulobacter and conjugated with the S17 E.coli to create the depletion strain (Pxylx::clpX clpX::Ω). The depletion strain was sucrose and spectinomycin resistant, and kanamycin sensitive.
CFU/ml/OD of *Caulobacter crescentus* decreases when ClpX is depleted

Since we had a successful construction of a ClpX depletion strain, we wanted to confirm that the growth of *Caulobacter* would arrest when ClpX is depleted. We depleted ClpX for 24 hours and measured the optical density (OD) and colony forming units (CFU) every 2 hours for a total of 24 hours (repeated in triplicate). As we can see from preliminary data shown in Figure 4, the depletion strain in xylose-containing media had stable CFU/ml/OD. The reason is that the *clpX* gene under control of the xylose-inducible P<sub>xylX</sub> promoter is expressed (Figure 5). However, the same strain in glucose-containing media exhibited a reduction in CFU/ml/OD after 21.5 hours of ClpX depletion. This is because there was no xylose in the media to induce the expression of *clpX* from P<sub>xylX</sub> promoter. Therefore, the viability of *Caulobacter crescentus* decreases or cells become larger as ClpX becomes depleted. Additional experiments will be needed to distinguish between these two possibilities. Even though the differences were not deemed significant by Student’s t-tests, it may be possible to obtain significant differences with more replicates. However, it is also possible that there is less CFU/ml/OD in *clpX* depleted cells since they become elongated overtime. The decline in viability in my ClpX depletion strain is different from those published by Jenal and Fuchs (1998). They used UJ200 (*P<sub>xylX</sub>::clpX clpX<sup>Ω</sup>*) and showed a significant reduction in biomass accumulation (monitored by optical density) and CFU/ml after 12 hours of ClpX depletion. Thus, they demonstrated that ClpX is essential for normal viability of *Caulobacter* cells (Jenal & Fuchs, 1998). Perhaps ClpX depleted more slowly in my experiment.

**Morphology and DNA content of Caulobacter cells with depleted ClpX**
Figure 4: Cell viability of *Caulobacter crescentus* decreases when ClpX is depleted in PYEG media. The depletion strain was grown in PYE media containing xylose or glucose. *Caulobacter* cells were viable when *clpX* was expressed under *P*xylX promoter in xylose-containing media. However, preliminary data suggesting that when ClpX was depleted in glucose containing media, the CFU/ml/OD of *Caulobacter* cells decreased. Each bar reflects the average from duplicate or triplicate trials. Outliers (due to pipetting error) were discarded. Even though the data is not deemed significant by Students’ T-tests, it may be possible to obtain significant differences with more replicates.
Figure 5: Inducible xylose promoter. clpX is an essential gene in Caulobacter crescentus. To create ClpX depletion strain, we placed the clpX gene under control of xylose-inducible Pxy/lX promoter. By growing the strain in glucose-containing media (in the absence of xylose), we were able to deplete ClpX.
Morphology of *Caulobacter* with depleted ClpX inducer            no inducer

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When ClpX is depleted, some *Caulobacter* cells elongate.
Figure 6: Morphology and DNA content of *Caulobacter crescentus* with depleted ClpX. *Caulobacter* cells were observed with DIC and fluorescence microscopy. There was no major difference in morphology of *Caulobacter* cells when they were grown in PYE media containing xylose (inducer) or glucose (no inducer) for 10 hours. However, some *Caulobacter* cells elongated when they were grown in glucose containing media for 12 hours compared to those grown in xylose containing media. No change observed in chromosome segregation when ClpX was depleted or expressed (This preliminary study was repeated in triplicate).
Since the CFU/ml/OD of *Caulobacter* cells decreased when ClpX was depleted, we assumed that ClpX depletion could affect the morphology and DNA content of the cells. To study the effect of ClpX depletion on the morphology of and chromosome segregation in *C. crescentus*, we examined the morphology of the cells with differential-interference contrast (DIC) microscopy and their DNA content stained with 4′, 6-diamidino-2-phenylindole (DAPI) with fluorescence microscopy. DAPI is a blue fluorescent dye that emits fluorescence when it is bound to AT rich regions of the DNA. As it is shown in Figure 6, there are no major changes in cell morphology or DNA content of *Caulobacter* cells during time point 0, 4, and 10 hours of cell growth in the absence or presence of the inducer. However, when ClpX was depleted for 12 hours (Figure 6O), some *Caulobacter* cells became elongated (as also reported by Jenal and Fuchs, 1998). Additionally, since ClpX depletion did not disturb the DNA content of the cells, we can conclude that ClpX is not required for chromosome segregation.

**ClpX depletion does not affect GcrA levels but increases CtrA levels**

To test whether ClpP-dependent degradation of GcrA requires the chaperone ClpX, we looked at the protein levels of ClpX, GcrA and CtrA when ClpX is depleted. Since we know that CtrA is degraded by ClpXP in stalked cells, we hypothesized that ClpXP might also be responsible for degradation of GcrA in swarmer cells. ClpP proteolysis requires a separate ATP-dependent partner such as ClpX or ClpA to unfold and present the target protein to proteolytic domain (ClpP) (Tsai & Alley, 2001); and since we already knew (from Figure 1) that GcrA degradation in swarmer cells is not ClpA-dependent, we only looked at the amount of GcrA levels depleted in cultures of ClpX. The depletion strain (P_xylX::clpX clpX::Ω) was grown in glucose containing media and equal cell masses
Figure 7: When ClpX is depleted, CtrA levels increase while GcrA levels remain unchanged. Western blot analysis suggests that ClpX may not be the chaperone assisting the ClpP protease in the degradation of GcrA. There was a 28% decrease in ClpX levels and a 44% increase in CtrA levels when ClpX was depleted in glucose containing media for 12 hours of growth, while no change was observed in GcrA levels (This is preliminary data repeated in triplicate). A longer depletion should be performed to confirm these results.
were analyzed for ClpX, GcrA, and CtrA (as our positive control) levels at 4, 10, and 12 hours of growth. As the Western blot in Figure 7 indicates, there was ~28% reduction in ClpX levels and ~44% increase in CtrA levels after 12 hours of growth, while no change was observed in GcrA levels (This preliminary data was repeated in triplicate). The 44% increase in CtrA levels confirmed that ClpP proteolysis of CtrA is ClpX-dependent since CtrA accumulates when there are low levels of ClpX. Moreover, no change in GcrA accumulation suggests that ClpX may not be the chaperone for ClpP-mediated proteolysis of GcrA. However, it is possible that ClpX may have a higher affinity for GcrA than for other proteins that accumulate to toxic levels. Therefore, it is possible that GcrA is degraded by ClpXP with such a high affinity that cells die before a difference in GcrA accumulation could be detected.

**Screen for a clpX bypass suppressor**

If GcrA is degraded by ClpXP with such a high affinity that cells die before we could observe any change in GcrA accumulation using our ClpX depletion strain, then we needed a mutation that would allow for *Caulobacter* to live without *clpX*. For that reason, we created merodiploid strains after conjugation of *clpXΩ/pNPTS138* into a wild type genetic background (Figure 8). First we performed UV mutagenesis on our merodiploid strain at different time exposures to obtain the killing curve. As it is shown in Figure 9, we got nearly 0% survival for 300 and 400 micro joules however we got 75% survival for 100 micro joules and 18% survival for 200 micro joules (repeated in triplicate). By increasing the microJules of UV exposure, we got lower survival rates. Then we grew cultures overnight in PYE broth and spread the UV treated cells on PYE plates containing spectinomycin, streptomycin, and sucrose to select for *clpXΩ* (spectinomycin and
Figure 8: Screen for \textit{clpX} bypass suppressor. The merodiploid strain was exposed to UV light for different time intervals and only 100 and 200 Micro Joules *100 UV treated cells were grown in PYE plates that contained streptomycin, spectinomycin, and sucrose to counter select against \textit{sacB} and select for \textit{clpX}. 10,000 colonies that grew were then patched on kanamycin plates to look for mutation in \textit{clpX}. Since all the colonies were resistant to kanamycin, they contained mutation in \textit{sacB} (This is preliminary data).
Figure 9: UV mutagenesis. The merodiploid strain was exposed to UV light at different time exposure to induce mutation in \textit{clpX}. The survival rate of \textit{Caulobacter} decreased as the Micro Joules of UV exposure increased. The number of sucrose-resistant spectinomycin-streptomycin resistant colonies checked from the two UV conditions are shown (This is preliminary data repeated in triplicate).
streptomycin resistant) and counter-select against sacB (sucrose sensitive). After colonies were grown they were patched on kanamycin plates to search for any clpX: Ω mutants. Sensitivity to kanamycin would suggest mutation in clpX; however, because all the colonies, ~10,000 (5,600 colonies from 100 micro joules and 4,300 colonies from 200 micro joules), were resistant to kanamycin, they all had sacB mutations (Figure 8). Therefore, UV mutagenesis suggests that it may not be possible to isolate a clpX bypass suppressor (This is preliminary data). This experiment should be repeated using cells in the 300 microjoule condition.
DISCUSSION

Overview of results

According to previous studies (Sean Murray, unpublished results) we know that degradation of GcrA in swarmer cells is ClpP-dependent. Generally ClpP works with a chaperone such as ClpX or ClpA to degrade Caulobacter proteins such as CtrA or McpA. We know that the Clp family proteases have two subunits, an ATP binding regulatory subunit (ClpA or ClpX) and a proteolytic subunit (ClpP). The regulatory subunits, ClpX or ClpA, are necessary for recognizing the substrate, unfolding, and presenting the amino acids to ClpP for degradation (Tsai and Alley, 2001). However, the chaperone that helps ClpP to degrade GcrA is not yet known. Therefore, we began series of experiment to determine if Clpx is the chaperone.

To examine the chaperone needed for GcrA proteolysis, we tested a number of chaperone mutants and found out that ClpA, ClpB, and ClpS were not the chaperones needed for ClpP proteolysis of GcrA. Therefore, we tested another chaperone, ClpX, by constructing ClpX depletion strain, PxylX::clpX clpX::Ω. Since clpX gene is an essential gene in Caulobacter crescentus, we needed to have a clpX mutant with functional copy of clpX under xylose-inducible promoter. We examined the effect of ClpX depletion on Caulobacter crescentus and confirmed that cells lose viability when ClpX is depleted. Not only does depletion of ClpX influence the viability of C. crescentus (Jenal and Fuchs, 1998), but it alters the morphology of the cell (Jenal and Fuchs, 1998; this study). Depletion of ClpX for 12 hours caused some cells to elongate, however ClpX depletion did not affect the DNA content of cells suggesting that ClpX is not required for chromosomal segregation (this study). Moreover, to test whether depletion of ClpX has
any effects on GcrA, CtrA, or ClpX levels, we conducted a Western blot experiment. Our Western blot experiments indicated a 28% decrease in ClpX levels, a 44% increase in CtrA levels, and no change in GcrA levels after 12 hours of ClpX depletion. This suggests that either ClpX is not the chaperone helping ClpP to degrade GcrA or that ClpXP has a higher affinity for GcrA than for other essential proteins. Therefore, we used our merodiploid strain for UV mutagenesis to induce a mutation in a gene other than clpX that would allow for *Caulobacter* to live without functional *clpX*. After screening 10,000 colonies, we did not find a *clpX* bypass suppressor.

**Possible explanations for GcrA degradation**

As a result of our findings we came out with possible explanations for GcrA degradation in swarmer cells. As explained before, it may be possible that a ~28% decrease in ClpX accumulation was not sufficient to observe any changes in GcrA protein levels because of their high affinity. Since depleted ClpX and mutated ClpA did not affect GcrA levels, perhaps there is a different chaperone that helps ClpP to degrade GcrA. It is also possible that two chaperones like ClpX and ClpA simultaneously contribute to ClpP degradation of GcrA. Moreover, it is possible that ClpP works alone when degrading GcrA and possibly GcrA has a partner protein in stalked cells and predivisional cells that protects it from degradation and this protein would be absent in swarmer cells (Figure 1).

A study on ATP independent proteolysis shows that ClpP, the protease subunit is able to degrade protein substrates (Casein) processively without the ATPase subunit of ClpA in *E. coli* (Jennings et al., 2008). Furthermore, this study shows that the size of the degraded protein subunits by ClpP is very similar to the size of the degraded subunits
Figure 1: Possibilities for GcrA degradation. It is possible that both ClpX and ClpA simultaneously help ClpP to degrade GcrA (yellow) (A). It is also possible that there is either an unknown chaperone (pink) working with ClpP (B) or ClpP works alone in breaking down GcrA (C). It is possible that GcrA has a partner protein (blue) in the stalked and predivisional cells that protects it from degradation (D).
that is made by ClpAP protease. However, the rate of the degradation of full-length unfolded protein by ClpP is very slow in the absence of the ClpA or ClpX. ClpA is able to translocate substrate at higher rate than ClpP alone and the reason is that the conformational changes of ClpA and ClpP together allows ClpA to speed up the translocation without altering the basic ClpP mechanism (Jennings et al., 2008).

**More speculation on GcrA degradation**

Protein degradation by ClpXP can be mediated by direct tag recognition or adaptor-mediated recognition. For example, SspBα in *E. coli* is the adaptor for ClpXP that binds to *ssrA*-tagged substrates and tethers the substrates to ClpXP. In a study of protein degradation with the help of adaptor, when both adaptor-dependent (*ssrA*-tagged substrates bound to SspBα) and adaptor-independent substrates (CtrA) were present, the SspBα adaptor increased the degradation of the former protein (*ssrA*-tagged protein) and decreased the degradation of the latter protein (CtrA) through competition (Chien et al., 2007). In the same manner, it is possible that when there are low amounts of ClpX, an adaptor protein such as SspBα binds to ClpXP and increases GcrA degradation. We could then say that the reason we didn’t observe high amount of GcrA protein when ClpX was depleted was due to increased proteolysis with the help of adaptor protein. It is possible that the adaptor protein compensated for the low amount of ClpX protein and we observed no change in GcrA protein levels. Figure 2 illustrates the function of an adaptor protein. It is also possible that a cofactor of ClpX plays a crucial role in increasing the proteolysis of GcrA even in the presence of low amounts of ClpX. From previous studies, we know that ClpX has two domains, a zinc-binding domain that forms dimers and an AAA+ ATP-binding domain that assembles into a hexamer. AAA+ domains
identify a broader series of particular sequences then zinc-binding domains. Mutational analysis and NMR determined the dimeric cofactor, SspB$_2$ on the zinc-binding domain of ClpX in *E. coli*. It was found that C-terminus of SspB$_2$ interacts with the hydrophobic area on the zinc-binding domain of ClpX. The SspB$_2$ cofactor increases the efficiency of degradation of SsrA-tagged proteins by ClpXP (Thibault *et al.*, 2006).

Since ClpXP is present constitutively during all stages of the cell cycle, it is possible that GcrA may be bound to a partner protein that inhibits its degradation by covering the recognition motif during those stages of the cell cycle that GcrA is normally present.

As a result, there are many possibilities for degradation of GcrA in the swarmer cells. However, we speculated that it is possible for GcrA protein to be degraded only by ClpP without an ATP binding regulatory subunit. Moreover, it is also possible that GcrA has a partner protein that protects the GcrA in stalked and predivisional cells but not in swarmer cells. It is also possible that different chaperones work together to present the GcrA to ClpP to be degraded. Bhat *et al.* (2013) biochemically identified ClpP substrates in *Caulobacter*, confirming that both CtrA and DnaA are ClpP substrates. However, they did not identify GcrA in their experiment. It is possible that GcrA is not directly degraded by ClpP. ClpP may control proteases that are involved in degradation of GcrA. Thus, it is possible that GcrA protease's activity is ClpP-dependent, but ClpP is not involved directly in degrading GcrA (Bhat, *et al.*, 2013). Therefore, there is a need for further investigation to conclude which of the possibilities if any would be the actual mechanism by which ClpP degrades GcrA. Therefore there is a need for further investigation to conclude which of the possibilities if any would be the actual mechanism by which ClpP...
degrades GcrA. To experimentally determine if ClpP is able to degrade GcrA without chaperones ClpA and ClpX; we can construct a clpA knockout strain and insert that into clpX depletion strain. By Western blot analysis of GcrA we can determine whether ClpP with low levels of ClpX and ClpA is able to degrade GcrA. However, there might be an unknown chaperone that helps ClpP to degrade GcrA. In order to perform the experiments in *vitro*, we need to purify the proteins such as ClpP, ClpA, ClpX, and GcrA. We can add all of them in a test tube to see if both chaperones are needed for the degradation of GcrA. We can also add GcrA and ClpP together to test whether ClpP alone can degrade GcrA without any chaperones. However, it is possible that conditions outside the living cells do not correspond to conditions inside the cells since there may be other factors that can affect the result. There may be a backup mechanism in the *Caulobacter* that we might not able to observe in *vitro*. For example in the absence of necessary amount of ClpX protein, another physiological pathway might play a crucial role in maintaining the cell cycle and compensating for the low amount of ClpX.

*Caulobacter crescentus* has four master regulators and one of these master regulators is GcrA that is degraded in swarmer cell. Previous experiments suggested that GcrA degradation is ClpP-dependent but it is not yet known if there is any chaperone that helps ClpP to degrade the GcrA. My project was to investigate if there was any chaperone such as ClpX or ClpA assisting ClpP in degradation process. By performing many experiments, we found out that neither of these chaperone may be responsible for the degradation of GcrA. It needs further investigation to answer the question of what proteases is/are responsible for the degradation of GcrA in swarmer cells.
Figure 2: Adaptor protein can increase substrates degradation by ClpXP. The ssrA tag of a protein substrate is recognized and attached to an adaptor protein such as SspB before the substrate is degraded by ClpXP protease. The adaptor protein enhanced the degradation process of ClpXP. This Figure is from (http://2011.igem.org/Team:UPO-Sevilla/Project/Improving_Flip_Flop/Proteolysis/Inhibition_system).
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