The physiological role and proteolytic regulation of DnaA in *Caulobacter crescentus* chromosome replication.

by

Boris Gorbatyuk

Department of Microbiology and Immunology

McGill University, Montreal

January 2004

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Your file Votre référence
ISBN: 0-612-98259-9
Our file Notre référence
ISBN: 0-612-98259-9

"One of the major revelations of this century is the universality of biochemistry. First observed in the near-identity of the pathways and mechanisms of alcohol fermentation in yeast and glycolysis in human muscle, this insight was reinforced by the discoveries of the intricate patterns of biosynthesis of amino acids, fatty acids, and nucleotides. It could have been expected that the devices for polymerizing these precursors would also be preserved throughout evolution. In fact, the replication of E. coli *and its phages and plasmids has proven prototypical. The variations on these basic themes in other prokaryotes and in eukaryotes are endlessly fascinating."*

Arthur Kornberg and Tania Baker, in "DNA Replication" published by W.H. Freeman and Company (New York), 1992.

ABSTRACT

Ph.D. Boris Gorbatyuk Microbiology and Immunology

Caulobacter crescentus is an asymmetrically dividing bacterium whose progeny differ in their ability to initiate chromosome replication. The stalked cell can initiate replication, whereas the motile swarmer cell must differentiate into a stalked cell prior to replication. This cell differentiation is obligate and essential for completion of the natural *C. crescentus* cell-cycle.

In model bacteria, such as *Escherichia coli*, replication is governed by the DnaA protein, however the role of DnaA in *C. crescentus* replication control has not been studied. To address the requirements for DnaA, a strain was constructed where *dnaA* was conditionally expressed in *C. crescentus*. DnaA's absence has many effects: Although swarmer cells are able to differentiate into stalked cells, these progressively filament as stalked cells without dividing. Most importantly, *C. crescentus* cannot initiate replication and loses viability in the absence of DnaA. Transcription from CtrA dependent promoters, e.g. flagellar promoters, methylation genes and *ctrA* itself, is turned off without DnaA, implying a checkpoint control of DNA replication upon developmental events.

The *E. coli* DnaA homologue is a stable protein whose acitivity is regulated by titration to high affinity binding sites and by stimulation of its intrinsic ATPase activity. However, unlike *E. coli* DnaA, *C. crescentus* DnaA is targeted for proteolysis and is even unstable in *E. coli*. In *C. crescentus,* DnaA is degraded in swarmer cells and stalked cells with different rates and in an ATP-dependent manner by the ClpP protease, but not the typical ClpP chaperone pairs. Most DnaA protein from one generation is absent from the

next generation. This ensures that newly formed stalked cells rely on *de novo* synthesized DnaA to initiate replication.

C. crescentus DnaA is also degraded in response to specific nutritional cues such as starvation for nitrogen or carbon, and in stationary phase. However, DnaA is not degraded during heat or cold shock, or in response to iron, magnesium or phosphate starvation. This is the first example in any bacterial system where the ubiquitous DnaA protein is targeted for proteolysis as a means of regulation.

RÉSUMÉ

Ph.D. Boris Gorbatyuk Microbiologie et Immunologie

Caulobacter crescentus est une bactérie se divisant de façon assymétrique dont les deux différents types de progéniture diffèrent par leur capacité à initier la réplication du chromosome. Le type cellulaire non-motile, le "stalked", peut initier la réplication tandis que le "swarmer", la cellule motile, doit, avant la réplication du chromosome, se différencier en cellule non-motile. Cette différenciation cellulaire est obligatoire et essentielle à la complétion du cycle cellulaire normal de *C. crescentus*.

Chez plusiers bactéries utilisées comme modèles, comme par example *Escherichia coli*, la réplication est sous le contrôle de la protéine DnaA, par contre le rôle de DnaA chez *C. crescentus* n'a pas été étudié. Afin de circonscrire la nécessité de DnaA, une souche où DnaA est exprimée conditionnellement a été construite. L'absence de DnaA a plusieurs effets: malgré le fait que les "swarmers" arrivent à se differencier en "stalked", ces dernières deviennent progressivement filamenteuses sans arriver à se diviser. De façon plus importante, *C. crescentus* ne peut initier la réplication et, de ce fait, perd sa viabilité en l'absence de DnaA. Aussi la transcription de promoteurs dépendants de CtrA, i.e. les promoteurs des gènes flagellaires, de méthylation et CtrA lui-même, est arrêtée en absence du DnaA, indiquant un point de contrôle de la réplication du point de vue développemental.

L'homologue de DnaA chez *E. coli* est une protéine stable dont l'activité est régulée par une titration de liaisons à des sites à haute affinité et par la stimulation de son activité ATPase intrinsèque. Par contre, contrairement au DnaA de *E. coli*, celui de *C.*

crescentus est ciblé pour la protéolyse et s'est même avéré instable lorsqu' exprimé chez *E. coli*. Chez *C. crescentus*, DnaA est dégradé dans les deux types cellulaires à des vitesses différentes et de manière dépendante à l'ATP par la protéase ClpP mais sans utiliser l'habituelle paire de chaperones ClpP. La plupart des protéines DnaA d'une génération est absente de la suivante. Ceci assure que les cellules "stalked" nouvellement formées ont besoin de la synthèse *de novo* de DnaA afin d'initier la réplication.

Le DnaA de *C. crescentus* est aussi dégradé en réponse à des signaux nutritionnels spécifiques comme la carence en azote ou en carbone et en phase stationnaire. Par contre, DnaA n'est pas dégradé pendant un choc thermique chaud ou froid, ni en réponse à la carence en fer, magnésium ou en phosphate. Ceci constitue le premier example chez tous les systèmes bactériens où la protéine ubiquitaire DnaA est ciblée par la protéolyse comme forme de régulation.

ACKNOWLEDGEMENTS

I am greatly indebted to the mentorship and support provided by my supervisor, Dr. Gregory Marczynski. His wealth of knowledge and motivation were instrumental in allowing me to expand upon my abilities, believe in myself and to develop this thesis. His openness to new ideas, accessibility and creative thinking are qualities that I admire and will always look up to.

Drs. James Coulton and Brian Driscoll served as members of my Ph.D advisory committee and their mentorship, suggestions, constructive criticisms and support throughout my Ph.D are greatly appreciated and valued. Drs. Lucy Shapiro, Swaine Chen, and Ann Reisenauer at Stanford University, Dr. Urs Jenal at the University of Basel in Switzerland, Dr. Yves Brun at Indiana University, Dr. Suely Gomes at the Universidade de São Paulo in Brazil, and Dr. Kirstin Skarstad at the Institute of Cancer Research in Oslo, Norway generously provided reagents, technical support and expertise.

Past and present members of the Marczynski laboratory, particularly William Spencer, Patrick Bastedo and Marie-Claude Ouimet served not only as excellent technical and scientific resources, but also as valuable colleagues and friends and created a great working atmosphere for me over the past 5 years. Marie-Claude Ouimet, it should be noted, translated the abstract into French and I acknowledge her efforts. Other present and past members of the Department of Microbiology and Immunology, particularly, Cezar Khursigara, Madani Thiam, Camil Sayegh, David Alexander, David Gregory, Deborah Stewart and Mark Arbing were inspiring to me as colleagues and as friends and supported me both emotionally and scientifically throughout my Ph.D. The 50's were

not only a great hockey organization but a life-style that I am proud to have been a part of.

My twin sister, Elana Gorbatyuk, has been my best friend for every day in all of the 28 years of my life and her wonderful spirit, zest and energy will always inspire me. My girlfriend, Debbie Finkelberg has stood beside me through the good and the bad and is a steadfast, caring and tremendously special individual. I am so appreciative of all of her encouragement and support. Finally, I would like to thank my parents, Val and Ella Gorbatyuk. They have inspired me to follow my goals and dreams and to strive for the best amidst the most difficult of challenges. They have sacrificed so much to create the best opportunities for myself and for my sister and are without question the most important people in my life. This thesis is as much theirs as it is mine.

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. Used genetic techniques to establish that the *dnaA* gene is essential for *Caulobacter crescentus* and its absence leads to a loss in *C. crescentus* viability.
2. Utilized a conditionally expressing genetic system to demonstrate through radioactive labeling of DNA and Fluorescent Activated Cell Sorting analysis that DnaA is essential for replication initiation in *C. crescentus*.
3. Demonstrated the physiological and morphological consequences that arise from depleting DnaA from *C. crescentus* such as filamentation and cell-cycle arrest.
4. Determined that DnaA activity is dominant or "epistatic" to positive contributors for replication initiation in *C. crescentus*, such as transcription from a replication promoter.
5. Identified for the first time, that a DnaA homologue is an unstable protein that is subject to regulated proteolytic degradation. This is in sharp contrast to the best studied *E. coli* DnaA homologue that is remarkably stable.
6. Showed that *C. crescentus* DnaA maintains its properties of instability even when it is expressed in *E. coli*. Therefore, the instability property of *C. crescentus* DnaA is specific for the protein and the stability of *E. coli* DnaA is probably not due to an absence of DnaA protein degradation specificity factors or the presence of inhibitory factors in *E. coli*.
7. Determined that *C. crescentus* DnaA is degraded primarily by the ClpP protease.

8. Determined that *C.crescentus* DnaA is not degraded by the typical ClpP chaperones, ClpX or ClpA chaperones. This implies that *C. crescentus* DnaA degradation may require a novel chaperone.
9. Demonstrated the cell-cycle pattern of DnaA degradation to be different from that of any other previously characterized *C. crescentus* proteins.
10. Characterized the *C. crescentus* cell-cycle arrest that develops under conditions that are limiting for a nitrogen source and demonstrated that this particular arrest leads to the loss of DnaA from the cells.
11. Identified the nutrient limiting and stress conditions that cue DnaA loss from *C. crescentus*. These conditions include nitrogen and carbon starvation as well as entry into the stationary phase of the *C. crescentus* growth cycle. They do not include heat or cold shock, active growth, or starvation for iron, magnesium or phosphate.
12. Used transcriptional reporters and immunoblot analysis to demonstrate that DnaA is not regulated by the master CtrA response-regulator that controls expression of 25% of all cell-cycle regulated genes, but CtrA transcription and expression is dependent upon DnaA.

CONTRIBUTION OF AUTHORS

I present the experimental portions of this thesis in manuscript format. Experimental chapters 2, 3 and 4 comprise manuscripts that either were published (Chapter 2), were submitted (Chapter 3) or are in final preparation (Chapter 4).

Chapters 2, 3 and 4 all contain their own Abstract (Chapters 3 and 4) or Summary (Chapter 2), Introduction, Materials and Methods (Chapters 3 and 4) or Experimental Procedures (Chapter 2), Results, Discussion, Acknowledgements and References sections. A preface serves as a connecting text between manuscripts and is found prior to Chapters 2, 3 and 4. A general introduction and an up-to-date review of literature are provided in Chapter 1 and general conclusions and opportunities for future research are presented in Chapter 5. References are found at the end of each chapter. Page numbers of this thesis are found at the bottom right of each page.

Below, I list the three chapters that are presented as manuscripts and I list the contributions of each author with respect to the experimental work. I am responsible for the rest of the experimental work and I composed the text of each manuscript. Dr. Gregory T. Marczynski edited each of the three manuscripts and all of my work was conducted in the laboratory of Dr. Gregory T. Marcynski and under his supervision.

1. **Gorbatyuk, B. and Marczynski, G. T.** 2001. Physiological consequences of blocked *Caulobacter crescentus dnaA* expression, an essential DNA replication gene. *Mol. Microbiol.* **40**: 485-497. (Presented as Chapter 2 of this thesis)

Dr. G. T. Marczynski provided the data for Figure 1 of this manuscript and assisted in writing the relevant section.

2. **Gorbatyuk, B. and Marczynski, G. T**. 2003. Programmed stability and degradation of chromosome replication proteins DnaA and CtrA. (Submitted; presented as Chapter 3 of this thesis)

Dr. G. T. Marczynski edited this manuscript. I wrote and provided all data for this chapter.

3. **Gorbatyuk, B. and Marczynski, G.T.** 2003. Environmental cues for *C. crescentus* DnaA degradation. (to be submitted; presented as Chapter 4 of this thesis)

Dr. G. T. Marczynski edited this manuscript. I wrote and provided all data for this chapter.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1:

CHAPTER 2:

CHAPTER 3:

CHAPTER 4:

LIST OF TABLES

CHAPTER 2:

CHAPTER 3:

CHAPTER 4:

CHAPTER 1:
LITERATURE REVIEW,
RATIONALE AND
OBJECTIVES OF THE THESIS

OVERVIEW OF THE LITERATURE REVIEW

This thesis deals with the topic of replication initiation in *Caulobacter crescentus.* This literature review will provide the background for understanding this topic and is divided into six main sections. The first section, "*Caulobacter crescentus*: a model developmental bacterium" deals exclusively with this unusual bacterium and why it is a good model for several fundamental biological problems. The second section, "The *C. crescentus* cell-cycle: an overview of development" deals with the established and hypothetical mechanisms by which *C. crescentus* regulates proteins that control development. A particular focus of this section is the CtrA protein, a global regulator of this cell-cycle. Since one of the major effects of CtrA is to limit replication initiation, the third section entitled, "Control of chromosome replication in *C. crescentus,*" deals with the current hypotheses about replication initiation in *C. crescentus,* and how replication is a checkpoint for development. This section will also describe the *C. crescentus* origin of replication, *Cori*, and the putative binding sites within *Cori* that provide clues as to the main protein players that regulate replication.

Since a putative binding site for the DnaA protein exists in *Cori*, the fourth main section will deal with the main model mechanism of replication initiation in bacteria and is entitled, "Replication initiation in bacteria: DnaA-dependent replication initiation." In this section, the *E. coli* DnaA-dependent replication initiation mechanism, the best understood of all bacteria, will be discussed. A particular importance will be placed upon the model of DnaA-dependent replication and upon the mechanisms that *E. coli* utilizes to ensure that DnaA-dependent replication initiation is restricted and balanced with cell growth.

This will lead into the fifth section of this literature review entitled, "DNA replication in eukaryotic cells: analogous features and mechanisms." The eukaryotic replication proteins, although distinct from bacterial replication proteins, make use of analogous mechanisms. In this section, the main eukaryotic replication initiation proteins will be described and compared to their analogous prokaryotic counterpart. In addition, since eukaryotic replication initiation is coupled to the cell-cycle, it is necessary to identify similarities to *C. crescentus* coordination of replication with the cell-cycle.

The sixth and final section is entitled, "Selective bacterial proteolysis." This section will focus upon bacterial ATP-dependent proteases, the diverse helper proteins and the cues to which they respond to target specific proteins for degradation. This section is particularly important because I discovered that *C. crescentus* DnaA is likewise targeted by similar proteases. This provides a thorough and up-to-date survey of information that pertains to this thesis topic: The role of *C. crescentus* DnaA protein in initiating chromosome replication and its regulation by proteolysis.

CAULOBACTER CRESCENTUS: A MODEL DEVELOPMENTAL BACTERIUM.

Caulobacter crescentus is a free-living, strictly aerobic Gram-negative bacterium that grows in dilute aqueous environments (Poindexter 1981). It is a member of the alpha-purple subdivision of proteobacteria (Stahl et al. 1992; Sly et al. 1999), a group that includes the intracellular parasite and causative agent of Typhus *Rickettsia prowazekii*, the bovine and human pathogen *Brucella abortus,* the plant symbiont *Sinorhizobium meliloti*, and the plant pathogen *Agrobacterium tumefaciens*. *Rickettsia prowazekii* is probably a close relative of the endosymbiont that evolved to become the eukaryotic mitochondrion (Andersson et al. 1998). Although members of this group have diverse behaviors, their genomes share striking similarities and conserved features. The amenability of non-pathogenic *C. crescentus* to laboratory manipulations makes it an ideal organism to study many functions common to this apparently diverse group of bacteria. The ability to synchronize *C. crescentus* cultures (Evinger and Agabian 1977) has made it an especially good model for cell-cycle processes such as chromosome replication (Marczynski et al. 1990; Marczynski and Shapiro 1992; Marczynski et al. 1995; Marczynski 1999), and this is exploited in this thesis.

Besides scientific interests, *C. crescentus* is also being utilized in biotechnology for applications that include surface antigen presentation for vaccine production, as well as for industrial protein overproduction (J. Smit, University of British Columbia). However, *C. crescentus* is primarily exploited for developmental and cell-cycle studies. During *C. crescentus* cell-division, obligate developmental events (Stove and Stanier 1962) are coupled to cell-division and to chromosome replication (Osley and Newton 1978). *C. crescentus* buds motile, flagella-bearing swarmer cells that first swim and

chemotax, but later differentiate into sessile stalked cells that initiate chromosome replication. This differentiation involves global changes, highlighted by morphological changes that include the shedding of the flagellum and the subsequent growth of a filamentous appendage in its stead, called the "stalk" or "prostheca." Once initiation of chromosome replication occurs, the stalked cell engages in an ordered series of events that lead to the asymmetric pre-divisional cell, which is a hybrid between a swarmer and a stalked cell. Subsequent asymmetric cell division yields a swarmer cell and a stalked cell progeny (Fig. 1). Since the swarmer cell always swims away in natural environments, this strategy couples cell growth and scattering to prevent overcrowding where nutrients are few. This unique cell-cycle resembles a eukaryotic developmental cycle and is therefore considered a model bacterial developmental system. Most *C. crescentus* research performed in university laboratories focuses on developmental events that are coupled to the *C. crescentus* cell-cycle (Fig. 2). For example, *C. crescentus* is a model bacterium for studying flagellar biosynthesis (Gober and England 2000), of which a great deal of information is known, as well as pili biosynthesis and stalk biosynthesis (Brun and Janakiraman 2000), of which more information about its regulation is emerging. In addition, *C. crescentus* serves as a model organism for studying cell-division and DNA replication, which will be described in greater detail below (Fig. 2). There have been many recent reviews devoted to this topic (Hung et al. 2000; Jenal and Stephens 2002; Marczynski and Shapiro 2002; Ryan and Shapiro 2003).

The *C. crescentus* genome has recently been sequenced (Nierman et al. 2001) and it provides many insights on *C. crescentus* regulation and adaptation to nutrient-poor environments. The circular G + C rich (>67%) *C. crescentus* genome is over 4 Mb and it

contains almost 4000 genes. *C. crescentus* has many signal transduction proteins (44 response regulators, 34 histidine kinases, and 27 histidine kinase/response regulator hybrids) (Nierman et al. 2001). It also has many TonB dependent transporters (65) that presumably transport dilute nutrients across the outer membrane. It does not have an exceptionally large number of high-affinity ATP dependent transporters across the cytoplasmic membrane. Since its environment is so nutritionally limiting, *C. crescentus* presumably uses high-affinity transporters to scavenge for nutrients and then use low-affinity transporters to acquire these nutrients (Nierman et al. 2001). It also has a large number of efflux and secretion systems. Approximately 2.5% of its genome is devoted solely to sensing and moving towards substrates in its environment (Nierman et al. 2001).

Since *C. crescentus* exists primarily in dilute, nutrient-poor environments, its rate of growth is typically slow in its natural environment, approximately 3 reproductive cycles per day (Poindexter et al. 2000). Unlike *Escherichia coli* and *Bacillus subtilis,* that can initiate replication more than once from the same origin before cell-division, *C. crescentus* stringently limits replication to once and only once per cell-division cycle (Marczynski 1999). In addition, whereas *B. subtilis* can enter a spore-forming phase only in response to starvation (and is considered an alternate developmental pathway), the *C. crescentus* swarmer to stalked cell development is an obligate part of its cell-cycle and not an alternate developmental pathway. The ease of growing *C. crescentus* (2 hour doubling time from a swarmer cell in "rich" PYE media, and 2.5 hour doubling time in minimal M2 media) and its amenability to synchronization studies (Evinger and Agabian 1977) makes it a model organism to study cell-cycle specific events. Specifically, since replication initiation is coupled to morphological differentiation, it is especially strategic

to focus the question of replication initiation into what prevents one cell type from replicating its chromosome, yet allows another cell-type to replicate its chromosome. This question is the main focus of the research conducted in Dr. Marczynski's laboratory.

THE *C. CRESCENTUS* CELL-CYCLE: AN OVERVIEW OF DEVELOPMENT

Much of the *C. crescentus* cell-cycle is comprised of ordered events, where the beginning of one event is contingent upon the successful completion of the previous event. Therefore, morphological development are under checkpoint control (Jenal and Stephens 2002). What are these ordered developmental events and how might they be regulated? Please refer to Figure 1 for a diagrammatic summary.

The swarmer cell is a motile cell capable of responding to nutritional chemo-attractants (chemotaxis). It has a single flagellum and an intricate chemosensory network of proteins that can respond to multiple nutritional stimuli. It is also able to attach to surfaces due to the presence of pili at the flagellum-bearing pole (Smit 1987). The flagellum is shed and the pili retract upon the decision to differentiate to stalked cells. In place of the flagellum, a filamentous appendage comprised of the cytoplasm devoid of ribosomes and DNA (Cohen-Bazire et al. 1966) and surrounded by the cell membrane grows in its place. This filamentous appendage is called the "prostheca" or "stalk" and its rigidity is provided by crossbands comprised mainly of peptidoglycan that attach the inner membrane and the outer membranes (Brun and Janakiraman 2000). The end of the stalk contains an adhesive holdfast which may have been present in the base of the flagella at the swarmer end of the pre-divisional cell stage, but upon flagellar shedding, acquired at the end of the stalk (Kurtz, Jr. and Smith 1992). This holdfast is primarily responsible for the rosette appearance that *C. crescentus* cells form in the wild (Stove and Stanier 1962; Poindexter 1964).

The stalked cell is immediately able to initiate chromosome replication. Following replication initiation, the newly duplicated origin of replication is rapidly

segregated to the pole opposite the stalk (Jensen and Shapiro 1999). Chromosome movement appears to be rapid since no intermediate positions are seen between the poles. As replication elongation proceeds, flagellar biosynthesis initiates and a single polar flagellum is built from orderly synthesized proteins at the pole opposite of the stalk (Gober and England 2000). This forms what is called the pre-divisional cell. Cell division proteins localize to the midpoint of the pre-divisional cell organizing a polymeric ring of proteins (Z ring) that may serve as both a scaffold and motor for cytokinesis. A physical barrier of compartmentalization appears between these two distinct cell types (Judd et al. 2003) and cytokinesis completes the asymmetric division program to yield two morphologically and functionally distinct progeny. The new swarmer cell assembles pili adjacent to the flagellum. Whereas, the newly divided stalked cell can immediately initiate chromosome replication, the newly divided swarmer cell must again differentiate into the stalked cell in order to initiate replication and cell division. How are these developmental events regulated?

Differential transcription, signal transduction, localization and proteolysis: Four methods of protein control throughout the *C. crescentus* cell-cycle.

At least four general mechanisms regulate *C. crescentus* development and ensure that specific proteins are both present and active at the correct temporal stage: 1) differential transcription, 2) signal transduction, 3) protein localization and 4) proteolysis. The following section will deal with these mechanisms.

Differential Transcription

Differential transcription ensures that mRNA is available only at specific times in the cell-cycle. The genome sequence suggests that there are as many as 16 sigma factors that can direct RNA polymerase to transcribe specific genes (Nierman et al. 2001). Prior to this report, only three sigma factors were identified. These were sigma-D, sigma-H and sigma-N. Sigma D is the principal Sigma 70 protein, although in *C. crescentus* it is slightly larger (sigma 73) and offers greater specificity than the enteric sigma 70 (Malakooti and Ely 1995). Sigma-H is a Heat shock sigma factor whose expression increases in response to elevated temperature (Wu and Newton 1996; Reisenauer et al. 1996) and Sigma-N (sigma 54) is involved in expression of genes in both flagellar and stalk biosynthesis (Brun and Shapiro 1992). The remaining 13 sigma factors resemble extracytoplasmic factor (ECF) sigma factors (Nierman et al. 2001) and may respond to periplasmic or extracellular stimuli in mediating changes in gene expression. However, their roles are not clear and have not yet been closely studied.

Sigma-N is itself regulated by the CtrA response regulator, the focus of discussion below. Sigma-N functions only in conjunction with temporally regulated activator proteins to more precisely regulate the transcription of specific temporally regulated genes such as Class III and Class IV flagellar genes (Ramakrishnan and Newton 1990; Wu et al. 1995; Marques et al. 1997). The FlbD response regulator (this family of proteins is discussed in greater detail below) is a protein that is an activator of Class III flagellar genes and binds to enhancer sequences (ftr) located approximately 100 base-pairs away from the transcription start site (Gober and Shapiro 1992). FlbD must act in concert with sigma 54 containing RNA polymerase for transcription to occur. The Class

III flagellar genes also contain an IHF binding site and this is believed to bring the distant FlbD protein into close proximity to the sigma 54 dependent promoter (Gober and Shapiro 1992). Interestingly, the FlbD response regulator is presumed to function in activation only after the flagella has undergone early assembly and formed the MS-ring, flagellar switch and the flagellum secretion apparatus (Muir and Gober 2001).

In *C. crescentus* approximately 15% of all genes are temporally regulated throughout the cell-cycle (Laub et al. 2000) and 25% of those genes are regulated through the response regulator CtrA (Laub et al. 2000) where the majority of them (70%) are directly regulated by binding of CtrA to their promoters (Laub et al. 2002). The CtrA protein is a response regulator and is the target of phosphorylation cascades involving several two-component phosphorylation proteins (to be discussed in greater detail below). So what is the scope of proteins regulated by CtrA? Please refer to Figure 3 throughout the discussion on CtrA.

CtrA, the master cell-cycle regulator in C. crescentus*: the scope of its activity*

CtrA is auto-regulated and the *ctrA* gene is transcribed from two promoters. The first promoter P1 is a weak promoter that is auto-repressed by phosphorylated CtrA (CtrA-P) (the mechanism by which CtrA is phosphorylated is covered in the section below). P1 is turned on in stalked cells and CtrA-P starts to accumulate in early pre-divisional cells. CtrA-P then represses transcription from P1 and activates the stronger P2 promoter to further drive its own expression in pre-divisional cells(Domian et al. 1999). This illustrates the dual role of CtrA as an activator and as a repressor. CtrA binds a

consensus recognition sequences TTAA-N7-TTAAC within various promoters, and this allows CtrA to serve as a global regulator of transcription.

CtrA is an activator of temporal genetic transcription involved in flagellar and pili biosynthesis and of the gene encoding the DNA methyltransferase (covered in greater detail below in describing replication control in *C. crescentus*) throughout the cell-cycle and a repressor of the early cell-division gene *ftsZ*, stalk biosynthetic genes and a promoter implicated in replication initiation control (Marczynski et al. 1995; Quon et al. 1996; Kelly et al. 1998; Laub et al. 2000; Skerker and Shapiro 2000; Laub et al. 2002). Therefore, CtrA's presence, and as will be shown, phosphorylation state is important for its activity in regulating cell-cycle events. It is fascinating that a single protein could be involved in regulating such a diversity of events and therefore some detail will be afforded to its regulation and activity throughout this literature review (Please see Fig. 3 for CtrA roles in *C. crescentus*).

Bacterial signal transduction

Signal transduction is another major means of regulating temporal gene expression in *C. crescentus* through CtrA. Bacterial signal transduction is called "two-component" when it involves paired sensor and receiver proteins: a sensor kinase protein that responds to stimuli and a response regulator that is the downstream signaling partner in this signaling cascade. For reviews please see (Stock et al. 1995; OSteras and Jenal 2000; Ohta et al. 2000). Typically, the sensor kinase autophosphorylates onto a histidine residue in its phosphor-transfer subdomain and then transfers the phosphoryl group onto an aspartate in the regulator domain of its response regulator partner. The conformational change that

accompanies response regulator phosphorylation activates the output domain of the response regulator and allows it to mediate its effector function in response to the upstream environmental stimulus. The output domain of response regulators is typically DNA-binding and can mediate transcription of genes that respond appropriately to the environment stimuli. Although, bacterial signal transduction is described as two-component, the modular nature of the histidine kinase and response regulators allows for a multi-component phosphorelay as seen in bacterial sporulation. In *B. subtilis* sporulation, the Spo0A master response-regulator is under control of a multi-component phosphorelay involving three separate histidine kinases, KinA, KinB and KinC as well as two modular proteins Spo0F and Spo0B. All of these phosphorelay components respond to distinct signals and allow for multi-level control of sporulation (Burkholder and Grossman 2000). The result of phosphorelay downstream signaling is phosphorylation of Spo0A and the expression of sporulation genes (Burkholder and Grossman 2000). The *C. crescentus* genome encodes 34 histidine protein kinase genes, 44 response regulator genes and 27 hybrid HPK/RR genes (Nierman et al. 2001). These signaling proteins can allow *C. crescentus* to process many signals that presumably also control growth and development.

Such signal transduction is essential for *C. crescentus* and unexpectedly, several essential signaling proteins are required for cell viability (Hecht et al. 1995; Quon et al. 1996; Jacobs et al. 1999; Fuchs et al. 2001; Cabantous et al. 2002). For example CtrA is essential presumably because it directly controls ~25% of the genes under cell cycle control (Laub et al. 2002). This response regulator was originally identified in a screen for temperature sensitive genes that control the earliest events in the flagellar

transcription cascade as well as essential events for cell cycle progression (Quon et al. 1996). A phosphorylation mutation in the Aspartic acid residue fails to complement a *ctrA Ts* allele or a CtrA null allele when expressed on a low copy number plasmid (Quon et al. 1996), but can do so when placed onto a high-copy plasmid (Domian et al. 1997). As well, it has been shown that phosphorylation of CtrA is temporally regulated in the cell-cycle and occurs in swarmer cells but not in stalked cells. CtrA becomes phosphorylated in mid S phase, following replication initiation (Domian et al. 1997). Phosphorylation stimulates a co-operative mode of CtrA binding between two sites, observed only within the replication origin (Siam and Marczynski 2000).

There are a number of potential histidine kinases that influence CtrA phosphorylation, and CtrA phosphorylation is probably not limited to only one histidine kinase or one signal transduction pathway (Fig. 3). An intriguing histidine kinase is the essential protein CckA. CckA was identified in a screen for temperature sensitive alleles that were defective in motility and pili formation (also CtrA-dependent functions), but could not be complemented by *ctrA* supplied on a plasmid (Jacobs et al. 1999). CckA is a "hybrid kinase" in that it contains a transmitter module with a histidine residue as well as a receiver module (like that found in response regulators) that contains an aspartic acid residue. At non-permissive temperatures, *C. crescentus* expressing the temperature-sensitive *cckA* allele is aberrant for cell-division as demonstrated by their filamentous shapes, as well as by over-replication, implied by excess DNA in FACS analysis (Jacobs et al. 1999). These observations resemble *ctrA Ts* cells grown at non-permissive temperature, suggesting that CckA and CtrA act in the same pathway. But most importantly, CtrA is not phosphorylated *in vivo* in *cckA Ts* cells at non-permissive

temperatures, and this clearly implicates CckA in the phosphorylation of CtrA (Jacobs et al. 1999; Jacobs et al. 2003). As well, CtrA phosphorylation follows the pattern of CckA phosphorylation in the cell-cycle (Jacobs et al. 2003). However, it has yet to be demonstrated that CckA can directly phosphorylate CtrA and due to the hybrid nature of CckA, it is plausible that it may function in a phosphorelay by phosphorylating a modular histidine phosphotransferase that in turn directly phosphorylates CtrA.

In addition to CckA, two additional kinases probably control CtrA phosphorylation (Jenal 2000; Ohta et al. 2000). PleC and DivJ are histidine kinases that both contribute to the phosphorylation of DivK, although neither PleC nor DivJ are essential. PleC is implicated in polar organelle development and cell motility and DivJ is involved in the location of stalk formation and cell division. DivK is an essential single domain response regulator that is also implicated in the phosphorylation of CtrA. This is due to the ability of a mutant *ctrA* allele called *sokA* to suppress a loss of viability and the cell-division defect of a *divK* null mutant (Wu et al. 1998). The *sokA* allele can also suppress the cell-division defect from a *divJ* histidine kinase (Wu et al. 1998) . Interestingly, a novel protein kinase was discovered in *C. crescentus,* DivL that becomes autophosphorylated onto a tyrosine residue (Wu et al. 1999a). This is unique because DivL looks like a histidine kinase but Tyrosine replaces the Histidine. DivL can phosphorylate CtrA directly *in vitro*, but does not phosphorylate DivK Therefore, CtrA is probably controlled by several phosphorelay networks that includes DivL, DivJ and CckA as separate histidine kinases that all serve to directly or indirectly target CtrA (Jenal 2000) (Fig. 3).

Localization of C. crescentus *signaling proteins*

Research in *C. crescentus* has revealed that not only is it essential to maintain these signal transduction regulatory proteins, but that the location of these regulatory proteins is vital to their function (Jacobs and Shapiro 1998; Jacobs et al. 1999; Jacobs et al. 2001; Viollier et al. 2002a; Hinz et al. 2003). A number of reviews have focused on this topic (Shapiro and Losick 1997; Wheeler et al. 1998; Jenal and Stephens 2002; Jensen et al. 2002). Histidine Kinases are both regulated by the cell-cycle for their localization, but also regulate the cell-cycle by their localization.

Protein localization emerged as an important paradigm from work on eukaryotic cells (Mochly-Rosen 1995). Protein localization was not considered important in small bacterial cells until it was shown that chemotaxis proteins localized to *C. crescentus* (Alley et al. 1992) and *E. coli* cells (Maddock and Shapiro 1993). Interestingly, immunoblots revealed that CckA was present throughout the cell-cycle and S^{35}-methionine pulse-chase experiments demonstrated that it was a stable protein (Jacobs et al. 1999). However, its phosphorylation and the consequent phosphorylation of CtrA was limited to a specific temporal period within the cell-cycle (Jacobs et al. 1999; Jacobs et al. 2003). It was important for CckA to anchor itself to the membrane in the pole opposite the stalk after replication initiation (Fig. 3). A *cckA* allele mutant for membrane anchoring could not complement a null *cckA* strain arguing that membrane localization is absolutely essential for CckA function. Interestingly, the membrane anchoring *cckA* mutant can complement a *cckA* Ts allele and argues for complementation through heterodimerization (Jacobs et al. 1999).

PleC and DivJ HPKs, as well as the DivK response regulator follow unique cell-cycle localization patterns (Fig. 3). PleC localizes to the flagellated pole in swarmer and pre-divisional cells. In contrast, PleC is dispersed throughout the cell-membrane in stalked cells (Wheeler and Shapiro 1999). DivJ, on the other hand, localizes to the stalked pole as swarmer cells shed their flagella and remains at that position for the duration of the cell-cycle (Wheeler and Shapiro 1999). DivK is not a membrane bound protein and is dispersed throughout the swarmer cell, but becomes bound to the stalked pole during swarmer to stalk transition and later additionally localizes to the opposite pole in predivisional cells. Its localization is dependent on DivJ and its release following cell-division is dependent upon PleC (Jacobs et al. 2001).

How these histidine kinases localize to the correct poles is addressed by research that was aimed at understanding pili biogenesis and positioning at the swarmer cell pole and holdfast formation during the swarmer to stalk transition. This research uncovered that PilA, the main subunit of the pilus subunit and a gene whose transcription is regulated by CtrA (Laub et al. 2000; Skerker and Shapiro 2000; Laub et al. 2002), accumulates at the swarmer pole of pre-divisional cells (Viollier et al. 2002a). The pilus secretion apparatus comprised of the CpaC outer membrane secretion channel and the CpaE assembly factor co-localize to the swarmer cell pole prior to pilus filament polymerization and therefore determine the site of pilin localization (Viollier et al. 2002a). PleC mutants yield symmetrically divided daughter cells that lack pili and PilA subunits fail to accumulate when the PleC site of autophosphorylation is mutated from a Histidine to an Alanine (Viollier et al. 2002a). The CpaE assembly factor is required for

PleC and CpaC localization to the swarmer cell pole, however, the mutant, non-autophosphorylated PleC cannot release PleC or CpaE from the swarmer cell pole during swarmer to stalk transition. In addition to the phosphorylation state of PleC, the localization of PleC is also important for its activity. The PodJ "polar development" protein is required for proper PleC formation and in the absence of PodJ, PleC fails to localize and pili fail to form (Viollier et al. 2002b; Hinz et al. 2003). Therefore, both activity and localization of a histidine kinase is important for proper cell-cycle progression.

Proteolysis during the C. crescentus *cell-cycle*

Bacterial proteolysis is a key regulatory step to ensure that unwanted proteins are not present at an improper time and place. In *C. crescentus*, 5% of identified proteins through 2-D gel electrophoresis are degraded within the course of the cell-cycle (Grunenfelder et al. 2001). Proteolysis is a key regulatory mechanism in *C. crescentus* and a number of well-studied proteins are subject to proteolytic regulation throughout the cell-cycle. For a more comprehensive look at the various bacterial proteases that exist, please refer to the section on Bacterial Proteases below. Please refer to Figure 1 of Chapter 3 for a diagrammatic representation of *C. crescentus* patterns of proteolysis.

The CtrA Response Regulator is subject to both temporal and spatial proteolysis (Fig. 1 and Fig. 3). CtrA is degraded in stalked cells upon swarmer to stalk transition (Domian et al. 1997). In addition, after being re-synthesized in early pre-divisional cells, CtrA is degraded exclusively in the stalked cell compartment just prior to cell-division,

but not in the swarmer cell compartment (Domian et al. 1997). CtrA is exclusively degraded by the ATP-dependent ClpXP protease (Jenal and Fuchs 1998) (Fig. 4). Both ClpX and ClpP are essential in *C. crescentus* and depletion of either ClpX or ClpP (from conditionally expressing strains) stabilized CtrA protein in the cell-cycle (Jenal and Fuchs 1998). However, immunoblots revealed that ClpX and ClpP levels are constant and present throughout the cell-cycle. Since CtrA is only degraded at precise spatial and temporal periods of the cell-cycle (Jenal and Fuchs 1998), additional specificity factors must direct CtrA to the ClpXP degradation machinery. The need for a specificity factor has also been suggested from recent work that identified that CtrA has a bipartite degradation signal required for temporally controlled proteolysis (Ryan et al. 2002). Here, the authors identify that in addition to a degradation signal previously identified in the C-terminus Alanine residues (Domian et al. 1997), an N-terminal degradation signal is present in the receiver domain of CtrA and that temporal degradation is independent of CtrA phosphorylation-state (Ryan et al. 2002). The authors suggest that whereas the C-terminal degradation signal is likely regulated by direct ClpX recognition, the N-terminal signal necessary for cell-cycle degradation likely interacts with a specificity factor. This hypothesis is strengthened by their *in vitro* data demonstrating that CtrA is not degraded by purified ClpXP, whereas other ClpXP substrates are directly recognized and degraded in this *in vitro* system (Ryan et al. 2002).

Another protein that has a similar cell-cycle pattern of degradation to CtrA is McpA. McpA is a chemoreceptor present in swarmer cells and it is degraded in stalked cells. Like CtrA, it is degraded by a ClpXP-dependent pathway, yet unlike CtrA whose ClpX recognition sequence is in the extreme C-terminal, the McpA degradation signal is

located 15 amino-acid residues from the C-terminus (Tsai and Alley 2001). Although McpA and CtrA appear to be degraded temporally by the same proteases, the mechanism by which they are degraded by ClpXP appears to differ. A deletion in the gene encoding a tmRNA called *ssrA* (a molecule that tags stalled peptides by adding an alanine rich tag and allows them to be recognized by some proteases – described in detail in the Bacterial Proteases section) causes a delay in swarmer to stalked cell transition. However, CtrA proteolysis was unaffected, whereas McpA proteolysis was delayed considerably (Keiler and Shapiro 2003) arguing that the specificity factors that temporally regulate their degradation must be different.

Several other proteins have been demonstrated to be degraded differentially throughout the *C. crescentus* cell-cycle. The CcrM, methyltransferase is degraded by Lon protease (Wright et al. 1996). Lon is present throughout the cell-cycle but CcrM is only synthesized in late pre-divisional cells. Therefore, the degradation of CcrM is limited to the point in the cell-cycle when it is synthesized and it is consequently degraded within 20 minutes of being synthesized in late pre-divisional cell. In another example, the FtsZ protein aggregates to form a cytokinetic ring at the midpoint of the cell (Quardokus et al. 2001). Following cytokinesis, this cytokinetic ring de-aggregates and is rapidly degraded when in this form (Kelly et al. 1998). Interestingly, the FtsZ protein is degraded from the swarmer cell compartment of pre-divisional cells but not from the stalked cell compartment. The protease that mediates this control is unknown. However, it is rationalized that this temporal and spatial degradation pattern ensures that only the stalked cell receives FtsZ molecules for subsequent cell division (Kelly et al. 1998).

The ClpAP protease is believed to degrade the FliF flagellar membrane anchor protein. ClpAP degrades FliF in a cell-cycle manner during swarmer to stalk transition (Hengge and Bukau 2003). Also, the FtsH protease is important for adaptation to stress and development. Cells growing without FtsH fail to grow at elevated temperatures and lose viability at long exposure to stationary phase (Fischer et al. 2002).

In summary, it is clear that proteolysis is important for cell cycle progression. Replication control proteins CtrA, and DnaA as described below (Chapters 3 and 4), are also targeted for proteolysis.

CONTROL OF CHROMOSOME REPLICATION IN *CAULOBACTER CRESCENTUS*

In *Caulobacter crescentus*, replication is initiated upon swarmer to stalked cell differentiation (Marczynski et al. 1990). Cloned origin of replication plasmids also demonstrate this temporal property of replication initiation (Marczynski and Shapiro 1992). Unlike *E. coli* and *B. subtilis*, *C. crescentus* replication initiation occurs once and only once per cell-division cycle (Marczynski 1999). Therefore, *C. crescentus* must have evolved unique regulatory controls to ensure that replication initiation is tightly controlled and coupled with the cell-cycle. The control of chromosome replication in *C. crescentus* is the first example of negative regulation by the binding a response regulator (CtrA) to its replication origin (Quon et al. 1998). I will highlight the role played by CtrA as a repressor of replication and also focus upon the *C. crescentus* origin of replication (*Cori*) as a site where this regulation takes place. In addition, I will describe the role played by DnaA as a positive regulator of chromosome replication (full details in Chapter 2).

The *C. crescentus* origin of replication (*Cori*)

Unlike other eubacteria, whose origins are centered around the *dnaA* gene, the *C. crescentus* origin of replication (*Cori*) is located within a gene cluster that is conserved among alpha-proteobacteria (Brassinga et al. 2001). This organization has *Cori* centered between the *hemE* gene, the first gene of a heme biosynthetic operon, and RP001, a gene of unknown but apparently conserved function.

The evidence that *Cori* is the replication origin came from several different approaches. Taking advantage of synchronous DNA replication initiation in stalked cells, DNA labeling experiments combined with pulse-field gel electrophoresis (PFGE) across the entire *C. crescentus* genome identified that replication occurred bi-directionally and that the earliest replicating region originated from a discreet region of the chromosome (Dingwall and Shapiro 1989). PFGE was later used to further isolate the earliest replicating region from a cosmid of DNA and to clone it (Marczynski and Shapiro 1992). This subclone was shown to support autonomous plasmid replication in a plasmid that normally cannot replicate in *C. crescentus* and sequencing revealed that this subclone contained regions that are conserved in *E. coli* origins of replication such as a putative DnaA binding site and AT-rich region (Fig. 4) (described in greater detail in this literature review) (Marczynski and Shapiro 1992). In addition, these plasmids temporally replicated in stalked cells and not in swarmer cells (Marczynski and Shapiro 1992), a distinction from broad-host range plasmids that had the ability to replicate in both swarmer and stalked cell-types (Marczynski et al. 1990). *Cori* was confirmed to be the actual replication origin by the Brewer and Fangman two-dimensional gel electrophoresis technique that further localized *Cori* to a 1.6 kb region (Brassinga and Marczynski 2001).

Cori *DNA Replication Elements*

Please refer to Fig. 4, the "map" of *Cori,* throughout this discussion of *Cori* DNA replication elements. The combination of two replication assays was used to identify *Cori* DNA replication elements. Point mutations, insertions and deletions within the *Cori*

region (Marczynski et al. 1995) were placed into non-replicating plasmids that rely on functional *Cori* for autonomous plasmid replication (Marczynski and Shapiro 1992). These mutagenized *Cori*-driven plasmids were tested for their ability to replicate and to follow a cell-cycle replication pattern. This assay is called the "autonomous plasmid replication assay." If a mutant did not allow for *Cori*-plasmid replication or perturbed the cell-cycle pattern, then this region would be considered important for replication or its control. These mutants could also be tested by the "allele exchange assay." By homologous recombination and *Cori* replacement, altered DNA is tested for the ability to replicate in the context of the chromosome (B. Yang and Marczynski, unpublished data). Therefore, if a mutant blocked plasmid replication or could not be exchanged on the chromosome, then it would be considered an essential *Cori* DNA replication element.

Cori has conserved DNA sequence elements, similar to other "model" replication origins (*E. coli*), and *Cori* also has unique sequence elements. Most notably, *Cori* has 5 binding sites for the CtrA response regulator protein (Quon et al. 1998), shown as sites "a', "b", "c", "d" and "e" in Fig. 4. CtrA binds to all five binding sites, except with different affinities. CtrA binds to sites "a" and "b" co-operatively, but independently of the other three sites, which in turn, are also bound independently by CtrA (Siam and Marczynski 2000). The co-operative binding to sites "a" and "b" is enhanced by CtrA-P and CtrA-P is believed to stimulate co-operative binding between two adjacent CtrA half-sites (Siam and Marczynski 2000). As well, phosphorylated CtrA binds to site "c" within *Cori* with forty-fold greater affinity than unphosphorylated CtrA and the apparent relative binding order of CtrA to its sites changed upon CtrA phosphorylation ("a" < "c", "b", "d", "e" vs "a" < "c", "d", "e" < "b") (Siam and Marczynski 2000). The binding of CtrA

to *Cori* sites is stronger than to any other genes in the CtrA regulon (Laub et al. 2002) and is most likely the first sites occupied and last sites released by CtrA.

In addition, *Cori* contains an AT rich region (Marczynski et al. 1995) that overlaps two CtrA binding sites, a binding site for the DNA bending protein, IHF, which competes with CtrA for binding (Siam et al. 2003), as well as putative binding sites for the DnaA replication initiator protein (Marczynski et al. 1995). There only appears to be one "good" putative DnaA binding site within *Cori*, compared to many DnaA binding sites in *E. coli* and *B. subtilis*. This putative DnaA binding site matches 8/9 base pairs of the *E. coli* DnaA binding site consensus. A detailed discussion of the model of bacterial DnaA-dependent replication will be covered in detail below. Mutagenesis in the one putative DnaA binding site, adjacent to CtrA binding site "e" reduced *Cori*- plasmid replication (Marczynski et al. 1995). In addition, mutagenesis of this entire putative DnaA box could never replace the wild-type DnaA box on the chromosome and abolished *Cori* plasmid replication (Dhabadhai and Marczynski, unpublished data). The genetic organization of the putative DnaA box adjacent to CtrA binding site "e" appears to be the only hallmark of *Cori* that is well conserved even amongst distant phylogenetic *Caulobacter* species (S.M. Shaheen and Marczynski, unpublished data), and most likely, the interplay between DnaA and CtrA on *Cori* is essential in setting the timing of replication. This however is speculative and deserves further attention.

Transcription can influence replication. *Cori* contains three transcription promoter regions, one which directs transcription of the hemE gene (Pw), a strong promoter region (Ps) which directs a non-translatable transcript and a divergent promoter (P3) that has not been well characterized but transcribes into the direction of RP001

(Marczynski et al. 1995). Whereas transcription from Pw is constitutive throughout the cell-cycle, transcription from Ps coincides temporally with replication initiation (Marczynski et al. 1995; Quon et al. 1998) and is believed to play an important role in replication control. Ps transcription is controlled by CtrA binding to the origin and the abolishment of CtrA binding sites upstream of Ps abolished cell-cycle transcription and caused unregulated replication initiation (Quon et al. 1998). The DnaA protein, however, is absolutely essential for replication initiation and as will be shown in Chapter 2, is epistatic to Ps transcription control in regulating replication (Gorbatyuk and Marczynski 2001). In other words, Ps does not by itself (without DnaA) trigger replication.

Some regions of *Cori* are not as well characterized. The AT rich region is a feature that is conserved amongst bacterial replication origins and as will be described below in DnaA-dependent replication initiation, is important for forming an "open-complex" that is essential for replication initiation. A purine-rich region is found adjacent to the AT rich region and its deletion is not tolerated on the chromosome (Marczynski and Shapiro 2002). While the AT rich region might hypothetically serve as the site where double-stranded DNA first unwinds, the significance of the purine-rich region is not known. An IHF binding site is also present on the chromosome and it is believed to play a role in replication control by potentially bending the DNA and bringing the DnaA box into close proximity to the AT rich region. IHF competes with CtrA for binding at CtrA site "c" (Siam et al. 2003). Increase in IHF transcription is co-incidental with swarmer to stalk transition and it may therefore play an antagonistic role to CtrA (Laub et al. 2000). This role in helping to regulate DnaA-mediated replication by IHF

however is speculative and based upon the *E. coli* model of replication initiation (Marczynski and Shapiro 2002)

In light of the uniqueness of *Cori* in that it is regulated by CtrA binding, it is still believed to initiate replication in a DnaA-dependent mode (Gorbatyuk and Marczynski 2001) (Please see chapter 2). However, the timing of replication initiation most likely depends upon the interplay between DnaA and CtrA binding in the origin, among other signals.

Methylation of the Caulobacter *chromosome*

In *E.coli,* the sequestration of the origin by SeqA protein binding to Dam hemi-methylated DNA is an important control of replication initiation. This is covered in great detail below. This provided the motivation to study the methylation of the *C. crescentus* chromosome. The *ccrM* gene is essential for *C. crescentus* and encodes a methyl-transferase that is essential for viability (Stephens et al. 1996). The *ccrM* gene's transcription is tightly controlled by CtrA in the cell-cycle (Reisenauer et al. 1999; Laub et al. 2000), such that it is transcribed in the pre-divisional cell (Zweiger et al. 1994) and the protein is *de novo* synthesized in the pre-divisional cell where it rapidly starts to methylate hemi-methylated DNA (Stephens et al. 1996). Hemi-methylated DNA starts to accumulate as chromosome replication proceeds throughout S-phase as newly replicated DNA is unmethylated, but is remethylated when CcrM appears. CcrM recognizes the GANTC sequence and methylates the adenine residue (Berdis et al. 1998). In fact, replication control in *C. crescentus* is so tightly controlled that methylation was used as

an assay for replication promiscuity. In this case, it was shown that the *C. crescentus* chromosome very rarely accumulated unmethylated DNA (more than once per cell-cycle replication) as opposed to plasmids that accumulate unmethylated DNA more frequently (Marczynski 1999). The activity of CcrM is limited to a specific period of the cell-cycle (only to late pre-divisional cells) due to its timely synthesis and then rapid and specific degradation by Lon protease (Wright et al. 1996). In the presence of Lon, CcrM has a 20 minute half-life and its activity is therefore restricted to the time and place where it is synthesized. In addition, in a *lon*$^-$ mutant, there is over-replication and hemi-methylated DNA never accumulates throughout the cell-cycle (Wright et al. 1996). Therefore, the timely regulation of methylation contributes to the cell-cycle timing of replication.

Dynamic Movement of Cori *and of the replisome*

Fluorescent *in situ* hybridization was used to study sub-cellular *Cori* localization. This study identified rapid *Cori* chromosome movement immediately following replication. A *Cori* specific fluorescently labeled probe was able to localize *Cori* at various points in the cell-cycle through fluorescence microscopy. In *C. crescentus*, *Cori* is localized to the stalked cell pole during S phase, and immediately following replication initiation, it is rapidly segregrated to the opposite pole, the future flagella-bearing pole of pre-divisional cells (Jensen and Shapiro 1999). This also suggests that *Cori*-proximal sequences may contain the binding sites for the mitotic-like elements. As well, it suggests that whatever proteins are present to repress chromosome replication after initiation (and restrict

replication to once per cell-cycle) may also be localized at the poles in order to act at *Cori*.

In *Bacillus subtilis*, it was observed through Green Fluorescent Proteins (GFPs) fused to replisome-proteins that the replisome was primarily localized to the mid-point of the cell and was stationary while newly replicated DNA was spooled to the opposite ends of the cell, presumably by the force generated from the replication factory (Lemon and Grossman 1998). In contrast, in *C. crescentus*, the replisome is mobile. In a similar GFP-tagged experiment, the *C. crescentus* replisome was shown to gradually move from the stalk-pole towards the mid-point of the cell. As DNA was pulled into the moving replisome, the newly replicated DNA was then extruded to both cell poles. Most likely, the accumulation of DNA at the cell poles excludes and propels the replisome from the ends of the cell and progressively allows it to move to the mid-point of the cell where it disassembles following completion of replication (Jensen et al. 2001).

Replication as a Checkpoint for Development

In *C. crescentus*, mutants with blocked chromosome replication arrested typically as unpinched, non-motile filamentous cells (Osley and Newton 1977). This suggested that there was checkpoint control in *C. crescentus* development where later developmental events, such as building the flagellum and acquiring motility, were contingent upon the successful completion of chromosome replication. The fact that replication mutants were observed to arrest with an unpinched phenotype also suggested that initiation of cell division was contingent upon chromosome replication. The observation that replication mutants were non-motile suggested that flagellar transcription was contingent upon

successful chromosome replication. In this next section, I will discuss replication checkpoint control in the regulation of proper cell-division and flagellar transcription.

Flagellar gene transcription

In *C. crescentus,* flagellar gene transcription is regulated through an ordered cascade where functional expression of class II genes (encoding the MS ring, switch and type III secretion apparatus) activates transcription of class III flagellar genes (that encode basal-body rods) and functional expression of class III proteins in turn activates transcription of class IV flagellin genes. Flagellar gene transcription is also coupled to chromosome replication. Originally, class IV flagellin protein synthesis was observed to require DNA synthesis. This requirement is now attributed to a regulatory coupling between class II genetic transcription and DNA synthesis. The *fliL* (Stephens and Shapiro 1993), *fliQ* (Dingwall et al. 1992) and *ccrM* (Stephens et al. 1995) genes turn off their transcription when *C. crescentus* is either treated with hydroxyurea or in a *dnaC* Ts mutation background (Ohta et al. 1990)(in *C. crescentus dnaC* is a replication elongation protein) at non-permissive temperature. All three genes are regulated by CtrA: *fliL* and *fliQ* during temporal flagellar biosynthesis and *ccrM,* late in the cell-cycle. Their promoters bind CtrA, but with different affinities, where *ccrM* has the weakest binding to CtrA of the three. Presumably this explains why *ccrM* transcription is turned on later than *fliQ* in the cell-cycle (Reisenauer et al. 1999). In this scheme, the *ctrA* gene is considered a "class I flagellar gene", and its transcription also requires DNA synthesis. Although the coupling mechanism is not understood, it may involve DNA methylation and a

requirement of the replication fork to pass through the *ctrA* promoter (Reisenauer and Shapiro 2002).

Cell-division gene transcription and correct cell-division protein positioning

The unpinched phenotype observed in replication mutants suggested that cell-division proteins either do not localize or are not activated when replication is blocked. A series of experiments addressed this phenomenon. First insights into how cell-division is coupled to replication and the cell-cycle were through transcriptional studies aimed at understanding the synthesis pattern of the cell-division initiator, FtsZ. FtsZ, is an early-acting cell-division protein that structurally resembles eukaryotic tubulin. FtsZ forms a mid-cell ring that presumably provides both a scaffold and a contractile mechanism for septum formation (Addinall and Holland 2002). CtrA is a repressor of *ftsZ* transcription (Kelly et al. 1998). Transcription of *ftsZ* is coupled to the cell-cycle where the proteolysis of CtrA during the swarmer to stalked cell transition relieves the repression of *ftsZ* and allows it to be transcribed early in S phase (Kelly et al. 1998). Whereas *ftsZ* is repressed by CtrA, *ftsQ* and *ftsA* encode late cell-division proteins that are activated by CtrA later in S-phase (Sackett et al. 1998; Wortinger et al. 2000). Transcription of *ftsQA* was off when DNA replication was inhibited by hydroxyurea (Wortinger et al. 2000). Inhibiting DNA replication prevents the accumulation of CtrA in pre-divisional cells by blocking the transcription of the *ctrA P2* promoter and consequently *ftsQA* transcription. Therefore, replication is an important checkpoint for the expression of cell-division as well as flagellar proteins.

How FtsZ is localized to the midpoint of the bacterial cell is an interesting question that is beyond the scope of this literature review. Several intriguing models exist in *E. coli* for this mechanism, but in *C. crescentus* even less is known. It is clear that the FtsZ cytokinetic ring accumulates to the midpoint of the cell (Quardokus et al. 2001). However, an apparent role for replication initiation in FtsZ ring positioning became apparent from our own physiological characterization of a conditional *dnaA* strain where cells blocked for *dnaA* expression start to show polar constrictions (Please see chapter 2) (Gorbatyuk and Marczynski 2001). This was later confirmed through fluorescence microscopy studies that showed that FtsZ directly mislocalizes in these *dnaA* blocked cells (Quardokus and Brun 2002). DnaA-dependent replication initiation is now currently believed to be a checkpoint for the correct positioning of FtsZ rings at the mid-point, however it is not clear how this checkpoint is mediated.

REPLICATION INITIATION IN BACTERIA: DNAA-DEPENDENT REPLICATION INITIATION

In 1966, when Kohiyama and other researchers (in the group led by Francois Jacob) identified *E. coli* temperature sensitive mutants for DNA replication, they grouped these mutants into two classes: delayed-stop mutations and fast-stop mutations (Kohiyama et al. 1966; Kohiyama 1968). Fast-stop mutations immediately ceased replicating when shifted to non-permissive temperature, whereas delayed-stop mutations ceased replication with slower kinetics that are consistent with the completion of ongoing replication. These and other observations implied that delayed-stop genes encode proteins that initiate chromosome replication. Only three genes showed delayed-stop alleles and they were given the names *dnaA, dnaB* and *dnaC.* We now know that the *dnaA* gene encode the replication initiator (Fuller et al. 1981; Fuller and Kornberg 1983), *dnaB* encodes the replicative helicase (Baker et al. 1986) and *dnaC* encodes the helicase loader (Lanka and Schuster 1983).

Three years earlier in 1963, Jacob *et al.* published the famous replicon hypothesis where they proposed a model outlining the basic elements that are required for replication control (Jacob et al. 1963). Their model included the replicator or the site from where replication would begin (the *cis* element), and the initiator that would catalyze the replication initiation process (the *trans* element). DnaA was later equated with the "initiator", and this is supported by experiments where, for example, alterations in DnaA concentration vary the cell volume at which replication takes place (Lobner-Olesen et al. 1989). The *oriC* region on the chromosome was identified to be the "replicator" and this

is supported by its ability to allow extra-chromosomal replication of an F plasmid in an Hfr strain. This region was originally called *poh* or permissive on hfr prior to being called *oriC,* the conventional current name (Hiraga 1976). The *oriC* was able to support autonomous plasmid replication on plasmids that did not contain a replication origin (Messer et al. 1978). The minimal DNA sequences required for replication were identified as 245 specific base pairs that support autonomous plasmid replication (Oka et al. 1980), and that also required DnaA for replication both *in vivo* and *in vitro* (Fuller et al. 1981; Tabata et al. 1983). It is primarily the interaction between DnaA and its cognate binding sites within *oriC* that mediates the mechanism of replication initiation in *E. coli.* This mechanism is described below.

The model of Replication Initiation in *E. coli*

E. coli oriC replication initiation is mediated by the DnaA protein (Bramhill and Kornberg 1988a), which is remarkably well-conserved amongst eubacteria (Skarstad and Boye 1994). This suggests a conserved mechanism, however, most of our knowledge on DnaA comes from *E. coli* studies, and it is not clear what properties of DnaA are universal and what properties are unique to *E. coli.* Please refer to Fig. 5 for a schematic of the DnaA dependent replication initiation model in *E. coli.* DnaA protein binds to binding sites (DnaA boxes) within the *E. coli oriC.* DnaA boxes have a consensus recognition sequence in *E. coli* as 5'- TTATNCACA. *E. coli oriC* has 5 DnaA boxes. Each binding site has a different affinity for the DnaA protein, and DnaA binds to these sites as monomers. The binding to these sites is ordered but non co-operative (Margulies and Kaguni 1996) and whereas most DnaA boxes appear to be bound throughout the cell-

cycle, one of these binding sites (the lowest affinity site of the five) is occupied by DnaA only around the time of replication initiation in the *E. coli* cell-cycle (Cassler et al. 1995). Electron microscopy experiments estimate that 20-30 DnaA proteins bind to the origin during open complex formation (Fuller et al. 1984), surface plasmon resonance estimates 18 DnaA monomers per *oriC* during the initial complex stage (Messer et al. 2001) whereas a recent and quantitative immublot assay estimates the number to be 5 during the initial complex stage (Carr and Kaguni 2001). Although, DnaA can bind as monomers to the origin initially, it is believed to oligomerize after binding as monomers in order to initiate replication (Simmons et al. 2003).

DnaA can bind to both ATP and ADP, and is both functionally (Su'etsugu et al. 2001) and structurally (Erzberger et al. 2002) considered a member of the AAA+ ATPase family of proteins (Neuwald et al. 1999). AAA+ family members have several conserved motifs including the Walker A (involved in ATP binding) and Walker B (involved in ATP hydrolysis) (Walker et al. 1982) motifs. (A brief discussion of AAA+ family of proteins is covered below). DnaA protein can bind to the origin as DnaA-ATP or DnaA-ADP, although only DnaA-ATP has the ability to initiate replication (Sekimizu et al. 1987). In order to initiate replication, the DnaA-ATP monomers induce a bend in the DNA structure estimated to be of 40° (Schaper and Messer 1995). The binding of DnaA at this stage is considered the initial complex. As DNA bending molecules like HU, IHF or FIS bind to DNA and induce further bending within the *oriC* DNA, and as transcription is initiated by RNA Polymerase to form a negatively supercoiled template, DnaA protein melts the double stranded DNA at three tandem 13 mers located within an AT-rich region at the leftward end of the replication origin (Bramhill and Kornberg

1988b; Hsu et al. 1994). The formation of ssDNA in the AT-rich region is called Open-Complex formation.

In addition to the 9-mer DnaA boxes, DnaA can also recognize 6-mer binding sites when it is bound to ATP but not ADP. These 6-mer binding sites were called DnaA-ATP boxes (Speck et al. 1999) and three are located in the 13 mer AT-rich region. DnaA-ADP cannot melt the 13 mer AT-rich region (Katayama and Crooke 1995). During melting of the AT-rich region, DnaA-ATP binds to 6 mer single stranded binding sites with a high-affinity. It binds to these 6 mer double stranded binding sites with low affinity, but co-operatively with DnaA-ATP bound to a 9 mer DnaA box (Speck and Messer 2001). It is not clear how DnaA unwinds the AT-rich DNA, although it is believed to be stimulated by IHF binding (Hwang and Kornberg 1992; Grimwade et al. 2000). The SSB, which binds to single stranded DNA, enlarges the open-complex of *E. coli oriC* (Krause and Messer 1999) and thus appears to play a positive role in maintaining a single stranded DNA template for future replication.

This unwound "open-complex" with stably bound DnaA and SSB, is a substrate for DnaB helicase loading by DnaC to form the "Pre-priming Complex." DnaB is loaded onto the open complex with the help of an ATP-dependent helicase loader, DnaC. DnaB and DnaC form two separate 6:6 complexes; the intrinsic helicase activity of DnaB and its ATPase activity is inhibited when in this complex (Wahle et al. 1989). DnaC must bind to ATP to form this complex and prevent DnaB from binding non-specifically (to non-origin sites). However, upon DnaC interaction with single-stranded DNA (Learn et al. 1997) and DnaA, DnaC ATP hydrolysis selectively releases DnaB onto the unwound open-complex (Wahle et al. 1989). It was recently demonstrated that DnaB helicase does

not randomly load onto unwound DNA in a DnaA-dependent manner, but rather DnaB is directed to the bottom DNA strand first and then to the top strand by DnaA bound to DnaA box R1 (Weigel and Seitz 2002). DnaB helicase activity is then stimulated and the two helicases translocate past each other in a 5' to 3' direction from the sites where they were loaded to the replication start sites; they must move at least 65 nucleotides in order to recruit primase (Fang et al. 1999).

DnaB then interacts with DnaG primase, and DnaG then lays down an RNA primer and primes both leading and lagging strand synthesis in bi-directional bacterial replication (Hiasa and Marians 1994). Primase then promotes the "clamp loading" proteins to form a homodimer of the β subunit of DNA polymerase III, the "sliding clamp", around *oriC* DNA. As will be shown below, this sliding clamp also negatively regulates DnaA activity. After DNA Polymerase III is added, the "replisome" then can replicate the entire *E. coli* chromosome.

AAA+ Family of Proteins

Since DnaA belongs to a family of proteins called AAA+ and since many proteins (bacterial proteases, chaperones, and replication initiators and eukaryotic replication initiators) covered in this literature review and thesis also belong to this family, I will focus a brief discussion onto the AAA+ family.

AAA stands for ATPases associated with a variety of cellular activities (Neuwald et al. 1999). The classic AAA family of proteins was involved in a variety of cellular activities in bacteria and eukaryotes that included proteins like the regulatory subunit of the eukaryotic 26S proteasome, proteins involved in membrane trafficking, mitochondrial

membrane assembly, signal transduction and mitotic spindle formation. These activities were typically carried out by chaperone proteins that assist the non-covalent assembly of proteins or protein complexes. Chaperones are considered "quality control" mechanisms that either help facilitate the proper folding of proteins or target misfolded or mistranslated proteins for degradation. The AAA designation changed to AAA+ after recent genomic searches significantly broadened the number of AAA family members to include "molecular matchmaking proteins", proteins that assist in substrate remodeling onto primed DNA (an example of a type of AAA+ protein that performs this is a clamp loader or a helicase loader) and many of these proteins include proteins involved in replication (*E. coli* γ- complex loads the β-clamp that slides along DNA during replication while bound to PolIII, Eukaryotic RFC that loads the PCNA (Proliferating cell nuclear antigen) clamp) (Neuwald et al. 1999; Davey et al. 2002).

The AAA+ family now also includes proteins like DnaA, DnaC, and NtrC-like transcription factors (FlbD described in this thesis). It also includes the subunits that form the MCM hexamer (the presumed eukaryotic helicase), three of the ORC subunits (the eukaryotic replication initiator protein) and the Clp family of chaperones (Fig. 8 and covered in Bacterial proteases section). The AAA+ proteins are associated in assembly (clamp loaders), remodeling (NtrC to remodel the closed complex to open complex in transcription, and DnaA to remodel DNA), and disassembly (chaperones like ClpA disassembles RepA dimers into monomers during replication of P1 bacteriophage (Pak and Wickner 1997; Neuwald et al. 1999)

The domain organization common to AAA+ proteins is shown in Fig. 6. The region from BoxVII to the end of Sensor II defines the AAA+ superfamily. Along with

the Walker A motif (or P loop) that is involved in ATP binding and the Walker B motif that is involved in ATP hydrolysis, there is a highly conserved arginine in the BoxVII motif that is believed to hydrolyze ATP by contacting its gamma phosphate, potentially, after a priming interaction (such as DNA binding by DnaC to release DnaB as described in the previous section). In addition, structural studies combined with biochemical studies have proposed mechanisms by which these AAA+ proteins oligomerize and consequently hydrolyze ATP within these higher ordered structures (Davey et al. 2002). In the case of one AAA+ heterohexamer, MCM (the presumed replicative helicase in eukaryotes), it is proposed that the interaction between a P-loop of one subunit with the conserved arginine of another subunit contributes to the ATP hydrolysis in the higher ordered complex (Davey et al. 2003). This study implies that there is a conserved mechanism among AAA+ proteins to mediate ATP hydrolysis when in a higher ordered complex, even if their roles are different and specific for their biological systems.

DnaA Protein Structure and Domain Organization

The key role played by DnaA in directing replication initiation begs for a better structural understanding of DnaA, its domain organization and the roles played by each domain in regulating DNA replication. It is also important to understand how DnaA-mediated replication is limited to once-per-generation. These two issues are addressed below.

A great aid to understanding mechanism of DnaA function has been the solving of the DnaA crystal structure (Erzberger et al. 2002) and its combination and corroboration

with genetic mutant studies. DnaA can be divided into four functional domains (Messer et al. 1999). Domain I (the first 1-86 amino-acids), is a self-oligomerization domain, and it includes residues that interact with DnaB helicase (Sutton et al. 1998; Seitz et al. 2000). Gluteraldehyde cross-linking experiments followed by immunoblots with mutant N-terminal DnaA and with a truncated N-terminal DnaA confirmed that the N-terminus is involved in oligomerization as these mutants failed to form oligomeric structures at the same rate and abundance as wild-type DnaA (Simmons et al. 2003).

DnaA Domain II (between Amino-acids 87-134 in *E. coli*) has a great variability amongst bacteria. It is very long in *Streptomyces lividans* (Majka et al. 1997), yet appears to be completely absent from the thermophilic bacteria, *Aquifex aeolicus*, which was used to elucidate DnaA crystal structure (Erzberger et al. 2002). The C-proximal portion of Domain II may also interact with DnaB, as antibodies raised against residues 111-148 interfered with DnaA-DnaB interactions (Marszalek and Kaguni 1994). However, this domain is the most variable, and therefore may be the least important to DnaA function amongst bacteria.

DnaA Domain III (between Amino Acids 130-350 in *E. coli*) contains AAA+ homologies including, the essential ATP-binding region, the Walker A-motif. It also contains the Walker B motif that is the intrinsic ATPase (Neuwald et al. 1999; Davey et al. 2002). The ATP-binding and ATPase activities of DnaA are required for *oriC* replication. How intrinsic ATP hydrolysis regulates DnaA will be discussed below. Many temperature sensitive alleles map to Domain III. For example, 6 of the 14 classic *dnaA* mutants mapped to a change in amino-acid 184 from Alanine to Valine (five contained an additional second mutation) (Skarstad and Boye 1994; Erzberger et al.

2002). This includes the classical *dnaA5 Ts* allele (Hupp and Kaguni 1993) as well as the *dnaA46 Ts* allele (Carr and Kaguni 1996). In addition, the classical *dnaAcos* allele is an intergenic cold-sensitive suppressor of *dnaA46*. *dnaAcos* causes over-initiation under a down-shift in temperature and it cannot bind ATP and ADP and thus cannot regulate precise replication (Katayama 1994; Simmons and Kaguni 2003). Clearly ATP binding and hydrolysis are very important for DnaA regulation in *E. coli*.

In addition to its critical role in nucleotide binding and hydrolysis, Domain III also plays a key role in oligomerization. This has been demonstrated by surface plasmon resonance where there was a reduced dissociation rate when a part of Domain III was included in the assay (Messer et al. 2001). As well, the structure of DnaA was modeled to accommodate a AAA+-oligomerization state of p97, a eukaryotic AAA+ protein that is involved in chaperone activity during membrane fusion (Zhang et al. 2000). AAA+ oligomeric proteins have a conserved mode of assembly where one protein projects amino acids from the Box VII motif into the nucleotide binding cleft of a partner protein with which it forms an oligomer. Specifically, an Arginine residue makes contact with the gamma phosphate of ATP in the adjacent protein. AAA+ proteins form similar oligomeric structures regardless of the ATP/ADP/AMP state. According to the modeling study comparing DnaA to p97, it was proposed that two highly conserved Arginines (R281 and R285 present in 66 out 67 DnaA homologues) project to a nucleotide binding cleft of an adjacent DnaA monomer (Erzberger et al. 2002). The only DnaA homologue that did not have this conserved residue was *C. crescentus*. This could be significant, because it implies that *C. crescentus* DnaA may not oligomerize in the same way as *E. coli* DnaA. Interestingly, *Cori* appears to have only one DnaA box and, unlike the *E. coli*

origin, is void of other strong putative DnaA boxes (Marczynski et al. 1995). Since oligomerization of DnaA may rely upon the proximity of DnaA boxes to each other in *E. coli*, it has been proposed that DnaA monomers once bound to these boxes may form a higher ordered structure by oligomerization like other AAA+ family members involved in replication (Davey et al. 2002).

DnaA domain IV (comprising the last 94 C-terminal amino-acids in *E. coli*) is primarily involved in DNA binding. The C-terminal domain is made up of a helix-turn-helix motif that is linked to a long connector helix (Erzberger et al. 2002). The authors performed a search to reveal the closest structural homologue to DnaA domain IV and identified the trp operon repressor. They therefore modeled DnaA binding to DNA on the trp repressor with DNA structure (Otwinowski et al. 1988). Their modeling study identified the DnaA signature motif in the helix-turn-helix and a basic loop region that is adjacent to the helix-turn-helix. These motifs contained amino acids, previously identified through mutational studies, that are required for DNA binding (Sutton and Kaguni 1997; Blaesing et al. 2000). In addition, the model predicted that for DnaA protein to selectively contact DNA, a conformation change is required so that Domain III and Domain IV do not clash with eachother. The authors propose that there is great flexibility between these two domains (Erzberger et al. 2002).

Most recently, a crystal structure was obtained where the *E. coli* DnaA binding domain IV was complexed to a 13 bp oligonucleotide that contains a DnaA box. This structure provides insights into how DnaA recognizes its DnaA boxes (Fujikawa et al. 2003). The helix-turn-helix (α3-α4-α5) domain binds to the major groove of DNA specifically making contact between the α5 alpha helix and the fourth T and sixth C in

the DnaA box TTATCCACA. Interestingly Threonine 435 of DnaA makes contact with the sixth C of the DnaA box and corroborates the mutagenesis of T435 study where a T435M mutant of DnaA lost DNA binding specificity (Sutton and Kaguni 1997). As well, they identified that Arginine 407 and Lysine 415 of α3 make contacts with the DNA backbone phosphate groups of the fourth T and the complementary T of the ninth A respectively, and Threonine 436 makes contacts with the phosphate backbone of the sixth C. All of these contacts are consistent with DnaA mutagenic studies in DNA binding (Blaesing et al. 2000). As well, contacts were recognized between residues in the α3 helix and the DNA minor groove. The authors also identified a 28° bend in DNA when complexed with DnaA protein (Fujikawa et al. 2003), corroborating DnaA's bending effect when studied in solution (Schaper and Messer 1995).

E. coli's strategies to limit replication to once and only once

Certainly DnaA in *E. coli* is a replication initiator, and the above discussions point to the mechanism used to initiate replication. However, bacteria must also regulate the frequency of replication initiation. *E. coli* has evolved at least three main strategies to regulate replication and these strategies target either the activity of DnaA directly, its free soluble concentration, its accessibility to DnaA boxes, and its ability to form open complexes. (Later, I will also review strategies employed by eukaryotes to regulate replication precisely and in co-ordination with the cell-cycle. *E. coli* utilizes three specific strategies to regulate DNA replication: sequestration of *oriC*, Regulated Inactivation of DnaA (RIDA) by the DNA polymerase sliding clamp component and the

Hda protein, and by titration of DnaA by high affinity binding sites within the *E. coli* genome (Reviewed in (Messer 2002) and (Boye et al. 2000)).

Sequestration of oriC

In *E. coli oriC* DNA is hemi-methylated following replication, and it is methylated on GATC sites by Dam methyltransferase in order to serve as a template for replication (Boye 1991a). Whereas methylation is rapid at most GATC sites around the chromosome, it is conspicuously slow (a 13 minutes delay following replication) around *oriC* and the *dnaA* promoter. This delay is caused by the "sequestration" mechanism which prevents the pre-mature initiation of replication (Campbell and Kleckner 1990; Lu et al. 1994). It was also observed that replication is inhibited *in vivo* when a hemi-methylated *oriC* template is transfected (Russell and Zinder 1987), but not *in vitro* (Boye 1991b). This argued that a new protein may restrict access to hemi-methylated *oriC* DNA and to the *dnaA* promoter. This protein was identified as SeqA. *seqA*$^-$ mutants caused extra replication while overexpressing SeqA suppressed the over-replication phenotype of the *dnaA*^{cos} mutant (Lu et al. 1994). In the absence of SeqA, initiation of replication can occur several times during each cell-cycle (Boye et al. 1996). Therefore, SeqA is a negative regulator, while DnaA is a positive regulator of replication.

How does SeqA interfere with the function of DnaA? Torheim and Skarstad identified that the SeqA protein inhibited open-complex formation but did not inhibit the binding of DnaA to *oriC* plasmids (Torheim and Skarstad 1999). The authors found that the topology of DNA changed when SeqA was bound to DNA, in that it did not allow Topoisomerase I to relax negative supercoils. Therefore, SeqA may inhibit DnaA

function without competing for its binding sites. Support for that notion comes from electron-microscopy work of DnaA complexes bound to DNA. Here, it was demonstrated that SeqA does not interfere with DnaA binding to the origin (Skarstad et al. 2000). It has also been suggested that even if SeqA does not interfere with the binding of DnaA to the origin, that it might affect its ability to form pre-priming complexes (Wold et al. 1998).

Further insights on how SeqA bound to *oriC* came from the crystal structure of the C-terminal SeqA-hemi-methylated DNA complex (Guarne et al. 2002). It was reported that SeqA binds to hemi-methylated GATC sites on DNA when there are at least 2 GATC sites on the fragment. It binds to each site asymmetrically, most probably due to the presence of the methylated adenine. SeqA needs cooperative interactions to stabilize its DNA-binding (Brendler and Austin 1999). The crystal structure revealed that the C-terminal half of SeqA makes hydrogen bonds and van der Waals interactions with the methylated Adenine, and that it makes additional contacts with the other bases and the DNA phosphate backbone. The crystal structure also revealed that SeqA can bind DNA as two dimers that make contacts with two other DNA-bound dimers. The authors propose that hemimethylated *oriC* then acts as a high-avidity complex for SeqA and restricts Dam methylase and DnaA function, but as replication proceeds and more hemi-methylated GATC's become exposed, available SeqA decreases to a critical concentration and ceases to sequester *oriC* (Guarne et al. 2002). SeqA is membrane associated (Shakibai et al. 1998). The motivation to find the membrane-associated components that bind hemi-methylated *oriC* stemmed from earlier work that found that hemi-methylated *oriC* DNA associated with the membrane (Landoulsi et al. 1990).

Therefore, the SeqA mechanism of preventing DnaA to form higher order structures by sequestering and maintaining a hemi-methylated origin bound to the membrane is one strategy that *E. coli* utilizes to regulate DnaA activity. Also, sequestration appears to limit *dnaA* transcription immediately after replication initiation and lower the number of newly synthesized DnaA molecules that are present immediately prior to initiation (Theisen et al. 1993).

Stimulation of intrinsic DnaA-ATP hydrolysis to regulate DnaA activity

As mentioned above, DnaA bound to ATP is able to initiate replication in an *in vitro* reconstituted system, whereas DnaA-ADP is not. This ATP/ADP nucleotide switch in DnaA is a means to regulate replication initiation. The importance of ATP hydrolysis for DnaA regulation was also shown by experiments that identified a soluble protein extract that could specifically inactivate the ability of DnaA to initiate chromosome replication *in vitro* (Katayama and Crooke 1995). The protein extract contained a 150 kDa fraction (IdaA – Inactivation of DnaA factor) that was unable to block over-initiation by the $DnaA^{cos}$ mutant (Katayama and Crooke 1995), which is unable to bind to nucleotide (Katayama et al. 1995).

RIDA or Regulatory Inactivation of DnaA is the conversion of DnaA-ATP to DnaA-ADP. This conversion of ATP to ADP involves DNA polymerase III β subunit and another protein fraction called IdaB, recently discovered to be the Hda protein (Kato and Katayama 2001). The β subunit of DNA Polymerase III, encoded by DnaN, is the sliding clamp component of PolIII and is functional for inactivation of DnaA-ATP when

it is in the presence of the other assembled components of DNA Pol III (specifically the γ complex which is involved in loading the β subunit onto DNA), the IdaB protein as well as DNA (Katayama et al. 1998). Interestingly, the conversion of DnaA-ATP to ADP required that replication be ongoing and therefore, dependent upon replication elongation (Katayama et al. 1998).

In accordance with this *in vitro* data, *in vivo* analysis of the *E. coli* cell-cycle demonstrated that DnaA-ATP levels increase around the time of replication initiation, but decrease rapidly after the initiation of chromosome replication (Kurokawa et al. 1999). The increase in DnaA-ATP was dependent on *de novo* protein synthesis, suggesting that newly synthesized DnaA binds to ATP. In the absence of DnaN, an accumulation of the ATP form of DnaA was observed *in vivo*, supporting the *in vitro* RIDA data (Kurokawa et al. 1999).

The Hda protein is also involved in RIDA and mediates the interaction of DnaA with the β subunit of DNA Pol III (Kato and Katayama 2001). The *hda* gene stands for homologous to *dnaA*, as its DNA sequence is very similar to the third domain of DnaA. The *hda* gene was originally identified as a multi-copy extragenic suppressor for a mutation in the *dnaN* gene (the β-subunit sliding clamp). The *dnaA* gene however could not serve as a multi-copy suppressor for *dnaN* and thus in spite of the sequence homology to *hda*, appears to play an opposite role to *hda.* Hda was found to be essential and a negative regulator of replication. Its role in replication is in limiting over-initiation as *hda* mutant strains accumulate DnaA-ATP and *oriC* chromosome DNA (Kato and Katayama 2001). The *hda*$^-$ protein extract was unable to support RIDA activity, but was complemented by the IdaB fraction previously described (Katayama et al. 1998).

Therefore, the Hda protein was identified as the active protein in the IdaB fraction (Kato and Katayama 2001).

The characterization of DnaA mutants which could bind ATP but not hydrolyse ATP (DnaA R334A and R334H), and which were refractory to RIDA and intrinsic hydrolysis demonstrated the importance of this domain for stabilization of a water molecule near the ATP binding pocket and its consequent role in ATP hydrolysis (Su'etsugu et al. 2001; Nishida et al. 2002). More importantly, these mutants both showed an over-replication phenotype and reflect the role of RIDA in regulating over-replication.

Sequestration limits the ability of DnaA to form open-complexes through topological changes to the origin due to SeqA binding, while RIDA limits the number of replication competent DnaA-ATP molecules by stimulating its intrinsic ATP hydrolysis. However, neither of these mechanisms serves to limit the number of DnaA proteins that could initiate chromosome replication in *E. coli*. The third known mechanism for limiting replication in *E. coli* is called titration of DnaA to high-affinity sites on the chromosome. .

Titration of DnaA by binding to high-affinity sites on the chromosome

It is believed that replication can occur only when active DnaA reaches a critical concentration. This is called "initiation potential". It is estimated that there are about 1000-2000 DnaA molecules in each *E. coli* cell (Hansen et al. 1991) and the concentration of these proteins in the cell remains fairly constant even at varying growth

rates. This is owed, in part, to the fact that DnaA can autoregulate its own transcription based on its intracellular concentration (Messer and Weigel 1997). However, only 40 or so DnaA molecules are required to bind to the origin in order to initiate replication, so how is the intracellular concentration of DnaA limited to prevent promiscuous replication initiation?

One mechanism that appears to function to regulate free DnaA protein concentration is titration via specific high-affinity DnaA binding sites. High affinity DnaA binding sites were discovered that could bind eight-fold more DnaA molecules than *oriC* (Kitagawa et al. 1996), and were called the *datA* locus to stand for "DnaA titration" (Kitagawa et al. 1998). A *datA* null mutant grows normally, exhibiting the same doubling times and growth rates in both minimal and rich media. However, the *datA* null mutant exhibited asynchronous initiations when grown in all media as confirmed both by FACS analysis of total DNA content and by Southern hybridization to measure *oriC/terC* ratios. This asynchronous replication is mediated through inability of *datA* to bind to DnaA as mutations in a DnaA binding site created the asynchronous replication (Kitagawa et al. 1998).

E. coli has 308 consensus DnaA boxes throughout its genome. Use of a solid-phase DNA binding assay identified which of these boxes are important for binding DnaA with high affinity (Roth and Messer 1998). The solid phase assay made use of biotinilated DnaA domain IV to isolate chromosomal DNA fragments with high affinity to DnaA domain IV. Among the high affinity sites, only the *datA* site on the chromosome affects the timing of replication initiation as mutations in seven other high-affinity sites had no effect on replication (Ogawa et al. 2002).

The competition model for co-ordination of chromosome replication with cell growth

The combination of SeqA sequestration, RIDA and titration has been proposed to form the "competition model for the coordination of chromosome replication with cell-growth." This model attempts to explain how *E. coli* initiates replication at a particular time and only when the cells reach a critical map (Donachie and Blakely 2003).

This model assumes that DnaA-ATP and DnaA-ADP compete for binding to DnaA boxes at *oriC*. All newly synthesized DnaA molecules bind to ATP due to the predominance of ATP molecule concentration in the cell. All new DnaA-ATP molecules, following initiation of replication, are converted to DnaA-ADP molecules by RIDA. This conversion, coupled with the sequestration of the origin and the *dnaA* promoter during the "eclipse period", serves to limit the amount of DnaA-ATP molecules present in the cell immediately following replication, and conversely maintains high total DnaA-ADP levels. As cells grow, after the eclipse period, new DnaA molecules are synthesized and new DnaA-ATP molecules start to accumulate. As the conversion of DnaA-ATP to ADP is not stimulated during ongoing replication, DnaA-ATP concentration approaches equilibrium with DnaA-ADP. DnaA-ATP then competes for binding with DnaA-ADP, and at a critical concentration, DnaA-ATP then can initiate replication, presumably by the *in vitro* model of DnaA dependent replication initiation. In addition, titration of both forms of DnaA is removed as chromosome numbers increase during replication elongation. This is important since new DnaA-ADP is not actively synthesized (from DnaA-ATP hydrolysis) in the cell following the eclipse period, and some of the DnaA-ADP binds to the finite number of high-affinity DnaA binding sites in

the chromosome and is titrated prior to new DnaA-ATP ever being synthesized. Therefore, there are less titration sites for new DnaA-ATP to bind to since mostly free DnaA-ADP and some old DnaA-ATP occupies these titration sites. However, DnaA-ATP is constantly being synthesized following eclipse period and as chromosome replication continues, free DnaA-ATP concentration approaches the total number of free DnaA-ADP and can then compete for binding to the binding sites in *oriC* (Donachie and Blakely 2003).

DNA REPLICATION IN EUKARYOTIC CELLS: ANALOGOUS FEATURES AND MECHANISMS.

Eukaryotes also form special protein and DNA complexes, but at many replication origins on the genome. These complexes are called the pre-replicative complexes (pre-RCs) and also involve an ordered assembly of several replication factors. In addition to the ordered assembly of these factors, the pre-RC awaits cell-cycle cues to initiate replication and to terminate the activities of the replication proteins until the next cell-cycle. *C. crescentus* makes use of the DnaA replication initiator in promoting replication but also receives cell-cycle cues through CtrA and other signaling molecules. In this section, I will address the proteins that assemble to form the pre-RC in eukaryotes and draw comparisons to prokaryotic replication initiator proteins. I will also address the cell-cycle control of replication timing and draw comparisons back to *C. crescentus* and *E. coli* cell-cycle control.

The licensing factor hypothesis

In eukaryotes, replication initiation is limited to the S phase and is dependent upon protein complexes that assemble in G1 phase. Following S phase, there is a preparatory phase called G2, prior to M phase (cell division) (Fig. 8). Experiments by Blow and Laskey performed on *Xenopus* extracts suggested the "licensing hypothesis" which, in part, explains why replication initiation occurs only once in the cell-cycle, during early S phase and not after or before (Blow and Laskey 1988). When protein synthesis was inhibited in *Xenopus* extracts, the nuclei only undergo a single round of replication

without division and arrest in G2 phase as opposed to undergoing multiple rounds of replication and division under normal conditions. However, when these arrested nuclei were permeablised with detergent, this allowed replication to proceed without the need for proceeding through M phase and dividing. The authors argued that a key protein that promotes replication initiation must become available to the nucleus after the cells complete M phase and the nuclear membrane breaks down. They also argued that since G2 phase cells lack this ability to replicate, they must also lack this key replication promoting protein. They called this protein "the licensing factor"(LF) and they hypothesized that LF is consumed (or inactivated) by replication and that the timely initiation of replication must only occur after M phase, when the licensing factor would become accessible to the nucleus (Blow and Laskey 1988). Therefore, LF is required for replication initiation and is also consumed by replication. LF is placed in the cytoplasm and enters and binds DNA in M phase following nuclear breakdown. Please see Figure 7.

As will be demonstrated below, replication initiation in eukaryotes is complex and the "licensing" is now viewed as the sequential loading of MCM proteins with the help of Cdt1 protein onto origins that are pre-bound by ORC and Cdc6. This provides the license to initiate replication and forms what is called the pre-replicative complex (pre-RC). After MCM is loaded, Cdc6 is removed and after initiation of replication, Cdt1 is removed from the origin and degraded by an ubiquitin specific system, to ensure that licensing only occurs after M phase and not before (Zhong et al. 2003). Licensing of the origin, in combination with cell-cycle cues through cyclins and Cdks coordinate to ensure that replication is properly temporally regulated.

The components of the pre-replicative complex and their regulation:

In the simple unicellular eukaryotes like the budding yeast, *Saccharomyces cerevisiae*, replication requires four 10-15 base-pair sequences spread over 100-150 bp, called autonomously replicating sequences (ARSs). All ARS elements have a highly conserved 11-bp ARS consensus sequence (A-element) and several B-element (involved in unwinding) found within the sequence (Bell 2002). The minimum required origin sequence varies with different eukaryotes and in some cases there does not appear to be a conserved sequence requirement for *cis*-acting elements at all, as in some metazoan examples like *Xenopus* embryos. The formation of the pre-replicative complex (pre-RC) at origin sites is extremely important in initiating replication within the proper time in the eukaryotic cell-cycle, and it is obviously important to select the correct replication site from which to initiate replication. The protein that selects the site of initiation is the origin recognition complex (ORC) (Bell 2002).

The Origin Recognition Complex and comparison to DnaA

The hexameric Origin Recognition Complex, composed of six different protein subunits, recognizes the A-element in *S. cerevisiae* origins and is conserved in all eukaryotes (Bell and Dutta 2002). The ORC complex requires all six subunits for replication and viability although only Orc1p-Orc5p and not Orc6p are required to bind to DNA in *S. cerevisiae.* However, ATP binding of ORC is absolutely essential for DNA binding (Bell and Stillman 1992), and ORC can also bind to single stranded DNA (Lee et al. 2000). Three ORC proteins are AAA+ family members and ATP hydrolysis is absolutely essential for

ORC function. Like *E. coli* DnaA, *S. cerevisiae* ORC can also bind and recognize single stranded DNA, although its specificity for ssDNA does not depend on specific sites (DnaA-ATP boxes), but rather on length of ssDNA (80 bases vs. 30 bases) and is independent of the ATP-bound state (Bell and Dutta 2002). ORC is in the ATP bound state throughout its association with double stranded DNA. Single-stranded DNA stimulates the ATP-hydrolysis of ORC and this ATP hydrolysis is believed to trigger a transition between replication initiation in eukaryotes and replication elongation (Lee et al. 2000). The role of ORC as a binding protein is reminiscent of DnaA and its role in binding single-stranded DNA is similar to that exhibited by DnaA-ATP. Interestingly, the DnaA protein is triggered to hydrolyze its ATP in order to prevent re-initiation and ORC hydrolysis of ATP is believed to be a trigger from replication initiation to elongation (Bell 2002). Also like DnaA, ORC is bound to DNA throughout the yeast cell-cycle, and it is ORC that recruits other proteins to form higher-ordered complexes with the Cdc6 protein, Cdt1 protein, MCM2-7 and finally Cdc45 protein. The role of these proteins in replication control will be covered in greater detail.

An interesting observation came from studies that looked upon association of ORC subunits with chromatin during the cell-cycle. Whereas *S. cerevisiae* ORC appears to constitutively associate itself with the origin, *Homo sapiens* Orc1p has been shown to dissociate from chromatin during the end of S phase and is degraded following replication initiation (Kreitz et al. 2001; Mendez et al. 2002). In addition, the Hamster Orc1p subunit is not associated with chromatin in M phase (Natale et al. 2000). Hamster Orc1p is ubiquitinated during S phase, and then deubiquitinated and rebound to chromatin during G1 to S transition (Li and DePamphilis 2002). The ubiquitinated form

of Orc1p is targeted for degradation by the 26S proteosome during S phase. Orc1p removal during the S phase may prevent multiple rounds of replication initiation. Therefore, proteolysis appears to be a mechanism of regulating some ORC subunits during some eukaryotic cell-cycles and not others. Interestingly, proteolysis has not been a mechanism that is utilized by the *E. coli* prokaryotic DnaA model system to regulate replication, however, as will be shown in Chapters 2 and 3 of this thesis, proteolysis is a mechanism that regulates *C. crescentus* DnaA. This demonstrates the value of studying comparative model systems to identify mechanisms of regulating replication initiators.

Cdc6 Protein

The affinity of ORC binding to its binding sites appears to be regulated in part by another protein, Cdc6p (Mizushima et al. 2000). Cdc6p is also a member of the AAA+ family of proteins and is very homologous to Orc1p. Its ATP hydrolysis and ATP binding is also important for its interaction with ORC and is believed to be required to limit the binding of ORC to non-specific DNA (Mizushima et al. 2000). Cdc6p also causes a conformational change in ORC and this is believed to help recruit the MCM complex, to be described in more detail below, to the origin and to be the key step in regulating replication intiation and providing licensing.

The ATP binding and hydrolysis of Cdc6 is important for its role in loading the MCM complex. Mutations in both the Walker A box and the Walker B box are unable to support cell growth and a mutation in the Walker B box (important for ATP hydrolysis) prevented the loading of a protein comprising the MCM complex (Weinreich et al. 1999). As will be described, the MCM complex is believed to be the eukaryotic helicase and in

this case, the Cdc6 protein is considered to be helicase loader. Therefore, the importance of Cdc6 ATP hydrolysis in loading the helicase has been proposed to be analogous to the role of DnaC helicase loader in loading the DnaB helicase in bacterial models (Lee and Bell 2000).

How is Cdc6p regulated throughout the cell-cycle? Cdc6p is unstable. It is synthesized late in G1 phase just prior to S phase (Piatti et al. 1995) and is absent from the cells immediately following replication initiation. It is targeted by cyclin-dependent kinase phosphorylation and ubiquitin-dependent degradation. There are three modes of Cdc6 proteolysis throughout the cell-cycle (Drury et al. 2000). The first mode of Cdc6 degradation occurs in early G1 phase, where Cdc6 has a 15 minute half-life. This degradation is independent of the main ubiquitination proteins (SCF and APC) as well as the Cdc28 cyclin-dependent kinase. The second mode of Cdc6 degradation occurs near the end of G1 phase, where Cdc6 has a short half-life of approximately 2 minutes. This degradation requires SCF for polyubiquitination and 26S proteasome targeting, and it also requires the presence of the Cdc28 cyclin-dependent kinase (and its association with G1 cyclins but not S cyclins). Therefore, this rapid degradation occurs during G1 phase and not S phase. The third mode of Cdc6 degradation occurs later in the cell-cycle in M-phase, where Cdc6 has a 15 minute half-life. This degradation also requires SCF for polyubiquitination and is dependent upon Cdc28, but apparently is different from the second mode because the phosphorylation occurs on a different site of Cdc6 (Drury et al. 2000; Perkins et al. 2001).

Cdt1 Protein

It was originally discovered in the fission yeast *Schizosaccharomyces pombe* that an essential gene for replication was *cdt1* (Hofmann and Beach 1994). In fission yeast, Cdt1 is able to form a complex with Cdc6 (Cdc18 in *S. pombe*) and accumulates in the nucleus in G1 (Nishitani et al. 2000). Cdt1 accumulates in the same cell-cycle pattern as Cdc6 and is degraded during S-phase in (Li et al. 2003); it is believed to co-operate with Cdc6 to promote DNA replication initiation by recruiting MCM complex, but is not believed to be important for the later stages of replication (Nishitani et al. 2000). Cdt1 is conserved amongst lower and higher eukaryotes and appears to provide the license for cells to initiate replication after mitosis (Bell and Dutta 2002). Cdt1 is bound tightly by a protein called Geminin (Wohlschlegel et al. 2000), an inhibitor of DNA replication in higher eukaryotes (McGarry and Kirschner 1998). Geminin is absent during G1 phase, but starts to accumulate in S, G2 and M phases. Geminin is degraded by APC mediated ubiquitination during M phase (McGarry and Kirschner 1998). The absence of Geminin during G1 phase thereby allows Cdt1 to license the origin by interacting with Cdc6 to recruit the MCM complex, the critical event in replication initiation in eukaryotes and the presence of Geminin after initiation of replication is believed to prevent intitiations to occur after the G1 to S transition.

Mcm2-7 Protein Complex, Cdc45, Cyclin-dependent Kinases and Cdc7-Dbf4b

The MCM protein complex is made up of six subunits that are all AAA+ family members (Tye 1999). A recent study proposed a model for how the MCM heterohexamer would arrange itself and identified an architecture where the catalytic Arginine finger motif in

one subunit would catalyze the hydrolysis of ATP bound to a P loop motif in an adjacent monomer (Davey et al. 2003). Each of the MCM subunits is unique and essential for *S. cerevisiae* (Tye 1999). The MCM proteins are recruited to the nucleus during G1 and S phase when in a complex due to their nuclear localization sequences and exported out of the nucleus in S phase, G2 and M (Young et al. 1997; Nguyen et al. 2000). It is believed that the MCM complex functions as a replicative helicase like DnaB in *E. coli*, however this is not entirely clear in eukaryotes (Lee and Bell 2000; Davey et al. 2002; Bell and Dutta 2002).

The MCM complex is present in the preRC and bound to the chromatin (Zou and Stillman 1998). It then interacts directly with two other proteins on the chromatin, Cdc45 (Zou and Stillman 1998) and Cdc7-Dbf4 (Lei et al. 1997). The Cdc45 protein is believed to be important for interacting with DNA Polymerase α and the primase, DNA Polymerase ε and Cdc7-Dbf4 (Tye 1999). Cdc7-Dbf4 is a kinase that, in concert with another kinase, Cdc28-Clb5, promotes S phase and recruits DNA polymerase α to the replication origin. Cdc45 is required for entry into S phase and in addition, Cdc45 is important for elongation of replication after initiation (Tercero et al. 2000). Cdc7-Dbf4 is a serine/threonine kinase (Cdc7 is the catalytic subunit and Dbf4 is the regulatory subunit that is expressed in G1 phase until the end of S phase) that phosphorylates a specific MCM protein. It is believed that this phosphorylation of MCM stabilizes its interaction with Cdc45 and allows for replication initiation (Bell and Dutta 2002).

The second kinase involved is Cdc28-Clb5, where Cdc28 is the kinase and Clb5 is one of the S-phase cyclins. It can promote entry into S-phase and allow for loading of Cdc45 late in G1 phase at a yeast origin, but cannot do so when Cdc28 is coupled to a G1

phase cyclin (Zou and Stillman 1998; Zou and Stillman 2000). However, it is unclear as to which pre-RC complex protein is the target of Cdc28-Cln5 phosphorylation. Cdc28-Cln5 is functional in G1 to S transition due to the degradation of the Cyclin-dependent kinase inhibitor, Sic1p (which normally binds to S-phase Cdc28 and restricts is activity). This release of functional Cdc28-Cln5 is due to Sic1p phosphorylation by Cdc28-G1 cyclin complex and the subsequent ubiquitin-dependent proteolysis of Sic1p (Hoyt 1997). Therefore, even if the mechanism of action is quite different, the Sic1p protein may be looked upon as being analogous to *C. crescentus* CtrA in that it is a negative regulator of replication in G1 phase and that its proteolysis allows for replication initiation to occur.

Taken together, the eukaryotic mechanism of replication requires a co-ordinated set of actions: The ORC complex sets the specificity for where replication can initiate from, but the timely set of events is regulated by multiple phosphorylation and proteolytic events. These events eventually lead to the timely recruitment of Cdc6 and Cdt1 to the origin and the eventual loading and recruitment of MCM complex to the yeast origins. The MCM complex then associates with Cdc45 and the phosphorylation by Cdc28-Clb5 and Cdc7-Dbf4 then allows for the entry into S phase by further recruitment of primase and other polymerases that allow for leading and lagging strand synthesis.

Restricting replication to only once per cell-cycle requires multiple events that include proteolysis of Orc1p, ORC ATP hydrolysis, the binding of Geminin to Cdt1 in higher eukaryotes, as well as the timely phosphorylation and proteolysis of Cdc6p. The nuclear export of MCM during S phase and its import following M-phase also contributes to temporal replication.

How does the eukaryotic mechanism of replication regulation compare to E. coli?

Whereas several different proteins are negatively regulated in eukaryotes, only DnaA protein has been identified as the target for negative regulation in *E. coli*. DnaA is analogous to the ORC complex and its ATP hydrolysis is stimulated through RIDA following replication initiation. ORC also hydrolyses ATP to ADP. The SeqA system of sequestering the origin during the eclipse period to the membrane does not appear to be a conserved mechanism in prokaryotes and is believed to be specific for only some bacteria. It is unlikely that *C. crescentus* has SeqA. The datA region of DnaA titration in *E.coli* also is specific for *E. coli*, and there doesn't appear to a region with analogous high affinity binding sites for ORC in eukaryotes. Whereas many proteins are regulated in eukaryotes, only the DnaA protein is regulated in *E. coli* to limit replication to once and only once. However, we know comparatively little about DnaA and the regulation of chromosome replication in other bacteria.

How do the cell-cycle controls of replication in eukaryotes compare to C. crescentus?

Replication control in *C. crescentus* is primarily in the hands of CtrA and DnaA (Chapter 2). *C. crescentus* DnaA presumably undergoes intrinsic ATP hydrolysis based on the presence of putative RIDA proteins in the genome (W. Spencer and G.T. Marczynski, unpublished data) and conserved Walker motifs in DnaA sequence. However, unlike *E. coli* DnaA, *C. crescentus* DnaA is targeted for proteolysis like hsORC (Chapter 3 and 4). In addition to focusing control upon DnaA, *C. crescentus* also utilizes the CtrA protein to regulate replication. CtrA is phosphorylated in the cell-cycle and is also degraded just prior to the start of S-phase. This is analogous to Sic1 protein that is phosphorylated and

consequently degraded just prior to S-phase. Eukaryotes utilize phosphorylation by cyclin-dependent kinases that are present at different times of the cell-cycle to trigger transitions into specific cell-cycle stages. In *C. crescentus* specific kinases are differentially localized at different cell-cycle stages to help regulate cell-cycle events. *C. crescentus* cell-cycle is more complex than that of *E. coli* and it is not surprising that like eukaryotes, the limitation of replication in *C. crescentus* is placed in the hands of several proteins and that the mechanism of limiting DnaA activity is also apparently more complex.

BACTERIAL PROTEASES

As described above in discussing the *C. crescentus* cell-cycle, proteolysis ensures that unwanted proteins are not present at improper times, and examples of selective proteolysis included McpA, CcrM and CtrA. In this section, I will discuss specific bacterial proteases that are used for selective protein degradation. I will also focus upon how bacteria use proteases in response to environmental stress and nutrient limitation. In general, bacterial proteases can recognize a protein for degradation and then processively degrade that recognized substrate. This is different from eukaryotic proteases that recognize ubiquitin tagged proteins as substrates for degradation through a single 26S proteasome. In bacteria, the degradation is provided by the protease that is either equipped with or partnered with an ATPase chaperone that provides the substrate specificity. 90% of all bacterial proteolysis is ATP dependent and 80% of these are degraded by ClpXP and Lon (Gottesman and Maurizi 1992). An ATP-independent protease, DegP functions in the periplasm of Gram-negative bacteria to respond to pH, osmotic, elevated temperature and hydrogen peroxide stress (Hengge and Bukau 2003). Interestingly, it can switch between its role as a chaperone and protease at elevated temperatures (Spiess et al. 1999; Hengge and Bukau 2003). This section will only deal with ATP-dependent proteases. Please refer to Figure 7 for a comparative schematic of the bacterial proteases.

The ClpXP protease

ClpX, an AAA+ ATPase, is the ATP-dependent chaperone that provides some of the specificity in ClpP protein degradation. The ClpP proteolytic core is a barrel-like structure made up of two ClpP heptamers that hydrolyze proteins after hydrophobic residues. The structure of the ClpP protease was solved and it identified the serine active site within the center of the protease (Wang et al. 1997). The ClpX chaperone component is made up of two sets of hexamers in a ring formation. This ClpX ring is bound to either both ends or only to one end of the ClpP protease. Interestingly, the ClpXP protease may be either polar (1:1 ClpX: ClpP) or bipolar (2:1 ClpX: ClpP). For bipolar ClpXP, even if substrate is bound to both ClpX sides, only one substrate protein can be translocated into the ClpP core at a particular time (Ortega et al. 2002).

Whereas ClpP can degrade short peptides on its own, it cannot degraded folded proteins and requires ClpX mediated ATP hydrolysis to unfold the protein and then spool or translocate the polypeptide into the proteolytic core. ClpX has only one Walker Box motif (Walker A and Walker B), which distinguishes it from ClpA that has two Walker Box motifs (Fig. 8). As will be described below, the ClpAP and ClpXP proteases differ in substrate specificity. However, both ClpA and ClpX have a ClpP recognition motif composed of [LIV]G[FL] that allows it to recognize and dock with ClpP (Kim et al. 2001).

ssrA/tmRNA tagging for degradation

The ssrA/tmRNA quality control system in *E. coli* tags partially synthesized proteins on stalled ribosomes with a specific degradation signal so that the translated polypeptide acquires a degradation tag at the C-terminus (Keiler et al. 1996). This degradation signal is provided by the *ssrA* tmRNA molecule which has the components of both mRNA and tRNA (Keiler et al. 1996). The tRNA component of *ssrA* acquires the growing polypeptide chain in the ribosome and then proceeds to add its 11 amino-acid (alanine-rich) tag that is encoded in its mRNA component to the nascent polypeptide chain. The ssrA tagged polypeptide is then degraded mostly by both ClpAP and ClpXP cytoplasmic proteases although other proteases also contribute to their degradation (Gottesman et al. 1998). SsrA-tagged polypeptides were shown to be stabilized *in vivo* by *clpA*$^-$, *clpX*$^-$ and *clpP*$^-$ mutants and *in vitro* to be degraded by ClpXP and ClpAP (Gottesman et al. 1998). Interestingly, ClpX and ClpA recognize the tags independently, however ClpX and its specificity factor SspB (described below) were able bind to the same sites on the SsrA tag as ClpA, thereby modulating each other's activity (Flynn et al. 2001).

Trans-targeting

The ssrA/tmRNA system can also degrade full-length proteins. In Mu transposable bacteriophage, the Mu repressor, whose degradation (and consequent de-repression) is important for lytic phage growth is also dependent upon the *ssrA*-tagged peptide under carbon starvation and stationary phase onset (O'Handley and Nakai 2002). Interestingly, in this system a phenomenon called trans-targeting occurs where a mutant form of the Mu repressor acquires a C-terminal *ssrA* tag and can then oligomerize with a non-mutant

form and targets the non-mutant form for ClpP-dependent degradation (O'Handley and Nakai 2002).

Specificity factors for ClpXP

Whereas tagging by *ssrA* provides a partial explanation for ClpXP specificity, the discovery of a ribosome-associated protein, SspB provided even greater enhanced specificity towards ClpXP degradation (Levchenko et al. 2000). Analytical centrifugation identified that SspB sedimented with a molecular weight of a dimer and when bound to ClpX, SspB stimulated ClpX intrinsic ATP hydrolysis, allowed it to bind more tightly to its substrate and allowed it be proteolyzed by ClpXP at a lower concentration (Wah et al. 2002). As well, the ternary complex with ClpX hexamer was determined to contain an SspB dimer and two *ssrA*-tagged protein monomers (Wah et al. 2002). SspB is an example of a specificity factor for ClpXP and this is significant for *C. crescentus* because both McpA and CtrA are hypothesized to require a specificity factor for their degradation (Tsai and Alley 2001).

Common features of proteins recognized by ClpXP

A non-proteolytic *clpP* mutant called $clpP^{trap}$ was used to find proteins that were translocated into ClpP in a ClpX-dependent manner. Any trapped protein was then co-purified with $ClpP^{trap}$ and analyzed. From this study, approximately 60 ClpX substrates were identified. Analysis of the substrates suggested that there were five specificity determinants for ClpXP degradation. Two of the determinants mapped to the C-terminal

domain, with one resembling MuA C-terminus RRKKAI and the other resembling the ssrA tag (Flynn et al. 2003). The other determinants mapped to the N-terminal domain and were segregated into three groups. The proteins recognized by ClpXP were sorted into several categories. These groups included transcription regulators like sigma factors, ribosomal proteins, chaperones, motility and transport proteins and metabolism and energy production proteins (Flynn et al. 2003). The significance of targetting sigma factors and ribosomal proteins will be discussed in the proteolysis during stress and starvation section below.

Lon protease

The Lon serine protease is a single polypeptide. The serine active site is near the C-terminus and the ATPase domain is in the middle of the protein. The ATP component of Lon is important in destabilizing secondary structures of proteins as it was shown that compact proteins are refractory to Lon proteolysis, yet become sensitive to Lon proteolysis in an ATP independent manner (Van Melderen et al. 1996). Lon protease is phylogenetically conserved. A Lon homologue was found in humans where it appears to have a mitochondrial localizing tag (Wang et al. 1993) as well as in yeast (van Dijl et al. 1998), and most bacteria (Wright et al. 1996). Lon is not essential in *E. coli* but is important for exit out of the SOS response to DNA damage as it targets degradation of SulA, a repressor of cell division (Higashitani et al. 1997). Lon is also not essential in *C. crescentus,* but it selectively degrades CcrM at the correct point of the cell-cycle (Wright et al. 1996). During starvation, *E. coli* Lon plays a very important role in degrading ribosomal proteins and will be discussed below (Kuroda et al. 2001). In the development

bacterium, *Myxococcus xanthus,* two Lon homologues are found. The *lonV* gene is essential for vegetative *M. xanthus* growth (Tojo et al. 1993b) whereas the *lonD* gene is dispensable for vegetative growth but is essential for development (Tojo et al. 1993a).

Lon and ClpXP proteolysis during stress and starvation:

Bacteria have precise mechanisms to respond to nutrient limitation and other stresses. In this section, I will focus upon two distinct mechanisms that regulate proteolysis. The stabilization of the stationary phase sigma factor by regulating the phosphorylation state of its specificity regulator for ClpXP is one mechanism, whereas the degradation of ribosomal proteins in response to nutrient limitation by ClpXP and by Lon is another mechanism.

Stationary Phase σ^s:

The σ^s protein is the sigma factor induced under stress conditions for *E. coli* such as reduced growth rate, high cell density, low temperature and high osmolarity. This is reviewed in (Hengge-Aronis 2002). This sigma factor directs the transcription of more than 70 genes that can then deal with these stresses. However, it is the regulation of σ^s by ClpXP proteolysis that will be the focus of this section.

σ^s cannot be degraded by ClpXP directly but requires the help of a recognition factor, RssB. RssB is a two-component response regulator and null *rssB* stabilizes an otherwise highly unstable σ^s. Phosphorylation of RssB is believed to facilitate the interaction between RssB and σ^s. The RssB regulator in complex with σ^s makes contact with ClpXP and then RssB is released from the complex. Under high osmolarity, carbon,

nitrogen and phosphorus starvation, high temperature, and low pH, the proteolysis of σ^s is inhibited. This is an interesting situation since it allows for multiple levels of control in regulating the degradation of σ^s, in other words many regulatory inputs control σ^s. For example, the level of control could be regulated at the level of an unidentified cognate histidine kinase for RssB or at its unidentified phosphatase since controlling the phosphorylation state is important in the binding of RssB with σ^s. As well, carbon starvation may act directly upon the ClpXP protease and not upon RssB phosphorylation since the RssB:: σ^s complex forms normally under these conditions, yet σ^s proteolysis is effected (Becker et al. 2000).

Amino-acid starvation and ribosomal protein degradation

E. coli cells starved for amino-acids accumulate polyphosphate which in turn influences proteolysis (Kuroda et al. 2001). Polyphosphate (PolyP) is a linear polymer that accumulates when *E. coli* cells enter stationary phase. In addition to its role as a storage molecule, PolyP has many vaguely defined roles identified with survival during stress and starvation. The synthesis of PolyP was associated with amino-acid starvation when a mutant for the synthetic enzyme responsible for PolyP (PolyP kinase) was shifted to a poorer growth media (Kuroda et al. 1999). The shift to the poor growth medium resulted in a lag in growth that was reversed when excess amino-acids were added. This suggested that lack of PolyP was responsible for growth lag and that PolyP promotes the acquisition of amino acids. In addition, *lon*$^-$ and *clpXP*$^-$ showed a similar lag when shifted to a poor medium and this was reversible by addition of amino-acids (Kuroda et al. 2001). Lon binds to PolyP and the addition of protein fractions to Lon and PolyP

identified specific ribosomoal proteins that were degraded by Lon only in the presence of PolyP. The S2 ribosomal protein is associated with the 30S subunit, and it was shown that Lon can only degrade this protein when PolyP is present (Kuroda et al. 2001). Therefore, this data suggests that starvation, as signaled by the accumulation of PolyP, stimulates Lon protease to degrade abundant ribosomal proteins in order to release amino-acids for other cell functions (Kuroda et al. 2001).

Interestingly, in a separate *in vivo* study aimed to identify substrates recognized directly by ClpXP, six specific ribosomal proteins all part of the 50S ribosomal subunit were shown to contain domains that are targeted for ClpXP degradation (Flynn et al. 2003). The authors rationalized that these proteins were trapped during late exponential growth and suggest that ClpXP also targets ribosomal proteins for degradation during starvation and releases their amino-acids for energy and metabolism.

ClpAP / ClpCP protease

The ClpA and ClpC chaperones are very homologous. ClpC was found to be the *B. subtilis* Clp100/Hsp homologue to ClpAP. They both contain two AAA ATPase domains that each contains a Walker A and Walker B motif. In addition, both of these proteins have ClpP recognition motifs in the C-terminus adjacent to the second ATPase domain; this is unlike a very similar chaperone ClpB which does not contain this motif (Dougan et al. 2002). ClpA can unfold native proteins in the presence of ATP (Weber-Ban et al. 1999) and can direct these unfolded peptides into ClpP (Reid et al. 2001). ClpAP can complex with an adaptor protein, ClpS and this association has recently been crystallized (Zeth et al. 2002). This study identified two residues that are important for ClpA

interaction with ClpS and for formation of a ternary complex with ClpP. In addition, the binding of ClpS to ClpA mediates a switch in substrate specificity from degrading primarily ssrA-tagged proteins to changing substrate specificity and then degrading aggregated proteins (Zeth et al. 2002).

In addition, the ClpC chaperone must bind to an adaptor protein, MecA, in order degrade a competence transcriptional regulator, ComK (Turgay et al. 1998). ComK controls the transcription of genes that encode proteins (like DNA transporters) that allow *B. subtilis* to uptake naked DNA (induce competence), an ability that *B. subtilis* acquires during late logarithmic phase. ComK positively activates its own transcription, but this role is inhibited when it is in a ternary complex with MecA and ClpC. During high cell density, as a response to quorum sensing, the ComS protein becomes synthesized and dissociates the ternary MecA:ComK:ClpC complex. The ClpCP protease can degrade ComK when escaping competence but only in the presence of MecA (Turgay et al. 1998). However, ClpCP can degrade MecA when ComS is present in response to quorum sensing, allowing ComK to be functional and therefore differential proteolysis can rapidly regulate the onset of an adaptation to environmental stress. This is another example of how specificity factors help to regulate the activity of proteases to degrade key global regulators depending upon the environmental signals that the cell receives.

HslUV / ClpYQ protease:

The ClpYQ protease is a hybrid protease. The ClpY chaperone is very similar to ClpX in that it has a single AAA motif but does not contain a ClpP recognition motif (Kim et al. 2001). Instead, its partner protease is ClpQ which is a threonine protease. Interestingly,

the ClpYQ protease appears to be redundant to Lon for substrate recognition as it appears to target the similar substrates for degradation (Wu et al. 1999b). The ClpYQ protease is homologous to the eukaryotic proteasome 20S subunits (Rohrwild et al. 1996) and its structure has been solved and determined to structurally resemble the 20S subunits of the 26S proteasome in eukaryotes (Bochtler et al. 1997). The least is known about HslUV relative to the other bacterial proteases.

FtsH protease:

Like the Lon protease, FtsH (HflB) is a single polypeptide that contains the AAA motif. Interestingly, FtsH is a metalloprotease and requires zinc for its stimulation. FtsH also contains two membrane spanning domains that anchor it to inside of the cytoplasmic membrane (Tomoyasu et al. 1995). The Zinc binding site and the AAA motif of FtsH are exposed within the cytoplasmic domain and the crystal structure revealed that FtsH ATPase domain forms a hexameric ring like the other AAA proteases (Niwa et al. 2002). FtsH is ubiquitous amongst bacteria and is essential in *E. coli* but not in *B. subtilis* for its vegetative growth (Deuerling et al. 1997). However, in *B. subtilis*, FtsH is important for spore formation and adaptation to high temperatures (Deuerling et al. 1997). In *C. crescentus,* FtsH is not essential, but is important for survival during stationary phase and in modulating the heat shock response (Fischer et al. 2002). Another main difference amongst FtsH in comparison to ClpXP or the other proteases mentioned is that it lacks a good "unfoldase" activity, and cannot degrade thermo-stable proteins. Instead, FtsH targets proteins that have low thermodynamic stability and that are not completely

"folded" (Herman et al. 2003). FtsH can also target proteins with SsrA tags especially at elevated temperatures (Herman et al. 1998).

The most is known about how FtsH degrades the cytoplasmic heat shock sigma factor, σ^{32}. This heat shock sigma is brought to FtsH for degradation by DnaK/J chaperone system, but at elevated temperatures, the heat induced misfolded proteins are believed to titrate away these chaperones and thereby interfere with the delivery of σ^{32} to FtsH (Tomoyasu et al. 1998). This increased stability of σ^{32} partly accounts for the increased transcription of heat-shock (σ^{32}) genes. Although, FtsH is ubiquitous amongst bacteria, relatively little is known about its functions in comparison to some of the other bacterial proteases.

How ClpX and ClpA selectively complex with ClpP

Interestingly, it was demonstrated that a ClpP recognition signal is present in some Clp/Hsp 100 chaperones but not in others (Kim et al. 2001). Based on sequence comparisons in different organisms, it was identified that all ClpA and ClpX homologues contained this recognition signal whereas ClpB and HslU, proteases that do not interact with ClpP, did not have this motif (Kim et al. 2001). This sequence is a tripeptide of [LIV]-G-[FL] and was found between the Box VII and Sensor I domain of the AAA+ ATPase motif in the chaperone. This is a criterion for predicting potential ClpP partners in bacteria.

Other Chaperones and degradation

If ClpP is only postulated to bind to specific chaperone partners that have conserved motifs as described above, does that exclude the possibility for other chaperones to present proteins for ClpP-dependent degradation? An interesting scenario arises through work with GroEL and a fusion protein called CRAG, where *in vivo* experiments in *E. coli* demonstrated that this degradation required GroEL and ClpP but not ClpX,A or B (Kandror et al. 1994) or all three ATPases together (Kandror et al. 1999). As well, Lon protease appears to play a secondary role in the degradation of CRAG, when ClpP is absent from the cells (Kandror et al. 1999). CRAG degradation was ATP-dependent, but not through the usual ATP-binding and hydrolyzing protein (Kandror et al. 1994). It was later shown that CRAG is recognized and degraded by GroEL when it is in complex with Trigger Factor, a protein believed to play a role in assisting GroEL to bind to newly-synthesized polypeptides and assist in their folding (Kandror et al. 1995). The association of Trigger Factor with GroEL is probably a general mechanism to assist proteins to fold properly (Kandror et al. 1995).

A model was proposed to explain how paradoxically GroEL can facilitate degradation of CRAG when GroEL's primary role is to assist proper protein folding (Kandror et al. 1999). The model proposed that because CRAG is an artificial fusion protein, it is improperly folded and is recognized by GroEL and Trigger Factor to help facilitate its refolding. Following a period of time when CRAG is associated with GroEL, ATP binding and hydrolysis by the GroES subunit facilitates the release of the protein from GroEL. However, CRAG doesn't assume a proper folded conformation and is partially unwound. This partially unwound intermediate is degraded by ClpP, as ClpP can degrade the unfolded portion of the protein, but not the folded form. This

degradation is non-processive but directional (C-terminal degradation). The fragmented CRAG intermediate then reassociates with GroEL and through a GroES and ATP-dependent mechanism the fragmented CRAG that is further unwound is released and again non-processively degraded by ClpP. Therefore, while GroES/L is trying to fold CRAG by cycles of binding and release, GroEL exposes CRAG amino-acids to ClpP for partial degradation (Kandror et al. 1999). This is an example of an ATP-dependent degradation of a protein by ClpP that is independent of the usual suspects: ClpA and ClpX. This is important because it identifies for the first time that ClpP can degrade proteins without its typical chaperone pairs and implies that other non-ClpP partnered chaperones may be involved in recognizing substrate specificity for protein degradation.

RATIONALE AND OBJECTIVES OF THIS THESIS

In bacteria, replication initiation is hypothesized to be universally mediated by DnaA protein (Messer 2002). I set out to determine whether DnaA played a similar role in *C. crescentus* replication control, and to identify mechanisms by which DnaA is regulated in *C. crescentus*. Previous work suggested that excess *dnaA* transcription in *C. crescentus* might cause extra replication, but did not conclusively demonstrate this property (Zweiger and Shapiro 1994).

My first objective was to determine whether *dnaA* was essential for *C. crescentus* viability, and whether DnaA was required for chromosome replication. As presented in Chapter 2, I exploited molecular genetics techniques to test and confirm these hypotheses. I then addressed the physiological consequences of turning off *dnaA* expression for *C. crescentus*. The data presented in chapter 2 also document unexpected physiological consequences for the *C. crescentus* cell-cycle. For example, Chapter 2 demonstrates that DnaA activity is epistatic to the previously proposed positive replication controls by transcription from inside the *Cori* replication origin.

Once I identified that DnaA is a key protein in replication control, I set out to analyze mechanisms by which DnaA is regulated throughout the cell-cycle. In *C. crescentus*, many proteins are regulated by targeted proteolysis to ensure that proteins are not present at the wrong place or at the wrong time (Gottesman 1999). In Chapter 3, I set out to perform cell-cycle stability and synthesis experiments and unexpectedly discovered that DnaA was targeted for proteolysis. This was the first demonstration that DnaA is regulated by proteolysis in any bacterial system. I then set out to identify the protease that is involved in this degradation by testing DnaA stability in different protease-

depleted or protease-null *C. crescentus* genetic backgrounds. I also identified the modes of DnaA proteolysis throughout the different stages of the *C. crescentus* cell-cycle. Since growth and replication are coupled, I tested if DnaA degradation was stimulated under unfavorable growth conditions such as starvation for essential nutrients. I tested nitrogen starvation in mediating cell-cycle and growth arrest and also tested DnaA stability under these conditions as well as CtrA stability. I discuss the regulation of DnaA proteolysis in the *C. crescentus* cell-cycle in Chapter 3 of this thesis.

In Chapter 4, I performed experiments that ask which stresses stimulate DnaA degradation. I subjected *C. crescentus* to a variety of nutritional and environmental stresses and assayed DnaA protein stability under theses conditions. I positively identified specific stresses that contribute to DnaA loss. In addition I also tested whether these nutritional degradation cues were mediated by the same protease that degrades DnaA in the cell-cycle. Since CtrA is such a global regulator of gene expression and its absence causes an over-replication stress, I tested whether DnaA loss was dependent upon CtrA absence (and vice versa). In the last part of Chapter 4, I set out to identify an abundant protein product that was pulled down with anti-DnaA antiserum. I used molecular biology technique to positively identify this protein and to speculate on its potential role in concert with DnaA.

This thesis therefore characterizes the positive role played by DnaA in *C. crescentus* replication. This thesis also identifies proteolysis as a mechanism for degrading DnaA and this thesis addresses how (which protease?), when (what point of the cell-cycle?), where (which cell-types?) and why (response to cell-cycle and nutritional starvation cues) DnaA is degraded in *C. crescentus*.

References

1. Addinall, S. G. and Holland, B. 2002. The tubulin ancestor, FtsZ, draughtsman, designer and driving force for bacterial cytokinesis. *J. Mol. Biol.* **318**: 219-236.

2. Alley, M. R., Maddock, J. R., and Shapiro, L. 1992. Polar localization of a bacterial chemoreceptor. *Genes Dev.* **6**: 825-836.

3. Andersson, S. G., Zomorodipour, A., Andersson, J. O., Sicheritz-Ponten, T., Alsmark, U. C., Podowski, R. M., Naslund, A. K., Eriksson, A. S., Winkler, H. H., and Kurland, C. G. 1998. The genome sequence of *Rickettsia prowazekii* and the origin of mitochondria. *Nature* **396**: 133-140.

4. Baker, T. A., Sekimizu, K., Funnell, B. E., and Kornberg, A. 1986. Extensive unwinding of the plasmid template during staged enzymatic initiation of DNA replication from the origin of the *Escherichia coli* chromosome. *Cell* **45**: 53-64.

5. Becker, G., Klauck, E., and Hengge-Aronis, R. 2000. The response regulator RssB, a recognition factor for sigmaS proteolysis in *Escherichia coli*, can act like an anti-sigmaS factor. *Mol. Microbiol.* **35**: 657-666.

6. Bell, S. P. 2002. The origin recognition complex: from simple origins to complex functions. *Genes Dev.* **16**: 659-672.

7. Bell, S. P. and Dutta, A. 2002. DNA replication in eukaryotic cells. *Annu. Rev. Biochem.* **71**: 333-374.

8. Bell, S. P. and Stillman, B. 1992. ATP-dependent recognition of eukaryotic origins of DNA replication by a multiprotein complex. *Nature* **357**: 128-134.

9. Berdis, A. J., Lee, I., Coward, J. K., Stephens, C., Wright, R., Shapiro, L., and Benkovic, S. J. 1998. A cell cycle-regulated adenine DNA methyltransferase from *Caulobacter crescentus* processively methylates GANTC sites on hemimethylated DNA. *Proc. Natl. Acad. Sci. U. S. A* **95**: 2874-2879.

10. Blaesing, F., Weigel, C., Welzeck, M., and Messer, W. 2000. Analysis of the DNA-binding domain of *Escherichia coli* DnaA protein. *Mol. Microbiol.* **36**: 557-569.

11. Blow, J. J. and Laskey, R. A. 1988. A role for the nuclear envelope in controlling DNA replication within the cell cycle. *Nature* **332**: 546-548.

12. Bochtler, M., Ditzel, L., Groll, M., and Huber, R. 1997. Crystal structure of heat shock locus V (HslV) from *Escherichia coli. Proc. Natl. Acad. Sci. U. S. A* **94**: 6070-6074.

13. Boye, E. 1991a. A turnstile for initiation of DNA replication. *Trends Cell Biol.* **1**: 107-109.

14. Boye, E. 1991b. The hemimethylated replication origin of *Escherichia coli* can be initiated in vitro. *J. Bacteriol.* **173**: 4537-4539.

15. Boye, E., Lobner-Olesen, A., and Skarstad, K. 2000. Limiting DNA replication to once and only once. *EMBO Rep.* **1**: 479-483.

16. Boye, E., Stokke, T., Kleckner, N., and Skarstad, K. 1996. Coordinating DNA replication initiation with cell growth: differential roles for DnaA and SeqA proteins. *Proc. Natl. Acad. Sci. U. S. A* **93**: 12206-12211.

17. Bramhill, D. and Kornberg, A. 1988a. A model for initiation at origins of DNA replication. *Cell* **54**: 915-918.

18. Bramhill, D. and Kornberg, A. 1988b. Duplex opening by DnaA protein at novel sequences in initiation of replication at the origin of the *E. coli* chromosome. *Cell* **52**: 743-755.

19. Brassinga, A. K. and Marczynski, G. T. 2001. Replication intermediate analysis confirms that chromosomal replication origin initiates from an unusual intergenic region in *Caulobacter crescentus*. *Nucleic Acids Res.* **29**: 4441-4451.

20. Brassinga, A. K., Siam, R., and Marczynski, G. T. 2001. Conserved gene cluster at replication origins of the alpha-proteobacteria *Caulobacter crescentus* and *Rickettsia prowazekii*. *J. Bacteriol.* **183**: 1824-1829.

21. Brendler, T. G. and Austin, S. 1999. Binding of SeqA protein to DNA requires interaction between two or more complexes bound to separate hemimethylated GATC sequence. *EMBO J.* **18**: 2304-2310.

22. Brun,Y.V. and Janakiraman,R.S. 2000. The dimorphic life cycle of *Caulobacter* and stalked bacteria. In *Prokaryotic development* (eds. Y.V.Brun and L.J.Shimkets), pp. 297-317. American Society for Microbiology Press, Washington, DC.

23. Brun, Y. V. and Shapiro, L. 1992. A temporally controlled sigma-factor is required for polar morphogenesis and normal cell division in *Caulobacter. Genes Dev.* **6**: 2395-2408.

24. Burkholder,W.F. and Grossman,A.D. 2000. Regulation of the initiation of endospore formation in *Bacillus subtilis.* In *Prokaryotic Development* (eds. Y.V.Brun and L.J.Shimkets), pp. 151-166. American Society for Microbiology Press, Washington, DC.

25. Cabantous, S., Guillet, V., Ohta, N., Newton, A., and Samama, J. P. 2002. Characterization and crystallization of DivK, an essential response regulator for cell division and differentiation in *Caulobacter crescentus. Acta Crystallogr. D. Biol. Crystallogr.* **58**: 1249-1251.

26. Campbell, J. L. and Kleckner, N. 1990. *E. coli oriC* and the *dnaA* gene promoter are sequestered from *dam* methyltransferase following the passage of the chromosomal replication fork. *Cell* **62**: 967-979.

27. Carr, K. M. and Kaguni, J. M. 1996. The A184V missense mutation of the *dnaA5* and *dnaA46* alleles confers a defect in ATP binding and thermolability in initiation of *Escherichia coli* DNA replication. *Mol. Microbiol.* **20**: 1307-1318.

28. Carr, K. M. and Kaguni, J. M. 2001. Stoichiometry of DnaA and DnaB protein in initiation at the *Escherichia coli* chromosomal origin. *Journal of Biological Chemistry* **276**: 44919-44925.

29. Cassler, M. R., Grimwade, J. E., and Leonard, A. C. 1995. Cell cycle-specific changes in nucleoprotein complexes at a chromosomal replication origin. *EMBO J.* **14**: 5833-5841.

30. Cohen-Bazire, G., Kunisawa, R., and Poindexter, J. S. 1966. The internal membranes of *Caulobacter crescentus*. *J. Gen. Microbiol.* **42**: 301-308.

31. Davey, M. J., Indiani, C., and O'Donnell, M. 2003. Reconstitution of the Mcm2-7 heterohexamer, subunit arrangement, and ATP site architecture. *Journal of Biological Chemistry* **278**: 4491-4499.

32. Davey, M. J., Jeruzalmi, D., Kuriyan, J., and O'Donnell, M. 2002. Motors and switches: AAA+ machines within the replisome. *Nat. Rev. Mol. Cell Biol.* **3**: 826-835.

33. Deuerling, E., Mogk, A., Richter, C., Purucker, M., and Schumann, W. 1997. The *ftsH* gene of *Bacillus subtilis* is involved in major cellular processes such as sporulation, stress adaptation and secretion. *Mol. Microbiol.* **23**: 921-933.

34. Dingwall, A. and Shapiro, L. 1989. Rate, origin, and bidirectionality of *Caulobacter* chromosome replication as determined by pulsed-field gel electrophoresis. *Proc. Natl. Acad. Sci. U. S. A* **86**: 119-123.

35. Dingwall, A., Zhuang, W. Y., Quon, K., and Shapiro, L. 1992. Expression of an early gene in the flagellar regulatory hierarchy is sensitive to an interruption in DNA replication. *J. Bacteriol.* **174**: 1760-1768.

36. Domian, I. J., Quon, K. C., and Shapiro, L. 1997. Cell type-specific phosphorylation and proteolysis of a transcriptional regulator controls the G1-to-S transition in a bacterial cell cycle. *Cell* **90**: 415-424.

37. Domian, I. J., Reisenauer, A., and Shapiro, L. 1999. Feedback control of a master bacterial cell-cycle regulator. *Proc. Natl. Acad. Sci. U. S. A* **96**: 6648-6653.

38. Donachie, W. D. and Blakely, G. W. 2003. Coupling the initiation of chromosome replication to cell size in *Escherichia coli*. *Curr. Opin. Microbiol.* **6**: 146-150.

39. Dougan, D. A., Mogk, A., Zeth, K., Turgay, K., and Bukau, B. 2002. AAA+ proteins and substrate recognition, it all depends on their partner in crime. *FEBS Lett.* **529**: 6-10.

40. Drury, L. S., Perkins, G., and Diffley, J. F. 2000. The cyclin-dependent kinase Cdc28p regulates distinct modes of Cdc6p proteolysis during the budding yeast cell cycle. *Curr. Biol.* **10**: 231-240.

41. Erzberger, J. P., Pirruccello, M. M., and Berger, J. M. 2002. The structure of bacterial DnaA: implications for general mechanisms underlying DNA replication initiation. *EMBO J.* **21**: 4763-4773.

42. Evinger, M. and Agabian, N. 1977. Envelope-associated nucleoid from *Caulobacter crescentus* stalked and swarmer cells. *J. Bacteriol.* **132**: 294-301.

43. Fang, L., Davey, M. J., and O'Donnell, M. 1999. Replisome assembly at *oriC*, the replication origin of *E. coli*, reveals an explanation for initiation sites outside an origin. *Mol. Cell* **4**: 541-553.

44. Fischer, B., Rummel, G., Aldridge, P., and Jenal, U. 2002. The FtsH protease is involved in development, stress response and heat shock control in *Caulobacter crescentus*. *Mol. Microbiol.* **44**: 461-478.

45. Flynn, J. M., Levchenko, I., Seidel, M., Wickner, S. H., Sauer, R. T., and Baker, T. A. 2001. Overlapping recognition determinants within the ssrA degradation tag allow modulation of proteolysis. *Proc. Natl. Acad. Sci. U. S. A* **98**: 10584-10589.

46. Flynn, J. M., Neher, S. B., Kim, Y. I., Sauer, R. T., and Baker, T. A. 2003. Proteomic Discovery of Cellular Substrates of the ClpXP Protease Reveals Five Classes of ClpX-Recognition Signals. *Mol. Cell* **11**: 671-683.

47. Fuchs, T., Wiget, P., OSteras, M., and Jenal, U. 2001. Precise amounts of a novel member of a phosphotransferase superfamily are essential for growth and normal morphology in Caulobacter crescentus. *Mol. Microbiol.* **39**: 679-692.

48. Fujikawa, N., Kurumizaka, H., Nureki, O., Terada, T., Shirouzu, M., Katayama, T., and Yokoyama, S. 2003. Structural basis of replication origin recognition by the DnaA protein. *Nucleic Acids Res.* **31**: 2077-2086.

49. Fuller, R. S., Funnell, B. E., and Kornberg, A. 1984. The DnaA protein complex with the *E. coli* chromosomal replication origin (*oriC*) and other DNA sites. *Cell* **38**: 889-900.

50. Fuller, R. S., Kaguni, J. M., and Kornberg, A. 1981. Enzymatic replication of the origin of the *Escherichia coli* chromosome. *Proc. Natl. Acad. Sci. U. S. A* **78**: 7370-7374.

51. Fuller, R. S. and Kornberg, A. 1983. Purified DnaA protein in initiation of replication at the *Escherichia coli* chromosomal origin of replication. *Proc. Natl. Acad. Sci. U. S. A* **80**: 5817-5821.

52. Gober,J.W. and England,J.C. 2000. Regulation of flagellum biosynthesis and motility in *Caulobacter.* In *Prokaryotic development* (eds. Y.V.Brun and L.J.Shimkets), pp. 319-339. American Society for Microbiology Press, Washington, DC.

53. Gober, J. W. and Shapiro, L. 1992. A developmentally regulated *Caulobacter* flagellar promoter is activated by 3' enhancer and IHF binding elements. *Mol. Biol. Cell* **3**: 913-926.

54. Gorbatyuk, B. and Marczynski, G. T. 2001. Physiological consequences of blocked *Caulobacter crescentus dnaA* expression, an essential DNA replication gene. *Mol. Microbiol.* **40**: 485-497.

55. Gottesman, S. 1999. Regulation by proteolysis: developmental switches. *Curr. Opin. Microbiol.* **2**: 142-147.

56. Gottesman, S. and Maurizi, M. R. 1992. Regulation by proteolysis: Energy-dependent proteases and their targets. *Microbiol. Rev.* **56**: 592-621.

57. Gottesman, S., Roche, E., Zhou, Y., and Sauer, R. T. 1998. The ClpXP and ClpAP proteases degrade proteins with carboxy-terminal peptide tails added by the SsrA-tagging system. *Genes Dev.* **12**: 1338-1347.

58. Grimwade, J. E., Ryan, V. T., and Leonard, A. C. 2000. IHF redistributes bound initiator protein, DnaA, on supercoiled *oriC* of *Escherichia coli*. *Mol. Microbiol.* **35**: 835-844.

59. Grunenfelder, B., Rummel, G., Vohradsky, J., Roder, D., Langen, H., and Jenal, U. 2001. Proteomic analysis of the bacterial cell cycle. *Proc. Natl. Acad. Sci. USA* **98**: 4681-4686.

60. Guarne, A., Zhao, Q., Ghirlando, R., and Yang, W. 2002. Insights into negative modulation of *E. coli* replication initiation from the structure of SeqA-hemimethylated DNA complex. *Nat. Struct. Biol.* **9**: 839-843.

61. Hansen, F. G., Atlung, T., Braun, R. E., Wright, A., Hughes, P., and Kohiyama, M. 1991. Initiator (DnaA) protein concentration as a function of growth rate in *Escherichia coli* and *Salmonella typhimurium*. *J. Bacteriol.* **173**: 5194-5199.

62. Hecht, G. B., Lane, T., Ohta, N., Sommer, J. M., and Newton, A. 1995. An essential single domain response regulator required for normal cell division and differentiation in *Caulobacter crescentus*. *EMBO J.* **14**: 3915-3924.

63. Hengge, R. and Bukau, B. 2003. Proteolysis in prokaryotes: protein quality control and regulatory principles. *Mol. Microbiol.* **49**: 1451-1462.

64. Hengge-Aronis, R. 2002. Signal transduction and regulatory mechanisms involved in control of the sigma-S (RpoS) subunit of RNA polymerase. *Microbiology and Molecular Biology Reviews* **66**: 373-395.

65. Herman, C., Prakash, S., Lu, C. Z., Matouschek, A., and Gross, C. A. 2003. Lack of a Robust Unfoldase Activity Confers a Unique Level of Substrate Specificity to the Universal AAA Protease FtsH. *Mol. Cell* **11**: 659-669.

66. Herman, C., Thevenet, D., Bouloc, P., Walker, G. C., and D'Ari, R. 1998. Degradation of carboxy-terminal-tagged cytoplasmic proteins by the *Escherichia coli* protease HflB (FtsH). *Genes Dev.* **12**: 1348-1355.

67. Hiasa, H. and Marians, K. J. 1994. Primase coupled leading- and lagging-strand DNA synthesis from *oriC*. *Journal of Biological Chemistry* **269**: 6058-6063.

68. Higashitani, A., Ishii, Y., Kato, Y., and Koriuchi, K. 1997. Functional dissection of a cell-division inhibitor, SulA, of Escherichia coli and its negative regulation by Lon. *Mol. Gen. Genet.* **254**: 351-357.

69. Hinz, A. J., Larson, D. E., Smith, C. S., and Brun, Y. V. 2003. The *Caulobacter crescentus* polar organelle development protein PodJ is differentially localized and is required for polar targeting of the PleC development regulator. *Mol. Microbiol.* **47**: 929-941.

70. Hiraga, S. 1976. Novel F prime factors able to replicate in *Escherichia coli* Hfr strains. *Proc. Natl. Acad. Sci. U. S. A* **73**: 198-202.

71. Hofmann, J. F. and Beach, D. 1994. *cdt1* is an essential target of the Cdc10/Sct1 transcription factor: requirement for DNA replication and inhibition of mitosis. *EMBO J.* **13**: 425-434.

72. Hoyt, M. A. 1997. Eliminating all obstacles: regulating proteolysis in the eukaryotic cell cycle. *Cell* **91**: 149-151.

73. Hsu, J., Bramhill, D., and Thompson, C. M. 1994. Open complex formation by DnaA initiation protein at the *Escherichia coli* chromosomal origin requires the 13-mers precisely spaced relative to the 9-mers. *Mol. Microbiol.* **11**: 903-911.

74. Hung,D., McAdams,H.H., and Shapiro,L. 2000. Regulation of the *Caulobacter* cell cycle. In *Prokaryotic development* (eds. Y.V.Brun and L.J.Shimkets), pp. 361-378. American Society for Microbiology Press, Washington, DC.

75. Hupp, T. R. and Kaguni, J. M. 1993. DnaA5 protein is thermolabile in initiation of replication from the chromosomal origin of *Escherichia coli*. *Journal of Biological Chemistry* **268**: 13128-13136.

76. Hwang, D. S. and Kornberg, A. 1992. Opening of the replication origin of *Escherichia coli* by DnaA protein with protein HU or IHF. *Journal of Biological Chemistry* **267**: 23083-23086.

77. Jacob, F., Brenner, S., and Cuzin, F. 1963. On the regulation of DNA replication in bacteria. *Cold Spring Harb. Symp. Quant. Biol.* **28**: 329-348.

78. Jacobs, C., Ausmees, N., Cordwell, S. J., Shapiro, L., and Laub, M. T. 2003. Functions of the CckA histidine kinase in *Caulobacter* cell cycle control. *Mol. Microbiol.* **47**: 1279-1290.

79. Jacobs, C., Domian, I. J., Maddock, J. R., and Shapiro, L. 1999. Cell cycle-dependent polar localization of an essential bacterial histidine kinase that controls DNA replication and cell division. *Cell* **97**: 111-120.

80. Jacobs, C., Hung, D., and Shapiro, L. 2001. Dynamic localization of a cytoplasmic signal transduction response regulator controls morphogenesis during the *Caulobacter* cell cycle. *Proc. Natl. Acad. Sci. U. S. A* **98**: 4095-4100.

81. Jacobs, C. and Shapiro, L. 1998. Microbial asymmetric cell division: localization of cell fate determinants. *Curr. Opin. Genet. Dev.* **8**: 386-391.

82. Jenal, U. 2000. Signal transduction mechanisms in *Caulobacter crescentus* development and cell cycle control. *FEMS Microbiol. Rev.* **24**: 177-191.

83. Jenal, U. and Fuchs, T. 1998. An essential protease involved in bacterial cell-cycle control. *EMBO J.* **17**: 5658-5669.

84. Jenal, U. and Stephens, C. 2002. The *Caulobacter* cell cycle: timing, spatial organization and checkpoints. *Curr. Opin. Microbiol.* **5**: 558-563.

85. Jensen, R. B. and Shapiro, L. 1999. The *Caulobacter crescentus smc* gene is required for cell cycle progression and chromosome segregation. *Proc. Natl. Acad. Sci. U. S. A* **96**: 10661-10666.

86. Jensen, R. B., Wang, S. C., and Shapiro, L. 2001. A moving DNA replication factory in *Caulobacter crescentus*. *EMBO J.* **20**: 4952-4963.

87. Jensen, R. B., Wang, S. C., and Shapiro, L. 2002. Dynamic localization of proteins and DNA during a bacterial cell cycle. *Nat. Rev. Mol. Cell Biol.* **3**: 167-176.

88. Judd, E. M., Ryan, K. R., Moerner, W. E., Shapiro, L., and McAdams, H. H. 2003. Fluorescence bleaching reveals asymmetric compartment formation prior to cell division in *Caulobacter*. *Proc. Natl. Acad. Sci. U. S. A* **100**: 8235-8240.

89. Kandror, O., Busconi, L., Sherman, M., and Goldberg, A. L. 1994. Rapid degradation of an abnormal protein in *Escherichia coli* involves the chaperones GroEL and GroES. *Journal of Biological Chemistry* **269**: 23575-23582.

90. Kandror, O., Sherman, M., and Goldberg, A. 1999. Rapid degradation of an abnormal protein in *Escherichia coli* proceeds through repeated cycles of association with GroEL. *Journal of Biological Chemistry* **274**: 37743-37749.

91. Kandror, O., Sherman, M., Rhode, M., and Goldberg, A. L. 1995. Trigger factor is involved in GroEL-dependent protein degradation in *Escherichia coli* and promotes binding of GroEL to unfolded proteins. *EMBO J.* **14**: 6021-6027.

92. Katayama, T. 1994. The mutant DnaAcos protein which overinitiates replication of the *Escherichia coli* chromosome is inert to negative regulation for initiation. *Journal of Biological Chemistry* **269**: 22075-22079.

93. Katayama, T. and Crooke, E. 1995. DnaA protein is sensitive to a soluble factor and is specifically inactivated for initiation of in vitro replication of the *Escherichia coli* minichromosome. *Journal of Biological Chemistry* **270**: 9265-9271.

94. Katayama, T., Crooke, E., and Sekimizu, K. 1995. Characterization of *Escherichia coli* DnaAcos protein in replication systems reconstituted with highly purified proteins. *Mol. Microbiol.* **18**: 813-820.

95. Katayama, T., Kubota, T., Kurokawa, K., Crooke, E., and Sekimizu, K. 1998. The initiator function of DnaA protein is negatively regulated by the sliding clamp of the *E. coli* chromosomal replicase. *Cell* **94**: 61-71.

96. Kato, J. and Katayama, T. 2001. Hda, a novel DnaA-related protein, regulates the replication cycle in *Escherichia coli*. *EMBO J.* **20**: 4253-4262.

97. Keiler, K. C. and Shapiro, L. 2003. TmRNA is required for correct timing of DNA replication in *Caulobacter crescentus*. *J. Bacteriol.* **185**: 573-580.

98. Keiler, K. C., Waller, P. R., and Sauer, R. T. 1996. Role of a peptide tagging system in degradation of proteins synthesized from damaged messenger RNA. *Science* **271**: 990-993.

99. Kelly, A. J., Sackett, M. J., Din, N., Quardokus, E., and Brun, Y. V. 1998. Cell cycle-dependent transcriptional and proteolytic regulation of FtsZ in *Caulobacter*. *Genes Dev.* **12**: 880-893.

100. Kim, Y. I., Levchenko, I., Fraczkowska, K., Woodruff, R. V., Sauer, R. T., and Baker, T. A. 2001. Molecular determinants of complex formation between Clp/Hsp100 ATPases and the ClpP peptidase. *Nat. Struct. Biol.* **8**: 230-233.

101. Kitagawa, R., Mitsuki, H., Okazaki, T., and Ogawa, T. 1996. A novel DnaA protein-binding site at 94.7 min on the *Escherichia coli* chromosome. *Mol. Microbiol.* **19**: 1137-1147.

102. Kitagawa, R., Ozaki, T., Moriya, S., and Ogawa, T. 1998. Negative control of replication initiation by a novel chromosomal locus exhibiting exceptional affinity for *Escherichia coli* DnaA protein. *Genes Dev.* **12**: 3032-3043.

103. Kohiyama, M. 1968. DNA synthesis in temperature sensitive mutants in *E. coli*. *Cold Spring Harb. Symp. Quant. Biol.* **43**: 317-324.

104. Kohiyama, M., Cousin, D., Ryter, A., and Jacob, F. 1966. Mutants thermosensibles d'*E. coli* K-12. Isolement et chraracterisation rapide. *Ann. Inst. Pasteur* **110**: 465-486.

105. Krause, M. and Messer, W. 1999. DnaA proteins of *Escherichia coli* and *Bacillus subtilis*: coordinate actions with single-stranded DNA-binding protein and interspecies inhibition during open complex formation at the replication origins. *Gene* **228**: 123-132.

106. Kreitz, S., Ritzi, M., Baack, M., and Knippers, R. 2001. The human origin recognition complex protein 1 dissociates from chromatin during S phase in HeLa cells. *Journal of Biological Chemistry* **276**: 6337-6342.

107. Kuroda, A., Nomura, K., Ohtomo, R., Kato, J., Ikeda, T., Takiguchi, N., Ohtake, H., and Kornberg, A. 2001. Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in *E. coli*. *Science* **293**: 705-708.

108. Kuroda, A., Tanaka, S., Ikeda, T., Kato, J., Takiguchi, N., and Ohtake, H. 1999. Inorganic polyphosphate kinase is required to stimulate protein degradation and for adaptation to amino acid starvation in *Escherichia coli*. *Proc. Natl. Acad. Sci. U. S. A* **96**: 14264-14269.

109. Kurokawa, K., Nishida, S., Emoto, A., Sekimizu, K., and Katayama, T. 1999. Replication cycle-coordinated change of the adenine nucleotide-bound forms of DnaA protein in *Escherichia coli*. *EMBO J.* **18**: 6642-6652.

110. Kurtz, H. D., Jr. and Smith, J. 1992. Analysis of a *Caulobacter crescentus* gene cluster involved in attachment of the holdfast to the cell. *J. Bacteriol.* **174**: 687-694.

111. Landoulsi, A., Malki, A., Kern, R., Kohiyama, M., and Hughes, P. 1990. The *E. coli* cell surface specifically prevents the initiation of DNA replication at *oriC* on hemimethylated DNA templates. *Cell* **63**: 1053-1060.

112. Lanka, E. and Schuster, H. 1983. The DnaC protein of *Escherichia coli*. Purification, physical properties and interaction with DnaB protein. *Nucleic Acids Res.* **11**: 987-997.

113. Laub, M. T., Chen, S. L., Shapiro, L., and McAdams, H. H. 2002. Genes directly controlled by CtrA, a master regulator of the *Caulobacter* cell cycle. *Proc. Natl. Acad. Sci. U. S. A* **99**: 4632-4637.

114. Laub, M. T., McAdams, H. H., Feldblyum, T., Fraser, C. M., and Shapiro, L. 2000. Global analysis of the genetic network controlling a bacterial cell cycle. *Science* **290**: 2144-2148.

115. Learn, B. A., Um S.J., Huang, L., and McMacken, R. 1997. Cryptic single-stranded-DNA binding activities of the phage lambda P and *Escherichia coli* DnaC replication initiation proteins faciliate the transfer of *E. coli* DnaB helicase onto DNA. *Proc. Natl. Acad. Sci. U. S. A* **94**: 1154-1159.

116. Lee, D. G. and Bell, S. P. 2000. ATPase switches controlling DNA replication initiation. *Curr. Opin. Cell Biol.* **12**: 280-285.

117. Lee, D. G., Makhov, A. M., Klemm, R. D., Griffith, J. D., and Bell, S. P. 2000. Regulation of origin recognition complex conformation and ATPase activity: differential effects of single-stranded and double-stranded DNA binding. *EMBO J.* **19**: 4774-4782.

118. Lei, M., Kawasaki, Y., Young, M. R., Kihara, M., Sugino, A., and Tye, B. K. 1997. Mcm2 is a target of regulation by Cdc7-Dbf4 during the initiation of DNA synthesis. *Genes Dev.* **11**: 3365-3374.

119. Lemon, K. P. and Grossman, A. D. 1998. Localization of bacterial DNA polymerase: evidence for a factory model of replication. *Science* **282**: 1516-1519.

120. Levchenko, I., Seidel, M., Sauer, R. T., and Baker, T. A. 2000. A specificity-enhancing factor for the ClpXP degradation machine. *Science* **289**: 2354-2356.

121. Li, C. J. and DePamphilis, M. L. 2002. Mammalian Orc1 protein is selectively released from chromatin and ubiquitinated during the S-to-M transition in the cell division cycle. *Mol Cell Biol.* **22**: 105-116.

122. Li, X., Zhao, Q., Liao, R., Sun, P., and Wu, X. 2003. The SCF(Skp2) ubiquitin ligase complex interacts with the human replication licensing factor Cdt1 and regulates Cdt1 degradation. *Journal of Biological Chemistry* **278**: 30854-30858.

123. Lobner-Olesen, A., Skarstad, K., Hansen, F. G., von Meyenburg, K., and Boye, E. 1989. The DnaA protein determines the initiation mass of *Escherichia coli* K-12. *Cell* **57**: 881-889.

124. Lu, M., Campbell, J. L., Boye, E., and Kleckner, N. 1994. SeqA: a negative modulator of replication initiation in *E. coli*. *Cell* **77**: 413-426.

125. Maddock, J. R. and Shapiro, L. 1993. Polar localization of the chemoreceptor complex in the *Escherichia coli* cell. *Science* **259**: 1717-1723.

126. Majka, J., Messer, W., Schrempf, H., and Zakrzewska-Czerwinska, J. 1997. Purification and characterization of the *Streptomyces lividans* initiator protein DnaA. *J. Bacteriol.* **179**: 2426-2432.

127. Malakooti, J. and Ely, B. 1995. Principal sigma subunit of the *Caulobacter crescentus* RNA polymerase. *J. Bacteriol.* **177**: 6854-6860.

128. Marczynski, G. T. 1999. Chromosome methylation and measurement of faithful, once and only once per cell cycle chromosome replication in *Caulobacter crescentus*. *J. Bacteriol.* **181**: 1984-1993.

129. Marczynski, G. T., Dingwall, A., and Shapiro, L. 1990. Plasmid and chromosomal DNA replication and partitioning during the *Caulobacter crescentus* cell cycle. *J. Mol. Biol.* **212**: 709-722.

130. Marczynski, G. T., Lentine, K., and Shapiro, L. 1995. A developmentally regulated chromosomal origin of replication uses essential transcription elements. *Genes Dev.* **9**: 1543-1557.

131. Marczynski, G. T. and Shapiro, L. 1992. Cell-cycle control of a cloned chromosomal origin of replication from *Caulobacter crescentus*. *J. Mol. Biol.* **226**: 959-977.

132. Marczynski, G. T. and Shapiro, L. 2002. Control of chromosome replication in *Caulobacter crescentus*. *Annu. Rev. Microbiol.* **56**: 625-656.

133. Margulies, C. and Kaguni, J. M. 1996. Ordered and sequential binding of DnaA protein to *oriC*, the chromosomal origin of *Escherichia coli*. *Journal of Biological Chemistry* **271**: 17035-17040.

134. Marques, M. V., Gomes, S. L., and Gober, J. W. 1997. A gene coding for a putative sigma 54 activator is developmentally regulated in *Caulobacter crescentus*. *J. Bacteriol.* **179**: 5502-5510.

135. Marszalek, J. and Kaguni, J. M. 1994. DnaA protein directs the binding of DnaB protein in initiation of DNA replication in *Escherichia coli*. *Journal of Biological Chemistry* **269**: 4883-4890.

136. McGarry, T. J. and Kirschner, M. W. 1998. Geminin, an inhibitor of DNA replication, is degraded during mitosis. *Cell* **93**: 1043-1053.

137. Mendez, J., Zou-Yang, X. H., Kim, S. Y., Hidaka, M., Tansey, W. P., and Stillman, B. 2002. Human origin recognition complex large subunit is degraded by ubiquitin-mediated proteolysis after initiation of DNA replication. *Mol Cell* **9**: 481-491.

138. Messer, W, Egan, J. B., Gille, A., Holz, A., Schaefer, C., and Woelker, B. 1978. Minichromosomes: plasmids which carry the *E. coli* replication origin. *Mol. Gen. Genet.* **162**: 269-275.

139. Messer, W. 2002. The bacterial replication initiator DnaA. DnaA and *oriC*, the bacterial mode to initiate DNA replication. *FEMS Microbiol. Rev.* **26**: 355-374.

140. Messer, W., Blaesing, F., Jakimowicz, D., Krause, M., Majka, J., Nardmann, J., Schaper, S., Seitz, H., Speck, C., Weigel, C., Wegrzyn, G., Welzeck, M., and Zakrzewska-Czerwinska, J. 2001. Bacterial replication initiator DnaA. Rules for DnaA binding and roles of DnaA in origin unwinding and helicase loading. *Biochimie* **83**: 5-12.

141. Messer, W., Blaesing, F., Majka, J., Nardmann, J., Schaper, S., Schmidt, A., Seitz, H., Speck, C., Tungler, D., Wegrzyn, G., Weigel, C., Welzeck, M., and Zakrzewska-Czerwinska, J. 1999. Functional domains of DnaA proteins. *Biochimie* **81**: 819-825.

142. Messer, W. and Weigel, C. 1997. DnaA initiator - also a transcription factor. *Mol. Microbiol.* **24**: 1-6.

143. Mizushima, T., Takahashi, N., and Stillman, B. 2000. Cdc6p modulates the structure and DNA binding activity of the origin recognition complex in vitro. *Genes Dev.* **14**: 1631-1641.

144. Mochly-Rosen, D. 1995. Localization of protein kinases by anchoring proteins: a theme in signal transduction. *Science* **268**: 247-251.

145. Muir, R. E. and Gober, J. W. 2001. Regulation of late flagellar gene transcription and cell division by flagellum assembly in *Caulobacter crescentus*. *Mol. Microbiol.* **41**: 117-130.

146. Natale, D. A., Li, C. J., Sun, W. H., and DePamphilis, M. L. 2000. Selective instability of Orc1 protein accounts for the absence of functional origin recognition complexes during the M-G(1) transition in mammals. *EMBO J.* **19**: 2728-2738.

147. Neuwald, A. F., Aravind, L., Spouge, J. L., and Koonin, E. V. 1999. AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome Res.* **9**: 27-43.

148. Nguyen, V. Q., Co, C., Irie, K., and Li, J. J. 2000. Clb/Cdc28 kinases promote nuclear export of the replication initiator proteins Mcm2-7. *Curr. Biol.* **10**: 195-205.

149. Nierman, W. C., Feldblyum, T. V., Laub, M. T., Paulsen, I. T., Nelson, K. E., Eisen, J. A., Heidelberg, J. F., Alley, M. R., Ohta, N., Maddock, J. R., Potocka, I., Nelson, W. C., Newton, A., Stephens, C., Phadke, N. D., Ely, B., DeBoy, R. T., Dodson, R. J., Durkin, A. S., Gwinn, M. L., Haft, D. H., Kolonay, J. F., Smit, J., Craven, M. B., Khouri, H., Shetty, J., Berry, K., Utterback, T., Tran, K., Wolf, A., Vamathevan, J., Ermolaeva, M., White, O., Salzberg, S. L., Venter, J. C., Shapiro, L., Fraser, C. M., and Eisen, J. 2001. Complete genome sequence of *Caulobacter crescentus*. *Proc. Natl. Acad. Sci. U. S. A* **98**: 4136-4141.

150. Nishida, S., Fujimitsu, K., Sekimizu, K., Ohmura, T., Ueda, T., and Katayama, T. 2002. A nucleotide switch in the Escherichia coli DnaA protein initiates chromosomal replication: evidnece from a mutant DnaA protein defective in regulatory ATP hydrolysis in vitro and in vivo. *Journal of Biological Chemistry* **277**: 14986-14995.

151. Nishitani, H., Lygerou, Z., Nishimoto, T., and Nurse, P. 2000. The Cdt1 protein is required to license DNA for replication in fission yeast. *Nature* **404**: 625-628.

152. Niwa, H., Tsuchiya, D., Makyio, H., Yoshida, M., and Morikawa, K. 2002. Hexameric ring structure of the ATPase domain of the membrane-integrated metalloprotease FtsH from Thermus thermophilus HB8. *Structure. (Camb.)* **10**: 1415-1423.

153. O'Handley, D. and Nakai, H. 2002. Derepression of bacteriophage mu transposition functions by truncated forms of the immunity repressor. *J. Mol. Biol.* **322**: 311-324.

154. Ogawa, T., Yamada, Y., Kuroda, T., Kishi, T., and Moriya, S. 2002. The *datA* locus predominantly contributes to the initiator titration mechanism in the control of replication initiation in *Escherichia coli*. *Mol. Microbiol.* **44**: 1367-1375.

155. Ohta,N., Grebe,T.W., and Newton,A. 2000. Signal transduction and cell cycle checkpoints in developmental regulation of *Caulobacter*. In *Prokaryotic development* (eds. Y.V.Brun and L.J.Shimkets), pp. 341-359. American Society for Microbiology Press, Washington, DC.

156. Ohta, N., Masurekar, M., and Newton, A. 1990. Cloning and cell cycle-dependent expression of DNA replication gene *dnaC* from *Caulobacter crescentus*. *J. Bacteriol.* **172**: 7027-7034.

157. Oka, A., Sugimoto, K., Takanami, M., and Hirota, Y. 1980. Replication origin of the *Escherichia coli* K-12 chromosome: the size and structure of the minimum DNA segment carrying the information for autonomous replication. *Mol. Gen. Genet.* **178**: 9-20.

158. Ortega, J., Lee, H. S., Maurizi, M. R., and Steven, A. C. 2002. Alternating translocation of protein substrates from both ends of ClpXP protease. *EMBO J.* **21**: 4938-4949.

159. Osley, M. A. and Newton, A. 1977. Mutational analysis of developmental control in *Caulobacter crescentus*. *Proc. Natl. Acad. Sci. U. S. A* **74**: 124-128.

160. Osley, M. A. and Newton, A. 1978. Regulation of cell cycle events in asymmetrically dividing cells: functions required for DNA initiation and chain elongation in *Caulobacter crescentus*. *J. Bacteriol.* **135**: 10-17.

161. OSteras, M. and Jenal, U. 2000. Regulatory circuits in *Caulobacter*. *Curr. Opin. Microbiol.* **3**: 171-176.

162. Otwinowski, Z., Schevitz, R. W., Zhang, R. G., Lawson, C. L., Joachimiak, A., Marmorstein, R. Q., Luisi, B. F., and Sigler, P. B. 1988. Crystal structure of the trp repressor/operator complex at atomic resolution. *Nature* **335**: 321-329.

163. Pak, M. and Wickner, S. 1997. Mechanism of protein remodeling by ClpA chaperone. *Proc. Natl. Acad. Sci. U. S. A* **94**: 4901-4906.

164. Perkins, G., Drury, L. S., and Diffley, J. F. 2001. Separate SCF(CDC4) recognition elements target Cdc6 for proteolysis in S phase and mitosis. *EMBO J.* **20**: 4836-4845.

165. Piatti, S., Lengauer, C., and Nasmyth, K. 1995. Cdc6 is an unstable protein whose de novo synthesis in G1 is important for the onset of S phase and for preventing a 'reductional' anaphase in the budding yeast *Saccharomyces cerevisiae*. *EMBO J.* **14**: 3788-3799.

166. Poindexter, J. S. 1964. Biological properties and classification of the *Caulobacter* group. *Bacteriological Reviews* **28**: 231-295.

167. Poindexter, J. S. 1981. The *Caulobacters*: ubiquitous unusual bacteria. *Microbiol. Rev.* **29**: 123-179.

168. Poindexter, J. S., Pujara, K. P., and Staley, J. T. 2000. *In situ* reproductive rate of freshwater *Caulobacter* spp. *Appl. Environ. Microbiol.* **66**: 4105-4111.

169. Quardokus, E. M. and Brun, Y. V. 2002. DNA replication initiation is required for mid-cell positioning of FtsZ rings in *Caulobacter crescentus*. *Mol. Microbiol.* **45**: 605-616.

170. Quardokus, E. M., Din, N., and Brun, Y. V. 2001. Cell cycle and positional constraints on FtsZ localization and the initiation of cell division in *Caulobacter crescentus*. *Mol. Microbiol.* **39**: 949-959.

171. Quon, K. C., Marczynski, G. T., and Shapiro, L. 1996. Cell cycle control by an essential bacterial two-component signal transduction protein. *Cell* **84**: 83-93.

172. Quon, K. C., Yang, B., Domian, I. J., Shapiro, L., and Marczynski, G. T. 1998. Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin. *Proc. Natl. Acad. Sci. USA* **95**: 120-125.

173. Ramakrishnan, G. and Newton, A. 1990. FlbD of *Caulobacter crescentus* is a homologue of the NtrC (NRI) protein and activates sigma 54-dependent flagellar gene promoters. *Proc. Natl. Acad. Sci. U. S. A* **87**: 2369-2373.

174. Reid, B. G., Fenton, W. A., Horwich, A. L., and Weber-Ban, E. U. 2001. ClpA mediates directional translocation of substrate proteins into the ClpP protease. *Proc. Natl. Acad. Sci. U. S. A* **98**: 3768-3772.

175. Reisenauer, A., Mohr, C. D., and Shapiro, L. 1996. Regulation of a heat shock sigma32 homolog in *Caulobacter crescentus*. *J. Bacteriol.* **178**: 1919-1927.

176. Reisenauer, A., Quon, K., and Shapiro, L. 1999. The CtrA response regulator mediates temporal control of gene expression during the *Caulobacter* cell cycle. *J. Bacteriol.* **181**: 2430-2439.

177. Reisenauer, A. and Shapiro, L. 2002. DNA methylation affects the cell cycle transcription of the CtrA global regulator in *Caulobacter*. *EMBO J.* **21**: 4969-4977.

178. Rohrwild, M., Coux, O., Huang, H. C., Moerschell, R. P., Yoo, S. J., Seol, J. H., Chung, C. H., and Goldberg, A. L. 1996. HslV-HslU: A novel ATP-dependent protease complex in *Escherichia coli* related to the eukaryotic proteasome. *Proc. Natl. Acad. Sci. U. S. A* **93**: 5808-5813.

179. Roth, A. and Messer, W. 1998. High-affinity binding sites for the initiator protein DnaA on the chromosome of *Escherichia coli*. *Mol. Microbiol.* **28**: 395-401.

180. Russell, D. W. and Zinder, N. D. 1987. Hemimethylation prevents DNA replication in *E. coli*. *Cell* **50**: 1071-1079.

181. Ryan, K. R., Judd, E. M., and Shapiro, L. 2002. The CtrA response regulator essential for *Caulobacter crescentus* cell-cycle progression requires a bipartite degradation signal for temporally controlled proteolysis. *J. Mol. Biol.* **324**: 443-455.

182. Ryan, K. R. and Shapiro, L. 2003. Temporal and spatial regulation in prokaryotic cell cycle progression and development. *Annu. Rev. Biochem.* **72**: 367-394.

183. Sackett, M. J., Kelly, A. J., and Brun, Y. V. 1998. Ordered expression of *ftsQA* and *ftsZ* during the Caulobacter crescentus cell cycle. *Mol. Microbiol.* **28**: 421-434.

184. Schaper, S. and Messer, W. 1995. Interaction of the initiator protein DnaA of *Escherichia coli* with its DNA target. *Journal of Biological Chemistry* **270**: 17622-17626.

185. Seitz, H., Weigel, C., and Messer, W. 2000. The interaction domains of the DnaA and DnaB replication proteins of *Escherichia coli*. *Mol. Microbiol.* **37**: 1270-1279.

186. Sekimizu, K., Bramhill, D., and Kornberg, A. 1987. ATP activates DnaA protein in initiating replication of plasmids bearing the origin of the *E. coli* chromosome. *Cell* **50**: 259-265.

187. Shakibai, N., Ishidate, K., Reshetnyak, E., Gunji, S., Kohiyama, M., and Rothfield, L. 1998. High-affinity binding of hemimethylated *oriC* by *Escherichia coli* membranes is mediated by a multiprotein system that includes SeqA and a newly identified factor, SeqB. *Proc. Natl. Acad. Sci. U. S. A* **95**: 11117-11121.

188. Shapiro, L. and Losick, R. 1997. Protein localization and cell fate in bacteria. *Science* **276**: 712-718.

189. Siam, R., Brassinga, A. K., and Marczynski, G. T. 2003. A dual binding site for integration host factor and the response regulator CtrA inside the *Caulobacter crescentus* replication origin. *J. Bacteriol.* **185**: 5563-5572.

190. Siam, R. and Marczynski, G. T. 2000. Cell cycle regulator phosphorylation stimulates two distinct modes of binding at a chromosome replication origin. *EMBO J.* **19**: 1138-1147.

191. Simmons, L. A., Felczak, M., and Kaguni, J. M. 2003. DnaA protein of *Escherichia coli*: oligomerization at the *E. coli* chromosomal origin is required for initiation and involves specific N-terminal amino acids. *Mol. Microbiol.* **49**: 849-858.

192. Simmons, L. A. and Kaguni, J. M. 2003. The DnaAcos allele of *Escherichia coli*: hyperactive initiation is caused by substitution of A184V and Y271H, resulting in defective ATP binding and aberrant DNA replication control. *Mol. Microbiol.* **47**: 755-765.

193. Skarstad, K. and Boye, E. 1994. The initiator protein DnaA: evolution, properties and function. *Biochim. Biophys. Acta* **1217**: 111-130.

194. Skarstad, K., Lueder, G., Lurz, R., Speck, C., and Messer, W. 2000. The *Escherichia coli* SeqA protein binds specifically and co-operatively to two sites in hemimethylated and fully methylated *oriC*. *Mol. Microbiol.* **36**: 1319-1326.

195. Skerker, J. M. and Shapiro, L. 2000. Identification and cell cycle control of a novel pilus system in *Caulobacter crescentus*. *EMBO J.* **19**: 3223-3234.

196. Sly, L. I., Cox, T. L., and Beckenham, T. B. 1999. The phylogenetic relationships of *Caulobacter*, *Asticcacaulis* and *Brevundimonas* species and their taxonomic implications. *Int. J. Syst. Bacteriol.* **49 Pt 2**: 483-488.

197. Smit, J. 1987. Localizing the subunit pool for the temporally regulated polar pili of *Caulobacter crescentus*. *J. Cell Biol.* **105**: 1821-1828.

198. Speck, C. and Messer, W. 2001. Mechanism of origin unwinding: sequential binding of DnaA to double- and single-stranded DNA. *EMBO J.* **20**: 1469-1476.

199. Speck, C., Weigel, C., and Messer, W. 1999. ATP- and ADP-dnaA protein, a molecular switch in gene regulation. *EMBO J.* **18**: 6169-6176.

200. Spiess, C., Beil, A., and Ehrmann, M. 1999. A temperature-dependent switch from chaperone to protease in a widely conserved heat-shock protein. *Cell* **97**: 339-347.

201. Stahl, D. A., Key, R., Flesher, B., and Smit, J. 1992. The phylogeny of marine and freshwater caulobacters reflects their habitat. *J. Bacteriol.* **174**: 2193-2198.

202. Stephens, C., Reisenauer, A., Wright, R., and Shapiro, L. 1996. A cell cycle-regulated bacterial DNA methyltransferase is essential for viability. *Proc. Natl. Acad. Sci. U. S. A* **93**: 1210-1214.

203. Stephens, C. M. and Shapiro, L. 1993. An unusual promoter controls cell-cycle regulation and dependence on DNA replication of the *Caulobacter fliLM* early flagellar operon. *Mol. Microbiol.* **9**: 1169-1179.

204. Stephens, C. M., Zweiger, G., and Shapiro, L. 1995. Coordinate cell cycle control of a *Caulobacter* DNA methyltransferase and the flagellar genetic hierarchy. *J. Bacteriol.* **177**: 1662-1669.

205. Stock,J.B., Surette,M.G., Levit,M., and Park,P. 1995. Two-component signal transduction systems: structure-function relationships and mechanisms of catalysis. In *Two-component signal transduction* (eds. J.A.Hoch and T.J.Silhavy), pp. 25-51. American Society for Microbiology Press, Washington, DC.

206. Stove, J. L. and Stanier, R. Y. 1962. Cellular differentiation in stalked bacteria. *Nature* **196**: 1189-1192.

207. Su'etsugu, M., Kawakami, H., Kurokawa, K., Kubota, T., Takata, M., and Katayama, T. 2001. DNA replication-coupled inactivation of DnaA protein in vitro: a role for DnaA arginine-334 of the AAA+ Box VIII motif in ATP hydrolysis. *Mol. Microbiol.* **40**: 376-386.

208. Sutton, M. D., Carr, K. M., Vicente, M., and Kaguni, J. M. 1998. *Escherichia coli* DnaA protein. The N-terminal domain and loading of DnaB helicase at the *E. coli* chromosomal origin. *Journal of Biological Chemistry* **273**: 34255-34262.

209. Sutton, M. D. and Kaguni, J. M. 1997. Threonine 435 of *Escherichia coli* DnaA protein confers sequence-specific DNA binding activity. *Journal of Biological Chemistry* **272**: 23017-23024.

210. Tabata, S., Oka, A., Sugimoto, K., Takanami, M., Yasuda, S., and Hirota, Y. 1983. The 245 base-pair *oriC* sequence of the *E. coli* chromosome directs bidirectional replication at an adjacent region. *Nucleic Acids Res.* **11**: 2617-2626.

211. Tercero, J. A., Labib, K., and Diffley, J. F. 2000. DNA synthesis at individual replication forks requires the essential initiation factor Cdc45p. *EMBO J.* **19**: 2082-2093.

212. Theisen, P. W., Grimwade, J. E., Leonard, A. C., Bogan, J. A., and Helmstetter, C. E. 1993. Correlation of gene transcription with the time of initiation of chromosome replication in *Escherichia coli*. *Mol. Microbiol.* **10**: 575-584.

213. Tojo, N., Inouye, M., and Komano, T. 1993a. The *lonD* gene is homologous to the *lon* gene encoding an ATP-dependent protease and is essential for the development of *Myxococcus xanthus*. *J. Bacteriol.* **175**: 4545-4549.

214. Tojo, N., Inouye, S., and Komano, T. 1993b. Cloning and nucleotide sequence of the *Myxococcus xanthus lon* gene: Indespensability of *lon* for vegetative growth. *J. Bacteriol.* **175**: 2271-2277.

215. Tomoyasu, T., Gamer, J., Bukau, B., Kanemori, M., Mori, H., Rutman, A. J., Oppenheim, A. B., Yura, T., Yamanaka, K., Niki, H., and . 1995. *Escherichia coli* FtsH is a membrane-bound, ATP-dependent protease which degrades the heat-shock transcription factor sigma 32. *EMBO J.* **14**: 2551-2560.

216. Tomoyasu, T., Ogura, T., Tatsuta, T., and Bukau, B. 1998. Levels of DnaK and DnaJ provide tight control of heat shock gene expression and protein repair in *Escherichia coli*. *Mol. Microbiol.* **30**: 567-581.

217. Torheim, N. K. and Skarstad, K. 1999. *Escherichia coli* SeqA protein affects DNA topology and inhibits open complex formation at *oriC*. *EMBO J.* **18**: 4882-4888.

218. Tsai, J. W. and Alley, M. R. 2001. Proteolysis of the *Caulobacter* McpA chemoreceptor is cell cycle regulated by a ClpX-dependent pathway. *J. Bacteriol.* **183**: 5001-5007.

219. Turgay, K., Hahn, J., Burghoorn, J., and Dubnau, D. 1998. Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor. *EMBO J.* **17**: 6730-6738.

220. Tye, B. K. 1999. MCM proteins in DNA replication. *Annu. Rev. Biochem.* **68**: 649-686.

221. van Dijl, J. M., Kutejova, E., Suda, K., Perecko, D., Schatz, G., and Suzuki, C. K. 1998. The ATPase and protease domains of yeast mitochondrial Lon: roles in proteolysis and respiration-dependent growth. *Proc. Natl. Acad. Sci. U. S. A* **95**: 10584-10589.

222. Van Melderen, L., Thi, M. H., Lecchi, P., Gottesman, S., Couturier, M., and Maurizi, M. R. 1996. ATP-dependent degradation of CcdA by Lon protease. Effects of secondary structure and heterologous subunit interactions. *Journal of Biological Chemistry* **271**: 27730-27738.

223. Viollier, P. H., Sternheim, N., and Shapiro, L. 2002a. A dynamically localized histidine kinase controls the asymmetric distribution of polar pili proteins. *EMBO J.* **21**: 4420-4428.

224. Viollier, P. H., Sternheim, N., and Shapiro, L. 2002b. Identification of a localization factor for the polar positioning of bacterial structural and regulatory proteins. *Proc. Natl. Acad. Sci. U. S. A* **99**: 13831-13836.

225. Wah, D. A., Levchenko, I., Baker, T. A., and Sauer, R. T. 2002. Characterization of a Specificity Factor for an AAA+ ATPase. Assembly of SspB Dimers with ssrA-Tagged Proteins and the ClpX Hexamer. *Chem. Biol.* **9**: 1237-1245.

226. Wahle, E., Lasken, R. S., and Kornberg, A. 1989. The DnaB-DnaC replication protein complex of Escherichia coli. II. Role of the complex in mobilizing DnaB functions. *Journal of Biological Chemistry* **264**: 2469-2475.

227. Walker, J. E., Saraste, M., Runswick, M. J, and Gay, N. J. 1982. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J.* 945-951.

228. Wang, J., Hartling, J. A., and Flanagan, J. M. 1997. The structure of ClpP at 2.3 A resolution suggests a model for ATP-dependent proteolysis. *Cell* **91**: 447-456.

229. Wang, N., Gottesman, S., Willingham, M. C., Gottesman, M. M., and Maurizi, M. R. 1993. A human mitochondrial ATP-dependent protease that is highly homologous to bacterial Lon protease. *Proc. Natl. Acad. Sci. U. S. A* **90**: 11247-11251.

230. Weber-Ban, E. U., Reid, B. G., Miranker, A. D., and Horwich, A. L. 1999. Global unfolding of a substrate protein by the Hsp100 chaperone ClpA. *Nature* **401**: 90-93.

231. Weigel, C. and Seitz, H. 2002. Strand-specific loading of DnaB helicase by DnaA to a substrate mimicking unwound *oriC*. *Mol. Microbiol.* **46**: 1149-1156.

232. Weinreich, M., Liang, C., and Stillman, B. 1999. The Cdc6p nucleotide-binding motif is required for loading MCM proteins onto chromatin. *Proc. Natl. Acad. Sci. U. S. A* **96**: 441-446.

233. Wheeler, R. T., Gober, J. W., and Shapiro, L. 1998. Protein localization during the *Caulobacter crescentus* cell cycle. *Curr. Opin. Microbiol.* **1**: 636-642.

234. Wheeler, R. T. and Shapiro, L. 1999. Differential localization of two histidine kinases controlling bacterial cell differentiation. *Mol. Cell* **4**: 683-694.

235. Wohlschlegel, J. A., Dwyer, B. T., Dhar, S. K., Cvetic, C., Walter, J. C., and Dutta, A. 2000. Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. *Science* **290**: 2309-2312.

236. Wold, S., Boye, E., Slater, S., Kleckner, N., and Skarstad, K. 1998. Effects of purified SeqA protein on *oriC*-dependent DNA replication *in vitro*. *EMBO J.* **17**: 4158-4165.

237. Wortinger, M., Sackett, M. J., and Brun, Y. V. 2000. CtrA mediates a DNA replication checkpoint that prevents cell division in *Caulobacter crescentus*. *EMBO J.* **19**: 4503-4512.

238. Wright, R., Stephens, C., Zweiger, G., Shapiro, L., and Alley, M. R. 1996. *Caulobacter* Lon protease has a critical role in cell-cycle control of DNA methylation. *Genes Dev.* **10**: 1532-1542.

239. Wu, J., Benson, A. K., and Newton, A. 1995. Global regulation of a sigma 54-dependent flagellar gene family in *Caulobacter crescentus* by the transcriptional activator FlbD. *J. Bacteriol.* **177**: 3241-3250.

240. Wu, J. and Newton, A. 1996. Isolation, identification, and transcriptional specificity of the heat shock sigma factor sigma32 from *Caulobacter crescentus*. *J. Bacteriol.* **178**: 2094-2101.

241. Wu, J., Ohta, N., and Newton, A. 1998. An essential, multicomponent signal transduction pathway required for cell cycle regulation in *Caulobacter*. *Proc. Natl. Acad. Sci. U. S. A* **95**: 1443-1448.

242. Wu, J., Ohta, N., Zhao, J. L., and Newton, A. 1999a. A novel bacterial tyrosine kinase essential for cell division and differentiation. *Proc. Natl. Acad. Sci. U. S. A* **96**: 13068-13073.

243. Wu, W. F., Zhou, Y., and Gottesman, S. 1999b. Redundant *in vivo* proteolytic activities of *Escherichia coli* Lon and the ClpYQ (HslUV) protease. *J. Bacteriol.* **181**: 3681-3687.

244. Young, M. R., Suzuki, K., Yan, H., Gibson, S., and Tye, B. K. 1997. Nuclear accumulation of *Saccharomyces cerevisiae* Mcm3 is dependent on its nuclear localization sequence. *Genes Cells* **2**: 631-643.

245. Zeth, K., Ravelli, R. B., Paal, K., Cusack, S., Bukau, B., and Dougan, D. A. 2002. Structural analysis of the adaptor protein ClpS in complex with the N-terminal domain of ClpA. *Nat. Struct. Biol.* **9**: 906-911.

246. Zhang, X., Shaw, A., Bates, P. A., Newman, R. H., Gowen, B., Orlova, E., Gorman, M. A., Kondo, H., Dokurno, P., Lally, J., Leonard, G., Meyer, H., van Heel, M., and Freemont, P. S. 2000. Structure of the AAA ATPase p97. *Mol Cell* **6**: 1473-1484.

247. Zhong, W., Feng, H., Santiago, F. E., and Kipreos, E. T. 2003. CUL-4 ubiquitin ligase maintains genome stability by restraining DNA-replication licensing. *Nature* **423**: 885-889.

248. Zou, L. and Stillman, B. 1998. Formation of a preinitiation complex by S-phase cyclin CDK-dependent loading of Cdc45p onto chromatin. *Science* **280**: 593-596.

249. Zou, L. and Stillman, B. 2000. Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. *Mol Cell Biol.* **20**: 3086-3096.

250. Zweiger, G., Marczynski, G., and Shapiro, L. 1994. A *Caulobacter* DNA methyltransferase that functions only in the predivisional cell. *J. Mol. Biol.* **235**: 472-485.

251. Zweiger, G. and Shapiro, L. 1994. Expression of *Caulobacter dnaA* as a function of the cell cycle. *J. Bacteriol.* **176**: 401-408.

CHAPTER 1 FIGURE LEGENDS

Figure 1: The *C. crescentus* cell-cycle. The dimorphic *C. crescentus* cell-cycle is shown. The swarmer cell is motile and non-replicative. Swarmer to stalked cell differentiation, where the polar flagellum is shed and a stalk grown in its stead is coupled to replication initiation during G1 to S transition. The stalked cell replicates its chromosome and produces asymmetric cell-division yielding both a swarmer and stalked-cell progeny. The green shading indicates the CtrA protein, also shown in Figure 2, and described in Figure 3.

Figure 2: *C. crescentus* as a model bacterium. The different cellular functions for which *C. crescentus* is a model organism are shown in the context of the developmental cell-cycle. The amenability of *C. crescentus* for synchronization makes it ideal for cell cycle, morphological, and developmental studies. The CtrA response regulator's cell-specific distribution is shown in green. *C. crescentus* is a model bacterium for chromosome replication (upper left), differentiation and development (upper center), cell-division (upper right), stalk biosynthesis (lower left), pili formation (lower center) and flagellum biosynthesis (lower right).

Figure 3: The regulation of CtrA in the cell-cycle and the scope of its activity. The temporal and spatial cell-cycle degradation of CtrA is shown in green. The localization patterns of PleC (dark blue), DivJ (red), DivK(black) and CckA (light blue) is shown for the cell-cycle. The phosphorylation schemes for two-component and phosphorelay

proteins are shown, and their histidines (H1, H2), aspartates (D1, D2) and tyrosines (Y1) is designated. The role of CtrA-P as an activator (arrows) or repressor (perpendicular lines) of genes and their roles for the cell are shown. This figure is adapted from (Domian et al. 1999; Jacobs et al. 1999; Jenal 2000; Jacobs et al. 2001; Laub et al. 2002).

Figure 4: *Cori*, the *C. crescentus* replication origin. *Cori* is located between the first gene in the heme biosynthetic operon, *hemE,* and RP001, a gene of unknown function. *Cori* contains five CtrA binding sites (a-e) illustrated as paired circles. The putative DnaA box is illustrated as an arrow. CtrA sites ab are involved in regulating the transcription of the Ps, strong promoter, a cell-cycle regulated promoter. The AT rich region is shown to overlap the CtrA binding sites ab. The IHF protein binding sites is shown as the triangle.

Figure 5: The model of replication initiation in *E. coli*. DnaA binds to DNA boxes (black bars) in *oriC*. DnaA, when bound to ATP (pink circles), then can mediate open-complex formation in the AT-rich region of *oriC* and stabilize the ssDNA in the open-complex. Two DnaB hexamers (blue circles) are loaded onto the ssDNA by a DnaC (helicase loader) protein (grey circles). DnaB helicase then translocate ahead of the primase (blue circle) that lays down the primer for DNA replication and DNA Polymerase III that processively replicates the chromosome upon interaction with the sliding clamp (light blue ring). As elongation proceeds, the sliding clamp stimulates ATP hydrolysis of DnaA forming DnaA-ADP (green circles) that cannot initiate replication This figure is based upon the model illustrated in (Messer 2002).

Figure 6: **Protein domains common to all AAA+ members.** The motifs from Box VII to the end of Sensor II are the defining characteristics of AAA+. The Walker A (P loop) and Walker B motif are involved in ATP binding and hydrolysis respectively. The conserved arginine in BoxVII is believed to be involved in ATP hydrolysis during higher ordered structure formation. (Davey et al. 2002) (Neuwald et al. 1999).

Figure 7: The eukaryotic cell-cycle and its control. The G1 (pink), S (grey), G2 (blue) and M (green) stages of the cell-cycle are shown. Cell-division begins at the start of M phase. Replication licensing occurs at the end of M phase and the formation of the pre-RC complex occurs during G1 phase. The events that pertain to the formation of the pre-RC are shown. Replication initiates at the end of G1 phase , at the start of S phase and requires a co-ordinated activities between the pre-RC and cyclin-dependent kinases. As S phase proceeds, licensing is lost and the pre-RC complex disassembles.

Figure 8: A comparative schematic of bacterial proteases. The bacterial proteases are shown. The Walker A and Walker B motifs are shown as A and B (green lines) respectively. The proposed ClpP recognition motif within ClpX, ClpC and ClpA is shown as P (green line). The two AAA+ motifs in ClpA, B and C are shown as AAA-1 and AAA-2. The Serine active site in Lon protease is marked as S in the C-terminus. The trans-membrane domains in the FtsH protease are marked TM. This figure is based on one presented in (Dougan et al. 2002)

Figure 1: The *C. crescentus* cell-cycle

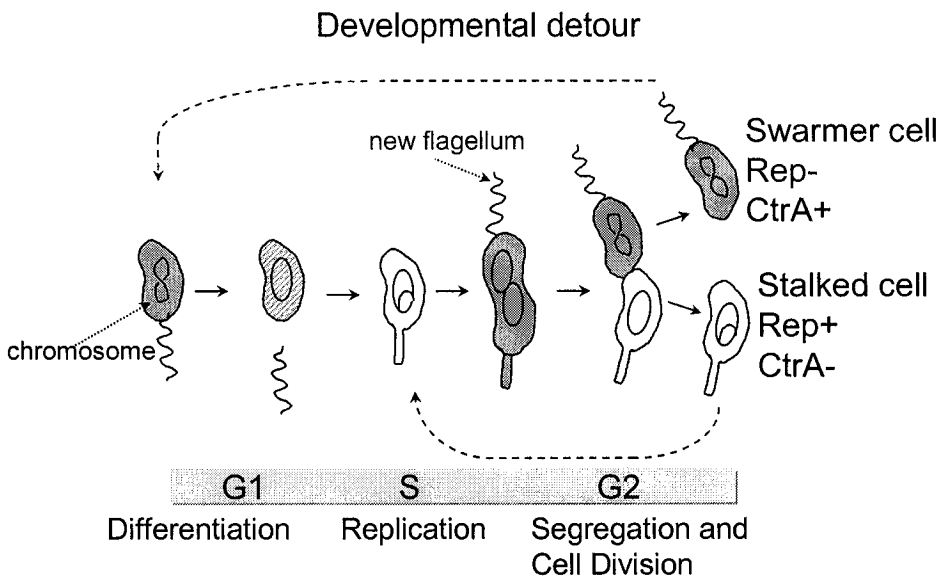


Figure 2: *C. crescentus* as a model bacterium.

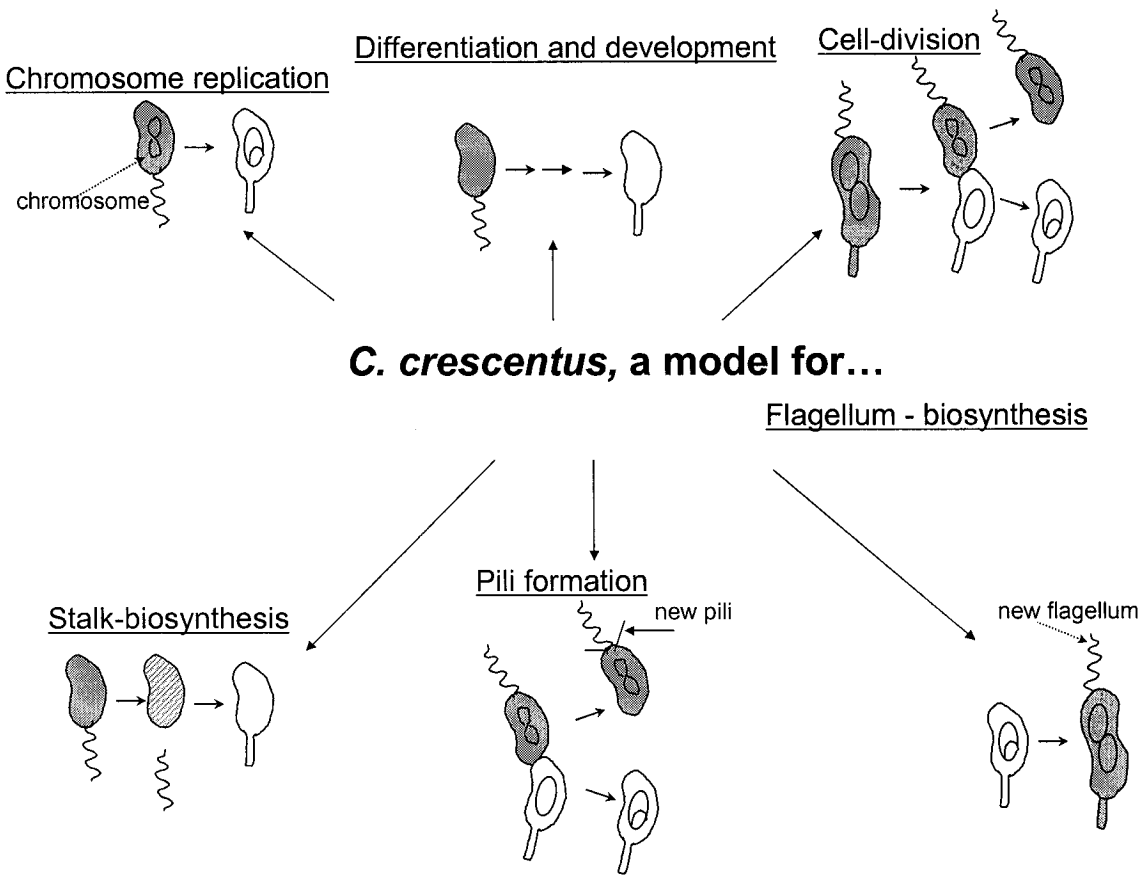


Figure 3: The regulation of CtrA in the cell-cycle and the scope of its activity.

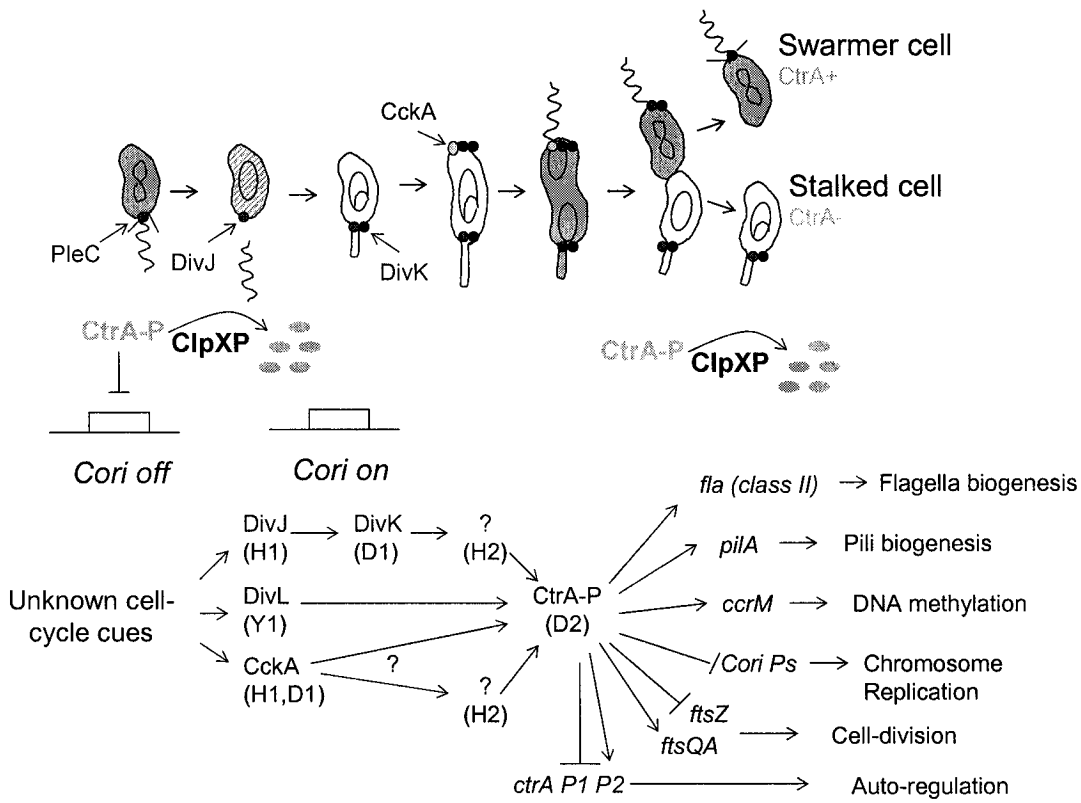


Figure 4: *Cori,* the *C. crescentus* replication origin.

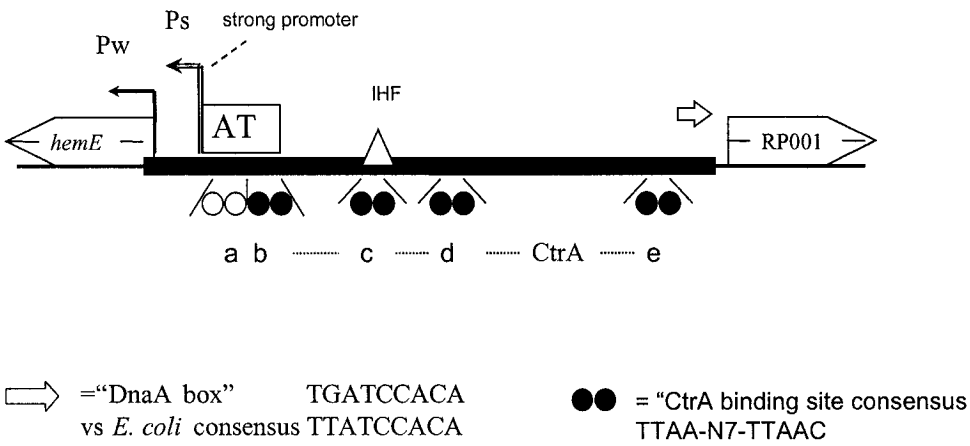


Figure 5: The model of replication initiation in *E. coli*.

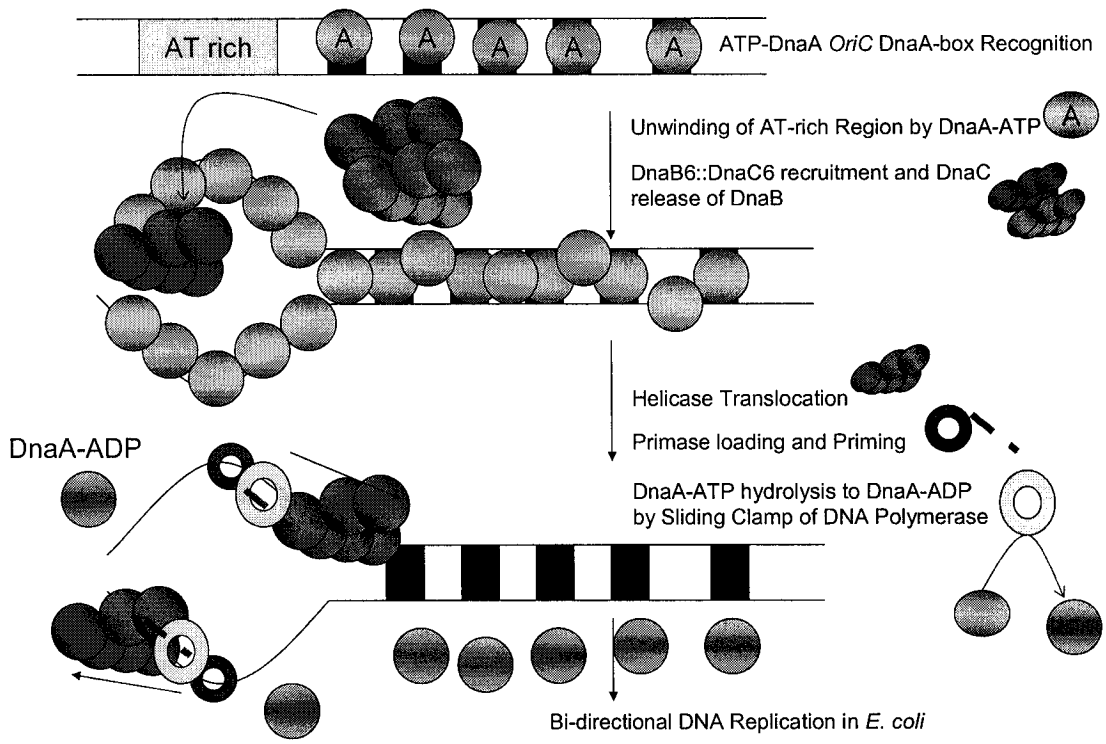


Figure 6: Protein domains common to all AAA+ members.

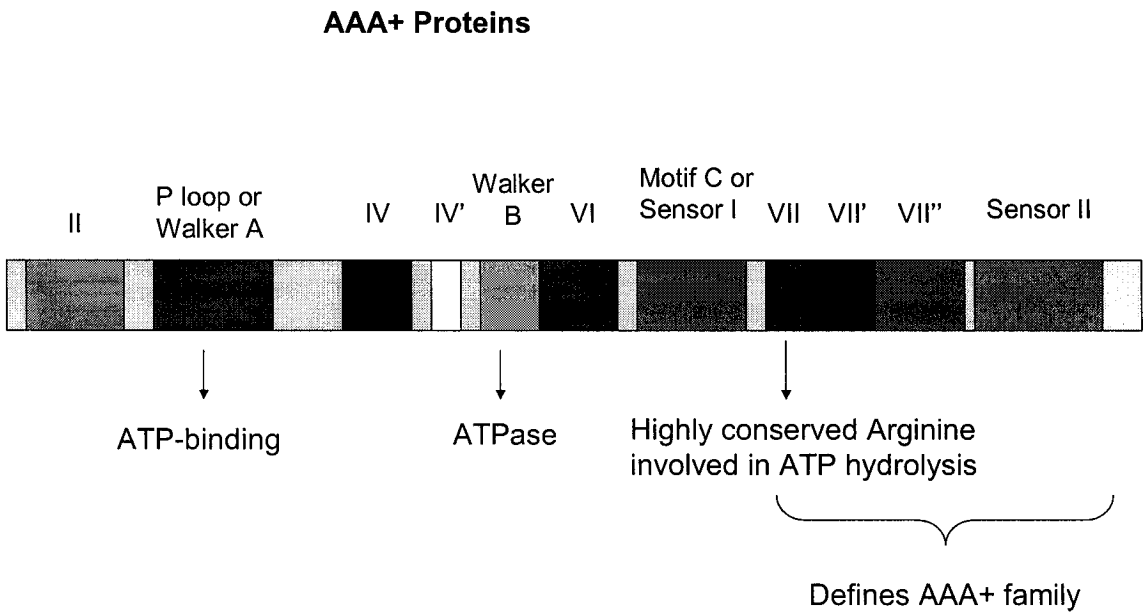


Figure 7: The eukaryotic cell-cycle and its control.

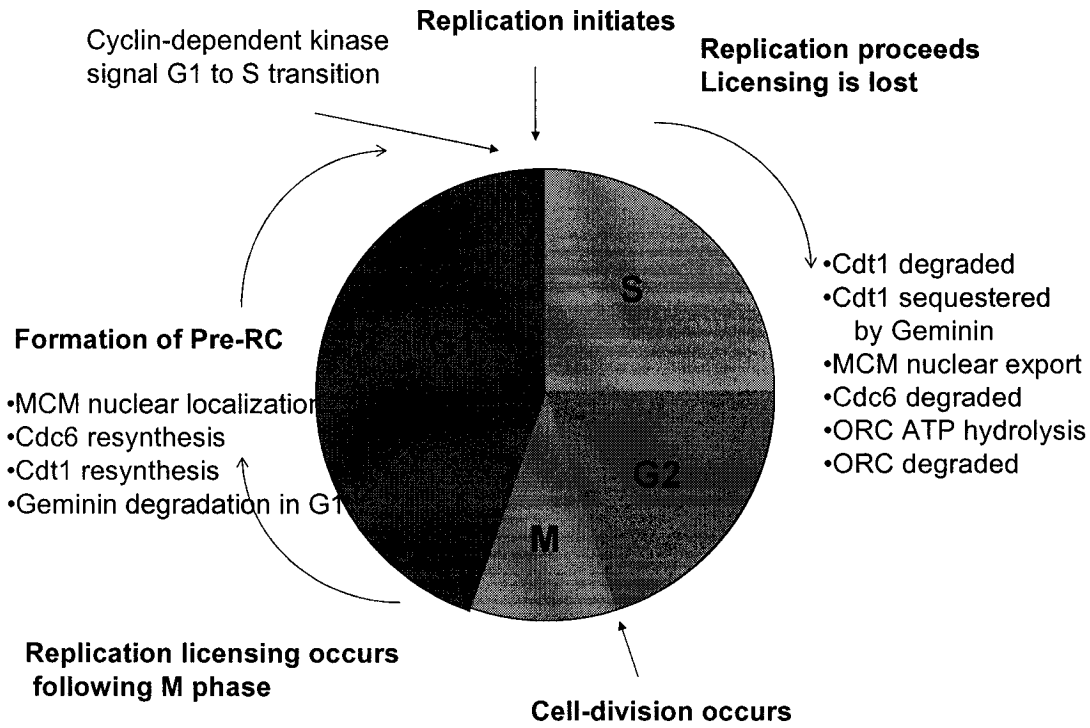
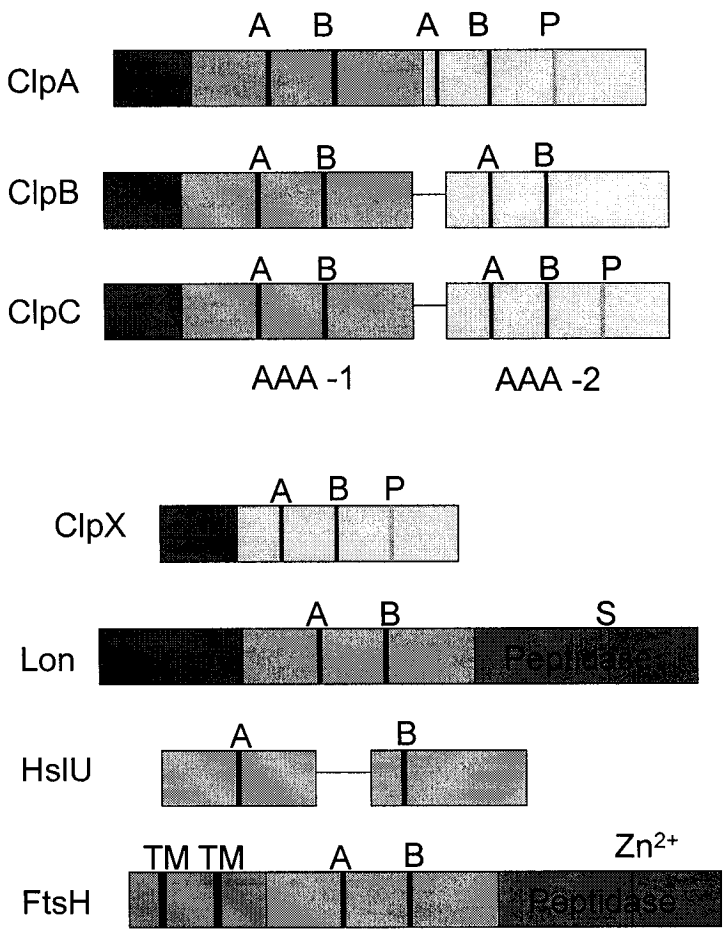


Figure 8: A comparative schematic of bacterial proteases.



PREFACE TO CHAPTER 2

E. coli replication is governed by the DnaA protein which is conserved amongst all bacteria. Therefore, DnaA is tacitly believed to be the replication initiator in all bacteria. The best characterized role for DnaA in replication initiation stems from work performed in *E. coli* that extensively analyzed its role as the key recognizer of the origin, DNA structure modifier, and the key recruiter of sequential replication initiation proteins. It is also targeted for regulation by several mechanisms to ensure that excessive replication initiation does not occur. In this chapter, I examine the role of DnaA in *C. crescentus*, a bacterium that uses unique mechanisms to restrict replication to the correct time during the cell-cycle. I first demonstrate that DnaA is an essential gene, and then I create a conditional system to regulate its expression in order to ascertain the physiological consequences that result from DnaA loss. I also employ transcription reporters to monitor the consequences of *dnaA* block on specific developmental stages. The role of DnaA as a key replication initiator for *C. crescentus* is demonstrated in this chapter.

Chapter 2: Physiological consequences of blocked *Caulobacter crescentus dnaA* expression, an essential DNA replication gene

Boris Gorbatyuk and Gregory T. Marczynski*

Dept. of Microbiology & Immunology

McGill University

Montreal, Quebec, Canada H3A 2B4

Running title: DNA Replication in *Caulobacter crescentus*

Keywords: DnaA, replication, *Caulobacter*, cell-cycle, CtrA.

*Mailing Address: Dept of Microbiology & Immunology
McGill University
3775 University Street
Montreal, Quebec, Canada H3A 2B4
Phone: (514) 398-3917
FAX: (514) 398-7052
Email:GMARCZYNSKI@NEXUS.MICROIMM.MCGILL.CA

Summary

Caulobacter crescentus chromosome replication is precisely coupled to a developmental cell-cycle. Like most eubacteria, *C. crescentus* has a DnaA homologue presumed to initiate chromosome replication. However, the *C. crescentus* replication origin (*Cori*) lacks perfect consensus *Escherichia coli* DnaA boxes. Instead, the *Cori* strong transcription promoter (Ps) may regulate chromosome replication through the CtrA cell-cycle response regulator. We therefore created a conditional *dna*A *C. crescentus* strain. Blocking *dna*A expression immediately decreased DNA synthesis which stopped after approximately one doubling period. Fluorescent flow cytometry confirmed that DNA synthesis is blocked at the initiation stage. Cell division also stopped, but not swarmer to stalked cell differentiation. All cells became stalked cells that grew as long filaments. Therefore, general transcription and protein synthesis continued while DNA synthesis stopped. However, transcription was selectively blocked from the flagellar *fli*Q and *fli*L, and methyltransferase *ccr*M promoters which require CtrA and are blocked by different DNA synthesis inhibitors. Interestingly, transcription from *Cori* Ps continued unaltered. Therefore, Ps transcription is not sufficient for chromosome replication. Approximately 6 to 8 hours following blocked *dna*A expression, cells exponentially lost viability. Coincidentally, β-galactosidase was induced from one transcription reporter, suggesting an altered physiology. We conclude that *C. crescentus* DnaA is essential for chromosome replication initiation, and perhaps also has a wider role in cell homeostasis.

Introduction:

The DnaA protein is essential for chromosome replication initiation in *Escherichia coli* (for recent reviews see Skarstad and Boye, 1994; Messer and Weigel, 1996; and Kaguni, 1997). *E. coli* DnaA binds four DnaA boxes within the *E. coli* origin of replication, *oriC*, and mediates open complex formation by making secondary contacts with three 13-mer motifs within an AT rich region. DnaA also recruits DnaB helicase to the open complex where it unwinds the origin and commits the chromosome for bi-directional replication (Bramhill and Kornberg, 1988; Baker and Wickner, 1992).

Although *dnaA* sequence homologies have been identified in many eubacteria (Skarstad and Boye, 1994), aside from *E. coli,* DnaA's role in replication control has been demonstrated genetically only in *B. subtilis* (Moriya *et al.,* 1990). DnaA homologues have been found in *Mycoplasma spp.* (Fraser *et al.,* 1995; Seto *et al.,* 1997), *Streptococcus pneumoniae* (Gasc *et al.,* 1998), *Rhizobium meliloti* (Margolin *et al.,* 1995), *Rickettsia Prowazeki* (Waite *et al.,* 1998), *C. crescentus* (Zweiger *et al.,* 1994), *Mycobacterium tuberculosis* (Rajagopalan *et al.,* 1995b), *Mycobacterium smegmatis* (Rajagopalan *et al.,* 1995a) and the cyanobacteria, *Synechocystis sp.* (Richter and Messer, 1995). It is assumed that DnaA is universally essential to initiate bacterial chromosome replication, however genetic disruption studies demonstrated that *dnaA* is not essential for *Synechocystis sp.* and its circadian cell cycle control (Richter *et al.,* 1998).

We wanted to test the assumption that DnaA is essential for chromosome replication in the phylogenetically distant Gram-negative, α-purple bacterium, *Caulobacter crescentus*. *C. crescentus* provides an exceptional model for studying the bacterial cell cycle. *C. crescentus* divides assymetrically to yield a motile, flagella-

bearing swarmer cell, and a sessile stalked cell (for recent reviews see Brun *et al.,* 1994; Shapiro and Losick, 1997; Hung *et al.,* 2000). Swarmer cells repress chromosome replication (Quon *et al.*, 1998), and in order for replication to proceed, they must shed their flagella and differentiate into stalk cells (Degnen and Newton, 1972b; Marczynski *et al.,* 1990). *C. crescentus* has one circular chromosome, and bi-directional chromosome replication (Dingwall and Shapiro, 1989) occurs once and only once per cell cycle (Marczynski, 1999).

The *C. crescentus* replication origin (*Cori*) uniquely supports autonomous *Cori*-plasmid replication in the stalked cell type (Marczynski and Shapiro, 1992). Several unique regulatory elements are present in *Cori.* There are five 9-mer GTTAA-N7-TTAA motifs (Marczynski and Shapiro, 1992) that are bound by the CtrA response regulator (Quon *et al.,* 1998; Siam and Marczynski, 2000). CtrA represses chromosome replication in swarmer cells (Quon *et al.*, 1998) and its degradation is thought to allow chromosome replication in stalk cells (Domian *et al.,* 1997). Several promoters have also been identified in *Cori* and transcription from the strong promoter (Ps) is believed to stimulate replication, as transcription from Ps is required for *Cori*-plasmid replication and only occurs in stalk cells (Marczynski *et al.*, 1995). Phosphorylated CtrA binds cooperatively to binding sites "a" and "b" of Ps in *Cori.* This presumably represses Ps transcription and consequent chromosome replication in swarmer cells (Siam and Marczynski, 2000).

Unlike the *B. subtilis* and *E. coli oriC*, as well as other bacterial replication origins which contain DnaA boxes that match the proposed consensus sequence, the *C. crescentus Cori* has no DnaA boxes that perfectly match the consensus (Marczynski and Shapiro, 1992). However, point mutations in one potential DnaA box, that matches 8/9

bp of the TTATCCACA *E. coli* consensus, abolish *Cori*-plasmid replication (Marczynski and Shapiro, 1992). This suggests a role for DnaA in *C. crescentus* replication control, but considering the lack of consensus DnaA boxes as well as other unique sequence features, this role may be significantly different.

Here, we report the physiological consequences of blocked *dnaA* expression on cell division and chromosome replication. We also studied the transcription of replication-dependent promoters and *Cori* promoters in response to blocked *dnaA* expression. We propose that constant transcription and translation of DnaA in *C. crescentus* is required for normal cell cycle progression.

Results

dnaA *is an essential gene for* C. crescentus

The chromosome *dna*A gene cannot be disrupted except when complemented by a plasmid *dna*A gene. The *dna*A gene was subcloned from a cosmid DNA spanning the entire replication origin (*Cori*) region (Fig. 1A). To disrupt the *dna*A gene, the indicated *Xba*I to *Bam*HI *dna*A fragment was ligated into plasmid pGM1360. This *Xba*I to *Bam*HI fragment contained the entire *dna*A gene, its promoter, as well as downstream sequences into the *alk*B gene that points in the opposite orientation (Fig. 1A). To create the *dna*A::Ω disruption plasmid (pGM1531), the omega cassette, encoding spectinomycin and streptomycin resistance genes (from pHP45), was ligated between two unique *Xho*I sites, and therefore disrupted and removed most of the *dna*A coding sequences (Fig. 1A). This *dna*A::Ω plasmid was conjugated with the synchronizable mutant *C. crescentus* strain NA1000, a derivative of wild type CB15. Southern blot analysis demonstrated that the resulting kanamycin (Km) resistant colonies acquired pGM1531 by homologous recombination at the *dna*A gene (Fig. 1B, and data not shown). These cells also acquired spectinomycin and streptomycin resistance from the marked *dna*A::Ω gene and sucrose sensitivity from the plasmid encoded *sac*B gene.

To test if the intact *dna*A gene could be exchanged by the *dna*A::Ω allele, we selected for a second homologous exchange that would remove integrated pGM1531 from the chromosome (Fig. 1B). These cells were plated on 3% sucrose, and surviving colonies were scored for antibiotic resistance. No spectinomycin and streptomycin resistant colonies were obtained (*dna*A::Ω, Fig. 1B), unless this strain also contained the

intact *dna*A gene on autonomously replicating pGM1522 (Fig. 1C). This implied allelic replacement (*dnaA*::Ω) was confirmed by Southern blot analysis (data not shown). The *dna*A::Ω cells also acquired stable tetracycline resistance (Tc) from pGM1522 (Fig. 1C), which is otherwise easily lost following subculture in PYE media without tetracycline. Since pGM1522 can not be lost from the *dna*A::Ω cells, when it provides the only copy of an intact *dna*A gene, this also argues that *dna*A is essential. Therefore, the natural *dnaA* gene can be disrupted only when another *dnaA* gene is present.

Conditional dnaA *expression also demonstrates that it is essential*

We used the conditional *xylX* promoter (P_{xylX}) and placed *dnaA* expression under both the transcriptional and translational control of the *xylX* operon. The transcription of P_{xylX} is induced by xylose and repressed by glucose (Meisensahl *et al.*, 1997). A 5' *EcoRI* site and a 3' *SpeI* site were introduced into *dnaA* by a PCR reaction that amplifed *dnaA* with oligonucleotide primers containing these sites (see experimental procedures) and cloned into the *EcoRI/SpeI* sites of the kanamycin (*nptII*) resistant plasmid, pNPT228XNE (Table 1). This created a translational fusion of *dnaA* with the first six amino acids of the first gene of the *xylX* operon, pGM2195 (Fig. 2A). Plasmid pGM2195 was integrated into the *xylX* locus of a wild type strain, NA1000, to create a strain, GM2409, with an asymmetric *xylX* gene duplication in the chromosome (Fig. 2A), confirmed by Southern blot hybridization (data not shown). The wild type *dnaA* gene, at the wild type locus was converted to the *dnaA*::Ω disruption by ϕCr30 transduction from strain GM2446 (Fig. 1C) into strain GM2409 (Fig. 2A). The resulting strain, GM2471, (Fig. 2B) was selected for

spectinomycin and streptomycin resistance. GM2471 was sensitive to tetracycline and non-viable when xylose was absent from PYE media or substituted by glucose. Occasionally, tetracycline resistance, due to *dnaA* plasmid pGM1522 was co-transduced with chromosome *dnaA*::Ω. These cells survived on glucose PYE media without xylose but did not lose tetracycline resistance. Therefore, GM2471 cells require xylose to grow and express the essential *dnaA* gene. Single copy P_{xylX} expression of *dnaA* complements the *dnaA*::Ω disruption apparently as effectively as multi-copy plasmid expression of *dnaA*.

Blocked dnaA *expression allows growth but blocks cell division*

To block *dnaA* expression, *C. crescentus*, strain GM2471 (*dnaA*::Ω, P*xylX*::*dnaA*) was grown in minimal (M2) media containing xylose as its sole carbon source (M2X), washed repetitively in M2 media lacking sugar, backdiluted to a low optical density and allowed to proceed growth in either M2G (glucose) or M2X (xylose) media. Cells shifted back to M2X media grew exponentially (Fig. 3A) with a growth rate comparable to wild type NA1000 cells. However, cells shifted to M2G media entered a linear growth phase indicative of blocked cell division (Fig. 3). Control wild type NA1000 cells shifted from xylose to glucose grew exponentially and maintained normal cell division (data not shown). Dark-field microscopy confirmed that M2G cells progressively filament as stalked cells (Fig. 4A-C). Some cells within this filamentous population developed a constriction near one cell pole. This constriction was also visible in some late GM2471 M2G cells (Fig. 4C), although cells were never able to complete cell division. These

GM2471 cells, when shifted to M2G maintained a normal morphology up to 3 hours after the shift, but subsequently failed to divide. Instead, their cell length progressively increased. However, washed cells returned to M2X maintained a typically wild-type morphology, for example at 10.5 hours after the shift (Fig. 4D). When synchronous swarmer populations of GM2471 were shifted to M2G, they all differentiated into stalked cells that likewise grew as stalked filaments. However, the duration of the swarmer to stalk transition was approximately 30 minutes longer than parallel swarmer cells shifted to M2X (data not shown).

Blocked dnaA *expression selectively and rapidly blocks DNA replication*

We examined whether filamentous GM2471 cells synthesize DNA. GM2471 cells were shifted for 7 hours to M2G or M2X media, and diluted to an optical density of 0.1. [α^{32}P]dCTP was added and [α^{32}P]DNA synthesis was measured during a 2.5 minute time course (Fig. 5A). Whereas the M2X culture demonstrated vigorous [α^{32}P]dCTP incorporation into DNA, the parallel M2G culture showed only a baseline level of [α^{32}P]dCTP incorporation (Fig. 5A). Since the M2G culture optical density continues to increase after 7 hours of the shift (Fig. 3) due to continued growth (Fig. 4A-C), this indicates that general metabolism, including RNA and protein synthesis persists while DNA synthesis is arrested.

We next examined how rapidly GM2471 glucose cultures arrested DNA synthesis. Cultures were sampled from 0 to 8 hours following the shift from xylose to glucose, normalized for cell number to an optical density of 0.1, and pulse labeled for 90

seconds with [α^{32}P]dCTP. This method provided a linear incorporation of [α^{32}P]dCTP that reflects the DNA synthetic rate (Fig. 5A and data not shown). The control shift, GM2471 M2X cells continued vigorous DNA synthesis (Fig. 5B Xyl). However, GM2471 M2G cells progressively decrease DNA synthesis and completely arrested DNA synthesis between 1.5 and 2 hours after the shift (Fig 5B Gluc).

C. crescentus *cells blocked in* dnaA *expression accumulate one chromosome*

We examined whether the DNA synthetic block described in Figure 5B is specific for replication initiation. GM2471 cells were grown in filtered M2X media overnight and shifted to M2G and M2X. Cells were sampled at various time points after the shift, treated with chloramphenicol for three hours, fixed in 70% ethanol, stained with Chromomycin A3 and analyzed by fluorescent flow cytometry as described in Experimental Procedures (Fig. 6) (Winzeler and Shapiro, 1995).

GM2471 shifted to M2X for O, 4 and 6 hours maintained populations of cells that had both one and two chromosomes (Fig. 6 "O Xyl, 4 Xyl, 6 Xyl"). Since cells were treated with chloramphenicol, all cells in this mixed population that initiated DNA replication were able to complete replication but were unable to consequently reinitiate replication. Thus, all cells that had the ability to initiate replication have exactly twice as much DNA content as cells that do not have the ability to initiate replication and are represented by the second major peak in the O Xyl, 4 Xyl and 6 Xyl panels. All cells grown in M2X that did not initiate replication (swarmer cells) are represented by the first major peak in the O Xyl, 4 Xyl and 6 Xyl panels.

However, although GM2471 cells shifted to M2G originally had relatively equal proportions of cells with both one and two chromosomes after chloramphenicol treatment (Fig. 6 "O Gluc"), they accumulated only one chromosome at 4 hours and 6 hours after shift to M2G (Fig. 6 "4 Gluc", "6 Gluc"). Since most cells blocked in *dnaA* expression accumulated one chromosome, these cells cannot initiate replication initiation. Therefore, blocked *dnaA* expression selectively blocks replication initiation.

Blocked dnaA *expression is bactericidal*

GM2471 cells were likewise shifted to M2X and M2G, periodically sampled, and plated on PYE media supplemented with xylose to reinduce DnaA. Whereas the M2X culture increased in cell numbers and plating efficiency, the M2G culture exhibited constant cell numbers and subsequently decreasing plating efficiency. Specifically, from 0 to 6 hours after the shift to M2G, viable cell numbers remained constant, but decreased exponentially between 6 to 14 hours (Fig 7). A 10-fold drop in viable cell numbers is observed within 10 hours after the shift, and a 100-fold drop is observed within 14 hours. Although optical density is increasing at a linear rate (Fig 3), due to increased cell length, the number of cells that can form colonies decreases significantly. This suggests that the longer M2G cells grow without DNA synthesis, the less likely they are to survive.

Effect of blocked dnaA *expression on* Cori *promoters*

Transcription from the *C. crescentus* replication origin (*Cori*) promoters may regulate chromosome replication (Marczynski *et al.,* 1995). We therefore studied three promoters within *Cori*: a CtrA regulated strong promoter (Ps) which transcribes towards the *hemE* gene, a constitutive weak promoter (Pw) which transcribes the *hemE* gene, and a divergent promoter (P3) (Siam and Marczynski, unpublished) which may overlap an essential DnaA box (Marczynski and Shapiro, 1992) (Fig. 8A). Transcription from Ps, apparently yields a non-translated RNA, that is restricted to the nascent stalked cells, and which may play a key role in replication initiation (Marczynski *et al.,* 1995). We introduced pRK290-derived plasmids containing the Ps (pGM1631), Pw (pGM1629), or P3 (pGM1630) promoters (Fig. 8A) fused to a *lacZ* reporter, into strain GM2471. After shifting the cultures from M2X to M2G or M2X as described above, we periodically sampled the cells and measured β-galactosidase activity. Promoter activity was calculated and expressed in both conventional Miller units (activity per cell mass) and total enzyme activity present in the culture.

Transcription from the constitutive Pw promoter, as expected, followed cell growth when shifted to M2X or M2G (Fig. 8B). However, the P3 promoter showed a selective drop in transcription as measured by Miller Units when shifted to M2G. Since total enzyme activity increased, the P3 promoter was not off. But, its transcription decreased relative to the cell mass (Fig. 8C). Interestingly, transcription from Ps remained strong and unchanged after the shift to M2G and its transcription was very active even 12 hours after the shift (Fig. 8D).

Blocked dnaA *expression effects transcription of DNA synthesis-dependent promoters*

Transcription from the *fliL* promoter (Stephens and Shapiro, 1993), the *fliQ* promoter (Dingwall *et al.,* 1992) and the *ccrM* promoter are selectively downregulated when DNA replication is blocked by hydroxyurea. We wanted to investigate whether these promoters would also be specifically downregulated in response to blocked *dnaA* expression. We therefore introduced pRK290-derived plasmids, containing the *fliQ* (pGM1574), *fliL* (pGM1576), or *ccrM* (pGM1612) promoters fused to a *lacZ* transcription reporter, into strain GM2471, shifted these cells from M2X to M2G, and measured β-galactosidase activity as described above.

Transcription from the *fliL* promoter stopped rapidly following the M2X to M2G shift. Total enzyme activity did not increase during growth under these conditions indicating that the *fliL* promoter was completely turned off (Fig. 9A). The *ccrM* promoter showed the same response (Fig. 9B). The *fliQ* promoter, like the *fliL* and the *ccrM* promoter halted transcription after the M2X to M2G shift, however, its activity dramatically increased following 8 hours of growth (Fig 9C). The constitutive *rsaA* promoter is not sensitive to treatment with hydroxyurea (Stephens and Shapiro, 1993) and served as a control promoter not influenced by chromosome replication. The *rsaA* promoter (pGM1575) showed an insignificant change in Miller units in cultures shifted to M2G because the total β-galactosidase activity tracked the increased cell mass (Fig. 9D).

Discussion

Genetic and biochemical experiments clearly established that DnaA is an initiator of DNA replication in *E. coli* and *B. subtilis*, but excluding our present study, direct genetic experiments have not been extended to other bacteria. However, the presence of *dnaA* in many eubacteria, coupled with some recent biochemical analysis, suggests conserved mechanisms. For example, the *Streptomyces lividans* DnaA protein binds DnaA boxes within the *S. lividans oriC* (Jakimowicz *et al.*, 1998), and the strongest binding occurs between DnaA and the TTGTCCACA DnaA box consensus that is only one base pair away from the *E. coli oriC* consensus TTATCCACA (Schaper and Messer, 1995). Although binding to consensus DnaA boxes suggests that the *S. lividans* DnaA performs the same function as the *E. coli* DnaA, it has yet to be demonstrated that the *S. lividans* DnaA mediates unwinding of *oriC* (Jakimowicz *et al.*, 1998). As well, in *M. smegmatis,* mutations of DnaA boxes severely impaired *M. smegmatis oriC* plasmid replication (Qin *et al.,* 1997). Point mutations in a near consensus DnaA box (TGATCCACA) suggested that the *C. crescentus* DnaA protein is also essential for replication of a uniquely organized origin, *Cori* (Marczynski and Shapiro, 1992). As well, transcription of the *C. crescentus dnaA* by an artificial Ptac promoter appears to stimulate chromosome replication (Zweiger and Shapiro, 1994), although whether DnaA plays an essential role for *C. crescentus* replication was not critically tested.

Therefore, we created a *C. crescentus* strain GM2471, whose *dnaA* expression requires xylose. We demonstrated that the *C. crescentus dnaA* is essential and that blocking *dnaA* expression causes rapid loss of DNA synthesis, an accumulation of cells

with only one chromosome, loss of transcription from replication-dependent *fliL* and *ccrM* promoters, and eventual loss in viability. Interestingly, *Cori* strong promoter (Ps) transcription remains active while DNA synthesis stops. These results are summarized in Figure 10.

Pulse labeling experiments demonstrated that DNA synthesis drops within a half hour after GM2471 cells are shifted from xylose to glucose, and DNA synthesis ceases within one generation time (Fig. 5B). These kinetics imply completion without the initiation of new chromosome replication which is necessary for entry into a new cell cycle. As well, fluorescent flow cytometry data (Fig. 6) reveals that the *dnaA* block is specific for replication initiation. Microscopic observations of synchronous GM2471 cells with blocked *dnaA* expression reveal that cells start to filament as stalked cells (Fig. 4 and data not shown). This filamentous phenotype is consistent with previous observations that DNA synthesis mutants filament as unpinched stalked cells (Osley and Newton, 1977). The appearance of constrictions near the cell pole in some of the cells in the filamentous M2G population suggests that a block in *dnaA* expression inhibits cell division at both the initiation and the progression stages. However, this point deserves further attention that is beyond the scope of this study. The *C. crescentus* cell cycle is conceptually organized into two pathways, a DNA synthesis pathway coupled to a cell division pathway (reviewed in Ohta *et al.*, 2000) where DNA synthetic mutants fail to enter the cell division pathway. It remains unclear how the chromosome replication pathway communicates with the *C. crescentus* cell division pathway. In *E. coli,* the SOS response protein, SulA, directly interacts with FtsZ and consequently blocks Z ring formation at the site of septation (Mukherjee *et al.*, 1998; Trusca *et al.,* 1998).

Analysis of *Cori* promoters revealed that strong promoter (Ps) transcription was ongoing in response to blocked *dnaA* expression (Fig. 7D). Transcription from Ps is required for *Cori*-plasmid replication, and transcription from Ps only occurs in stalk cells (Marczynski *et al.,* 1995). CtrA mediated repression of Ps transcription is believed to control replication initiation (Quon *et al.,* 1998; Siam and Marczynski, 2000). The fact that Ps transcription was ongoing when cells clearly stopped DNA replication indicates that if Ps plays a role in replication control, replication still requires DnaA.

It is unclear how Pw and P3 are involved in replication control. The Pw promoter is transcribed throughout the cell cycle (Marczynski *et al.,* 1995), and was unaffected by blocked *dnaA* expression (Fig. 7B). The P3 promoter may overlap an essential DnaA box, and P3 transcription decreased (Fig. 7C) relative to Pw, Ps, as well as other control promoters (Figs. 7, 8).

The mechanism by which DnaA mediates replication initiation in *C. crescentus* is unclear. In *E. coli*, DnaA binds to four DnaA boxes within the origin on a negatively supercoiled template and serially melts three AT-rich tandem repeats forming an open complex, and thereby setting the stage for replication initiation (reviewed in Bramhill and Kornberg, 1988; Messer and Weigel, 1996). An essential DnaA box was apparently identified in *Cori* where point mutations abolished plasmid replication (Marczynski and Shapiro, 1992). As well, several hypothetical DnaA boxes are also present inside *Cori* but they share even less sequence similarity with *E. coli* DnaA boxes (Marczynski and Shapiro, 1992). It remains to be tested whether *C. crescentus* DnaA protein binds to the hypothetical *C. crescentus* DnaA boxes.

Several cell cycle regulated promoters are selectively sensitive to blocked DNA replication in the presence of hydroxyurea or at the non-permissive temperature for the *dnaC* elongation mutant (Ohta *et al.,* 1990). This is true for the *ccrM* methyltransferase promoter (Stephens *et al.,* 1995), the *fliL* (Steph ens and Shapiro, 1993), and *fliQ* (Dingwall *et al.* 1992) class II flagellar promoters (for a review on flagellum biosynthesis in *C. crescentus* see Gober and England, 2000). All three replication sensitive promoters are regulated by the CtrA response regulator, which in turn is cell cycle regulated (Quon *et al.,* 1996; Domian *et al.,* 1997). The observation that flagellin synthesis is coupled to DNA synthesis was previously reported (Osley and Newton, 1977) and is now attributed to a dependence on the expression of genes early in the flagellar hierarchy (Dingwall *et al.,* 1992). The *fliL* gene product is essential for flagellum function but not assembly (Jenal *et al.,* 1994) and the *fliQ* gene product is believed to participate in flagellar-specific protein export (Zhuang and Shapiro, 1995).

We examined how the *ccrM, fliL*, and *fliQ* promoters respond to blocked *dnaA* expression, because in principle this treatment should block the initiation step of chromosome replication, while hydroxyurea treatment and growth at non-permissive temperature in the *dnaC* Ts mutation block the elongation step and may damage the DNA. Here, we show that the *fliL* and *ccrM* promoters are indeed sensitive to replication initiation blocks by blocked *dnaA* expression (Fig. 8A-B). Initially, the *fliQ* promoter construct demonstrated the same replication-sensitive response as the *fliL* and *ccrM* reporters, however transcription from this reporter became induced at approximately eight hours following the shift (Fig. 8C). This induction coincided with the drop in cell viability (Figs. 6,9). It is unclear whether the *fliQ* promoter itself or a different promoter

in the reporter construct induces transcription at this late period following blocked *dnaA* expression. However, these events clearly mark an altered cell physiology.

The loss of cell viability in response to blocked *dnaA* expression is a puzzling result. Loss of *E. coli* cell viability was reported for temperature sensitive *E. coli dnaA* (CRT46) following shift to non-permissive temperature, 40°C (Hirota *et al.,* 1968). If DnaA's only function is to bind DnaA boxes and then melt the AT rich region, then there are no covalent modifications or DNA strand breakage which could conspicuously cause cell death. Therefore, in principle *dnaA* initiation mutants should be different than for example, DNA polymerase mutants whose inactivation may leave nicked or broken DNA. Treatment with increasing concentrations of hydroxyurea and mitomycin C, two drugs that inhibit DNA synthesis but allow for cell growth and elongation, also decreased cell viability (Degnen and Newton, 1972a) and these treatments presumably damaged the DNA. We also observe a rapid loss in viability of *C. crescentus dnaC* temperature sensitive DNA synthesis mutants (Osley and Newton, 1977; Ohta *et al.,* 1990) when shifted to non-permissive temperature (data not shown). As well, DNA fragmentation correlates with drop in viability in the *rfc2* replication elongation mutant in the budding yeast, *S. cerevisiae* (Noskov *et al.*, 1998).

Direct loss of replication initiation may not be sufficient to lose viability as cells that stopped chromosome replication by 2 hours, following the xylose to glucose shift, remained viable up to 8 hours. So what is the difference between cells at 2 hours and those at 8 hours? The cells are filamenting and increasing in length, so they could increase past a threshold size. The repercussions of getting too big may be that even if the cells are able to re-synthesize DnaA and initiate chromosome replication, they may be

unable to segregate the newly replicated chromosome into the swarmer cell compartment because the distance may be too long for chromosome partitioning. Changes in cell membrane permeability have also been reported for *dnaA* mutants in *E. coli* (Wegrzyn *et al.*, 1999), and this may provide another possible explanation for the loss of viability.

In summary, we demonstrated that *C. crescentus dnaA* is absolutely required for chromosome replication. However, it is unclear and deserves further study just how DnaA-mediated replication coordinates with cell cycle cues to exercise tight and precise replication control.

Experimental Procedures

Bacterial strains and growth conditions

All bacterial strains and plasmids used in this study are shown in Table 1. *C. crescentus* strains were grown either in PYE complex (Poindexter, 1964) or M2G (0.2% glucose) or M2X (0.3% xylose) minimal media (Ely and Johnson, 1977). To shift unsynchronized GM2471 cells, fresh overnight cultures grown at 30°C in M2X were washed three times with M2 media with no sugar supplement and then seeded in either M2G or M2X media to a final Optical density (A_{660}) of 0.05-0.1 and allowed to grow at 30°C. We used 0.3% xylose in both PYE and M2 media to induce *dnaA* in GM2471 as it was previously shown to stimulate maximal induction of P_{xylX} (Meisenzahl *et al.*, 1997). Plasmids were mobilized to *C. crescentus* from *E. coli* S17-1 by conjugation (Simon *et al.*, 1983). To ascertain total number of viable cells, growing GM2471 cultures shifted to M2G or M2X were serially diluted in M2 medium without sugar and plated on PYE plates supplemented with 0.3% xylose.

Transcription analysis

β-galactosidase assays were performed as described (Miller, 1972) on cultures of *C. crescentus* strain GM2471 containing pRK290 *lacZ* reporter plasmids (Table 1) shifted to M2G or M2X as described above.

Microscopic techniques

C. crescentus strains were analyzed by darkfield light microscopy using a Leitz Dialux microscope at a magnification of 1000X. Bacterial samples were immobilized on glass slides by mixing cells quickly with 1% low-melt agarose that was kept molten at 35°C.

DNA manipulations and DNA radio-labeling

Standard cloning (Sambrook *et al.*, 1989) and PCR protocols (Dieffenbach and Dveksler, 1995) were used in this study. To create the *dnaA*::Ω disruption in pGM1380 and pGM1531 (Fig. 1A), pGM1360 (Table 1) was cut with *XhoI*, made blunt-ended with T4 DNA polymerase, and ligated with the *BamHI* cut antibiotic cassette, from pHP45, similarly made blunt-ended. PCR was used to introduce an *EcoRI* site to the 5' end of *dnaA* directly upstream of the first coding ATG to create a translational fusion with the *xylX* gene (pGM2195; Fig 2A). An *SpeI* site was introduced into the 3' end via PCR amplification of template pGM1181. The 5' primer containing the desired mutation was DNAA-UP (5'-CAG GAA TTC ATG ACC ATG AAG GGC GGG GT). The 3' primer containing the desired mutation was DNAA-DOWN (5'- GTC ACT AGT CCT GTC TCC AGA ACG ACC CT).

Generalized transduction was performed essentially as described (Ely, 1991). Phage lysates were UV irradiated by exposure to short wave UV light of a FischerBiotech FBUVLS-80 UV hand lamp (Fischer Scientific) for 1-3 minutes. Transduction with

bacteriophage ϕCr30 was used for introducing the *dnaA*::Ω disruption from phage lysates grown on GM2446 (Fig. 1C) into GM2409 (Fig. 2A) to create strain GM2471 (Fig. 2B).

Replication was assayed by DNA radio-labeling with [α-^{32}P]dCTP, as described (Marczynski and Shapiro, 1992) except that labeled dCTP (3,000 Ci/mmol) was used instead of dGTP. Prior to radio labeling, cells shifted to M2X and M2G and sampled at various time points were normalized to an optical density (A_{660}) of 0.1.

Flow Cytometry

All cultures analyzed using flow cytometry were grown in filtered M2G or M2X media at 30°C. All cells were sampled between an A_{660} of 0.05 to 0.2, and treated with 25 μg/ml chloramphenicol for 3 hours at 30°C. All samples were washed in ice cold water prior to fixation in order to avoid cell clumping. Samples were fixed by the addition of ice-cold ethanol to a final concentration of 70%. Cells were concentrated and stained with 10 μg/ml chromomycin A3 as described (Winzeler and Shapiro, 1995). 10,000 cells from each sampling were segregated into discreet bins according to Chromomycin A3 DNA fluorescence intensity. Cells were analyzed on a Becton Dickinson FACStar Plus machine (Becton Dickinson, San Jose, CA) equipped with an argon ion laser at the Stanford University Shared FACS Facility. Excitation was at 458 nm and fluorescence was measured at 495 nm. FACS data was analyzed using the FlowJo analytical program (Tree Star Inc., San Carlos, CA).

Acknowledgments

We thank Dr.'s M.R.K. Alley, U. Jenal, C. Stephens and Y. Brun for providing strains and plasmids. Dr. E.C.S. Chan and R. McLaughlin for kind help with dark-field microscopy. Ann Reisenauer and members of the Stanford University Shared FACS facility for invaluable help with Flow cytometry. S.L Chen for helpful discussion and critical reading. Members of the Marczynski laboratory and referees for critical reading.

This work was supported by the Medical Research Council of Canada (MRC) Grant MT-13453, and an MRC Scholarship Award 273-46 to G.T.M.

References

Baker, T.A., and Wickner, S.H. (1992) Genetics and enzymology of DNA replication in *Escherichia coli*. *Annu Rev Genet* **26:** 447-477.

Bramhill, D., and Kornberg, A. (1988) A model for initiation at origins of DNA replication. *Cell* **54:** 915-918.

Brun, Y.V., Marczynski, G., and Shapiro, L. (1994) The expression of asymmetry during *Caulobacter* cell differentiation. *Annu Rev Biochem* **63:** 419-450.

Colombi, D., and Gomes, S.L. (1999) An *alkB* gene homologue is differentially transcribed during the *Caulobacter crescentus* cell cycle. *J Bacteriol* **179:** 3139-3145.

Degnen, S.T., and Newton, A. (1972a) Dependance of cell division on the completion of chromosome replication in *Caulobacter crescentus*. *J Bacteriol* **110:** 852-856.

Degnen, S.T., and Newton, A. (1972b) Chromosome replication during development in *Caulobacter crescentus*. *J Mol Biol* **116:** 671-680.

Dieffenbach, C.W., and Dveksler, G.S. (1995) *PCR Primer: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Dingwall, A., and Shapiro, L. (1989) Rate, origin, and bidirectionality of *Caulobacter* chromosome replication as determined by pulsed-field gel electrophoresis. *Proc Natl Acad Sci USA.* **86:** 119-123.

Dingwall A., Zhuang, W., Quon, K., and Shapiro, L. (1992) Expression of an early gene in the flagellar regulatory hierarchy is sensitive to an interruption in DNA replication. *J Bacteriol* **174:** 1760-1768.

Ditta, G., Schmidthauser, T., Yakobson, E., Lu, P. Liang, X.W., Finlay, D.R., Guiney, D., and Helinski, D.R. (1985) Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. *Plasmid* **13:** 149-153.

Domian I.J., Quon, K.C., and Shapiro, L. (1997) Cell type-specific phosphorylation and proteolysis of a transcriptional regulator controls the G1-to-S transition in a bacterial cell cycle. *Cell* **90:** 415-424.

Ely, B. (1991) Genetics of *Caulobacter crescentus. Methods Enzymol* **204:** 372-384.

Ely, B. and Johnson, R.C. (1977) Generalized transduction in *Caulobacter crescentus. Genetics* **87:** 391-399.

Evinger, M., and Agabian, N. (1977) Envelope-associated nucleoid from *Caulobacter crescentus* stalked and swarmer cells. *J Bacteriol* **132:** 294-301.

Fraser, C.M., Gocayne, J.D., White, O., Adams, M.D., Clayton, R.A., Fleischmann, R.D., Bult, C.J., Kerlavage, A.R., Sutton, G., and Kelley, J.M. (1995) The minimal gene complement of *Mycoplasma genitalium*. *Science* **270:** 397-403.

Gasc, A.M., Giammarinaro, P., Richter, S., and Sicard, M. (1998) Organization around the dnaA gene of *Streptococcus pneumoniae*. *Microbiology* **144:** 433-439.

Gober, J.W., and Englund, J.C. (2000) Regulation of flagellum biosynthesis and motility in *Caulobacter*. In *Prokaryotic Development*. Brun, Y.V., and Shimkets, L.J. (eds). Washington, DC: American Society of Microbiology, pp 319-339.

Hirota, Y., Jacob, F., Ryter, A., Buttin, G., and Nakai, T. (1968) On the process of cellular division in *Escherichia coli* I. Asymmetrical cell division and production of Deoxyribonucleic Acid-less bacteria. *J Mol Biol* **35:** 175-192

Hung, D., McAdam, H., and Shapiro, L. (2000) Regulation of the *Caulobacter* cell cycle. In *Prokaryotic Development*. Brun, Y.V., and Shimkets, L.J. (eds). Washington, DC: American Society of Microbiology, pp 361-378.

Jakimowicz, D., Majka, J., Messer, W., Speck, C., Fernandez, M., Martin, M.C., Sanchez, J., Schauwecker, F., Keller, U., Schrempf, H., and Zakrzewska-Czerwinska, J.

(1998) Structural elements of the *Streptomyces oriC* region and their interactions with the DnaA protein. *Microbiology* **144:** 1281-1290.

Jenal, U., White, J., and Shapiro, L. (1994) *Caulobacter* flagellar function, but not assembly, requires FliL, a non-polarly localized membrane protein present in all cell types. *J Mol Biol* **243:** 227-244.

Kaguni, J.M. (1997) Escherichia coli DnaA protein: the replication initiator. *Molecules & Cells* **7:**145-57.

Marczynski, G.T. (1999) Chromosome methylation and measurement of faithful, once and only once per cell cycle chromosome replication in *Caulobacter crescentus*. *J Bacteriol* **181:** 1984-1993.

Marczynski, G.T., and Shapiro, L. (1992) Cell-cycle control of a cloned chromosome origin of replication from *Caulobacter crescentus*. *J Mol Biol* **226:** 959-977.

Marczynski, G.T., Dingwall, A., and Shapiro, L. (1990) Plasmid and chromosomal DNA replication and partitioning during the *Caulobacter crescentus* cell-cycle. *J Mol Biol* **212:** 709-722.

Marczynski, G.T., Lentine, K., and Shapiro, L. (1995) A developmentally regulated chromosomal origin of replication uses essential transcription elements. *Genes Dev* **9:** 1543-1557.

Margolin, W., Bramhill, D., and Long, S.R. (1995) The *dnaA* gene of *Rhizobium meliloti* lies within unusual gene arrangement. *J Bacteriol* **177:** 2892-2900.

Meisenzahl, A.C., Shapiro, L., and Jenal, U. (1997) Isolation and characterization of a xylose-dependent promoter from *Caulobacter crescentus*. *J Bacteriol* **179:** 592-600.

Messer, W., and Weigel, C. (1996) Initiation of chromosome replication. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology.* Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M., and Umbarger, H.E. (eds). Washington, DC: American Society of Microbiology, pp. 1555-1577.

Miller, J.H. (1972) *Experiments in molecular genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Moriya, S., Kato, K., Yoshikawa, H., and Ogasawara, N. (1990) Isolation of a *dnaA* mutants of *Bacillus subtilis* defective in initiation of replication: amount of DnaA protein determines cells' initiation potential. *EMBO J* **9:** 2905-2910.

Mukherjee, A., Cao, C., and Lutkenhaus, J. (1998) Inhibition of FtsZ polymerization by SulA, an inhibitor of septation in Escherichia coli. *Proc Natl Acad Sci USA* **95:** 2885-90.

Noskov, V.N., Araki, H., and Sugino, A. (1998) The RFC2 gene, encoding the third-largest subunit of the replication factor C complex, is required for an S-phase checkpoint in *Saccharomyces cerevisiae. Mol Cell Biol* **18:** 4914-4923.

Ohta, N., Masurekar, M., and Newton, A. (1990) Cloning and cell cycle-dependent expression of DNA replication gene *dnaC* from *Caulobacter crescentus. J Bacteriol* **172:** 7027-7034.

Ohta, N., Grebe, T.W., and Newton, A. (2000) Signal Transduction and cell cycle checkpoints in developmental regulation of *Caulobacter.* In *Prokaryotic Development.* Brun, Y.V., and Shimkets, L.J. (eds). Washington, DC: American Society of Microbiology, pp 341-359.

Osley, M-A., and Newton, A. (1977) Mutational analysis of developmental control in *Caulobacter crescentus. Proc Natl Acad Sci USA* **74:** 124-128.

Poindexter, J.S. (1964) Biological properties and classification of the *Caulobacter* group. *Bacteriol Rev* **28:** 231-295.

Prentki, H., and Krisch, H.M. (1984) *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* **29:** 303-313.

Qin, M.-H., Madiraju, M.V.V.S., Zachariah, S., and Rajagopalan, M. (1997) Characterization of the *oriC* region of *Mycobacterium smegmatis*. *J Bacteriol* **179:** 6311-6317.

Quon, K.C., Marczynski, G.T., and Shapiro, L. (1996) Cell cycle control by an essential bacterial two-component signal transduction protein. *Cell* **84:** 83-93.

Quon, K.C., Yang, B., Domian, I.J., Shapiro, L., and Marczynski, G.T. (1998) Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin. *Proc Natl Acad Sci USA* **95:** 120-125.

Rajagopalan, M., Qin, M.-H., Nash, D.R., and Madijaru, M.V.V.S. (1995a) *Mycobacterium smegmatis dnaA* region and autonomous plasmid replication activity. *J Bacteriol* **177:** 6527-6535.

Rajagopalan, M., Qin, M.-H., Steingrube, A., Nash, D.R., Wallace, Jr., R.J., and Madiraju, M.V.V.S. (1995b) Amplification and cloning of the *Mycobacterium tuberculosis dnaA* gene. *Gene* **163:** 75-79.

Richter S., and Messer, W. (1995) Genetic structure of the *dnaA* region of the cyanobacterium *Synechocystis* sp. strain PCC6803. *J Bacteriol* **177:** 4245-4251.

Richter, S., Hagemann, M., and Messer, W. (1998) Transcriptional analysis and mutation of a *dnaA*-like gene in Synechocystis sp. strain PCC 6803. *J Bacteriol* **180:** 4946-4949.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schaper, S., and Messer, W. (1995) Interaction of the initiator protein DnaA of *Escherichia coli* with its DNA targets. *J Biol Chem* **270:** 17622-17626.

Seto, S., Murata, S., and Miyata, M. (1997) Characterization of dnaA gene expression in *Mycoplasma capricolum*. *FEMS Microbiol Lett* **150:** 239-47.

Shapiro, L., and Losick, R. (1997) Protein localization and cell fate in bacterial. *Science* **276:** 712-718.

Siam, R., and Marczynski, G.T. (2000) Cell cycle regulator phosphorylation stimulates two distinct modes of binding at a chromosome replication origin. *EMBO J* **19:** 1138-1147.

Simon, R., Prieffer, U., and Puhler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Biotechnology* **1:** 784-790.

Skarstad, K., and Boye, E. (1994) The initiator protein DnaA: evolution, properties and function. *Biochim Biophys Acta* **1217:** 111-130.

Stephens, C.M., and Shapiro, L. (1993) An unusual promoter controls cell-cycle regulation and dependence on DNA replication of the *Caulobacter* fliLM early flagellar operon. *Mol Microbiol* **9:** 1169-1679.

Stephens, C.M., Zweiger, G., and Shapiro, L. (1995) Coordinate cell cycle control of a *Caulobacter* DNA methyltransferase and the flagellar genetic hierarchy. *J Bacteriol* **177:** 1662-1669.

Trusca, D., Scott, S., and Bramhill, D. (1998) Bacterial SOS checkpoint protein SulA inhibits polymerization of purified FtsZ cell division protein. *J Bacteriol* **180:** 3946-3953.

Waite, R.T., Shaw, E.I., Winkler, H.H., and Wood, D.O. (1998) Isolation and characterization of the *dnaA* gene of *Rickettsia prowazekii*. *Acta Virologica* **42:** 95-101.

Wegrzyn, A., Wrobel, B., and Wegrzyn, G. (1999) Altered biological properties of cell membranes in *Escherichia coli dnaA* and *seqA* mutants. *Mol Gen Genet* **261:** 762-769.

Winzeler, E., and Shapiro, L. (1995) Use of flow cytometry to identify a *Caulobacter* 4.5 S RNA temperature-sensitive mutant defective in the cell cycle. *J Mol Biol* **251:** 346-365.

Zhuang, W.Y., and Shapiro, L. (1995) *Caulobacter* FliQ and FliR membrane proteins, required for flagellar biogenesis and cell division, belong to a family of virulence factor export proteins. *J Bacteriol* **177:** 343-356.

Zweiger, G., and Shapiro, L. (1994) Expression of *Caulobacter dnaA* as a function of the cell cycle. *J Bacteriol* **176:** 401-408.

Figure Legends

Figure 1. Genetic disruptions at the *C. crescentus dnaA* locus.

A. The *C. crescentus dnaA* gene and its relationship to the replication origin (*Cori*), flanking genes and cloned DNA. This chromosome map is based on published data (Marczynski and Shapiro, 1992; Zweiger and Shapiro 1994; Colombi and Gomes 1999). The dotted line shows *dnaA* sequences cloned into pGM1360 and used to create the *dnaA*::Ω disruption in pGM1380 and subsequently pGM1531, as described in the text.

B. Plasmid pGM1531 (*dnaA*::Ω) integrated at the wild type *dnaA* locus, and frequencies of alternative (left or right side) plasmid excisions following 3% sucrose counter-selection and scoring 100 colonies for the Ω-cassette (100 μg/ml spectinomycin) antibiotic resistance.

C. Strain GM2446 created by *trans*-complementation of the *dnaA*::Ω chromosome disruption. Integrated plasmid pGM1531 (*dnaA*::Ω at the wild type *dnaA* locus), as before, and replicating plasmid pGM1522 (wild type *dnaA* gene). Frequencies of alternative pGM1531 excisions following 3% sucrose counter-selection and scoring 100 colonies for the Ω-cassette (100 μg/ml spectinomycin) antibiotic resistance.

Figure 2. Formation of a strain that conditionally expresses *dnaA*.

A. pGM2195 was constructed by PCR amplifying *dnaA*, as described in experimental procedure, such that an *EcoRI* site was created at the 5' end of the gene, directly upstream of the first Methionine codon. This allowed for a transcriptional and translational fusion

with the first six amino acids of the *xylX* operon as shown. pGM2195 is drawn integrated at the NA1000 *xylX* locus (Southern data not shown) to create strain GM2409.

B. In order to create strain GM2471, the *dnaA*::Ω disruption was transduced from GM2446 (Fig 1C) by ϕCr30 transducing phage into the wild type *dnaA* locus of GM2409. Km/Sp/Strp resistant and Tc sensitive colonies were selected for growth on PYE 0.3% xylose plates.

Figure 3. Growth of GM2471 following blocked *dnaA* expression. M2X (0.3% xylose) cultures were shifted either back to M2X (open circles) or to M2G (0.2% glucose), (closed circles). Subsequent growth was measured by absorbance at 660nm, and followed by microscopy (Fig. 4).

Figure 4. Morphology of GM2471 shifted to M2G or M2X. Dark-field micrographs at 1000X magnification are shown.

A, B, C. GM2471 shifted to M2G for 3, 7, and 10.5 hours.

D. GM2471 shifted back to M2X for 10.5 hours.

Figure 5. GM2471 shifted to M2G quickly arrest DNA synthesis.

A. GM2471 cells were shifted to M2X (open circles) or M2G (closed circles) for seven hours, normalized for cell number to an optical density (A_{660}) of 0.1 and labeled with [α^{32}-P]dCTP for 10, 30, 60, 90, 120 and 150 seconds. DNA synthesis was measured as described in experimental procedures.

B. GM2471 cells shifted to M2G and M2X were sampled periodically, normalized for cell number to optical density (A_{660}) of 0.1, and pulse labeled with [α^{32}P]dCTP for 90 seconds. Since sampled cells were normalized to an O.D of 0.1, we express cpm as cpm/O.D.

Figure 6. GM2471 shifted to M2G accumulate only one chromosome.
Flow cytometry was performed on GM2471 cells shifted to M2G and M2X. Cells were grown overnight in M2X and shifted to M2X and M2G for 0, 4 and 6 hours, treated with chloramphenicol for 3 hours and stained with Chromomycin A3. 10,000 cells from each sampling were segregated into discreet bins according to Chromomycin A3 DNA fluorescence intensity and are shown as density plots (0 Xyl, 4 Xyl, 6 Xyl, 0 Gluc, 4 Gluc, 6 Gluc).

Figure 7. GM2471 cells were shifted to M2G (closed circles) and M2X (open circles) and periodically sampled as described in experimental procedures. The number of viable cells is presented as colony forming units (CFU) per ml of culture.

Figure 8. Transcription from *Cori* promoters in response to blocked *dnaA* expression.
A. *Cori* promoters (Ps, Pw, P3) are shown with relation to CtrA binding sites (a,b,c,d,e), an essential DnaA box (filled arrowhead), the hemE gene, and an AT-rich region. Restriction sites *BglII* (Bg), *HindIII* (H), *EcoRI* (E), *PstI* (P) and *BamHI* (B) are shown. Plasmid *lacZ* reporters were constructed for Ps (pGM976), Pw (pGM1052) and P3

(pGM1054) by digesting with the appropriate enzymes and cloning the fragments into pGM915 (Table 1).

B, C, D. pGM1052 (Pw), pGM1054 (P3) or pGM976 (Ps) was conjugated into GM2471 and cultures were shifted to either M2G (closed circles) or M2X (open circles), sampled and assayed for β-galactosidase activity. The values are plotted for Miller Units (MU) and fold change in total enzyme activity.

Figure 9. Transcription from replication-dependent and control promoters

A, B, C. Replication-dependent promoter reporters pCS98 (P*fliL*::*lacZ*), pGZ22 (P*ccrM*::*lacZ*), and pWZ162 (P*fliQ*::*lacZ*).

D. Control promoter reporter pCS91 (P*rsaA*::*lacZ*).

Each reporter was conjugated into GM2471. Cultures were shifted to either M2G (closed circles) or M2X (open circles), periodically sampled and assayed for β-galactosidase activity. The values are plotted in Miller Units (MU) and fold change in total enzyme activity.

Figure 10. Summary of physiological consequences of blocked *dnaA* expression.

Table 1. Bacterial strains and plasmids

Strain/Plasmid	*Genotype or description*	*Sources*
E. coli		
S17-1	*E. coli* 294::RP4-2(Tc::Mu)(Km::Tn7)	Simon et al. (1983)
C. crescentus		
GM1609	NA1000 Δ*bla*	Marczynski et al. (1995)
GM2409	pGM2195 in *xylX* locus of NA1000, Km	This work
GM2415	pGM1531 in *dnaA* locus of NA1000 right crossover, Sp/St	This work
GM2446	*dna*A::Ω in chromosome from GM2415 after sucrose, pGM1522 complement, Sp/St, Tc	This work
GM2471	GM2446 ϕCr30 lysate transduced into GM2409Kn, Sp/St,	This work
NA1000	synchronizable mutant of w.t CB15	Evinger & Agabian (1977)
Plasmids		
pCS91	pRKlac290 P*rsaA*::*lacZ* transc. fusion, Tc	C. Stephens
pCS98	pRKlac290 P*flgF*::*gus* , P*fliL*::*lacZ*, Tc	C. Stephens
pGM384	Cosmid I, *Cori* DNA region, Tc	Marczynski &Shapiro (1992)
pGM915	pRKlac290 *lacZ*, Tc	Marczynski *et al.* (1995)
pGM976	pRKlac290 *Cori BamHI* to *HindIII* Ps::*lacZ*	Siam and Marczynski (2000)

pGM1052	pRKlac290 *Cori PstI* to *BglII*, Pw::*lacZ*	Marczynski *et al.* (1995)
pGM1054	pRKlac290 *Cori BamHI* to *EcoRI*, P3::*lacZ*	Marczynski *et al.* (1995)
pGM1181	pSK(+)-*dna*A, *Bam*HI from pGM384	This work
pGM1360	pUC BM21-*dna*A, *Xba*I-*Hind*III	
	from pGM1181	This work
pGM1380	pUC BM21-*dna*A::Ω, from pGM1360	This work
pGM1522	pRK404-*dna*A, *Bam*HI from pGM1360	This work
pGM1531	pNPTS138-*dna*A::Ω, from pGM1380	This work
pGM2195	pNPT228XNE -*dnaA*, PCR *EcoRI-SpeI*	
	from pGM1181	This work
pGZ22	pRKlac290 P*ccrM*::*lacZ*, Tc	C. Stephens
pHP45	Sp/St antibiotic cassette, Ap	Prentki and Krisch (1984)
pNPTS138	integrative, Km (nptI), *sac*B, *ori*T	M.R.K Alley
pNPT228XNE	integrative, Km (nptII) *xylX* locus	U. Jenal
pRK404	broad host-range RK2 based, Tc	Ditta et al. (1985)
pSK(+)	pBluescript II, cloning vector, Ap	Stratagene
pUC BM21	cloning vector, Ap	Boehringer Mannheim
pWZ162	pRKlac290 P*fliQ*::*lacZ* , Tc	Zhuang and Shapiro (1995)

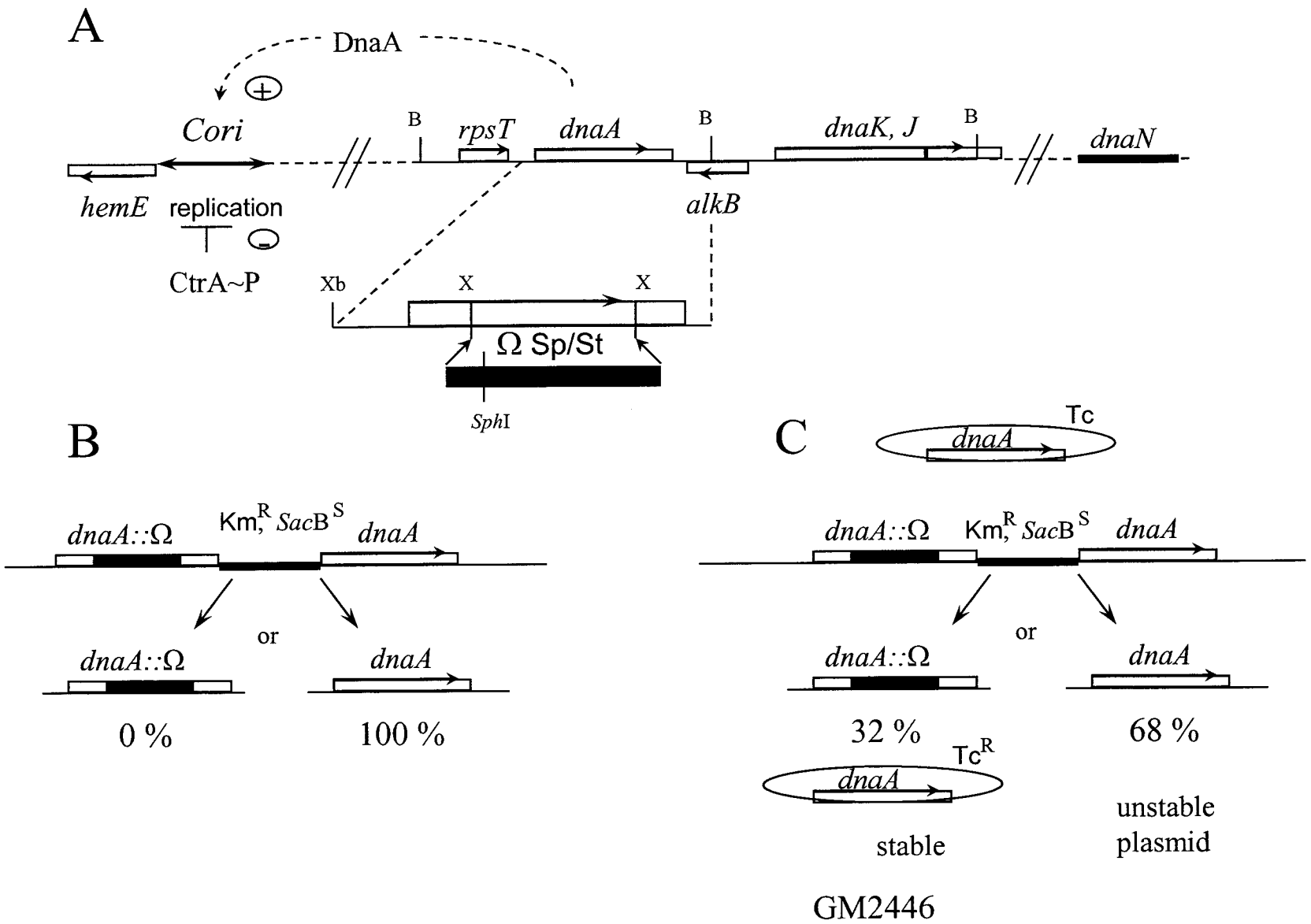
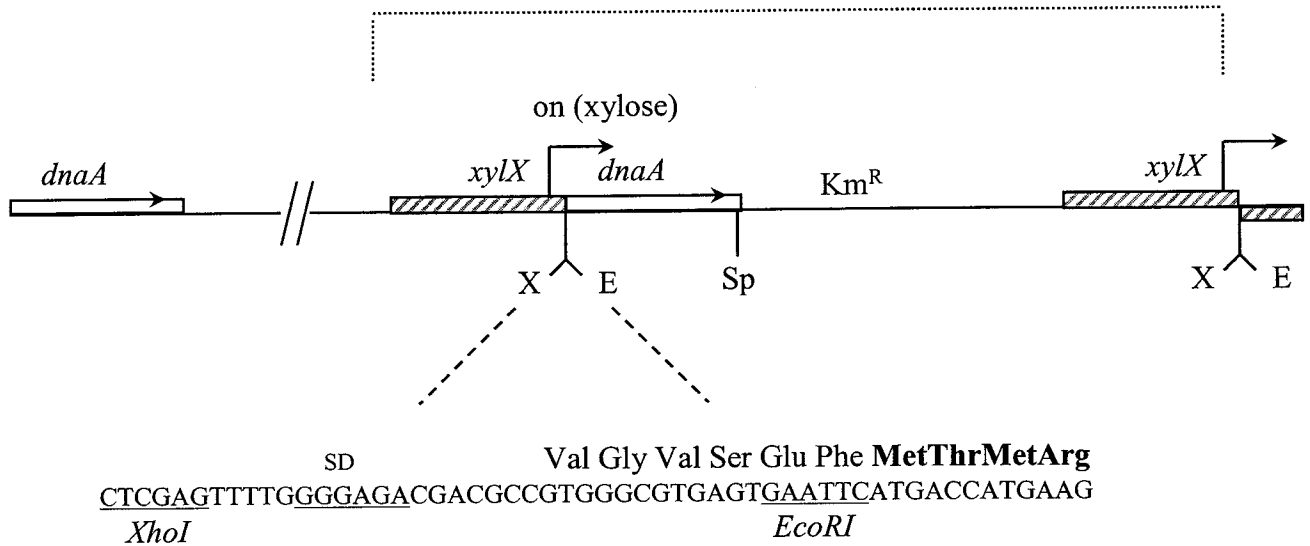

A
DnaA
Cori
B
rpsT
dnaA
B
dnaK, J
B
dnaN
hemE
replication
alkB
CtrA~P
Xb
X
X
Ω Sp/St
SphI
B
dnaA::Ω
Km,R SacB S
dnaA
or
dnaA::Ω
dnaA
0 %
100 %
C
Tc
dnaA
dnaA::Ω
Km,R SacB S
dnaA
or
dnaA::Ω
dnaA
32 %
68 %
TcR
dnaA
stable
unstable
plasmid
GM2446

Figure 1

A GM2409



B GM2471

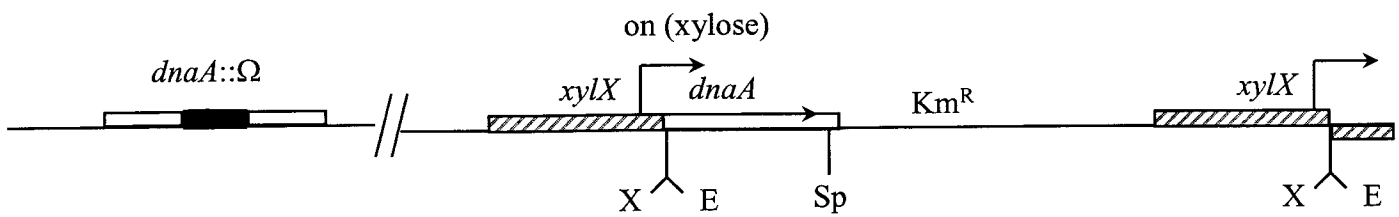


Figure 2

Figure 3

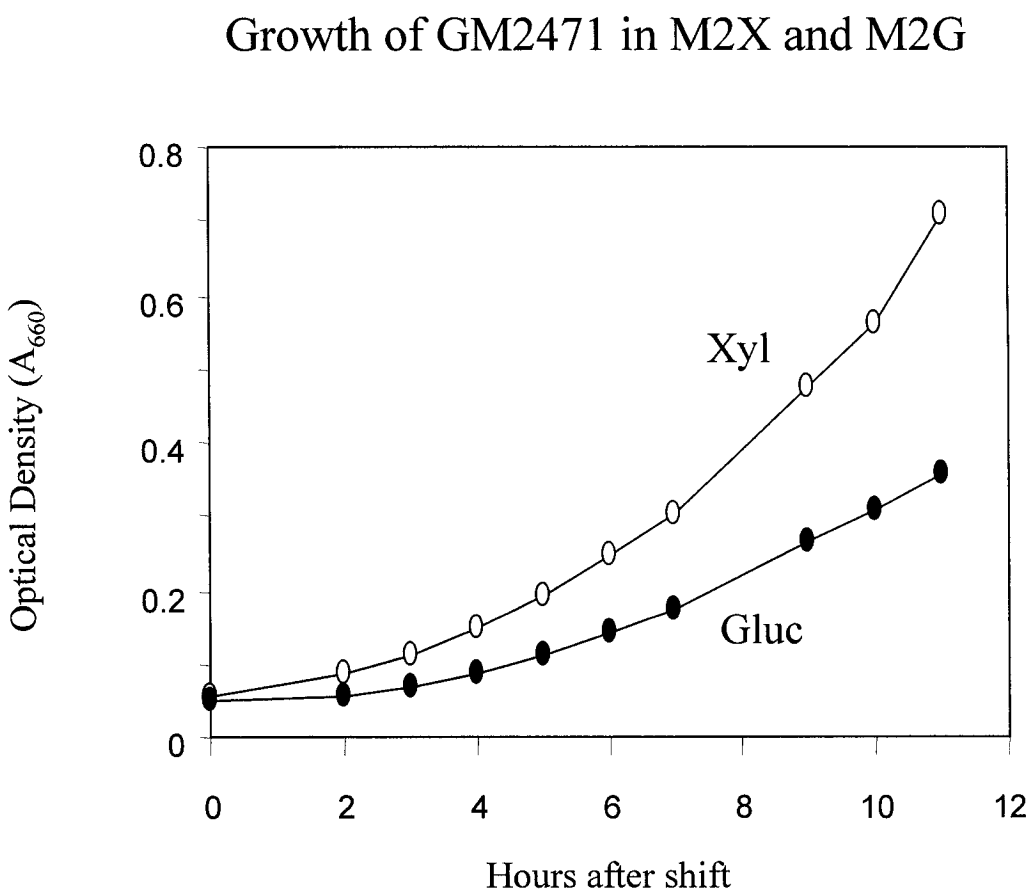

Growth of GM2471 in M2X and M2G
Optical Density (A_{660})
0.8
0.6
0.4
0.2
0
Xyl
Gluc
0
2
4
6
8
10
12
Hours after shift

Figure 4

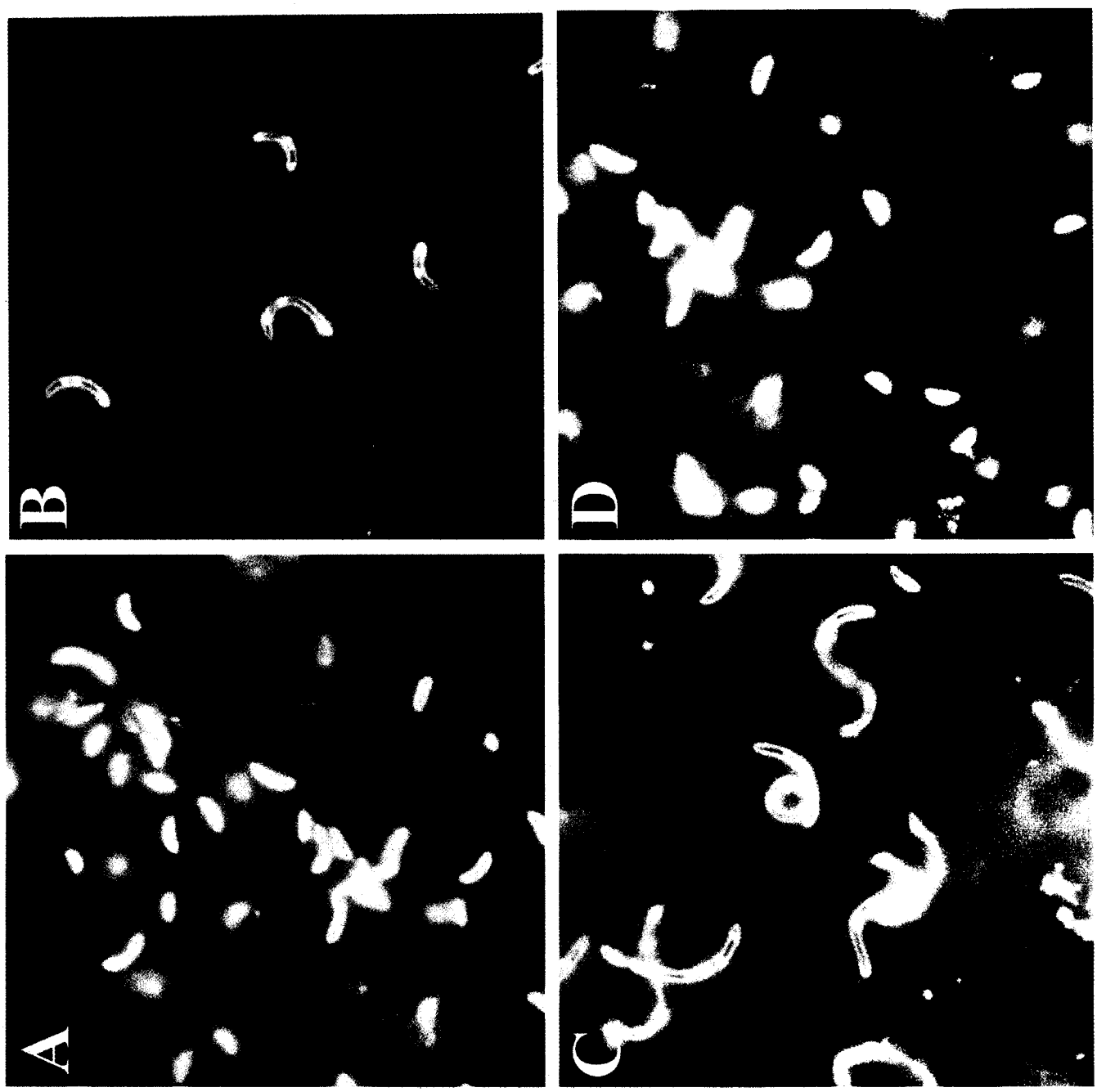
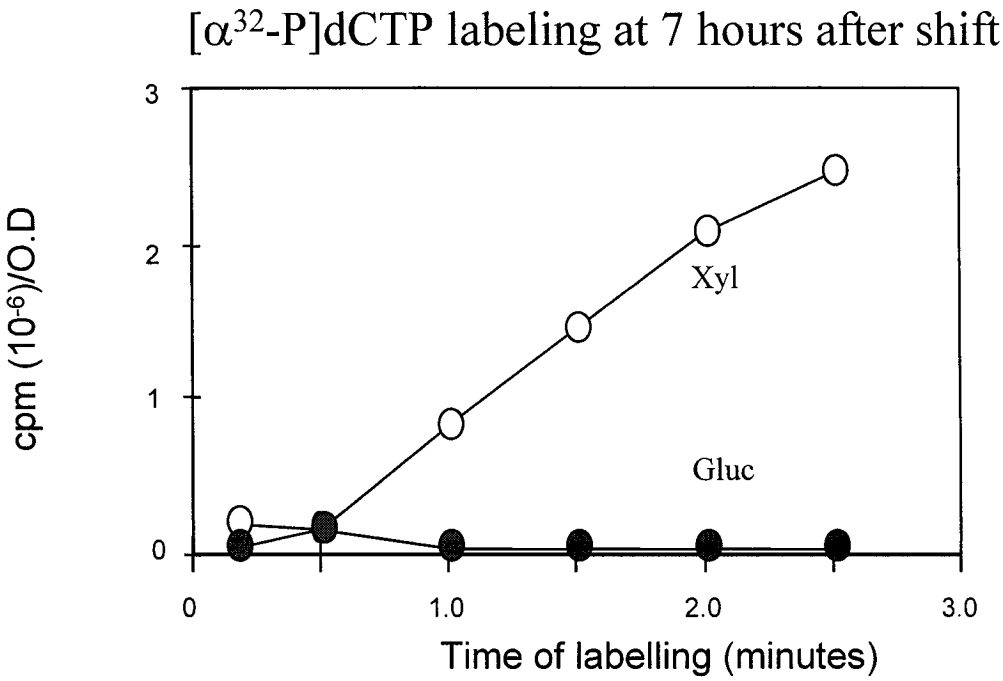


Figure 5

A


[α32-P]dCTP labeling at 7 hours after shift
cpm (10-6)/O.D
3
2
1
0
Xyl
Gluc
0
1.0
2.0
3.0
Time of labelling (minutes)

B

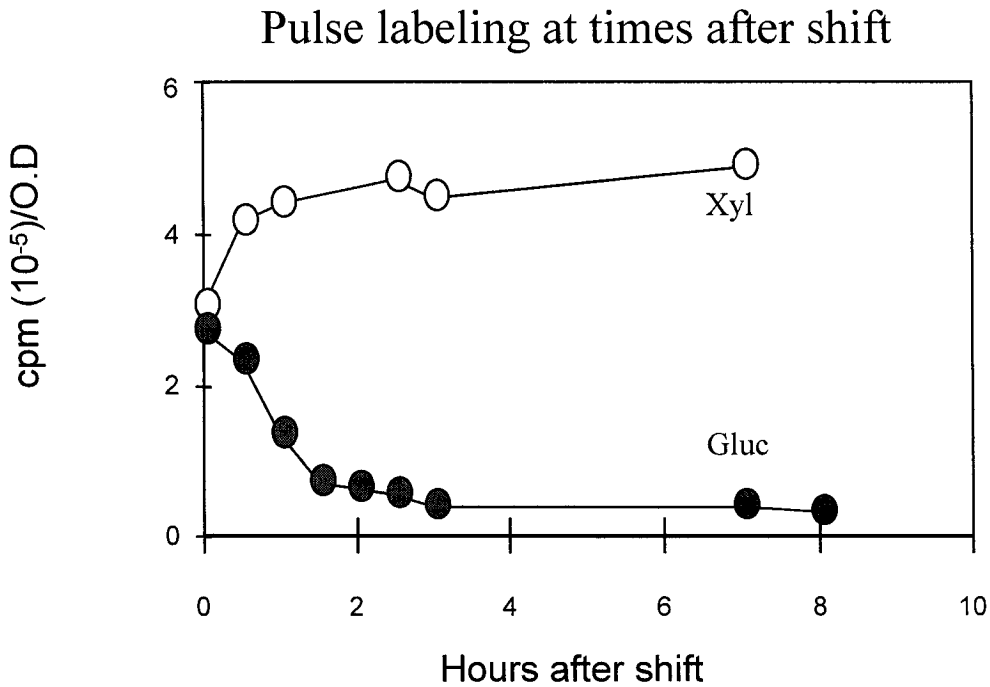

Pulse labeling at times after shift
cpm (10-5)/O.D
6
4
2
0
Xyl
Gluc
0
2
4
6
8
10
Hours after shift

Figure 6

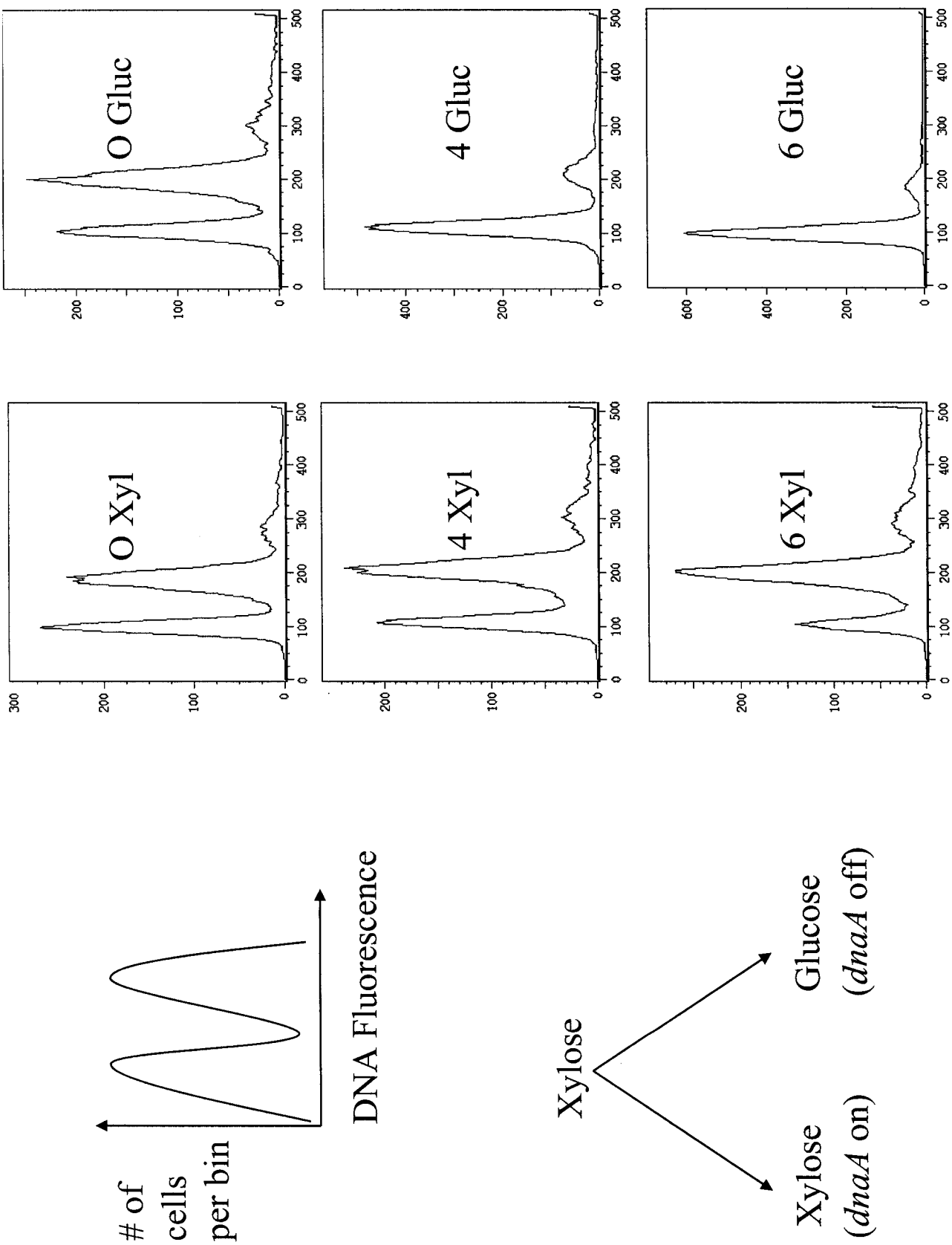
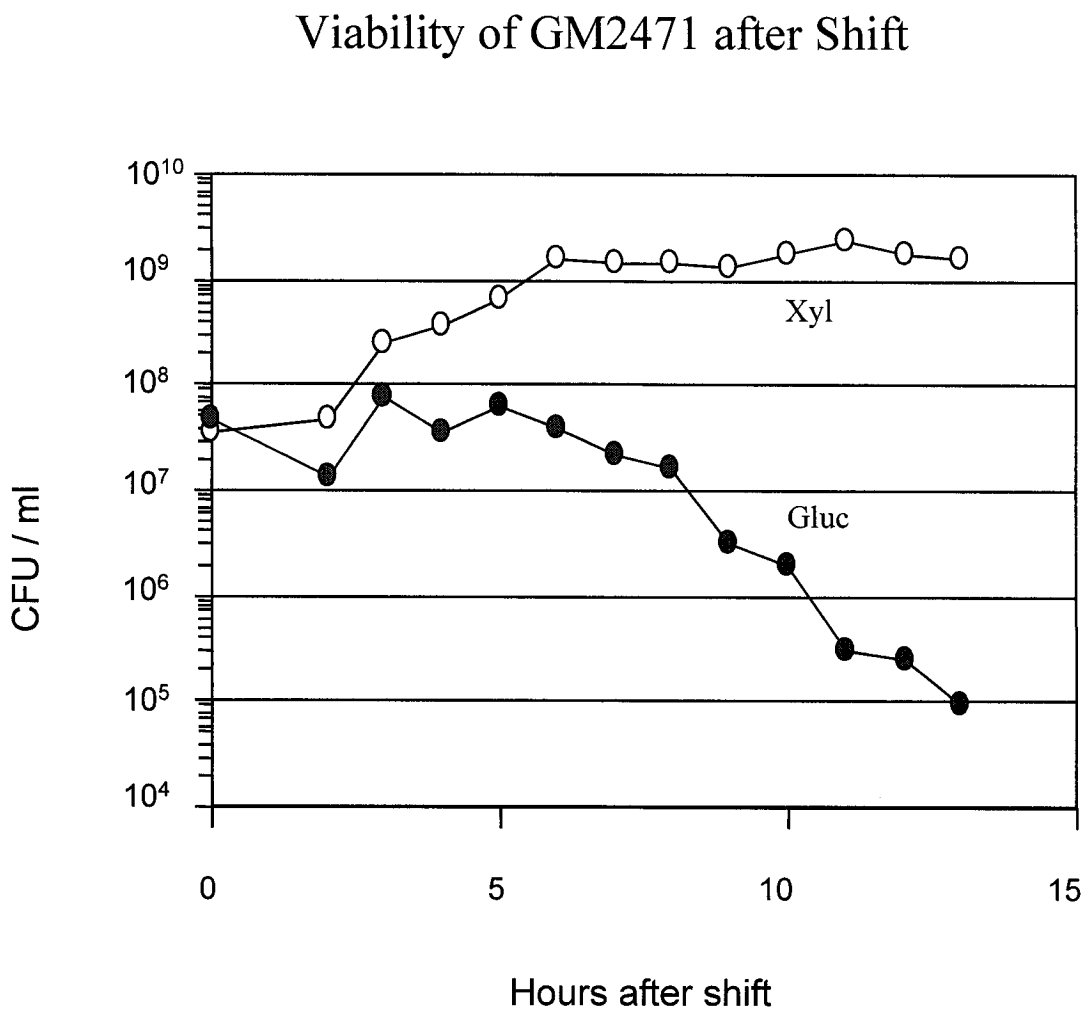

O Gluc
4 Gluc
6 Gluc
O Xyl
4 Xyl
6 Xyl
# of cells per bin
DNA Fluorescence
Xylose
Xylose (dnaA on)
Glucose (dnaA off)

Figure 7


Viability of GM2471 after Shift
CFU / ml
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
Xyl
Gluc
0
5
10
15
Hours after shift

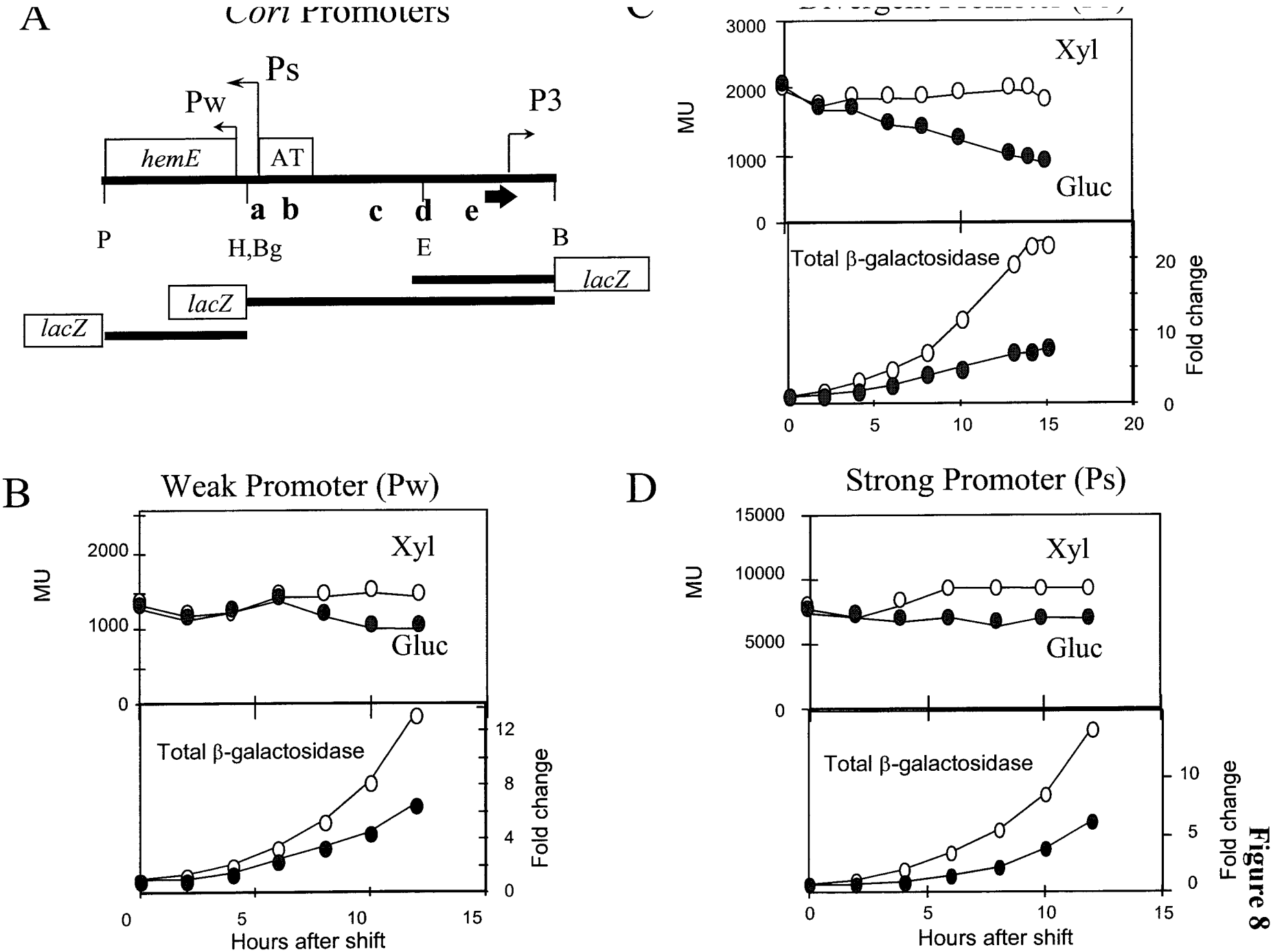
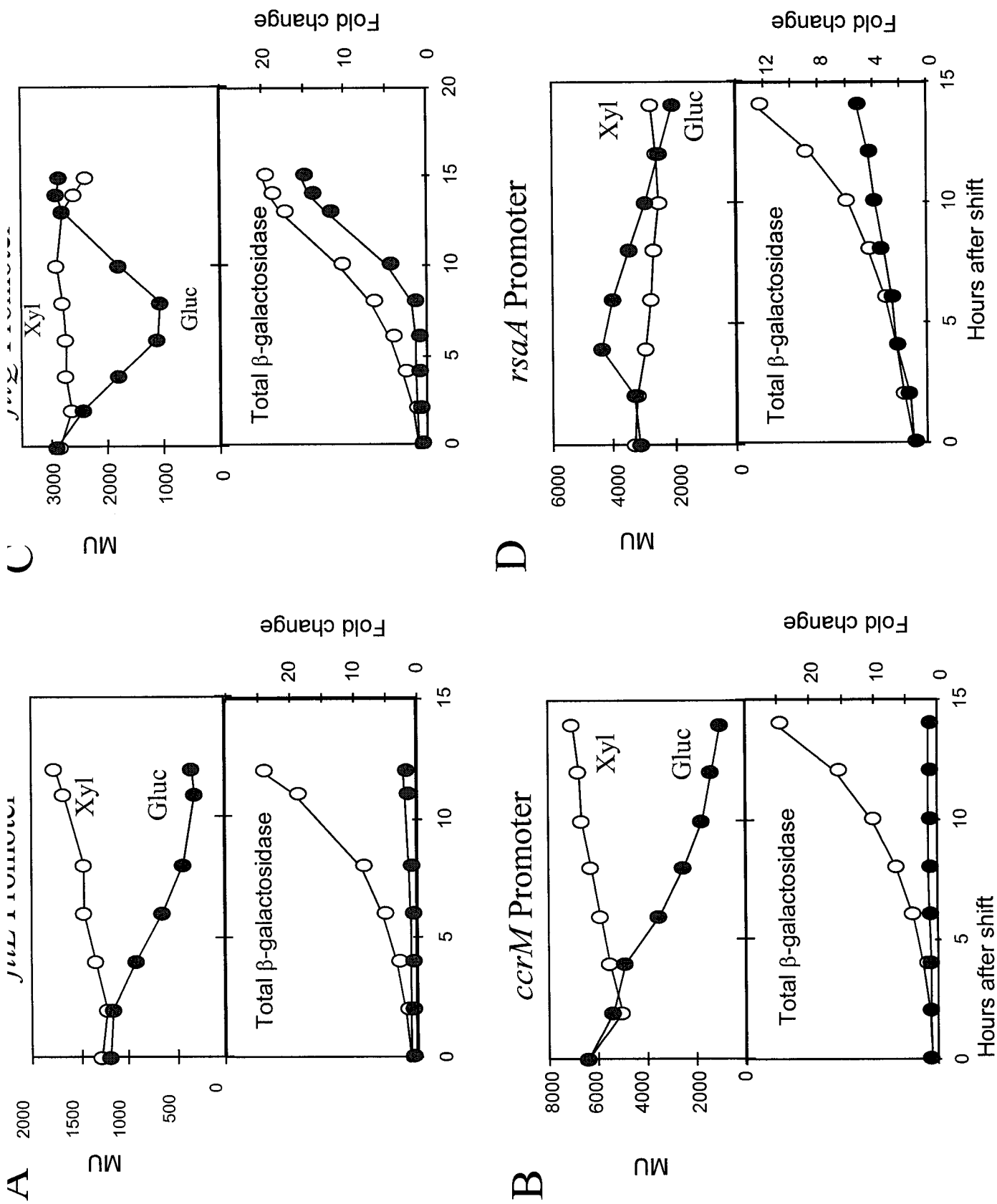

A
Ps
Pw
P3
hemE
AT
a b
c
d
e
P
H,Bg
E
B
lacZ
lacZ
lacZ
B
Weak Promoter (Pw)
MU
2000
1000
0
Xyl
Gluc
Total β-galactosidase
12
8
4
0
Fold change
0
5
10
15
Hours after shift
C
3000
2000
1000
0
Xyl
Gluc
MU
Total β-galactosidase
20
10
0
Fold change
0
5
10
15
20
D
Strong Promoter (Ps)
MU
15000
10000
5000
0
Xyl
Gluc
Total β-galactosidase
10
5
0
Fold change
0
5
10
15
Hours after shift

Figure 8

Figure 9


A
B
C
D
MU
Xyl
Gluc
Total β-galactosidase
Fold change
Hours after shift
ccrM Promoter
rsaA Promoter

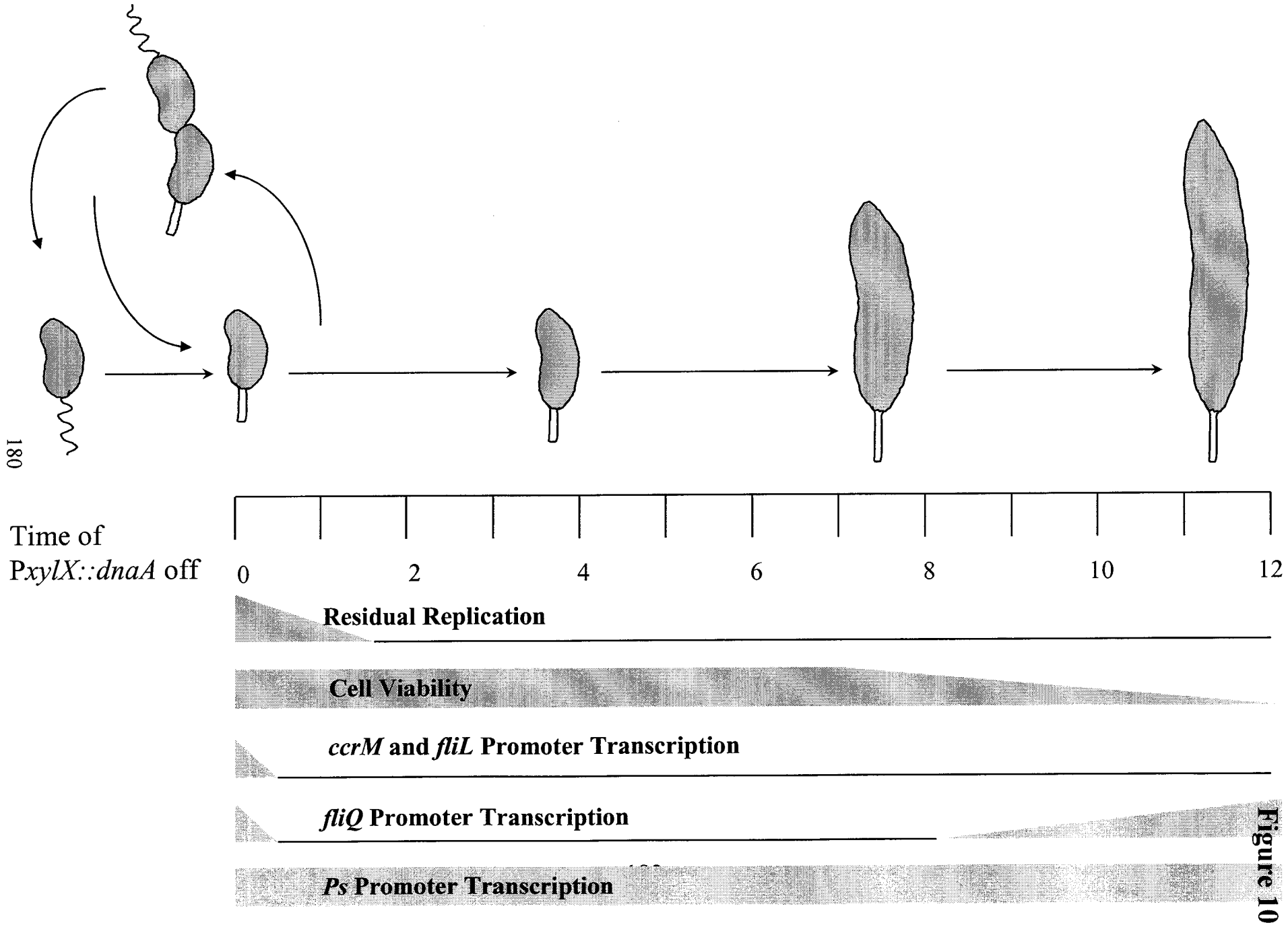

180
Time of
P*xylX::dnaA* off
0
2
4
6
8
10
12
Residual Replication
Cell Viability
ccrM and *fliL* Promoter Transcription
fliQ Promoter Transcription
Ps Promoter Transcription

Figure 10

PREFACE TO CHAPTER 3

In the previous chapter I found that DnaA is a key protein for *C. crescentus* replication control. I next wanted to identify how DnaA is regulated in the cell-cycle. Targeted proteolysis is a mechanism by which many *C. crescentus* proteins are regulated and 5% of all proteins are turned over during the course of one cell-cycle. I tested how DnaA is targeted for proteolysis. In this chapter, I performed protein stability assays in different protease mutant strains and in different *C. crescentus* cell types. I identified the protease that is involved in DnaA proteolysis, and I observed differential rates of DnaA proteolysis in the *C. crescentus* cell types. Interestingly, in this chapter, I identify that DnaA is degraded under starvation conditions, specifically nitrogen starvation, whereas CtrA is not. Therefore, DnaA and CtrA are inversely regulated by proteolysis under starvation conditions to limit replication under unfavorable conditions.

Chapter 3: Programmed stability and degradation of chromosome replication proteins DnaA and CtrA

Boris Gorbatyuk and Gregory T. Marczynski*

McGill University, Department of Microbiology and Immunology

*Correspondence to: Dr. G. T. Marczynski

McGill University, Department of Microbiology and Immunology

3775 University Street, Room 506

Montreal, Quebec, Canada. H3A 2B4

gmarczynski@nexus.microimm.mcgill.ca

Tel (+1) 514-398-3917; Fax (+1) 514-398-7052

Character count: 53,655 (with spaces)

Shortened running title: *C. crescentus* DnaA regulation by proteolysis

Key Words: DnaA, *Caulobacter*, CtrA, proteolysis, ClpP, starvation-response.

Abstract

DnaA is a ubiquitous bacterial protein that binds the replication origin to initiate chromosome replication, and DnaA is a key target for cell-cycle and cell growth controls. The dimorphic *Caulobacter crescentus* cell-cycle requires DnaA to initiate chromosome replication and to coordinate replication with cell differentiation. *C. crescentus* also requires CtrA to repress chromosome replication, and CtrA proteolysis through ClpXP, helps restrict chromosome replication to the dividing cell-type. We observe that the *C. crescentus* DnaA protein is targeted for proteolysis by cell-cycle specific and environment-specific cues. Like CtrA, DnaA is selectively degraded by the ClpP protease, but not through the standard ClpX or the ClpA chaperones. The cell-cycle proteolysis program ensures that only newly synthesized DnaA protein is present to initiate chromosome replication. Nitrogen starvation stimulates DnaA proteolysis, but conversely blocks CtrA proteolysis and maintains CtrA to repress replication. Proteolysis is a rapid mechanism to commit cells to distinct and alternative fates. We propose that at least two alternative proteolysis pathways are inversely coupled so as to spare or degrade key replication proteins in response to cell requirements.

Introduction

How cells maintain active growth in nutrient-rich environments and how they limit growth in nutrient-poor environments remains a challenging fundamental problem. Cells must integrate division and chromosome replication with networks that monitor and relay cellular status to key regulatory proteins. Bacteria such as *Caulobacter crescentus* provide an excellent model for cell-cycle and growth control (Marczynski and Shapiro 2002; Ryan and Shapiro 2003). *C. crescentus* evolved for efficient growth in nutrient-poor environments such as freshwater lakes that sustain only slow growth rates (Poindexter et al. 2000). However, *C. crescentus* grows rapidly in nutrient-rich media, and most importantly for cell-cycle studies, *C. crescentus* exhibits dimorphic cell division under both nutrient-poor and nutrient-rich conditions (Poindexter 1981).

Each *C. crescentus* cell division yields a distinct replicating and non-replicating progeny: a motile swarmer cell that delays cell division, and a distinct non-motile stalked cell that can immediately repeat asymmetric cell division (Fig. 1). Although the swarmer cell is non-replicating, with its polar flagellum and chemo-sensory systems, it is well suited for swimming and exploring the environment. In order to divide, the swarmer cell differentiates into a stalked cell. The swarmer cell ejects its flagellum and grows a "stalk" appendage. This new stalked cell then initiates chromosome replication precisely once and only once per cell-cycle (Marczynski 1999). Chromosome replication is integrated with a complex program of asymmetric cell division. The growing stalked cell builds a new flagellum and places new chemo-sensory proteins at the pole opposite the stalked pole. Therefore, *C. crescentus'* dimorphic progeny have two distinct growth strategies and two immediate options for confronting a nutrient-poor environment. For

cell-cycle studies, swarmer cells are isolated and analyzed as they synchronously differentiate into stalked cells, initiate chromosome replication, and divide asymmetrically in either nutrient-rich and nutrient-poor media (Poindexter 1981).

Selective protein synthesis and selective proteolysis also mark the dimorphic cell-cycle of *C. crescentus* (Fig. 1). For example, the chemo-sensory protein McpA is synthesized and localized to the flagellar pole. McpA is selectively degraded in non-motile stalked cells (Tsai and Alley 2001). Conversely, the cell-division protein FtsZ is selectively synthesized in stalked cells where the cytokinetic (Z) ring is required for septum formation. FtsZ is likewise selectively degraded in the non-dividing swarmer cells (Kelly et al. 1998).

The cell-cycle replication and transcription regulator CtrA also demonstrates selective synthesis and proteolysis. CtrA is a response regular protein at the end of a phospho-relay network that integrates as yet unidentified cell-cycle signals. CtrA controls the transcription of over 25% of the cell-cycle dependent mRNA (Laub et al. 2000). CtrA is present in swarmer cells, where it also represses chromosome replication, presumably by binding five DNA sites inside the chromosome replication origin (Quon et al. 1998). CtrA is selectively proteolyzed at the swarmer to stalked cell transition prior to chromosome replication. CtrA is later re-synthesized in dividing cells, but it is also proteolyzed in the stalked cell compartment during asymmetric division so that only progeny swarmer cells receive CtrA protein (Fig. 1; Domian et al. 1997). CtrA proteolysis requires the ClpX chaperone and ClpP protease which are present throughout the cell-cycle (Jenal and Fuchs 1998). Therefore, the temporal and spatial pattern of CtrA proteolysis also requires as yet unidentified cell-cycle factors.

Whereas CtrA is a repressor of chromosome replication, DnaA is a key activator, and DnaA is absolutely required for chromosome replication in *C. crescentus* (Gorbatyuk and Marczynski 2001). DnaA is highly conserved and present in all eubacteria. In *E. coli,* DnaA binds the chromosome replication origin, promotes open complex formation and recruits additional replication proteins (Bramhill and Kornberg 1988; Messer 2002). To ensure balanced replication during the cell cycle, *E. coli* DnaA activity is tightly regulated by at least three mechanisms. Regulatory Inactivation of DnaA (RIDA) stimulates the intrinsic ATPase activity of DnaA and prevents its reutilization for replication. Sequestration of the replication origin by the SeqA protein prevents DnaA-dependent open complex formation. Also, in *E. coli,* DnaA molecules are titrated by a high affinity chromosome locus *datA* that limits DnaA availability at the replication origin (Boye et al. 2000). Considering its ubiquity and its central role during chromosome replication, DnaA regulation may involve additional mechanisms, especially in other bacteria that evolved complex cell-cycle controls.

We report that unlike *E. coli*, the *C. crescentus* DnaA protein is targeted for proteolysis by cell-cycle-specific and environment-specific cues. *C. crescentus* DnaA is degraded faster in swarmer cells than in stalked cells. This cell-cycle proteolysis program ensures that only newly synthesized DnaA protein is present to initiate chromosome replication. Like CtrA, DnaA is selectively degraded by the ClpP protease, but not through the typical ClpX or the ClpA chaperones. We also demonstrate that nitrogen starvation stimulates DnaA proteolysis, and conversely that nitrogen starvation maintains CtrA by blocking its proteolysis. Our results suggest a nutritional monitor

superimposed over a cell-cycle system that directs proteins either towards or away from proteolysis according to cellular requirements.

Results

Radio immuno-precipitation assays for DnaA protein synthesis

To study DnaA protein synthesis and turnover, we first applied this radio immuno-precipitation assay to *C. crescentus* cells that conditionally control DnaA synthesis. In a previously described strain (GM2471), the wild-type *dnaA* is disrupted by an antibiotic resistance cassette and the only functional copy of *dnaA* is directed by the conditional promoter PxylX at the *xylX* chromosomal locus (Gorbatyuk and Marczynski 2001). Strain GM2471 requires xylose for cell viability and for chromosome replication. Therefore, we expect that GM2471 DnaA protein synthesis is "on" in the presence of xylose and "off" in the presence of glucose (Fig. 2A).

To confirm the conditional synthesis of DnaA, we grew GM2471 cells, wild type cells, and GM2471 cells trans-complemented with a *dnaA* plasmid (GM2726) in minimal media with xylose (M2X), and then shifted these cells either back to M2X or to minimal media with glucose (M2G). All cultures were then sampled and labeled with [^{35}S]-methionine as described in Materials and Methods. Labelled proteins were immunoprecipitated with *C. crescentus* DnaA antiserum, and resolved on SDS-PAGE (Fig. 2B). In wild type cells grown in both M2G and M2X, two major proteins migrated at approximately 58 kDa, consistent with the predicted size of *C. crescentus* DnaA protein. However, only the lower band (marked DnaA) requires xylose for synthesis in strain GM2471 (Fig. 2B), indicating that it is the *C. crescentus* DnaA. In strain GM2726 and wild type cells, DnaA protein is synthesized in both M2G and M2X (Fig. 2B).

The upper band was designated as a heat shock protein (marked Hsp) based on the following experiments. Wild-type cells were subjected to heat shock at 42°C and cold-shock at 15°C for 5 minutes prior to our radio immuno-precipitation assay (Fig. 2C). Only the upper band protein was heat-shock inducible and cold-shock repressible. *C. crescentus* DnaA synthesis is not significantly regulated by temperature.

DnaA synthesis can drive chromosome replication

We previously determined that blocked *dnaA* gene transcription prevents chromosome replication (Gorbatyuk and Marczynski 2001), and we therefore tested whether *de novo* DnaA protein synthesis could alone drive chromosome replication. Conditional PxylX::*dnaA* GM2471 cells were grown in M2X, and then shifted to M2G for 180 minutes to block DnaA synthesis, as outlined in Figure 3A. Under these conditions, GM2471 cells remained viable and grew as non-dividing stalked cells, but without chromosome replication (Fig. 3C). Upon re-addition of xylose at 180 minutes, both DnaA protein synthesis (Fig. 3B) and chromosome replication were measured (Fig. 3C). Within 15 minutes of xylose addition, DnaA protein synthesis resumes (Fig. 3B), and 15 minutes later, chromosome replication also resumed (Fig. 3C). DNA synthesis did not occur without xylose. The steep rise in the rate of [^{32}P] DNA synthesis suggests a synchronous burst of chromosome replication following a critical accumulation of DnaA.

C. crescentus *DnaA is synthesized throughout the cell-cycle*

Since *de novo* DnaA synthesis can initiate chromosome replication (Fig. 3), we tested whether *C. crescentus* uses this mechanism to initiate chromosome replication. Wild-type NA1000 Swarmer cells were isolated and allowed to proceed synchronously through the cell-cycle. Cells were sampled throughout the 120 minute cell-cycle and pulse labeled with [^{35}S]-methionine to measure DnaA protein synthesis (Fig. 4). We used flagellin and Hsp immuno-precipitation to track cell-cycle progression. As expected, anti-flagellin antibodies precipitated [^{35}S]-labelled proteins selectively from swarmer cells (0-30 min) and from late dividing cells (90-120 min). Interestingly, Hsp protein synthesis also has a distinctive cell-cycle pattern. The Hsp synthetic rate is low during most of the cell-cycle, but increases approximately 10-fold at 30 and 45 minutes (Fig. 4), approximately at the start of S-phase in stalked cells. In contrast to flagellin and Hsp synthesis, DnaA protein synthesis has only a subtle variation during the cell-cycle (Fig. 4). Although DnaA synthesis is continuous throughout the cell-cycle, its synthetic rates are approximately 2-fold higher in swarmer cells (0-30 minutes) than in the late dividing cells (90-120 minutes). However, a 2-fold synthetic change is not likely to account for the precise timing of chromosome replication.

C. crescentus *DnaA is a naturally unstable protein*

Several key *C. crescentus* cell-cycle proteins are unstable and regulated by selective proteolysis (Fig. 1). To test DnaA protein stability, we pulse labeled a culture of wild-

type NA1000 cells with [^{35}S]-methionine and chased these cells with unlabeled "cold" methionine. Cells were periodically sampled and analyzed by the DnaA radio immuno-precipitation assay. Whereas the Hsp protein is stable throughout the chase, the DnaA protein is unstable (Fig. 5). The half-life of DnaA in wild-type NA1000 cells is 60 minutes ($t_{1/2} \sim 60$ minutes), substantially shorter than the 150 minute doubling time. DnaA protein instability is comparable to other *C. crescentus* proteins (Fig. 1) that are targeted for proteolysis (Jenal and Hengge-Aronis 2003).

DnaA degradation requires ATP

Targeted proteolysis is often ATP-dependent. Accordingly, we tested *C. crescentus* DnaA degradation by poisoning ATP synthesis with sodium azide (Katayama et al. 1990; Wang et al. 1999). Protein stability experiments of wild-type NA1000 cells (as in Fig. 5) demonstrate that labeled DnaA protein is retained 90 minutes after azide treatment, while DnaA protein is degraded in the absence of azide (Fig. 6A). Since cells treated with azide also stop growing, we also treated NA1000 cells with chloramphenicol to inhibit cell growth without blocking ATP synthesis. The experiment in Figure 6B confirms that DnaA protein is degraded when cell growth is inhibited by blocking protein synthesis with chloramphenicol. This argues that DnaA is degraded by an energy-requiring, and presumably an ATP-dependent protease.

The ClpP protease degrades DnaA

We searched for the ATP-dependent protease of DnaA. Lon and ClpP are major ATP-dependent proteases in *E. coli.* In *C. crescentus*, Lon protease homologue selectively degrades CcrM methyltransferase (Wright et al. 1996), whereas the ClpXP chaperone-protease homologues selectively degrade CtrA (Jenal and Fuchs 1998). DnaA is efficiently degraded ($t_{1/2} \sim 95$ min) in a *lon* strain (LS1837), arguing that Lon protease is not a significant protease of DnaA (data not shown). The *clpP* gene is essential in *C. crescentus* and our strategy followed Jenal and Fuchs (1998) who demonstrated that depleting ClpP stabilized CtrA. In *C. crescentus* strain UJ199, the only functional copy of the *clpP* gene is under control of the conditional PxylX promoter (Figure 7A). We depleted the conditional ClpP strain for 12 hours by growing UJ199 in M2G, monitored ClpP levels by Immuno-blot (Fig. 7B) and then performed pulse-chase experiments to analyze DnaA stability (Fig. 7C). A 20-fold decrease in ClpP protein levels (Fig. 7B Gluc) significantly stabilized DnaA protein (Fig. 7C; $t_{1/2} \sim 6$ h). Conversely, when ClpP protein is conditionally expressed with xylose (Fig. 7B), its approximate 70 minute half-life (Fig. 7C Xyl) is consistent with DnaA stability in wild-type NA1000 cells ($t_{1/2} \sim 60$ minutes; Fig. 5). Since the residual decay of DnaA (Fig. 7C Gluc) is probably due to residual ClpP (Fig. 7B Gluc), this argues that DnaA is degraded primarily through the ClpP protease.

We also tested whether ClpP-dependent degradation of DnaA requires chaperones ClpX and ClpA. For example, CtrA degradation requires ClpX and ClpP (Jenal and Fuchs 1998). Whereas Lon protease contains both the ATP-dependent chaperone and

proteolytic components, ClpP protease requires a separate ATP-dependent chaperone such as ClpX or ClpA to unfold and present the target protein (Gottesman 1999). We conditionally induced a dominant negative allele of ClpX (*clpX**) from pMO88 (Table 1; Fig. 7D) for 2 hours and performed protein stability experiments, as described above. Whereas the CtrA protein is stabilized by ClpX* (Fig. 7E), DnaA protein remains unstable ($t_{1/2}$ ~ 60 minutes; Fig. 7F). Therefore, ClpP-dependent degradation of DnaA does not require ClpX. We next tested whether the ClpA chaperone aids DnaA degradation. Since the *clpA* gene is not essential, a DnaA protein stability experiment was performed with the *clpA* null mutant strain (UJ838; Table 1). DnaA was not significantly stabilized in the *clpA* null background ($t_{1/2}$ ~ 70 minutes; Fig. 7G). We also tested whether both ClpX and ClpA might cooperate during DnaA degradation. We therefore placed the conditional dominant negative *clpX** allele plasmid pM088 into the *clpA* null mutant (UJ838) and assayed DnaA stability following 2 hours of ClpX* induction (data not shown). DnaA remained ustable ($t_{1/2}$ ~ 70 minutes) demonstrating that ClpA and ClpX do not cooperate and do not independently aid DnaA degradation.

DnaA is degraded preferentially in swarmer cells

Since protein stability is regulated during the *C. crescentus* cell cycle (Fig. 1), we addressed whether DnaA degradation is likewise regulated during the cell cycle. Wild-type NA1000 cells swarmer cells were isolated, pulse-labeled with [^{35}S]-methionine and chased through the duration of the cell-cycle. DnaA synthesized in swarmer cells is first degraded rapidly in swarmer cells ($t_{1/2}$ ~ 45 minutes), and then degraded more slowly

when they differentiate into stalked cells ($t_{1/2}$ ~ 100 minutes; Figure 8A). Likewise, when we allowed swarmer cells to first differentiate into stalked cells and then pulse labeled stalked cells, DnaA protein is degraded with the slower stalked cell-specific rate ($t_{1/2}$ ~ 100 minutes; Fig. 8B).

DnaA is removed from divided cells

We tested whether DnaA synthesized prior to one S-phase is present for the next S-phase, i.e. whether old DnaA is retained after cell division. We pulse labeled synchronized swarmer cells at the G1 to S transition (30 minutes post-synchrony) and chased throughout the cell-cycle. At 120 minutes and 145 minutes into the chase, we isolated newly-divided swarmer cells and stalked cells. DnaA synthesized just prior to replication initiation is absent in both swarmer and stalked progeny (Fig. 8C). Therefore, DnaA protein from the start of one cell-cycle is removed prior to the second cell-cycle. This argues that DnaA protein is used to initiate chromosome replication only once.

Inverse regulation of DnaA and CtrA proteolysis during nitrogen starvation

The swarmer cell probes the environment and responds to nutrient status. For example, nitrogen starvation of swarmer cells blocks swarmer to stalk differentiation (Chiaverotti et al. 1981). We isolated swarmer cells and allowed them to grow in the presence or absence of ammonium which is the only nitrogen source in M2G media. These cultures were periodically sampled for immuno-blot analysis with antibodies to *C. crescentus*

DnaA and CtrA. Under standard nitrogen-rich conditions (+N), DnaA is present throughout the cell-cycle. There is ~2-fold more DnaA protein in stalked cells than in swarmer cells (Fig. 9A), and this is consistent with the greater DnaA protein stability in stalked cells (Fig. 8). As reported previously (Fig. 1), CtrA is present in swarmer cells, degraded upon swarmer to stalk cell differentiation and is resynthesized and stabilized in predivisional cells (Fig. 9A).

However, during nitrogen starvation (-N), swarmer to stalk differentiation is blocked and DnaA is rapidly cleared (Fig. 9B). Fifty percent of DnaA protein is cleared within 15 minutes of nitrogen starvation and total DnaA protein levels are undetectable within 120 minutes (Fig. 9B). However, CtrA protein is stabilized by nitrogen starvation and its temporal degradation pattern is altered (Fig. 9B). A two-fold drop in CtrA protein occurs within 15 minutes of nitrogen starvation. However, CtrA is clearly very stable once the cells adjust to nitrogen starvation and CtrA is detected even 150 minutes following nitrogen starvation. Therefore, under nitrogen starvation, where cells arrest as swarmer cells, the clearing of DnaA and the stabilization of CtrA are appropriate responses to conditions that require blocking chromosome replication such as nutrient starvation.

We next tested whether this proteolytic response was cell-type specific and also present in stalked cells. In a similar experiment, WT swarmer cells were allowed to differentiate into stalked cells and then starved for nitrogen (Fig. 9C). DnaA and CtrA levels were monitored by immuno-blots as described above. Stalked cells starved for nitrogen continue asymmetric growth, but do not divide, and arrest as late pre-divisional cells (data not shown). Fifty percent of DnaA protein is degraded within 30 minutes of

nitrogen starvation, and DnaA is undetected 180 minutes following nitrogen starvation (Fig. 9C). However, CtrA accumulates 180 minutes following nitrogen starvation in late predivisional cells (Fig. 9C) as it normally does during the cell-cycle (Domian et al. 1997). Therefore, upon nitrogen starvation, both swarmer cells and stalked cells respond with selective DnaA degradation, and with selective CtrA stabilization.

Discussion

We studied how DnaA protein synthesis and degradation are integrated with the dimorphic *C. crescentus* cell-cycle. Unlike *E. coli* DnaA protein, we discovered that the *C. crescentus* DnaA protein is unstable and targeted for proteolytic degradation. *C. crescentus* DnaA has a 100 minute half-life in stalked cells (Fig. 8B) and a 45 minute half-life in swarmer cells (Fig. 8A). In contrast, *E. coli* DnaA is stable ($t_{1/2} > 24$ h) and only temperature-sensitive *E. coli* DnaA protein is degraded (Torheim et al. 2000; Slominska et al. 2003). We propose that targeted DnaA proteolysis is a special adaptation to the needs of dimorphic growth. Selective DnaA proteolysis has not been documented in other bacteria. However, selective protein degradation is an obligate part of many developmental programs (Gottesman 1999), and other bacteria with sophisticated life cycles may also degrade DnaA and other replication proteins.

The mechanism of targetted DnaA degradation

C. crescentus DnaA degradation is very selective. Lon protease selectively degrades the CcrM protein (Wright et al. 1996), but Lon does not significantly degrade DnaA. Instead, *C. crescentus* DnaA is degraded primarily through the ClpP protease (Fig. 7C). Bacterial proteases often use an ATP dependent chaperone component that helps to recognize the protein, unfold it, and then directionally spool the polypeptide into the protease for degradation (Gottesman 1999). The ClpP protease can complex with ClpA or ClpX chaperones. However, DnaA is not degraded by ClpAP or ClpXP (Fig. 7D-G). As well, a *clpA*$^-$ strain expressing *clpX** did not stabilize *C. crescentus* DnaA. This

implies that DnaA degradation requires a novel ClpP chaperone. In contrast, *E. coli* wild type DnaA is stable, and the *E. coli* temperature sensitive DnaA204 mutant is degraded by three protease, ClpP, ClpQ, and by Lon (Slominska et al. 2003).

The precise cell-cycle and nutritional control of DnaA degradation suggests that a specificity factor may also direct DnaA to the ClpP protease, especially since ClpP is present throughout the cell-cycle (Jenal and Fuchs 1998). Specificity factors regulate proteases in *B. subtilis* and *E. coli* (Jenal and Hengge-Aronis 2003). Unique specificity factors for *C. crescentus* DnaA degradation are suggested by its unique cell-cycle pattern of degradation (Fig. 8A-B), that does not match that of CtrA, McpA or FtsZ (Fig. 1).

Unique cell-cycle pattern of DnaA degradation

The *C. crescentus* cell-cycle pattern of DnaA degradation is not shared by any of the other well-studied *C. crescentus* proteins (Fig. 1). For example, both CtrA and the chemotaxis protein, McpA, are stable in swarmer cells (Domian et al. 1997; Tsai and Alley 2001) where DnaA is particularly unstable (Fig. 8A). The pattern of DnaA degradation also differs from that of the cell division protein, FtsZ. Upon cell division, FtsZ protein is stabilized and retained in the progeny stalked cells, while FtsZ is actively degraded in the progeny swarmer cells (Kelly et al. 1998). In contrast, upon cell division, DnaA protein is degraded and removed from both progeny cells (Fig. 8C). This different pattern of protein degradation may reflect differences of FtsZ and DnaA protein re-utilization. FtsZ can presumably re-polymerize, and form a new Z-ring during the next cell-division cycle. However, in *E. coli* new DnaA protein synthesis is believed to

selectively promote new chromosome replication (Donachie and Blakely 2003). We propose that *C. crescentus* DnaA proteolysis promotes DnaA protein utilization only once per chromosome replication cycle. Likewise, new synthesis of *C. crescentus* DnaA triggers a wave of synchronous chromosome replication (Fig. 3C).

Cell-cycle control of DnaA synthesis and degradation

In *C. crescentus*, most cell-cycle transcription is interpreted according to a paradigm where genes are transcribed just prior to the utilization of their protein products (Laub et al. 2000). However, DnaA synthesis only partially fits this paradigm. Transcription of *dnaA* is cell-cycle modulated (Zweiger and Shapiro 1994; Laub et al. 2000), but *dnaA* is transcribed throughout the cell-cycle. Our protein synthesis data agree with these observations, as the rate of DnaA synthesis is higher early during the cell-cycle (Fig. 4). However, this approximately 2-fold modulation is not as great as for example that of the flagellin proteins or Hsp (Fig. 4), which have a conspicuous "on" or "off" pattern. As well, our immunoblot analysis shows that DnaA is present throughout the cell-cycle, but more DnaA is present in the stalked cells than in swarmer cells (Fig. 9A). Our immunoblot data is consistent with our DnaA synthesis data. Accordingly, more protein synthesis in the swarmer cells, prior to stalked cell formation (Fig. 4), and less proteolysis in stalked cells (Fig. 8A) causes DnaA protein accumulation in the stalked cells. We propose that proteolysis is an important "fine-tuning" mechanism for DnaA activity in the cell-cycle. DnaA synthesis and degradation rates are selectively changed during swarmer and stalked cell differentiation, but they are not conspicuously turned "on" or "off".

Cell division and DnaA degradation

Proteolysis is polarized during *C. crescentus* asymmetric cell division. For example, CtrA is preferentially degraded in the stalked cell compartment while FtsZ is preferentially degraded in the swarmer cell compartment of the pre-divisional cell (Fig. 1). Since DnaA is required for chromosome replication, we expected that DnaA might be removed only from the swarmer compartment and not the stalked compartment during cell division. However, we unexpectedly discovered that DnaA synthesized during the G1 to S transition was cleared from both swarmer and stalked cell compartments immediately following cell division (Fig. 8C). Since *de novo* synthesized DnaA is such a potent initiator of DNA replication (Fig. 3), we interpret DnaA proteolysis from both swarmer and stalked compartments of pre-divisional cells to mean that newly divided progeny must *de novo* synthesize DnaA in order to initiate chromosome replication. This interpretation is consistent with the current *E. coli* cell-cycle model requiring *de novo* DnaA for chromosome replication (Donachie and Blakely 2003).

However, these considerations create a paradox, since stalked cell degradation of DnaA is too slow to remove more than about 70% of the old DnaA prior to cell division. We therefore propose that proteolysis accelerates upon cell-division, and that this accounts for the absence of DnaA in both progeny cells (Fig. 8C). To support this idea, we blocked FtsZ synthesis and therefore cell division (Wang et al. 2001) and observed that cells maintain relatively slow DnaA proteolysis ($t_{1/2} \sim 150$ minutes) for over 6 hours

(data not shown). If cell division accelerates DnaA proteolysis, then we also propose that a check-point mechanism acts at or after the stage of FtsZ ring formation.

Nutrient status regulation of DnaA proteolysis and CtrA stability

Proteolysis is a rapid and efficient means for bacteria to respond to stress (Hengge-Aronis 2002; Jenal and Hengge-Aronis 2003), and proteolysis is the ultimate means to prevent unwanted protein function. Whereas we propose that DnaA proteolysis is a "fine-tuning" mechanism to regulate DnaA activity during the *C. crescentus* cell-cycle, DnaA proteolysis is clearly a "shut-off" mechanism for chromosome replication during nitrogen starvation. DnaA is rapidly removed from both swarmer and stalked cells with a very short 15 minute half-life (Fig. 9).

Simultaneous DnaA proteolysis and CtrA stabilization can account for blocked chromosome replication during nitrogen-starvation. The maintenance of CtrA protein in nitrogen starved cells presumably keeps the replication origin (*Cori)* occupied by CtrA. Five strong CtrA binding sites span the essential DNA of *Cori* (Siam and Marczynski 2000). Bound CtrA presumably keeps DnaA from binding *Cori* as putative DnaA boxes in *C. crescentus* overlap CtrA binding sites (Quon et al. 1998). As well, proteolysis of DnaA in swarmer cells may ensure that excess DnaA does not accumulate and compete with CtrA for binding to *Cori.*

Targeted DnaA and CtrA proteolysis is not the only system that integrates both cell-cycle and environmental cues in *C. crescentus.* For example, stalk biogenesis also

receives and integrates both cell-cycle (Quon et al. 1996) and environmental (phosphate limitation) signals (Gonin et al. 2000). Considering that *C. crescentus*, like most bacteria must navigate a variable natural environment, similarly integrated systems are probably the rule and not the exception.

Nutrient status and cell differentiation

The absence of DnaA on its own does not prevent cell differentiation, as swarmer cells blocked for *dnaA* expression differentiate into stalked cells, and continue to grow as filamentous stalked cells without cell division (Gorbatyuk and Marczynski 2001). Likewise, a CtrA mutant resistant to proteolysis does not prevent swarmer to stalk differentiation on its own (Domian et al. 1997). However, nitrogen starvation does block swarmer to stalked cell differentiation, and stalked cells arrest without cell division. Therefore, DnaA proteolysis and CtrA stabilization are important specific examples of a global response to nitrogen-limitation.

Contrast and comparison between C. crescentus *and* E. coli

Nitrogen starved *C. crescentus* also produce the global "alarmone" guanosine tetraphosphate (ppGpp) (Chiaverotti et al. 1981). In *E. coli*, ppGpp is produced when uncharged tRNA molecules (reflecting amino acid starvation) stall protein synthesis. *E. coli* ppGpp is a proposed negative regulator of *dnaA* transcription (Chiaramello and Zyskind 1990). We also observe that the *C. crescentus dnaA* promoter is negatively

regulated by nitrogen starvation (data not shown). Selective ribosome proteolysis also occurs in *E. coli* cells starved for amino acids (Kuroda et al. 2001). Under these conditions, *E. coli* stimulates the production of polyphosphate which complexes with Lon protease to selectively degrade ribosomal proteins. The accumulation of polyphosphate is directly related to ppGpp levels (Kuroda et al. 1997). Future experiments should address the relationships between polyphosphate, ppGpp and targeted proteolysis of DnaA and other cell-cycle regulators.

Proteolysis during C. crescentus *and phage development*

The switch from lysogenic to lytic phage growth also employs targeted proteolysis and is analogous to *C. crescentus'* cell-cycle differentiation as well as to *C. crescentus'* response to nitrogen starvation. For example, the *E. coli* bacteriophage Mu repressor protein maintains lysogeny by blocking transposase transcription. The proteolysis of the Mu repressor protein by ClpXP switches from lysogenic to lytic growth (Shapiro 1993; Marshall-Batty and Nakai 2003). Therefore, the *E. coli* Mu repressor is analogous to *C. crescentus* CtrA (also a negative regulator of replication) which is likewise degraded by ClpXP (Jenal and Fuchs 1998). Similarly, the *E. coli* lambda bacteriophage O replication protein is analogous to DnaA. O protein binds the lambda replication origin where it also initiates replication by unwinding the DNA and by recruiting replication proteins. Under poor growth conditions, rapid proteolysis of O protein through ClpXP also restricts lambda DNA replication and promotes lysogeny (Wegrzyn et al. 2000).

In summary, our data argues that targeted DnaA and CtrA proteolysis "shuts off" chromosome replication during conditions, such as nitrogen starvation when further growth is not possible. However, during the cell-cycle, targeted proteolysis probably "fine tunes" the mechanisms whereby the chromosome "decides" when to replicate and when not to replicate. Previous cell-cycle studies argue that CtrA proteolysis is an auxiliary mechanism to cell-cycle phosphorylation by histidine kinases (Domian et al. 1997). Likewise, we argue that DnaA proteolysis is an auxiliary mechanism to other cell-cycle controls. In addition to proteolysis, *C. crescentus* DnaA may be subject to Regulatory Inactivation of DnaA (RIDA), and as in *E. coli* it may interact with the sliding clamp of DNA polymerase to restrict its activity (Katayama et al. 1998). Also, in *E. coli*, the SeqA protein is a negative modulator of replication initiation (von Freiesleben et al. 1994; Lu et al. 1994; Torheim and Skarstad 1999). However, the apparent absence of a SeqA homologue in *C. crescentus* suggests that *C. crescentus* may have evolved DnaA proteolysis as at least one new way of restricting cell-cycle chromosome replication.

Materials and Methods

Radio immuno-precipitation assays

C. crescentus cells at an optical density (A_{660}) = 0.1 – 0.2 were pulse labeled with 2-20 x 10^6 cpm of [^{35}S]-Methionine / Cysteine mix (NEG-772 Easytag Express Protein Labeling Mix – New England Nuclear) for 5-10 minutes. The labeled cells were frozen and lysed in Wash Buffer with lysozyme (50 mM Tris pH=8; 0.45 M NaCl; 0.5% Triton X-100 (T-6878 Sigma Life Sciences); 10mg/ml lysozyme). The protein extract was pre-cleared with 10 µl of Recombinant Protein A immobilized on 6% fast-flow agarose (P-3476 Sigma Chemical, St. Louis, MO) and 5µl was TCA precipitated and scintillation counted in order to normalize all samples for [^{35}S]-Methionine counts. Protein extract samples were adjusted to contain equal amounts (1-2 x 10^6 cpm) of [^{35}S]-labeled proteins, and were incubated with antibodies at 4° C. 1 µl of Anti-DnaA, anti-CtrA and anti-Flagellin antiserum was added to 400 µl of adjusted protein extract (the antiserum were obtained as gifts from Lucy Shapiro's laboratory, Stanford University). Proteins were immunoprecipitated with 10 µl of Recombinant Protein A, washed three times in Wash Buffer, resuspended in protein loading buffer, boiled for 10 minutes and resolved by SDS-PAGE. 8% polyacrylamide gels resolved DnaA, and 10% polyacrylamide gels resolved CtrA and Flagellin proteins. Gels were dried and exposed to Phosphoimager screens and the screens were scanned by STORM (Molecular Dynamics, Amersham Biosciences). Protein levels were analysed using ImageQuant version 5.2 software (Molecular Dynamics, Amersham Biosciences).

For protein stability assays, *C. crescentus* cells were labeled as above and then chased for up to 150 minutes with 0.2% Tryptone, 0.02 % Cysteine and 1mM Methionine as described (Grunenfelder et al. 2001).

Strains and Growth Conditions

All bacterial strains and plasmids used in this study are summarized in Table 1. Plasmids were mobilized to *C. crescentus* by conjugation (Simon et al. 1983). All *C. crescentus* strains were grown at 30°C in either M2G (0.2% glucose) or M2X media (0.3% xylose) (Johnson and Ely 1977). In order to grow cells under nitrogen starved conditions, NH_4Cl was excluded from the M2G media. Stain UJ199 was depleted for ClpP by shifting an M2X UJ199 culture to M2G for 12 hours as described before (Jenal and Fuchs 1998). Strain GM2885 carrying a dominant negative copy of *clpX* (*clpX**) from pMO88 was induced for 2 hours by addition of 0.3% xylose to M2G prior to performing radio-immuno-precipitation assays.

Cell Synchrony and Immunoblot Analysis

WT NA1000 cells (GM1837) were synchronized using Ludox Density centrifugation to isolate swarmer cells as described (Evinger and Agabian 1977). Swarmer cells were released into M2G media or M2G media without NH_4Cl pre-warmed to 30°C. Cells sampled for immunoblot analysis were normalized to 1ml of A_{660}=0.1 per lane. To resolve DnaA and CtrA, proteins extracted by boiling cells in buffer were resolved on 8% and 10% SDS-PAGE, respectively. Resolved proteins were electro-transferred to Hybond-P PVDF membranes (Amersham Pharmacia Biotech). Anti-DnaA and anti-CtrA

antibodies were diluted 1:10,000. Anti-ClpP antibodies (a gift from Urs Jenal's laboratory, Biozentrum University of Basel) were diluted 1:5,000. Anti-Rabbit IgG conjugated to horseradish-peroxidase secondary antibodies were diluted 1:10,000. ECL-Plus Western Blotting Detection system was used to detect proteins (RPN2132 Amersham Biosciences UK Limited, Buckinghamshire, England).

DNA radiolabelling

Chromosomal replication was assayed by DNA radiolabelling exactly as described previously (Gorbatyuk and Marczynski 2001).

Acknowledgment

We are grateful to Urs Jenal, Dickon Alley, Lucy Shapiro and Yves Brun for strains and antibodies. We are also grateful to Sherif Tawfilis and Swaine Chen for advice regarding strains and reagents and to William Spencer and Patrick Bastedo for critical reading of this manuscript. This work was funded by the Canadian Institutes for Health Research (CIHR), Grant MT-13453.

References

1. Boye, E., Lobner-Olesen, A., and Skarstad, K. 2000. Limiting DNA replication to once and only once. *EMBO Rep.* **1**: 479-483.
2. Bramhill, D. and Kornberg, A. 1988. A model for initiation at origins of DNA replication. *Cell* **54**: 915-918.
3. Chiaramello, A. E. and Zyskind, J. W. 1990. Coupling of DNA replication to growth rate in *Escherichia coli*: a possible role for guanosine tetraphosphate. *J. Bacteriol.* **172**: 2013-2019.
4. Chiaverotti, T. A., Parker, G., Gallant, J., and Agabian, N. 1981. Conditions that trigger guanosine tetraphosphate accumulation in *Caulobacter crescentus*. *J. Bacteriol.* **145**: 1463-1465.
5. Domian, I. J., Quon, K. C., and Shapiro, L. 1997. Cell type-specific phosphorylation and proteolysis of a transcriptional regulator controls the G1-to-S transition in a bacterial cell cycle. *Cell* **90**: 415-424.
6. Donachie, W. D. and Blakely, G. W. 2003. Coupling the initiation of chromosome replication to cell size in *Escherichia coli*. *Curr. Opin. Microbiol.* **6**: 146-150.
7. Evinger, M. and Agabian, N. 1977. Envelope-associated nucleoid from *Caulobacter crescentus* stalked and swarmer cells. *J. Bacteriol.* **132**: 294-301.
8. Gonin, M., Quardokus, E. M., O'Donnol, D., Maddock, J., and Brun, Y. V. 2000. Regulation of stalk elongation by phosphate in *Caulobacter crescentus*. *J. Bacteriol.* **182**: 337-347.

9. Gorbatyuk, B. and Marczynski, G. T. 2001. Physiological consequences of blocked *Caulobacter crescentus dnaA* expression, an essential DNA replication gene. *Mol. Microbiol.* **40**: 485-497.

10. Gottesman, S. 1999. Regulation by proteolysis: developmental switches. *Curr. Opin. Microbiol.* **2**: 142-147.

11. Grunenfelder, B., Rummel, G., Vohradsky, J., Roder, D., Langen, H., and Jenal, U. 2001. Proteomic analysis of the bacterial cell cycle. *Proc. Natl. Acad. Sci. USA* **98**: 4681-4686.

12. Hengge-Aronis, R. 2002. Signal transduction and regulatory mechanisms involved in control of the sigma-S (RpoS) subunit of RNA polymerase. *Microbiol. Mol. Biol. Rev.* **66**: 373-395.

13. Jenal, U. and Fuchs, T. 1998. An essential protease involved in bacterial cell-cycle control. *EMBO J.* **17**: 5658-5669.

14. Jenal, U. and Hengge-Aronis, R. 2003. Regulation by proteolysis in bacterial cells. *Curr. Opin. Microbiol.* **6**: 163-172.

15. Johnson, R. C. and Ely, B. 1977. Isolation of spontaneously derived mutants of *Caulobacter crescentus*. *Genetics* **86**: 25-32.

16. Katayama, T., Kubota, T., Kurokawa, K., Crooke, E., and Sekimizu, K. 1998. The initiator function of DnaA protein is negatively regulated by the sliding clamp of the *E. coli* chromosomal replicase. *Cell* **94**: 61-71.

17. Katayama, Y., Kasahara, A., Kuraishi, H., and Amano, F. 1990. Regulation of activity of an ATP-dependent protease, Clp, by the amount of a subunit, ClpA, in the growth of *Escherichia coli* cells. *J. Biochem. (Tokyo)* **108**: 37-41.

18. Kelly, A. J., Sackett, M. J., Din, N., Quardokus, E., and Brun, Y. V. 1998. Cell cycle-dependent transcriptional and proteolytic regulation of FtsZ in *Caulobacter*. *Genes Dev.* **12**: 880-893.

19. Kuroda, A., Murphy, H., Cashel, M., and Kornberg, A. 1997. Guanosine tetra- and pentaphosphate promote accumulation of inorganic polyphosphate in *Escherichia coli*. *J. Biol. Chem.* **272**: 21240-21243.

20. Kuroda, A., Nomura, K., Ohtomo, R., Kato, J., Ikeda, T., Takiguchi, N., Ohtake, H., and Kornberg, A. 2001. Role of inorganic polyphosphate in promoting ribosomal protein degradation by the Lon protease in *E. coli*. *Science* **293**: 705-708.

21. Laub, M. T., McAdams, H. H., Feldblyum, T., Fraser, C. M., and Shapiro, L. 2000. Global analysis of the genetic network controlling a bacterial cell cycle. *Science* **290**: 2144-2148.

22. Lu, M., Campbell, J. L., Boye, E., and Kleckner, N. 1994. SeqA: a negative modulator of replication initiation in *E. coli*. *Cell* **77**: 413-426.

23. Marczynski, G. T. 1999. Chromosome methylation and measurement of faithful, once and only once per cell cycle chromosome replication in *Caulobacter crescentus*. *J. Bacteriol.* **181**: 1984-1993.

24. Marczynski, G. T. and Shapiro, L. 1992. Cell-cycle control of a cloned chromosomal origin of replication from *Caulobacter crescentus*. *J. Mol. Biol.* **226**: 959-977.

25. Marczynski, G. T. and Shapiro, L. 2002. Control of chromosome replication in *Caulobacter crescentus*. *Annu. Rev. Microbiol.* **56**: 625-656.

26. Marshall-Batty, K. R. and Nakai, H. 2003. Trans-targeting of the phage Mu repressor is promoted by conformational changes that expose its ClpX recognition determinant. *J. Biol. Chem.* **278**: 1612-1617.

27. Messer, W. 2002. The bacterial replication initiator DnaA. DnaA and *oriC*, the bacterial mode to initiate DNA replication. *FEMS Microbiol. Rev.* **26**: 355-374.

28. Poindexter, J. S. 1981. The *Caulobacters*: ubiquitous unusual bacteria. *Microbiol. Rev.* **29**: 123-179.

29. Poindexter, J. S., Pujara, K. P., and Staley, J. T. 2000. *In situ* reproductive rate of freshwater *Caulobacter* spp. *Appl. Environ. Microbiol.* **66**: 4105-4111.

30. Potocka, I., Thein, M., OSteras, M., Jenal, U., and Alley, M. R. 2002. Degradation of a *Caulobacter* soluble cytoplasmic chemoreceptor is ClpX dependent. *J. Bacteriol.* **184**: 6635-6641.

31. Quon, K. C., Marczynski, G. T., and Shapiro, L. 1996. Cell cycle control by an essential bacterial two-component signal transduction protein. *Cell* **84**: 83-93.

32. Quon, K. C., Yang, B., Domian, I. J., Shapiro, L., and Marczynski, G. T. 1998. Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin. *Proc. Natl. Acad. Sci. USA* **95**: 120-125.

33. Ryan, K. R. and Shapiro, L. 2003. Temporal and spatial regulation in prokaryotic cell cycle progression and development. *Annu. Rev. Biochem.* **72:** 367-94.

34. Shapiro, J. A. 1993. A role for the Clp protease in activating Mu-mediated DNA rearrangements. *J. Bacteriol.* **175**: 2625-2631.

35. Siam, R. and Marczynski, G. T. 2000. Cell cycle regulator phosphorylation stimulates two distinct modes of binding at a chromosome replication origin. *EMBO J.* **19**: 1138-1147.

36. Simon, R, Prieffer, U, and Puhler, A. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Biotechnology* **1**: 784-790.

37. Slominska, M., Wahl, A., Wegrzyn, G., and Skarstad, K. 2003. Degradation of mutant initiator protein DnaA204 by proteases ClpP, ClpQ and Lon is prevented when DNA is SeqA-free. *Biochem. J.* **370**: 867-871.

38. Torheim, N. K., Boye, E., Lobner-Olesen, A., Stokke, T., and Skarstad, K. 2000. The *Escherichia coli SeqA* protein destabilizes mutant DnaA204 protein. *Mol. Microbiol.* **37**: 629-638.

39. Torheim, N. K. and Skarstad, K. 1999. *Escherichia coli* SeqA protein affects DNA topology and inhibits open complex formation at *oriC*. *EMBO J.* **18**: 4882-4888.

40. Tsai, J. W. and Alley, M. R. 2001. Proteolysis of the *Caulobacter* McpA chemoreceptor is cell cycle regulated by a ClpX-dependent pathway. *J. Bacteriol.* **183**: 5001-5007.

41. von Freiesleben, U., Rasmussen, K. V., and Schaechter, M. 1994. SeqA limits DnaA activity in replication from *oriC* in *Escherichia coli*. *Mol. Microbiol.* **14**: 763-772.

42. Wang, L., Elliott, M., and Elliott, T. 1999. Conditional stability of the HemA protein (glutamyl-tRNA reductase) regulates heme biosynthesis in *Salmonella typhimurium*. *J. Bacteriol.* **181**: 1211-1219.

43. Wang, Y., Jones, B. D., and Brun, Y. V. 2001. A set of *ftsZ* mutants blocked at different stages of cell division in *Caulobacter*. *Mol. Microbiol.* **40**: 347-360.

44. Wegrzyn, A., Czyz, A., Gabig, M., and Wegrzyn, G. 2000. ClpP/ClpX-mediated degradation of the bacteriophage lambda O protein and regulation of lambda phage and lambda plasmid replication. *Arch. Microbiol.* **174**: 89-96.

45. Wright, R., Stephens, C., Zweiger, G., Shapiro, L., and Alley, M. R. 1996. *Caulobacter* Lon protease has a critical role in cell-cycle control of DNA methylation. *Genes Dev.* **10**: 1532-1542.

46. Zweiger, G. and Shapiro, L. 1994. Expression of *Caulobacter dnaA* as a function of the cell cycle. *J. Bacteriol.* **176**: 401-408.

Table 1. Bacterial Strains and Plasmids

Strain /plasmid	Genotype or description	Sources
E. coli		
S17-1	*E. coli* 294::RP4-2(Tc::Mu)(Km::Tn7)	(Simon et al. 1983)
C. crescentus		
GM2471	NA1000 *dnaA*::Ω *xylX*::*dnaA*	(Gorbatyuk and Marczynski 2001)
GM2726	GM2471 transcomplemented with pGM2049	This work
GM2885	NA1000 pMO88	This work
LS1837	NA1000 Δ*bla6 lon*::Ω	(Wright et al. 1996)
NA1000	Synchronizable mutant of WT CB15	(Evinger and Agabian 1977)
UJ199	NA1000 *clpP*::Ω *xylX*::pUJ174	(Jenal and Fuchs 1998)
UJ838	NA1000 *clpA*::Ω	U. Jenal
YB1585	NA1000 *ftsZ*163ΔC *xylX*::*ftsZ*	(Wang et al. 2001)
Plasmids		
pMO88	*clpX* with mutation in ATP binding site (*clpX**) under p*xylX* in pMR20	(Potocka et al. 2002)
pGM2049	pRK290*lacZ*::*dnaA* (*BamHI* A13)	(Marczynski and Shapiro 1992)

Figure Legends

Figure 1. The *C. crescentus* cell-cycle and selective proteolysis of CtrA, McpA and FtsZ.

CtrA (shading) and McpA (filled circle) are present in swarmer cells and selectively proteolyzed upon differentiation into stalked cells. McpA is localized to the swarmer cell pole in pre-divisional cells and CtrA is degraded in the stalked cell compartment of pre-divisional cells. FtsZ (ring) localizes as a ring-like structure at the midpoint of pre-divisional cells. Upon FtsZ ring constriction, FtsZ monomers are selectively degraded in the swarmer cell compartment but not the stalked-cell compartment.

Figure 2. Radio immuno-precipitation assay for DnaA protein synthesis.

A. GM2471, a conditional strain for expressing *dnaA* is shown (Gorbatyuk and Marczynski 2001). DnaA synthesis is controlled by the conditional PxylX promoter that is induced by xylose and repressed by glucose.

B. A radio immuno-precipitation assay with anti-DnaA antibodies was performed as described in *Materials and Methods*. DnaA and Hsp synthesis was monitored in NA1000 (WT), GM2471 and GM2471 + *dnaA* (pGM2049) strains grown either in M2G (G) or M2X (X) media.

C. DnaA and Hsp synthesis was measured in response to heat shock (10 min. at 42°C) and in response to cold shock at (10 min. at 15°C), as well as standard 30°C conditions.

Figure 3. *de novo* DnaA synthesis drives replication initiation.

A. GM2471 cells were blocked for DnaA synthesis for 180 minutes by shifting from M2X to M2G media. At 180 minutes, xylose was added to turn *dnaA* transcription back "on".

B. Radio immuno-precipitation assay of DnaA synthesis in GM2471 shifted to M2G for 180 minutes (X→G; *dnaA* off), and M2G + X (*dnaA* on) is shown. DnaA protein is depleted within 180 minutes of the X→G shift, and is *de novo* synthesized within 15 minutes of addition of xylose to the DnaA depleted culture.

C. DNA replication assay by [^{32}P] pulse labeling, performed on cells sampled in B.

Figure 4: Cell-cycle synthesis patterns of Flagellin, DnaA and Hsp proteins.

Synchronized NA1000 swarmer cells were isolated and allowed to proceed through the cell-cycle. At the indicated times, cells were labeled with [^{35}S]-methionine. Protein extracts were separately treated with either Flagellin or DnaA antibodies.

Figure 5: *C. crescentus* DnaA is an unstable protein.

A. NA1000 *C. crescentus* cells were labeled with [^{35}S]-methionine and chased with unlabelled amino-acids for up to 150 min. The DnaA radio immuno-precipitation assay was performed on cells sampled at the indicated times during the chase.

B. DnaA protein levels from A were quantified, normalized to total input radiolabeled proteins and plotted.

Figure 6: DnaA degradation is blocked by azide but not by chloramphenicol.

A. NA1000 *C. crescentus* were labeled with [^{35}S]-methionine and chased with unlabelled amino acids for 90 min in the presence of 8 μg/ml azide (+Az) or its absence (-Az). The DnaA radio immuno-precipitation assay was performed at 0 and 90 min during the chase.

B. Growth was measured in NA1000 cells by optical density (A_{660}) in response to the presence of 10 μg/ml of chloramphenicol (+CM) or its absence (-CM), as shown in the top panel. In the bottom panel, samples were [^{35}S]-methionine labelled and chased with unlabelled amino acids in the presence of chloramphenicol (+CM) or its absence (-CM). The DnaA radio immuno-precipitation assay was performed on cells sampled at the indicated times during the chase.

Figure 7: DnaA is degraded by ClpP protease but does not use ClpX or ClpA chaperones.

A. UJ199, a conditional *clpP* strain is shown (Jenal and Fuchs 1998). ClpP synthesis is turned on in the presence of xylose and off in the presence of glucose.

B. Anti-ClpP immunoblot reveals ClpP protein levels in UJ199 shifted to Glucose (Gluc) for 12 hours, and compared to a parallel culture maintained in xylose media (M2X).

C. The stabilities of DnaA were measured for UJ199 grown in Glucose (Gluc; diamonds), and for UJ199 grown in Xylose (Xyl; squares) and hence depleted for ClpP, as shown in B. At 12 hours of growth in either M2X or M2G, cells were [^{35}S]-methionine labelled and chased and the DnaA radio immuno-precipitation assay was performed on cells sampled at the indicated times during the chase.

D. GM2885, a strain conditionally expressing a dominant-negative form of ClpX (ClpX*) from pMO88 is shown. When grown with Xylose (+Xyl), ClpX*, a non-functional ClpX is expressed, and apparently poisons the ClpX protein complex.

E. Stability of CtrA and of F. DnaA in the presence of non-functional ClpX* (+Xyl; squares) or in the presence of functional ClpX (-Xyl; diamonds) is shown.

G. Stability of DnaA in a *clpA*$^-$ strain (squares) is compared to NA1000 (diamonds).

Figure 8: DnaA stability throughout the cell-cycle.

A. Stability of DnaA protein synthesized in swarmer cells is measured throughout the susequent cell-cycle (0-150 Min.). Swarmer cells were isolated and samples were [^{35}S]-methionine labelled and chased with unlabelled amino acids. The DnaA radio immuno-precipitation assay was performed on cells sampled at the indicated times during the chase. In a parallel culture, synthesis of Flagellin proteins at 0, 60, and 120 minutes in the cell-cycle tracks dimorphic development (upper panel). DnaA has a bimodal rate of degradation; swarmer cells degrade DnaA with a quicker rate ($t_{1/2} \sim 45$ minutes) than stalked cells ($t_{1/2} \sim 100$ minutes) (lower panel).

B. Stability of DnaA protein synthesized in stalked cells is measured throughout the cell-cycle. Swarmer cells differentiated into stalked cells and samples were likewise [^{35}S]-methionine labeled and chased. The rate of degradation of stalked-cell synthesized DnaA is $t_{1/2} \sim 100$ minutes.

C. The stability of DnaA protein synthesized in the G1 to S transition is measured throughout the subsequent cell-cycle. Late swarmer cells were likewise [^{35}S]-methionine labeled, chased throughout the subsequent cell-cycle and subjected to the radio immuno-precipitation assay. Upon completion of the cell-cycle, newly divided swarmer cells (Sw) and newly divided stalked cells (St) were isolated and segregated. DnaA protein is degraded from both the swarmer and stalked cell compartments following cell-division.

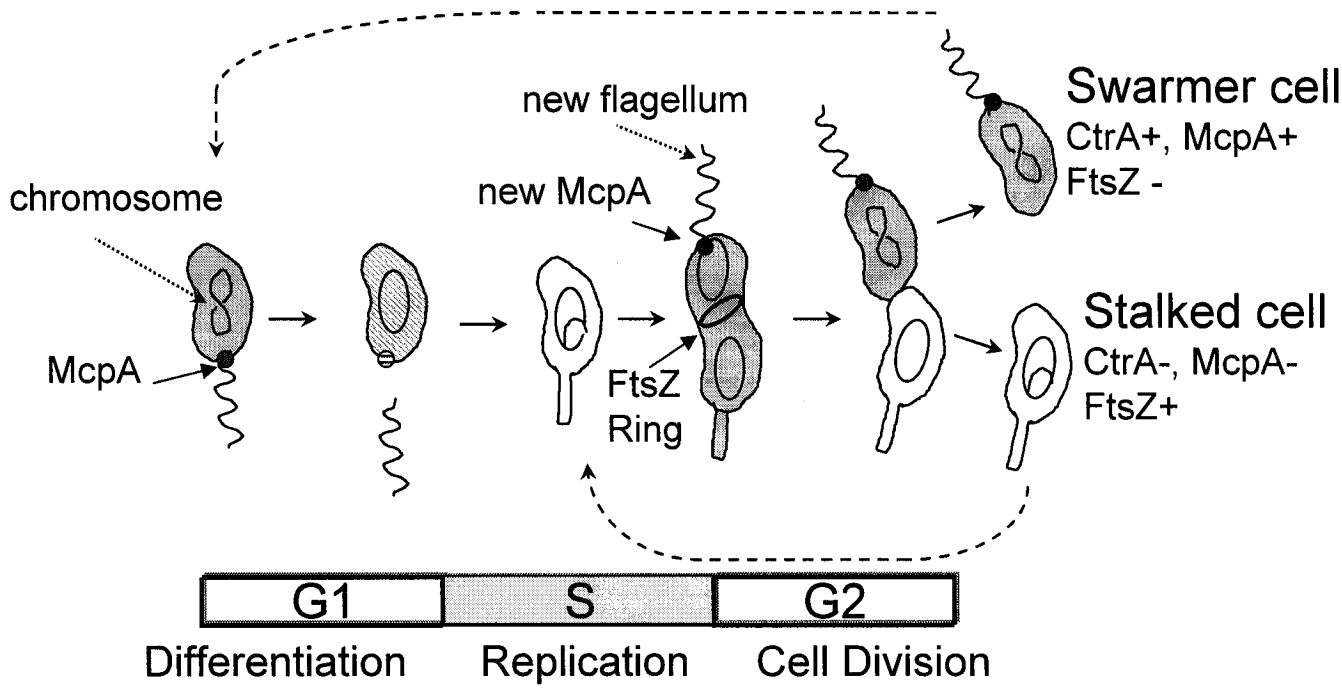
Figure 9: DnaA and CtrA levels throughout the cell-cycle and in response to nitrogen starvation.

A. WT NA1000 swarmer cells were isolated and allowed to proceed through the cell-cycle in standard M2G media with ammonium as the nitrogen source (+N). Cells were sampled at the indicated cell-cycle times, and immunoblots were performed separately with anti-DnaA and with anti-CtrA antiserum.

B-C. NA1000 swarmer cells (B) and stalked cells (C) were released into nitrogen starved conditions (-N; M2G media without ammonium). Cell-cycle progression under these conditions is shown schematically. Swarmer cells fail to differentiate into stalked cells (B) and stalked cells fail to complete cell-division (C) under nitrogen starved conditions. Cells were sampled at the indicated starvation times, and immunoblots were performed separately with anti-DnaA and with anti-CtrA antiserum.

Figure 1

C. crescentus cell cycle



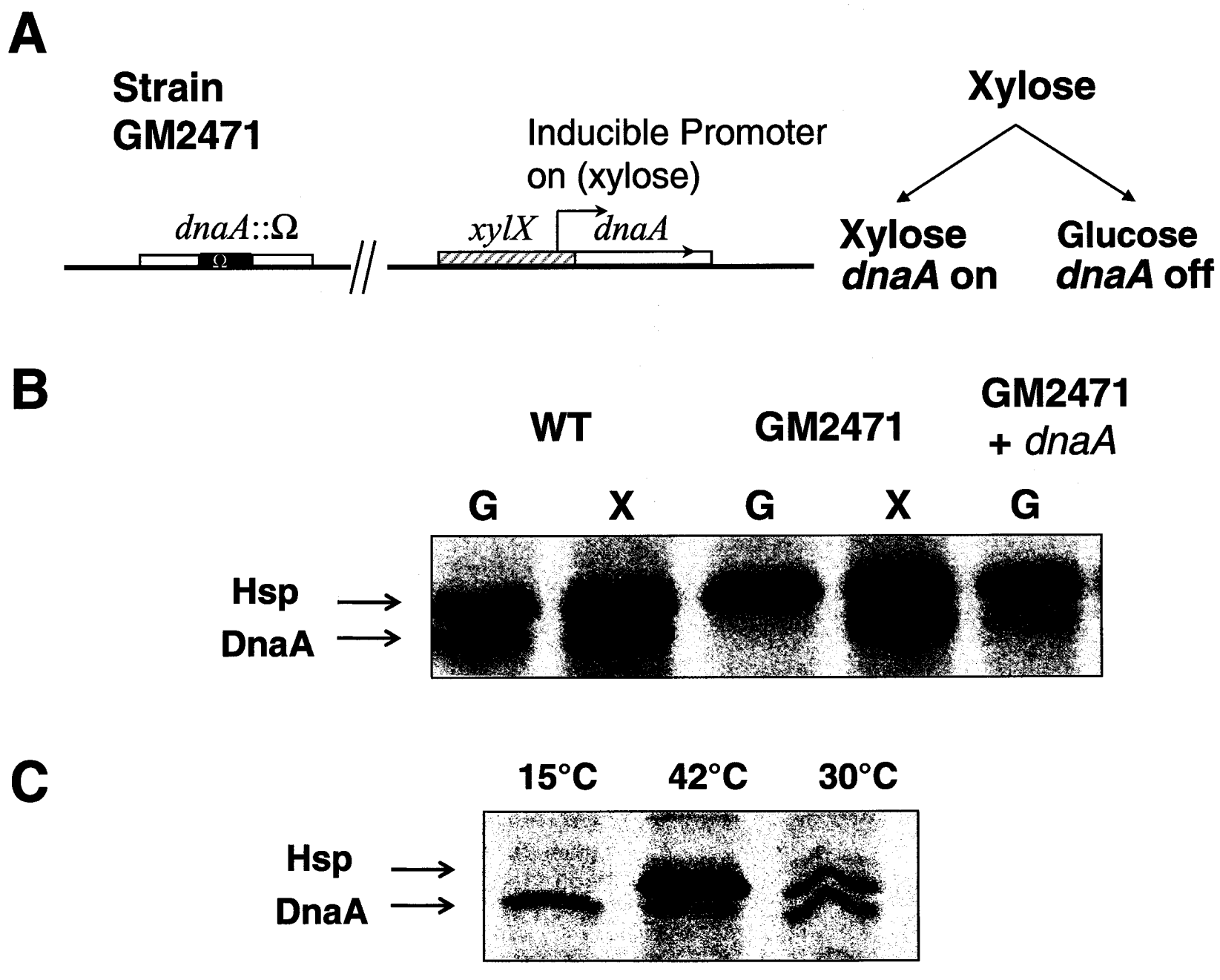

A
Strain
GM2471
Inducible Promoter
on (xylose)
dnaA::Ω
Ω
xylX
dnaA
Xylose
Xylose
dnaA on
Glucose
dnaA off
B
WT
GM2471
GM2471
+ dnaA
G
X
G
X
G
Hsp
DnaA
C
15°C
42°C
30°C
Hsp
DnaA

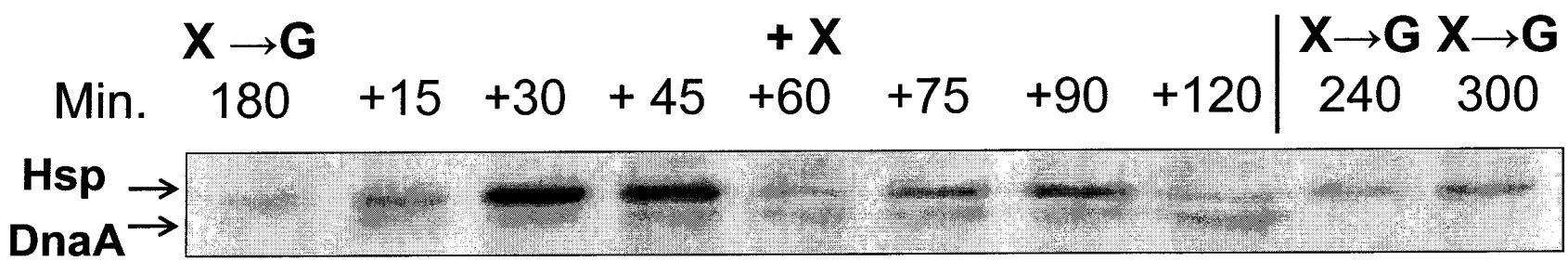
Figure 2

A

Strain GM2471

Xylose *dnaA* on → Glucose *dnaA* off (180) → Add Xylose *dnaA* on

B



C

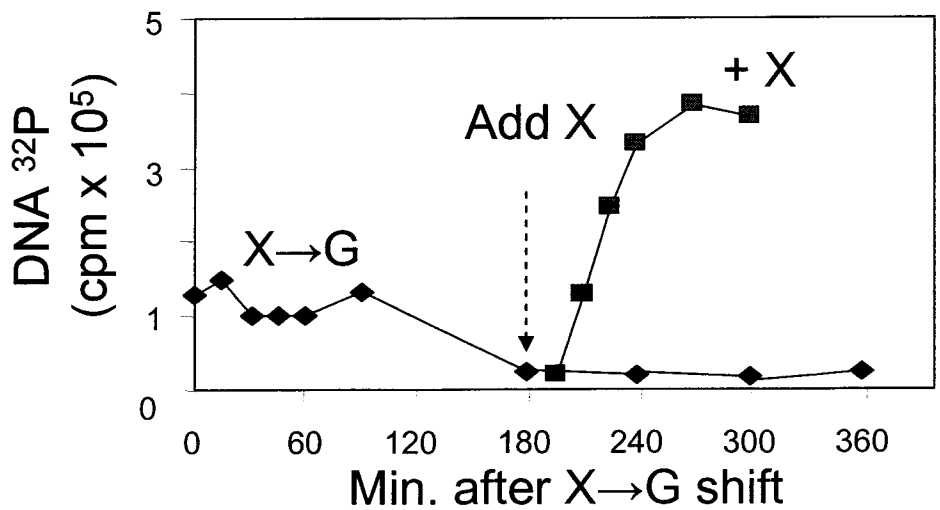


Figure 3

Figure 4

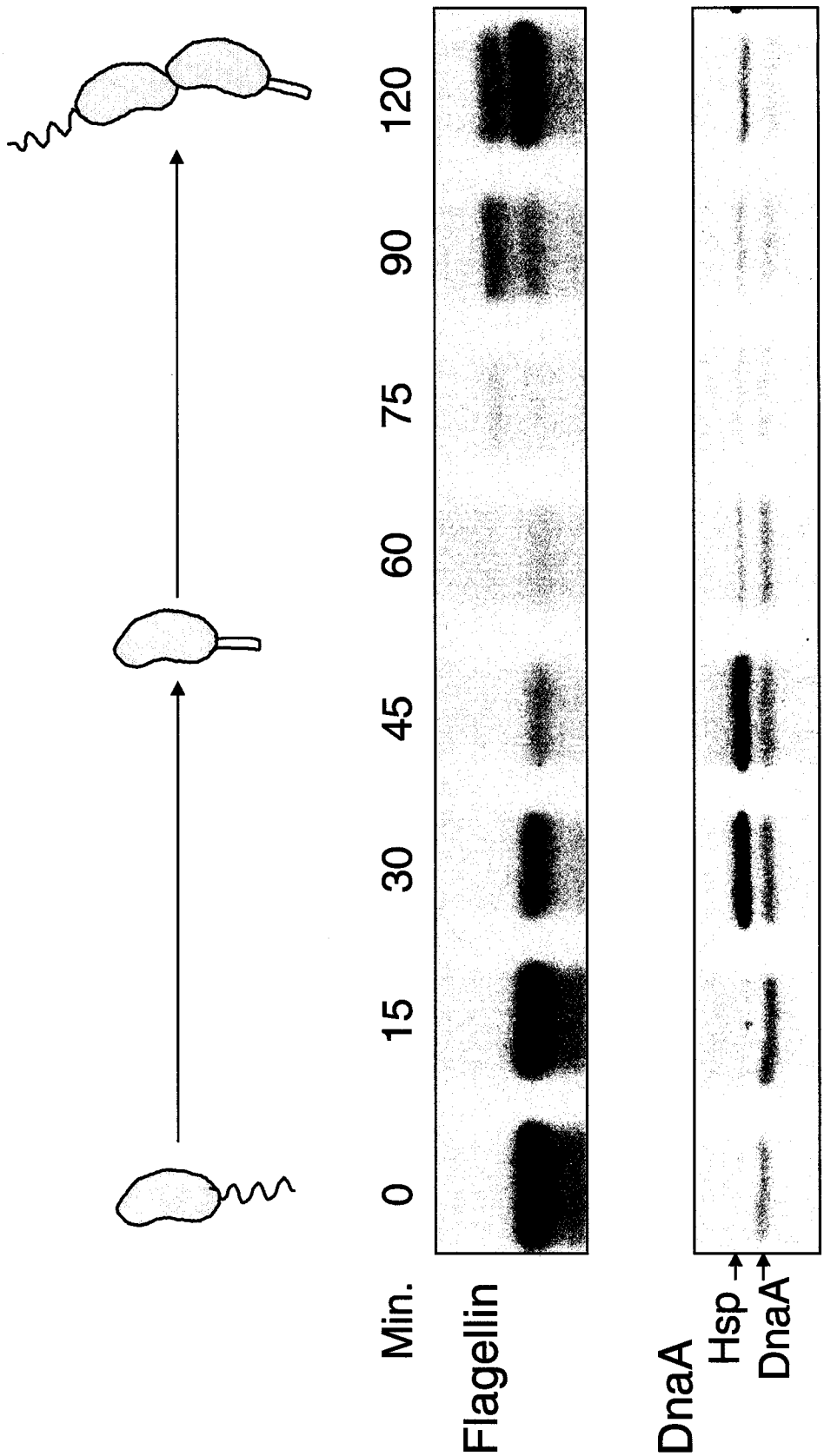
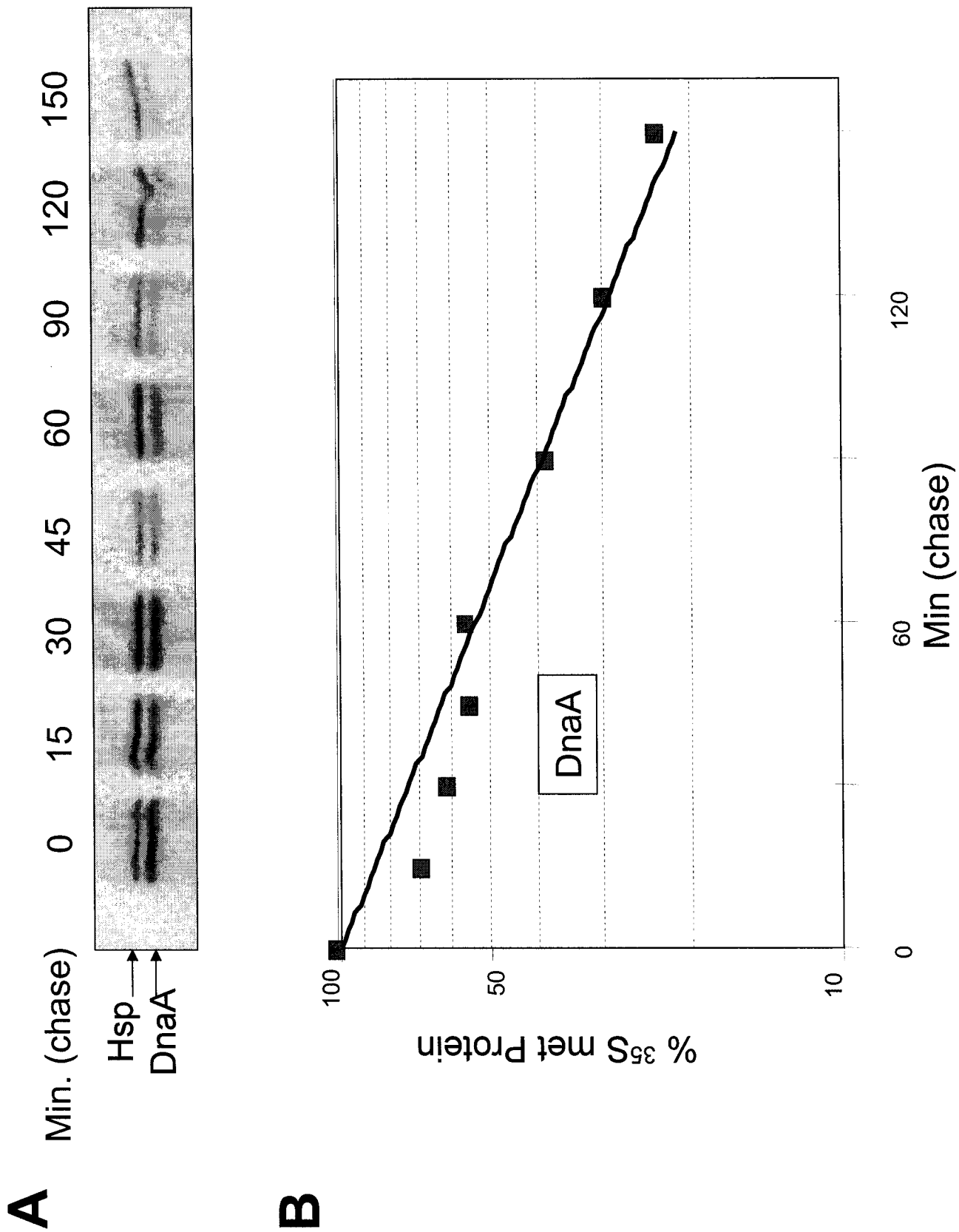


Figure 5


A
Min. (chase)
0
15
30
45
60
90
120
150
Hsp
DnaA
B
% 35S met Protein
100
50
10
DnaA
0
60
120
Min (chase)

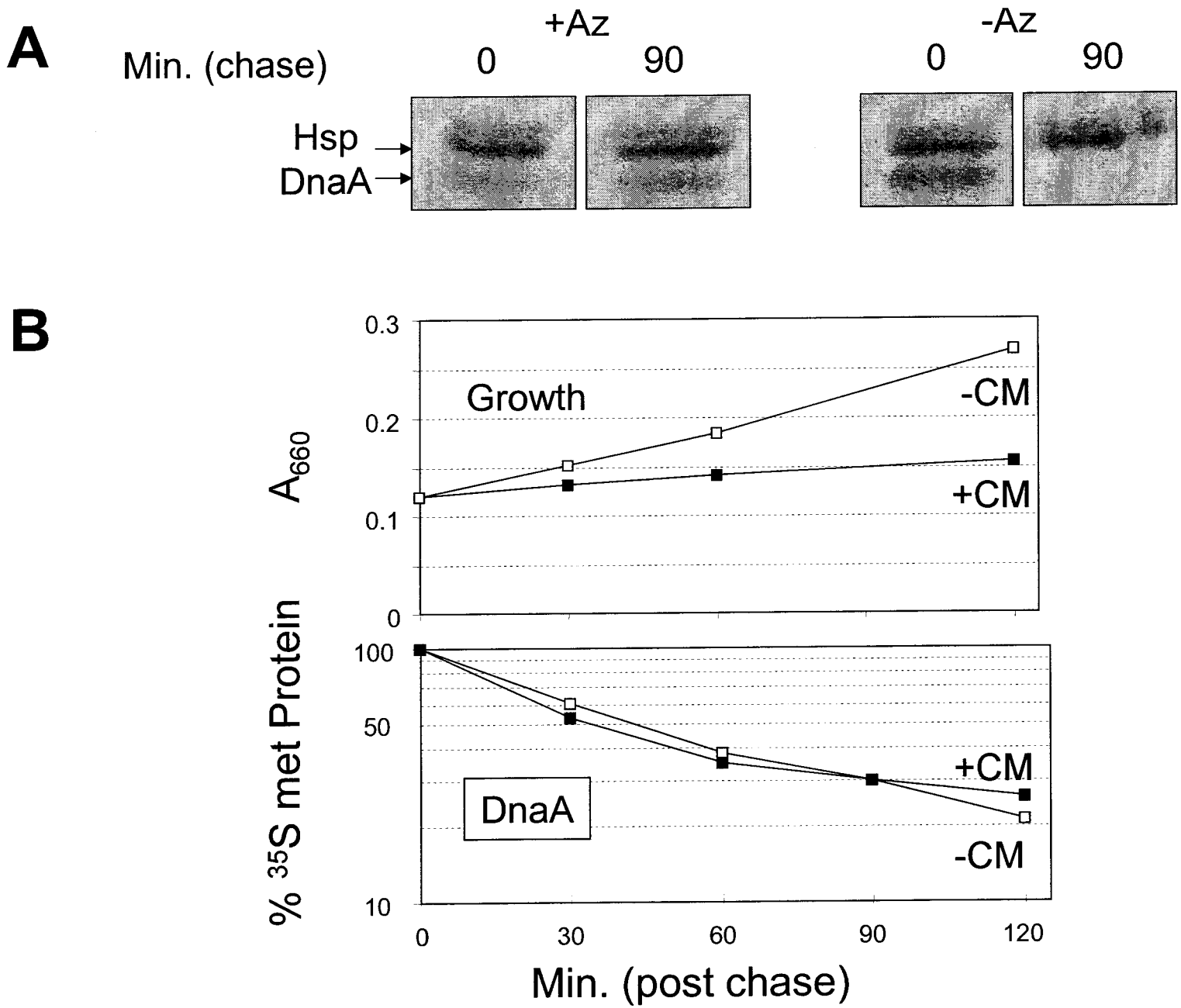

A
+Az
-Az
Min. (chase)
0
90
0
90
Hsp
DnaA
B
0.3
0.2
0.1
0
Growth
-CM
+CM
A_{660}
100
50
10
+CM
DnaA
-CM
% ^{35}S met Protein
0
30
60
90
120
Min. (post chase)

Figure 6

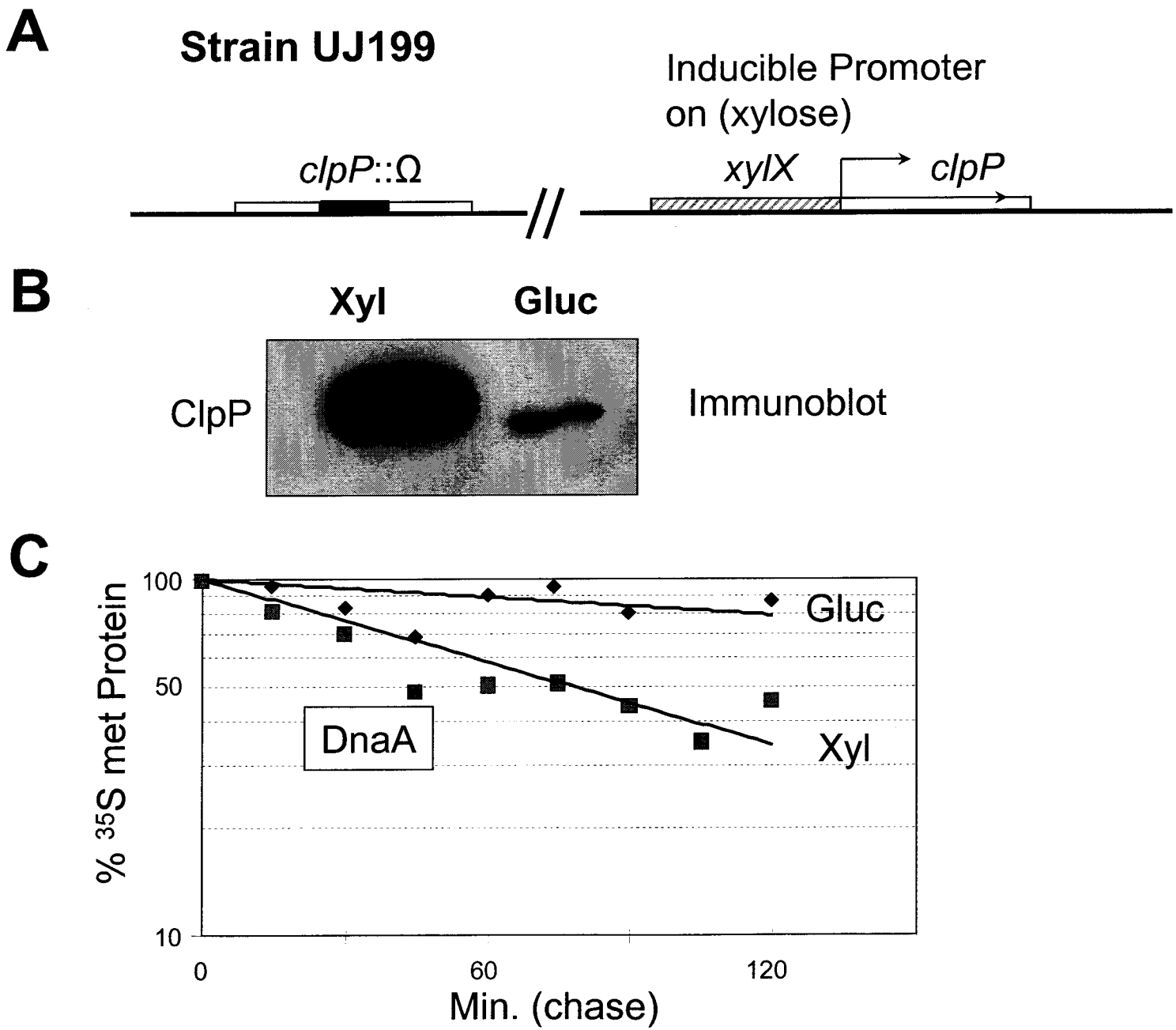

A
Strain UJ199
Inducible Promoter
on (xylose)
clpP::Ω
xylX
clpP
B
Xyl
Gluc
ClpP
Immunoblot
C
% 35S met Protein
100
50
10
0
60
120
Min. (chase)
Gluc
Xyl
DnaA

Figure 7

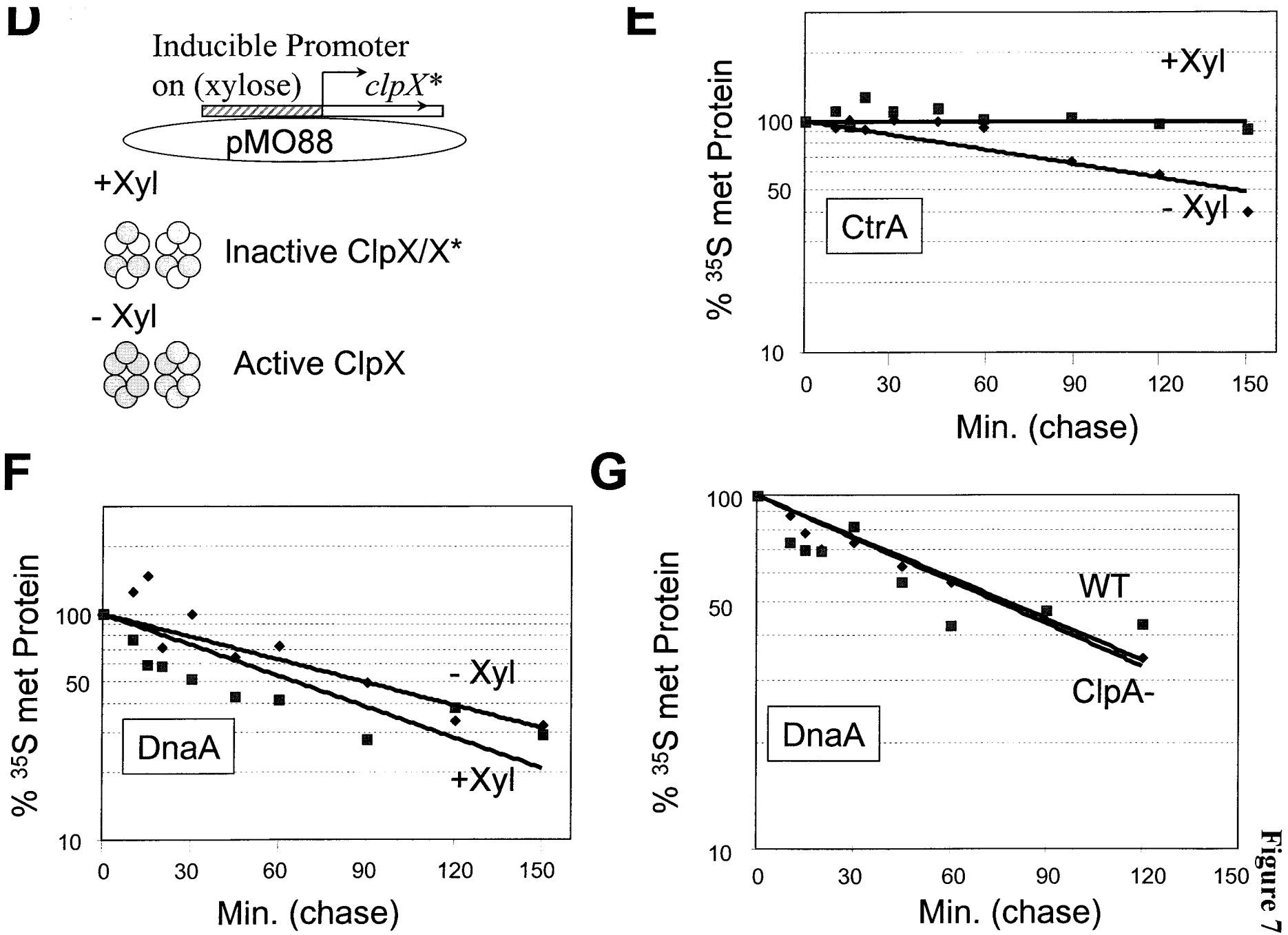

D
Inducible Promoter
on (xylose)
clpX*
pMO88
+Xyl
Inactive ClpX/X*
- Xyl
Active ClpX
E
+Xyl
- Xyl
CtrA
% 35S met Protein
100
50
10
0
30
60
90
120
150
Min. (chase)
F
DnaA
- Xyl
+Xyl
% 35S met Protein
Min. (chase)
G
WT
ClpA-
DnaA
% 35S met Protein
Min. (chase)

Figure 7

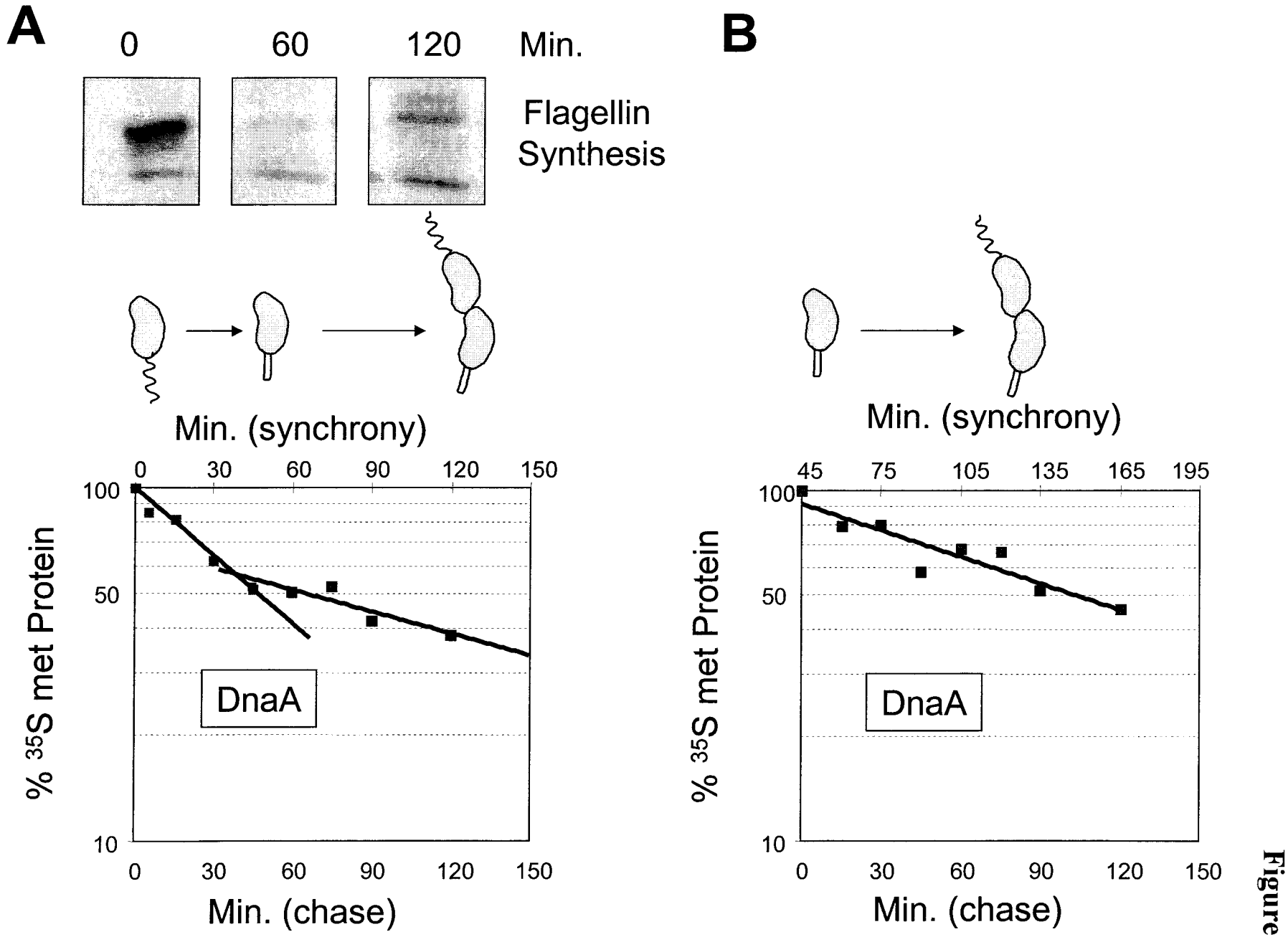

A
0
60
120
Min.
Flagellin Synthesis
Min. (synchrony)
0 30 60 90 120 150
100
50
10
% 35S met Protein
DnaA
0 30 60 90 120 150
Min. (chase)
B
Min. (synchrony)
45 75 105 135 165 195
100
50
10
% 35S met Protein
DnaA
0 30 60 90 120 150
Min. (chase)

Figure 8

Figure 8

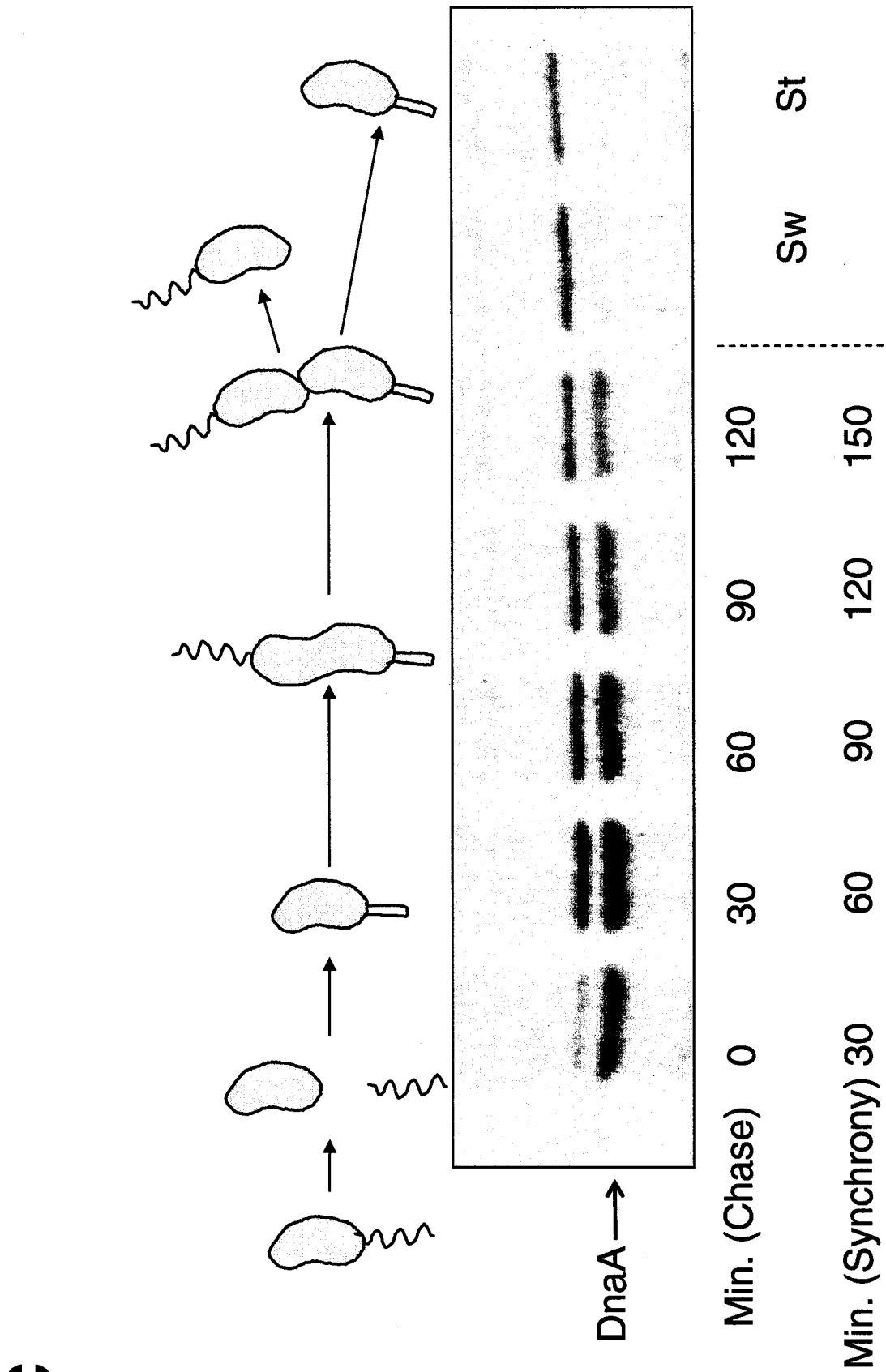

C
DnaA
Min. (Chase) 0 30 60 90 120
Min. (Synchrony) 30 60 90 120 150
Sw
St

Figure 9

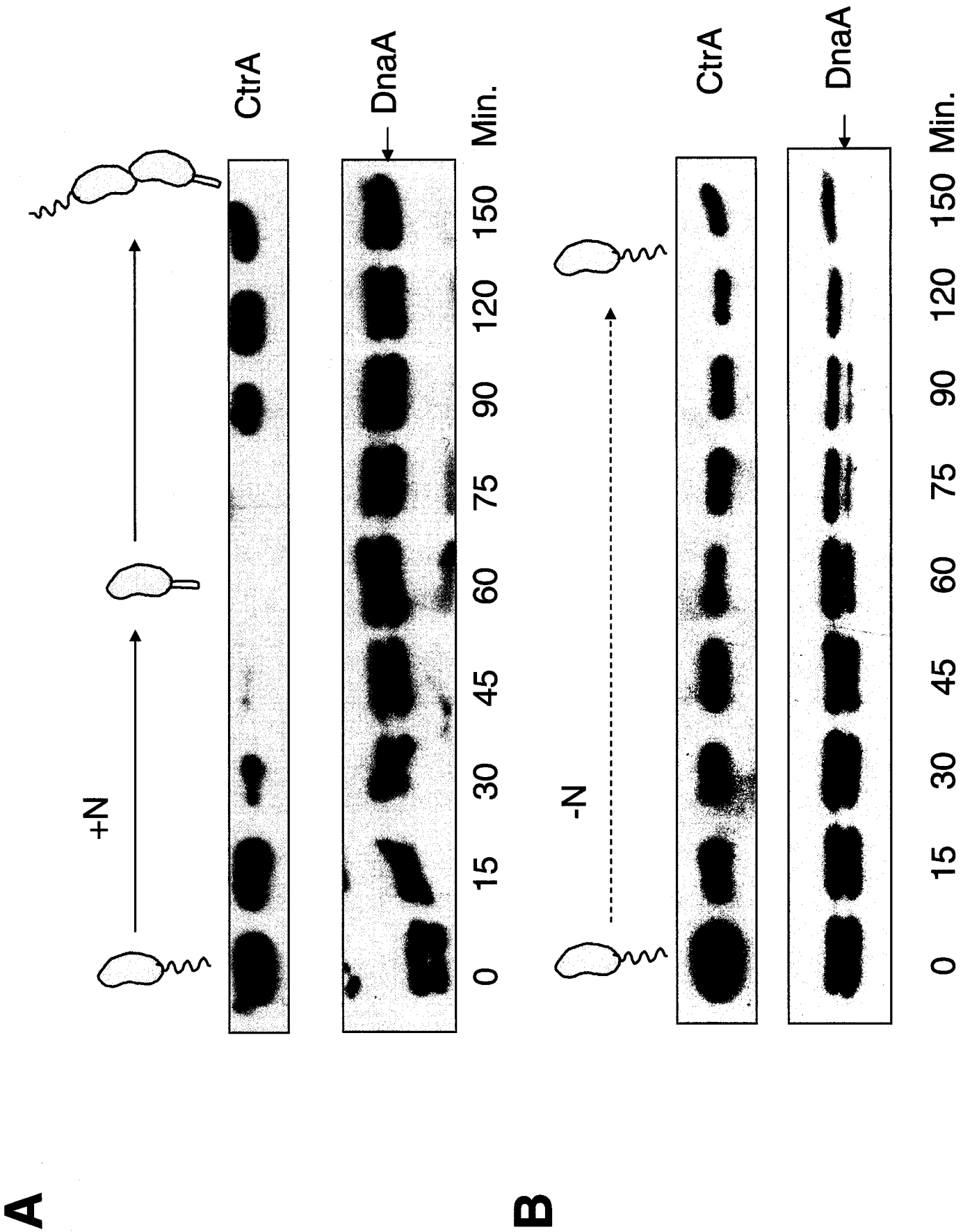
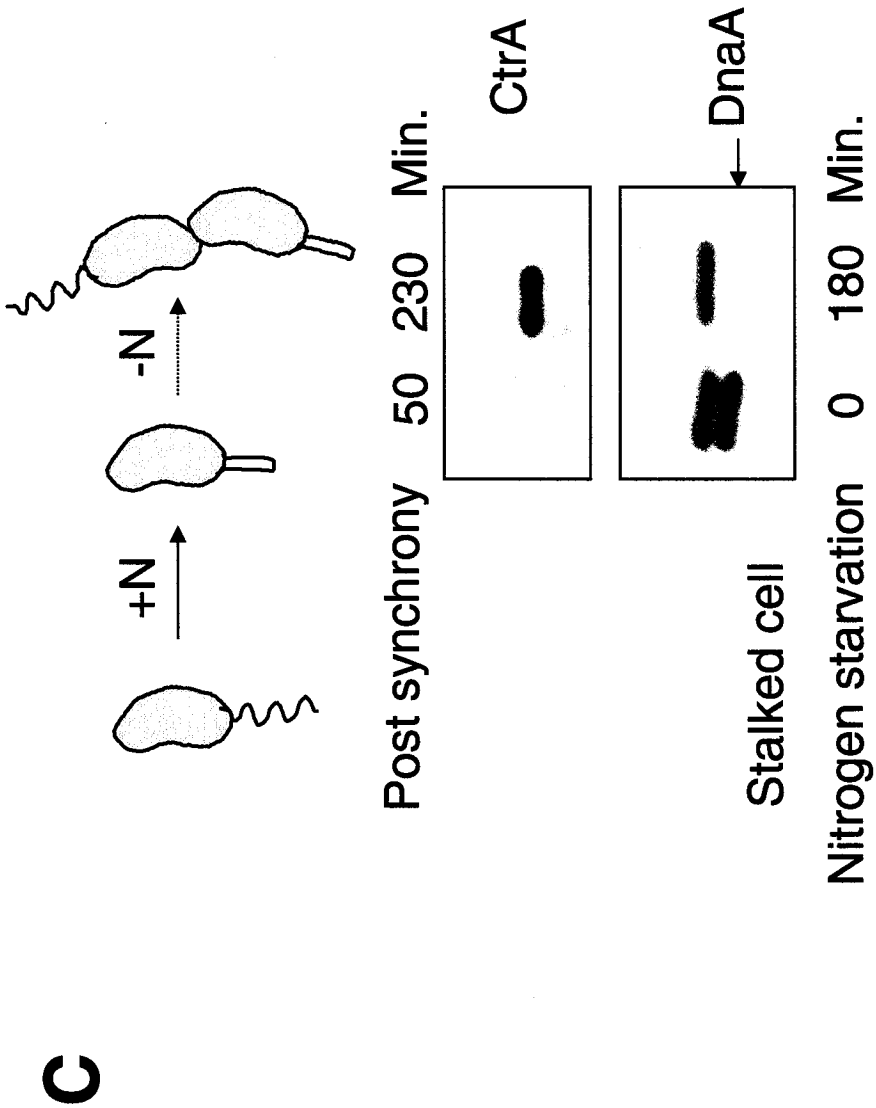

A
+N
CtrA
DnaA
Min.
0
15
30
45
60
75
90
120
150
B
-N
CtrA
DnaA
Min.
0
15
30
45
60
75
90
120
150

Figure 9


C
+N
-N
Post synchrony
50
230
Min.
CtrA
DnaA
Stalked cell
Nitrogen starvation
0
180
Min.

PREFACE TO CHAPTER 4

In the previous chapter, I demonstrated that DnaA is targeted by ClpP-dependent proteolysis and is differentially degraded in the cell-cycle, and that DnaA is degraded in response to nitrogen starvation. This study is a follow-up to the previous chapter. I seek to understand whether DnaA proteolysis is cued by general nutrient limitations and stresses or whether it is specific for only some nutrient limitations and not others. I also ask whether DnaA loss that is triggered by specific nutrient limitation is also degraded by ClpP as it is in response to cell-cycle cues. In addition, I examine the dependence of DnaA protein levels upon CtrA, and I test the converse, the dependence of CtrA upon DnaA. I also observe that *C. crescentus* DnaA is unstable in *E. coli* where the *E. coli* DnaA homologue is stable. This argues that DnaA holds *C. crescentus* proteolysis signals, and that the recognition proteins may be conserved.

Chapter 4: Nutritional cues for *C. crescentus* DnaA degradation.

Boris Gorbatyuk and Gregory T. Marczynski*

McGill University, Department of Microbiology and Immunology

*Correspondence to: Dr. G. T. Marczynski

McGill University, Department of Microbiology and Immunology

3775 University Street, Room 506

Montreal, Quebec, Canada. H3A 2B4

gmarczynski@nexus.microimm.mcgill.ca

Tel (+1) 514-398-3917; Fax (+1) 514-398-7052

Shortened running title: *C. crescentus* DnaA proteolysis and stress.

Key Words: DnaA, *Caulobacter*, proteolysis, ClpP, stationary-phase, starvation.

Abstract:

In *Caulobacter crescentus*, replication is initiated in the stalked cell type by the DnaA replication initiator protein. Unlike *Escherichia coli* DnaA, *C. crescentus* DnaA protein is is degraded during the cell-cycle by ClpP protease. In addition to cell-cycle cues, *C. crescentus* degrades DnaA in response to nitrogen starvation. Here we demonstrate that DnaA is degraded when *C. crescentus* is starved for nitrogen and carbon, but not for iron, magnesium or phosphate starvation. This carbon and nitrogen starved degradation of DnaA, like its degradation during the nutrient sufficient cell-cycle, is also performed by the ClpP protease. *C. crescentus* DnaA is also unstable when expressed in *E. coli*. DnaA is also degraded when cells enter stationary phase, and DnaA rapidly accumulates when cells are diluted into fresh media. In addition, DnaA protein levels do not change with heat shock, cold shock or during over-replication stress when grown at non-permissive temperature in a $ctrA^{Ts}$ strain. Therefore, DnaA proteins are not dependent upon the CtrA response-regulator, but interestingly *ctrA* transcription is dependent upon DnaA. We also identify the GroEL protein to be the heat shock protein immuno-precipitated with anti-DnaA anti-serum, and speculate on its role in DnaA degradation.

Introduction:

Caulobacter crescentus is an asymmetrically dividing, gram-negative bacterium that lives in dilute aquatic environments (Poindexter 1981). It divides to produce two distinct progeny, a motile flagella-bearing swarmer cell and a sessile stalked cell. The swarmer cell is unable to initiate chromosome replication and must undergo obligate differentiation where it sheds its single polar flagella and grows a filamentous appendage in its stead to become a stalked cell. This morphological differentiation is coupled to replication initiation and the stalked cell is able to initiate replication and cell division to divide asymmetrically and yield asymmetric progeny. This is a truly unique bacterial cell-cycle and its regulation has been the focus of recent reviews (Jenal and Stephens 2002; Marczynski and Shapiro 2002; Ryan and Shapiro 2003).

The inability of the swarmer cell to initiate chromosome replication is due, at least in part, to the presence of the CtrA master two-component response regulator that binds to the replication origin and represses replication (Quon et al. 1998; Siam and Marczynski 2000). CtrA is a global response regulator and controls the expression of one quarter of the cell-cycle regulated genes in *C. crescentus* (Laub et al. 2000). CtrA is present in swarmer cells, but is selectively degraded by the ClpXP chaperone/protease pair (Jenal and Fuchs 1998) in stalked cells but not in swarmer cells (Domian et al. 1997). In addition to this temporal pattern of regulation, CtrA is also degraded spatially only in the stalked cell compartment but not the swarmer cell compartment of pre-divisional cells (Domian et al. 1997). The selective absence of CtrA is not enough to explain the ability of stalked cells to initiate chromosome replication as the ubiquitous chromosome

replication initiator, DnaA (Messer 2002) is absolutely essential for replication initiation to proceed (Gorbatyuk and Marczynski 2001). Interestingly, the DnaA protein is also selectively degraded throughout the cell-cycle by the ClpP protease, but degradation does not require the typical ATP-binding chaperones, ClpA or ClpX (Gorbatyuk and Marczynski 2003). The cell-cycle degradation pattern of DnaA ensures that mostly newly-synthesized DnaA protein is available for stalked cells to initiate chromosome replication in each cell-cycle (Gorbatyuk and Marczynski 2003). Here, we demonstrate that the degradation of DnaA is a unique property of the *C. crescentus* protein, and can be degraded when expressed in the *E. coli* cell while the *E. coli* DnaA homologue is stable.

Although DnaA degradation is dependent upon its cell-cycle cues for degradation, it also is able to respond to nutritional cues like nitrogen starvation to trigger rapid degradation in both swarmer cells and stalked cells. This is in contrast to CtrA which is stabilized when either swarmer cells or stalked cells are starved for nitrogen (Gorbatyuk and Marczynski 2003). Therefore, the complete degradation of DnaA, the key replication initiator under unfavorable environmental conditions, and the stabilization of CtrA, the main replication repressor, is a mechanism by which *C. crescentus* responds to environmental stress to limit its growth. The presence or absence of DnaA in *C. crescentus* is a result of integrating cell-cycle and environmental/nutritional signals to identify favorable environmental conditions under which to replicate.

Targeted proteolysis due to environmental stress is a conserved mechanism by which bacteria adapt to poor environmental conditions. For example, in *E. coli*, many starvation signals including the end of the active growth phase and the onset of stationary phase lead to the targeted proteolysis of the anti-σ^s regulator protein, RssB by ClpXP, and

thereby allow for gene expression for adaptation to low nutrients and other stressful conditions in *E. coli* (Hengge-Aronis 2002). However, *E. coli* does not degrade DnaA under stationary phase conditions, in fact, DnaA accumulates under these conditions (Ali et al. 1999).

In this paper, we identify several stress conditions that promote *C. crescentus* DnaA degradation. We demonstrate that DnaA is degraded from *C. crescentus* when it is starved specifically for carbon or nitrogen and when *C. crescentus* enters stationary phase but not during active growth, a contrast to *E. coli*. In addition, we identify that DnaA is not targeted for proteolysis during heat-shock, cold-shock, active logarithmic growth or iron, magnesium or phosphate starvation. The degradation of DnaA under carbon and nitrogen starvation appears to be mediated by ClpP as it is during the cell-cycle under nutrient replete conditions. We also identify that DnaA protein levels do not depend on CtrA and do not respond to the over-replication stress caused by loss of $CtrA^{Ts}$ activity at non-permissive temperature, whereas *ctrA* transcription is affected by loss of DnaA. Finally, in this paper we identify that the upper-band heat shock protein that we previously detected in our radio immuno-assay (Gorbatyuk and Marczynski 2003) is GroEL and we comment on its potential role for DnaA degradation.

Results

C. crescentus DnaA is unstable when expressed in *E. coli* whereas *E. coli* DnaA is stable

We previously demonstrated *C. crescentus* DnaA protein instability in *C. crescentus*. We wanted to test whether *C. crescentus* DnaA would show instability when expressed in *E. coli*; *E. coli* DnaA was previously reported to be a stable protein (Slominska et al. 2003). *C. crescentus* DnaA cloned with a C-terminal His-tag in a pet21b vector was introduced into a BL21 *E. coli* strain (GM2504 or SLC). As the *C. crescentus dnaA* gene was cloned without its own promoter we took advantage of the leaky expression from pet21b when grown without IPTG. At t=0, we treated growing cells with Chloramphenicol (200 μg/ml) to block protein synthesis, sampled a normalized number of cells and performed either *C. crescentus* DnaA and *E. coli* DnaA immuno-blot analysis to measure protein stability over time.

As previously reported, we observe that *E. coli* DnaA protein is remarkably stable, even 24 hours after Chloramphenicol addition there is no significant decrease of DnaA (Fig. 1A). However, *C. crescentus* DnaA was unstable in *E. coli* ($t_{1/2}$ ~ 2.5 h) (Fig. 1B), but not as unstable as when expressed in *C. crescentus* ($t_{1/2}$ ~ 60 minutes) (Gorbatyuk and Marczynski 2003). Therefore, the instability of *C. crescentus* DnaA is specific to the protein, and the stability of *E. coli* DnaA is probably not due to an absence of DnaA protein degradation specificity factors or the presence of inhibitory factors in *E. coli*.

To rule out the possibility that anti- *C. crescentus* DnaA anti-serum crossreacted with anti- *E. coli* DnaA anti-serum, we induced *C. crescentus* DnaA in strain GM2504 with IPTG and performed immuno-blots with both anti-sera separately. Only the *C. crescentus* DnaA protein was induced with IPTG, whereas the *E. coli* DnaA protein remained unchanged when GM2504 was treated with IPTG (data not shown). Therefore, *E. coli* anti-DnaA anti-serum did not cross-react with *C. crescentus* anti-DnaA anti-serum and *vise versa.*

C. crescentus DnaA is absent from _C. crescentus_ Stationary Phase Cultures

In *E. coli*, DnaA accumulates when cells reach a high density and enter stationary phase (Ali et al. 1999). We tested whether *C. crescentus* DnaA behaved in this manner. We grew WT NA1000 *C. crescentus* at 30°C from a low optical density (A_{660} = 0.175) to a high optical density at stationary phase (Fig. 2A). It took approximately eight hours for the NA1000 cells to reach saturation and we maintained this culture in stationary phase for another 16 hours shaking at 30°C (24 hours after initial seeding). At this point the culture was diluted to its original starting cell density, and incubated for another two hours (Fig. 2A). We periodically sampled for anti-DnaA immunoblots (Fig. 2B) as described in Materials and Methods.

The immunoblots revealed that when *C. crescentus* reached stationary phase, DnaA protein levels significantly decreased (Fig. 2B, 8 hours), and DnaA was completely absent approximately 4 hours later, although cells continued to grow during stationary

phase (Fig. 2B, 12 and 24 h). After 24 hours, these stationary phase cells were diluted in fresh media to a lower optical density (A_{660} = 0.15), and DnaA protein rapidly reached exponential growth levels within 2 hours (Fig. 2B). Therefore, unlike *E. coli* DnaA that is stably maintained throughout both active growth and stationary phase, *C. crescentus* DnaA is absent in stationary phase cultures and its reappearance anticipates exit from stationary phase and entry into active growth phase.

Since *E. coli* DnaA actually increases during stationary phase (Ali et al. 1999), it is possible that *C. crescentus* has a secondary stationary phase function. A follow-up experiment argues against this hypothesis. We allowed NA1000 to enter stationary phase and remain in that state for 120 hours. We periodically sampled every 24 hours and performed anti-DnaA immuno-blots. *C. crescentus* DnaA was never present, even at late stationary phase (data not shown) where there is an altered physiology (Wortinger et al. 1998). This confirms that *C. crescentus* DnaA is only found in growing cells, and suggests that *C. crescentus* DnaA is only employed for chromosome replication.

DnaA is degraded during carbon and nitrogen starvation

We wanted to identify precise conditions that trigger DnaA loss in *C. crescentus*. We already identified that swarmer cells starved for ammonium (nitrogen-starved) arrested as swarmer cells and that nitrogen-starved stalked cells arrested as pre-divisional cells and did not divide to form swarmer and stalk progeny. These nitrogen-starved swarmer cells

and stalked cells rapidly degraded DnaA protein, implying a regulated proteolytic response.

We starved a mixed population of NA1000 cells for nitrogen as described in Materials and Methods, monitored their growth and performed immuno-blots to measure DnaA protein levels under these conditions. DnaA protein is completely absent from NA1000 within 60 minutes following nitrogen-starvation (Fig. 3B). This appears to be accelerated degradation since DnaA has a half-life of 60 minutes as measured by pulse-chase experiments. Following 120 minutes of nitrogen-starvation, cells were washed and resuspended in normal M2G media (+N) (Fig. 3A) and were sampled for immunoblots (Fig. 3C). Whereas DnaA is absent during nitrogen-starvation, upon re-suspension in media containing nitrogen, DnaA was rapidly re-synthesized and reached previous steady state levels within 30 minutes of growth in unstarved conditions (Fig. 3C). Therefore, the absence or presence of DnaA is directly co-ordinated with the presence or absence of nitrogen (ammonium-chloride) in the minimal growth medium.

Similarly, we starved a mixed population of NA1000 cells for carbon by washing and re-suspending an actively growing culture in M2 media without Glucose (M2 no G) as described in Materials and Methods. Interestingly, carbon-starved swarmer cells arrest as swarmer cells just like nitrogen-starved swarmer cells (data not shown) and we hypothesized that DnaA might also be degraded under these conditions. NA1000 immediately stops growing in the absence of a carbon source, but resumes active growth when glucose is added back to the media (Fig. 4A). Immuno-blots were performed to measure DnaA levels under these conditions. DnaA is completely absent from carbon-starved NA1000 cells between 30 and 60 minutes of starvation. Like, the nitrogen-

starved scenario, the re-addition of carbon re-synthesized DnaA protein within 30 minutes where the DnaA level reached steady-state (Fig. 4B). As well, the re-addition of Carbon and the re-synthesis of DnaA restores active growth to the previously-starved culture.

DnaA is degraded by selective degradation

Whereas carbon and nitrogen starvation leads to loss of DnaA protein from *C. crescentus*, this is not the case with iron, magnesium and phosphate starvation where DnaA is maintained and not lost from the cells (Fig. 5). Under phosphate starvation, cell growth completely stopped immediately, but cells proceeded through two doubling cycles without iron and magnesium prior to arresting growth. Therefore, DnaA loss from *C. crescentus* is not a general feature for all nutrient starvation and lack of cell growth but rather is specific for carbon and nitrogen.

Neither heat-shock nor cold-shock promote DnaA degradation from *C. crescentus*

We tested whether other environmental stresses such as elevated or lowered temperature (that suspends cell growth) would trigger degradation of DnaA from *C. crescentus*. We know from previous experiments that neither heat-shock nor cold-shock affects the synthesis of DnaA in *C. crescentus* (Gorbatyuk and Marczynski 2003). DnaA synthesis is not blocked under these stresses, nor is it stimulated, consistent with it not being a heat-shock protein.

NA1000 cells were actively grown at 30°C and then shifted to either 42°C (Fig. 6A) or 15°C (Fig. 6B) for 2 hours. During this temperature up-shift or down-shift, NA1000 was monitored for growth and sampled for anti-DnaA immuno-blot analysis. During the cold-shock, NA1000 abruptly stopped growing (data not shown) but DnaA levels remained unchanged throughout this shift (Fig. 6A). In addition, upon return to 30°C, no change in DnaA protein levels was observed (Fig. 6A) when these cells resumed growth. During the heat-shock, NA1000 cells kept growing (data not shown) and DnaA levels remained unchanged throughout the shift (Fig. 6B), and were unaffected by return to 30°C. Therefore, DnaA protein levels are not affected by elevated or lowered temperature stresses to *C. crescentus*.

The upper-band, that cross-reacted with anti-DnaA antibodies, was previously identified to be a heat-shock protein. Its abundance increased with heat-shock (Fig. 6B). In this paper, we identify this protein to be GroEL (Fig. 9).

Does ClpP degrade DnaA upon nitrogen and carbon starvation?

We previously demonstrated that DnaA is degraded through a ClpP-dependent pathway during nutrient sufficient cell-cycle growth. We next tested whether the degradation that is stimulated through carbon and nitrogen starvation was also dependent upon ClpP or whether a different protease was recruited.

To address this question we utilized strain UJ199 which conditionally expresses *clpP* via a xylose dependent transcription promoter (on in xylose; off in glucose) as previously described (Jenal and Fuchs 1998). UJ199 was grown in M2X overnight and

then seeded to A_{660} = 0.1 and allowed to grow in M2G; it was not allowed to reach saturation and was back-diluted periodically to maintain active growth in M2G and to deplete UJ199 of ClpP. After sixteen hours of active depletion, it was observed that UJ199 cells started to filament and at this point cells were sampled for anti-ClpP immunoblot analysis (Fig. 7A). It was observed that there was still some residual ClpP present in these cells, but the ClpP levels were much lower than prior to active depletion (Fig. 7A). As a control, a portion of ClpP-depleted UJ199 was released into M2G media with Chloramphenicol to measure the stability of CtrA in these cells over time by immuno-blots (Fig. 7B). As well, wild-type NA1000 cells were released into M2G with Chloramphenicol to measure CtrA stability (Fig. 7C). CtrA dependens on ClpP for its degradation (Jenal and Fuchs 1998). Our data confirm that CtrA is stabilized by ClpP-depletion in UJ199 (Fig. 7B) but degraded in wild-type NA1000 cells (Fig. 7C). Therefore, ClpP is sufficiently depleted from UJ199 to significantly reduce proteolysis.

The UJ199 cells that were depleted for ClpP for 16 hours were starved for nitrogen (Fig. 7D) and carbon (Fig. 7E) and compared to wild-type NA1000 cells starved for nitrogen (Fig. 7D) and carbon (Fig. 7E). If DnaA is degraded through ClpP under nitrogen or carbon starvation, then we expect it to be stabilized when ClpP levels are lower. In both nitrogen or carbon starvation, strain UJ199 depleted for ClpP shows a stabilized level of DnaA at the late starvation point (120 minutes in Fig. 7D and Fig. 7E) relative to the late starvation point in the parallel NA1000 cells (120 minutes in Fig. 7D. and Fig. 7E). However, in both nitrogen or carbon starvation, some DnaA was still degraded in UJ199 depleted for ClpP and we attribute this to the residual ClpP levels present in UJ199 (Fig. 7A).

Does over-replication and under-replication influence CtrA and DnaA protein turnover?

We first tested whether the stress of non-functional CtrA and its consequent over-replication (Quon et al. 1996) influenced DnaA protein levels. To inactivate out CtrA, a *ctrA* temperature-sensitive strain (LS2195) was shifted to non-permissive temperature and anti-DnaA immuno-blots were performed (Fig. 8A). DnaA protein levels remained unchanged when *ctrA Ts* was shifted to the non-permissive temperature (Fig. 8A). Previous studies demonstrated that these cells acquire extra chromosomes (Quon et al. 1998), implying that extra DnaA protein is not needed to support extra chromosome replication.

We then tested how loss of DnaA influenced *ctrA* transcription (Fig. 8B) and CtrA protein levels (Fig. 8C). Replication inhibition by hydroxyurea or by Ts DNA Polymersase was previously shown to turn off *ctrA* gene transcription (Wortinger et al. 2000). We test whether this effect extends to a controlled block of replication initiation. We introduced a plasmid carrying a *ctrA::lacZ* transcriptional reporter into GM2471 (GM2744), a conditional strain for *dnaA*. Expression of *dnaA* is turned off rapidly in GM2471 cells shifted from M2X to M2G and DnaA protein is absent one hour after the shift (data not shown). When *dnaA* expression was turned off, transcription of *ctrA* was also rapidly reduced (Fig. 8C).

We also monitored CtrA protein levels in GM2471 grown in both M2G (*dnaA* off) and M2X (*dnaA* on). GM2471 cells were grown in M2X, synchronized to isolate

swarmer cells and then shifted to M2G or M2X. Immuno-blots were performed for both CtrA and McpA proteins. Both CtrA and McpA are swarmer cell-specific proteins, both are degraded upon swarmer to stalk differentiation, and both are later resynthesized in pre-divisional cells. This wild-type pattern is observed in our xylose (*dnaA* on) culture. When *dnaA* is turned on, McpA and CtrA are degraded upon swarmer to stalk differentiation (Fig. 8C left panel t =60 min) and then resynthesized when pre-divisional cells reappear (Fig. 8C left panel t=120 min).

However, when *dnaA* expression was turned off, swarmer to stalk differentiation was delayed as was CtrA and McpA loss. Although, swarmer cells eventually differentiated into stalked cells (as seen by eventual loss of McpA and CtrA) and arrested as stalked cells that do not synthesize McpA or CtrA (Fig. 8C right panel). This is consistent with loss of *ctrA* transcription when *dnaA* expression was turned off (Fig. 8B). CtrA protein was absent from *dnaA* depleted cells, but unexpectedly re-appeared very late in the depletion (Fig. 8C right panel t = 720 min). We speculate that this increase in CtrA protein levels late in the *dnaA* block may be reflective of constant low *ctrA* transcription as well as possible CtrA stability.

Taken together, the immuno-blot and transcriptional assay data suggest that DnaA is not dependent upon CtrA for its expression, but that CtrA is dependent upon DnaA or chromosome replication for its expression.

GroEL is the Upper-Band detected in anti-DnaA Immunoprecipitations and Immuno-blots.

We identified the upper band that cross-reacted with anti-DnaA anti-serum in both immunoprecipitations (IPs) and immuno-blots and migrated to a slightly higher molecular weight than DnaA. The upper band is clearly a heat-shock protein (HSP), because its synthesis is induced upon temperature shift to 42°C from 30°C (Gorbatyuk and Marczynski 2003) and its molecular weight size of 60 kDa suggested that the upper band could be GroEL. To test if this upper band was GroEL, we performed an S^{35}-methionine pulse-chase experiment followed by anti-DnaA or anti-GroEL IPs on NA1000 cells (Fig. 9A). Since we previously demonstrated that DnaA was unstable and since the upper band was stable, we expected the major anti-GroEL IP product to migrate to the same molecular weight as the upper band from anti-DnaA Ips and we expected it to be a stable protein. Anti-sera for DnaA and for GroEL precipitate a 60 kDa stable protein (the upper band in both IPs) (Fig. 9A).

We also previously showed that the upper anti-DnaA IP product has a cell-cycle synthesis pattern where its synthesis is low in stalked cells, induced upon swarmer to stalk differentiation, low in late stalked / early pre-divisional cells, and then again induced slightly in late pre-divisional cells. We synchronized NA1000 cells, pulse-labelled the proteins and performed IPs with anti-DnaA and anti-GroEL antibodies. Flagellin IPs would mark the stage of the cell-cycle, as previously shown (Fig. 8B lower panel). The upper band from both DnaA and GroEL anti-sera has the same cell-cycle synthesis patter (Fig. 9B upper and middle panels).

In order to conclusively demonstrate that the upper band was GroEL, we performed the anti-GroEL and anti-DnaA IP assay on a conditional *groEL* strain (SG300) which expresses *groEL* in xylose only and not in glucose (Fig. 9C). We observed from anti-GroEL IPs that the upper band was synthesized when grown in xylose and not glucose in SG300 and that the exact same pattern was observed with the upper band from anti-DnaA IPs for SG300 (Fig. 9C). Therefore, both genetic and immunological criteria identify the upper Hsp band as *C. crescentus* GroEL protein.

Discussion

E. coli *DnaA stability and* C. crescentus *DnaA instability*

E. coli DnaA was reported to be a stable protein. Only mutant alleles of *dnaA* exhibited instability, and these were degraded by several different *E. coli* proteases (Slominska et al. 2003). Our data confirms that *E. coli* DnaA is indeed stable (Fig. 1A). We also demonstrate that *C. crescentus* DnaA is unstable when expressed in *E. coli* (Fig. 1B). *C. crescentus* DnaA instability is conserved even in a distantly related bacterium that normally does not degrade DnaA protein. Therefore, the instability determinants of *C. crescentus* DnaA probably lie within its own amino-acid sequence and it is unlikely that auxiliary factors are required for degradation.

Nutritional stress conditions and DnaA

E. coli DnaA and *C. crescentus* DnaA respond very differently to stress. For example, in *E. coli,* entrance into stationary phase leads to the stabilization and even accumulation of DnaA (Ali et al. 1999). This contrasts with our observations that *C. crescentus* DnaA is degraded upon entry to stationary phase and returns only when stationary phase cultures are back-diluted and allowed to grow exponentially (Fig. 2).

We have also identified the nutritional stress conditions that trigger the loss of DnaA from the *C. crescentus*. DnaA is degraded under starvation for nitrogen or carbon, but not iron, magnesium or phosphate. In addition, loss of DnaA is not stimulated by either heat shock or cold-shock. Therefore, the conditions that signal DnaA degradation are very specific. Blocking cell growth by cold-shock or by chloramphenicol, does not trigger DnaA loss.

DnaA degradation from nitrogen and carbon starvation appears to go through ClpP. It has recently been demonstrated that ClpP protease is important for adaptation to starvation conditions in *E. coli* (Weichart et al. 2003). c*lpP*⁻ mutants lost viability within prolonged starvation and several proteins accumulated in these mutants, in comparison to wild-type *E. coli,* in response to stationary phase starvation. Likewise, several proteins showed reduced stationary phase levels in *clpP*⁻ mutants, and therefore, there is reliance upon ClpP to most likely de-repress certain protein products (Weichart et al. 2003). Our data demonstrated that the starvation induced degradation of *C. crescentus* DnaA was through ClpP and therefore, ClpP likewise plays a role in starvation control by degrading DnaA and to thereby limit replication under these unfavorable conditions (Fig. 7).

The Dependence of DnaA upon CtrA and vice versa

The *dnaA* gene was not shown to be part of the CtrA regulon, and its transcription is not regulated by *ctrA Ts* under non-permissive temperature (Laub et al. 2000; Laub et al. 2002). Our analysis demonstrates that DnaA protein levels do not change when *ctrA Ts* was shifted to non-permissive temperature and indicates that DnaA protein level synthesis is not affected by CtrA or by the demand for extra chromosome replication (Fig. 8A). As well, it demonstrates that the over-replication stress is not a trigger for loss of DnaA through accelerated proteolysis as in the case of ammonium and carbon starvation and entry into stationary phase.

However, *ctrA* transcription is reduced when *dnaA* expression is blocked (Fig. 8B). This is consistent with previous observations that transcription from *ctrA* dependent promoters, *fliLM* and *ccrM,* is turned off in response to *dnaA* expression block

(Gorbatyuk and Marczynski 2001). Interestingly, a rise in CtrA protein levels was detected very late in *dnaA* blocked cultures (Fig. 8C). This increase in *ctrA* protein levels may help to explain why the transcription of a high-affinity *ctrA* promoter, *fliQ*, increased at late time-points following block in *dnaA* expression (Gorbatyuk and Marczynski 2001). However, at this late block in *dnaA* expression, cells lose viability and this altered physiology may not be a direct effect of *dnaA* block but rather an indirect artifact caused by loss of viability.

The GroEL chaperone and DnaA

Extragenic suppressor mutants of *dnaA46* in *E. coli* mapped to genes encoding GroEL and GroES (Fayet et al. 1986) suggesting an interaction between the heat-shock chaperones and DnaA. It was later found that GroEL helped to prevent self-aggregation of DnaA in the presence of ATP, and DnaA in the self-aggregated form cannot initiate replication (Banecki et al. 1998). Therefore, the GroEL chaperone was believed to have a positive role in helping DnaA to initiate replication.

Here, we identified the GroEL protein to be a major protein that was immuno-precipitated with anti-DnaA anti-serum. This was identified by cell-cycle synthesis pattern (Fig. 9B) that matches the pattern previously presented for GroEL in a variant strain (Baldini et al. 1998), and by conditional expression of GroEL from a strain that expresses it with xylose but not glucose (Fig. 9C) (da Silva et al. 2003). However, the GroEL protein does not appear to be dependent upon DnaA for immunoprecipitation as it is detected under conditions where DnaA is absent and *vice versa* (Gorbatyuk and Marczynski 2003).

Proteolysis of an artificial protein called CRAG was shown in *E. coli* to be dependent upon ClpP but to be independent of ClpA or ClpX, the major ClpP chaperone pairs in *E. coli*; instead proteolysis was shown to be dependent upon GroEL as the chaperone for ClpP (Kandror et al. 1999). We cannot help but draw parallels to the situation with DnaA degradation where it is independent of ClpA or ClpX, but dependent upon ClpP (Gorbatyuk and Marczynski 2003). This coincidental identification of GroEL in our DnaA-antibody dependent assays potentially points to testing whether GroEL is involved in DnaA-dependent degradation, but any suggestion that GroEL is involved in DnaA degradation at this point would be highly speculative and premature.

Materials and Methods

Immuno-blots

To ensure that the same relative amounts of total proteins were loaded, we normalized all growing samples to 1 ml of $A_{660} = 0.1$, pellet the cells, froze the samples and resuspended all samples in an equal volume of loading dye. Samples were lysed by boiling and loaded into 8% SDS-PAGE run out using the BioRAD Protean III system and then transferred onto PVDF membrane. Anti- *E. coli* DnaA antiserum (obtained as a gift from Kirsten Skarstad), anti- *C. crescentus* DnaA, anti-CtrA, anti-McpA (obtained as gifts from Lucy Shapiro's laboratory), and anti-ClpP antiserum (obtained as a gift from Urs Jenal's laboratory) were diluted to 1:10,000. Secondary anti-Rabbit IgG coupled to HRP was used as a secondary antibody at 1:10,000 and proteins were detected by ECL-Plus chemilluminescent reagents. Chemilluminescence was detected directly on Kodak film or by the Bio-Rad Versadoc CCD-coupled system and analyzed by Quantity One 4.4 software.

Protein labeling and Immunoprecipitations

We pulse labeled NA1000 and SG300 cells at an optical density (A_{660}) = 0.1-0.2 with 2-20 x 10^6 cpm of [^{35}S]-Methionine / Cysteine mix (NEG-772 Easytag Express Protein Labeling mix – New England Nuclear) for 5-10 minutes. All labeled cells were lysed in

Wash buffer with lysozyme (50 mM Tris pH = 8; 0.45 M NaCl; 0.5% Triton X-100 (T-6878 Sigma Life Sciences); 10 mg/ml lysozyme). The protein extract was pre-cleared with 10 μg/ml of Recombinant Protein A immobilized on 6% fast-flow agarose (P-3476 Sigma Chemical, St. Lousi, MO) and 5 μl was TCA precipitated (5% TCA) and scintillation counted in order to normalize all samples for [^{35}S]-Methionine counts. All extracts were adjusted to contain equal counts (1-2 x 10^6 cpm) of [^{35}S]-labeled proteins, and were incubated with 1 μl of anti-GroEL (a gift from Suely Gomes laboratory), anti-DnaA or anti-Flagellin (gifts from Lucy Shapiro's laboratory) antibodies at 4°C. Proteins were immunoprecipitated with 10 μl of Recombinant Protein A, washed three times in Wash Buffer, resuspended in protein loading buffer, boiled for 10 minutes and resolved by SDS-PAGE. 8% polyacrylamide gels resolved DnaA, and 10% polyacrylamide gels resolved CtrA and Flagellin proteins. Gels were dried and exposed to Phosphoimager screens and the screens were scanned by STORM (Molecular Dynamics, Amersham Biosciences). Protein levels were analysed using ImageQuant version 5.2 software (Molecular Dynamics, Amersham Biosciences).

For protein stability assays, *C. crescentus* cells were labeled as above and then chased for up to 150 minutes with 0.2% Tryptone, 0.02 % Cysteine and 1mM Methionine as described (Grunenfelder et al. 2001).

Growth Conditions

Where needed, Chloramphenicol was added to 200 μg/ml in *E. coli* and 25 μg/ml in *C. crescentus* for stability assays. IPTG was added to 1mM for induction controls. *E. coli* GM2504 cells were grown in LB broth with Ampicillin. *C. crescentus* was typically grown in M2G media with the appropriate antibiotics when needed. GM2471 was depleted for *dnaA* as previously described (Gorbatyuk and Marczynski 2001).

To starve NA1000 for carbon, overnight cultures were back-diluted and allowed to actively grow for 2 hours prior to being washed in M2 media without carbon and then released into M2 media without sugar. To starve NA1000 for nitrogen, M2 Salts were prepared without NH_4Cl and then added to standard $FeSO_4$, $MgSO_4$, $CaCl_2$ and Glucose to make M2G –N media. NA1000 was grown in M2G media overnight, back-diluted and allowed to grow actively for two hours prior to being washed in M2G – N media and released into M2G –N media at 30°C. Likewise, to starve for iron and magnesium, $FeSO_4$ and $MgSO_4$ were components that were excluded from standard M2G media. In the event of phosphate starvation, KH_2PO_4 and $NaPO_4$ were excluded from the M2 salts preparation.

Acknowledgements

We are grateful to Kirstin Skarstad (Institute for Cancer Research, Oslo, Norway), Suely Gomes (Universidade de São Paulo, Brazil), Urs Jenal (University of Basel, Switzerland) for and to Lucy Shapiro (Stanford University) for providing strains and reagents. We would also like to thank Patrick Bastedo for his technical assistance. This work was funded by the Canadian Institutes for Health Research (CIHR), Grant MT-13453.

References

1. Ali, Azam T., Iwata, A., Nishimura, A., Ueda, S., and Ishihama, A. 1999. Growth phase-dependent variation in protein composition of the *Escherichia coli* nucleoid. *J. Bacteriol.* **181**: 6361-6370.

2. Baldini, R. L., Avedissian, M., and Gomes, S. L. 1998. The CIRCE element and its putative repressor control cell cycle expression of the *Caulobacter crescentus groESL* operon. *J. Bacteriol.* **180**: 1632-1641.

3. Banecki, B., Kaguni, J. M., and Marszalek, J. 1998. Role of adenine nucleotides, molecular chaperones and chaperonins in stabilization of DnaA initiator protein of *Escherichia coli*. *Biochim. Biophys. Acta* **1442**: 39-48.

4. da Silva, A. C. A, Simao, R. C. G, Susin, M. F., Baldini, R. L., Avedissian, M., and Gomes, S. L. 2003. Downregulation of the heat shock response is independent of DnaK and ó^{32} levels in *Caulobacter crescentus*. *Mol. Microbiol.* **49**: 541-553.

5. Domian, I. J., Quon, K. C., and Shapiro, L. 1997. Cell type-specific phosphorylation and proteolysis of a transcriptional regulator controls the G1-to-S transition in a bacterial cell cycle. *Cell* **90**: 415-424.

6. Evinger, M. and Agabian, N. 1977. Envelope-associated nucleoid from *Caulobacter crescentus* stalked and swarmer cells. *J. Bacteriol.* **132**: 294-301.

7. Fayet, O., Louarn, J. M., and Georgopoulos, C. 1986. Suppression of the *Escherichia coli dnaA46* mutation by amplification of the *groES* and *groEL* genes. *Mol. Gen. Genet.* **202**: 435-445.

8. Gorbatyuk, B. and Marczynski, G. T. 2001. Physiological consequences of blocked *Caulobacter crescentus dnaA* expression, an essential DNA replication gene. *Mol. Microbiol.* **40**: 485-497.

9. Gorbatyuk, B. and Marczynski, G. T. 2003. Programmed stability and degradation of chromosome replication proteins DnaA and CtrA. *Chapter 3 of this thesis.*

10. Grunenfelder, B., Rummel, G., Vohradsky, J., Roder, D., Langen, H., and Jenal, U. 2001. Proteomic analysis of the bacterial cell cycle. *Proc. Natl. Acad. Sci. USA* **98**: 4681-4686.

11. Hengge-Aronis, R. 2002. Signal transduction and regulatory mechanisms involved in control of the sigma-S (RpoS) subunit of RNA polymerase. *Microbiology and Molecular Biology Reviews* **66**: 373-395.

12. Jenal, U. and Fuchs, T. 1998. An essential protease involved in bacterial cell-cycle control. *EMBO J.* **17**: 5658-5669.

13. Jenal, U. and Stephens, C. 2002. The *Caulobacter* cell cycle: timing, spatial organization and checkpoints. *Curr. Opin. Microbiol.* **5**: 558-563.

14. Kandror, O., Sherman, M., and Goldberg, A. 1999. Rapid degradation of an abnormal protein in *Escherichia coli* proceeds through repeated cycles of association with GroEL. *Journal of Biological Chemistry* **274**: 37743-37749.

15. Laub, M. T., Chen, S. L., Shapiro, L., and McAdams, H. H. 2002. Genes directly controlled by CtrA, a master regulator of the *Caulobacter* cell cycle. *Proc. Natl. Acad. Sci. U. S. A* **99**: 4632-4637.

16. Laub, M. T., McAdams, H. H., Feldblyum, T., Fraser, C. M., and Shapiro, L. 2000. Global analysis of the genetic network controlling a bacterial cell cycle. *Science* **290**: 2144-2148.

17. Marczynski, G. T. and Shapiro, L. 2002. Control of chromosome replication in *Caulobacter crescentus*. *Annu. Rev. Microbiol.* **56**: 625-656.

18. Messer, W. 2002. The bacterial replication initiator DnaA. DnaA and *oriC*, the bacterial mode to initiate DNA replication. *FEMS Microbiol. Rev.* **26**: 355-374.

19. Poindexter, J. S. 1981. The *Caulobacters*: ubiquitous unusual bacteria. *Microbiol. Rev.* **29**: 123-179.

20. Quon, K. C., Marczynski, G. T., and Shapiro, L. 1996. Cell cycle control by an essential bacterial two-component signal transduction protein. *Cell* **84**: 83-93.

21. Quon, K. C., Yang, B., Domian, I. J., Shapiro, L., and Marczynski, G. T. 1998. Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin. *Proc. Natl. Acad. Sci. USA* **95**: 120-125.

22. Ryan, K. R. and Shapiro, L. 2003. Temporal and spatial regulation in prokaryotic cell cycle progression and development. *Annu. Rev. Biochem.* **72**: 367-394.

23. Siam, R. and Marczynski, G. T. 2000. Cell cycle regulator phosphorylation stimulates two distinct modes of binding at a chromosome replication origin. *EMBO J.* **19**: 1138-1147.

24. Simon, R, Prieffer, U, and Puhler, A. 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Biotechnology* **1**: 784-790.

25. Slominska, M., Wahl, A., Wegrzyn, G., and Skarstad, K. 2003. Degradation of mutant initiator protein DnaA204 by proteases ClpP, ClpQ and Lon is prevented when DNA is SeqA-free. *Biochem. J.* **370**: 867-871.

26. Weichart, D., Querfurth, N., Dreger, M., and Hengge-Aronis, R. 2003. Global role for ClpP-containing proteases in stationary-phase adaptation of *Escherichia coli*. *J. Bacteriol.* **185**: 115-125.

27. Wortinger, M., Sackett, M. J., and Brun, Y. V. 2000. CtrA mediates a DNA replication checkpoint that prevents cell division in *Caulobacter crescentus*. *EMBO J.* **19**: 4503-4512.

28. Wortinger, M. A., Quardokus, E. M., and Brun, Y. V. 1998. Morphological adaptation and inhibition of cell division during stationary phase in *Caulobacter crescentus*. *Mol. Microbiol.* **29**: 963-973.

Table 1. Bacterial Strains and Plasmids

Strain /plasmid	Genotype or description	Sources
E. coli		
S17-1	*E. coli* 294::RP4-2(Tc::Mu)(Km::Tn7)	(Simon et al. 1983)
GM2504	BL21 with pet21b::*dnaA*	S.L. Chen and Lucy Shapiro
C. crescentus		
GM2471	NA1000 *dnaA*::Ω *xylX*::*dnaA*	(Gorbatyuk and Marczynski 2001)
GM2744	GM2471 with *ctrA*::*lacZ* reporter	This work
SG300	NA1000 *xylX*::*groESL*	(da Silva et al. 2003)
NA1000	Synchronizable mutant of WT CB15	(Evinger and Agabian 1977)
UJ199	NA1000 *clpP*::Ω *xylX*::pUJ174	(Jenal and Fuchs 1998)
LS2195	*ctrA401TS* isogenic to NA1000	(Quon et al. 1996)

Figure Legends:

Figure 1: *E. coli* DnaA is stable and *C. crescentus* DnaA is unstable in *E. coli.* GM2504 was treated with 200 μg/ml Chloramphenicol and protein stability was measured as described.

A. *E. coli* DnaA immunoblots after chloramphenicol treatment are shown and

B. *C. crescentus* DnaA immunoblots after chloramphenicol treatment are shown for the same samples as A.

Figure 2: *C. crescentus* DnaA is absent from stationary phase cultures.

A. A growth curve of wild-type CB15N cells (NA1000) shows that cells reach stationary phase eight hours following seeding at 30°C. After 24 hours of seeding, NA1000 was back-diluted to A660 = 0.15 and allowed to grow at 30°C. Cells start to increase their optical density between 1 and 2 hours of back-dilution.

B. A parallel DnaA immuno-blot was performed on the samples for all time points sampled in A. DnaA total protein levels decrease as soon as cells reach stationary phase and are completely absent while cells are maintained in stationary phase. Following exit from stationary phase, DnaA protein levels return to steady-state levels.

Figure 3: DnaA is absent from nitrogen-starved cultures.

A. *C. crescentus* NA1000 actively growing cells were washed and re-suspended in nitrogen-starved media and their growth was monitored by optical density (A_{660}). After 120 minutes of nitrogen-starvation, the cells were washed and resuspended in nitrogen containing media.

B. Anti-DnaA immuno-blots are shown for NA1000 cells starved for nitrogen. DnaA is completely absent from NA1000 nitrogen-starved cells within 60 minutes.

C. After 120 minutes of nitrogen-starvation, NA1000 was resuspended in normal M2G media (+N) and DnaA immuno-blots are shown. The re-addition of nitrogen triggers DnaA re-synthesis and DnaA reaches steady state-levels within 30 minutes of nitrogen re-addition.

Figure 4: DnaA is absent from carbon-starved cultures.

A. *C. crescentus* NA1000 actively growing cells were washed and re-suspended in M2 (no G) carbon-starved media and growth (A_{660}) was monitored. After 120 minutes, glucose was added back to the media and growth resumed.

B. Anti-DnaA immuno-blots were performed to measure DnaA protein levels under conditions where NA1000 was carbon starved and when carbon was added back to a 120

minute carbon-starved culture. Whereas DnaA is absent from carbon-starved cells, it reaches steady state levels within 30 minutes of carbon re-addition.

Figure 5: DnaA is not lost from iron, magnesium or phosphate starved *C. crescentus* cultures.

A. NA1000 was starved for phosphate, B. iron and C. magnesium for the times indicated as described in material and methods. Anti-DnaA immunoblots were performed under these conditions as described. DnaA is retained under these starvation conditions.

Figure 6: DnaA is not lost under Heat-shock or Cold-Shock.

A. NA1000 actively growing cells at 30°C were subjected to cold-shock and were grown at 15°C. After 90 minutes of growth under cold-shock, NA1000 was returned to grow at 30°C and throughout this shock anti-DnaA immuno-blots measured DnaA protein levels. DnaA levels are unchanged throughout the cold-shock.

B. NA1000 actively growing cells at 30°C were subjected to heat-shock and were grown at 42°C. After 120 minute of growth under these conditions, NA1000 was returned to 30°C. Immuno-blots measured total DnaA protein levels and show that DnaA protein is not lost under heat-shock.

Figure 7: DnaA appears to be degraded through ClpP during nitrogen and carbon starvation.

A. Anti-ClpP immuno-blots for UJ199 depleted for 16 hours in M2G in comparison to undepleted UJ199.

B. Stability of CtrA in a ClpP depleted culture is shown. Anti-CtrA immuno-blots for UJ199 depleted for 16 hours in M2G and treated with 25 μg/ml chloramphenicol is shown.

C. Stability of CtrA in NA1000 is shown through CtrA immuno-blots in NA1000 treated with 25 μg/ml chloramphenicol.

D. Anti-DnaA immuno-blots are shown for UJ199 (Gluc 16 h) and NA1000 cells that were starved for nitrogen for the times indicated. NA1000 cells starved for nitrogen for 120 minutes were returned to nitrogen-containing media (+N) to identify that loss of DnaA is specific for nitrogen.

E. Anti-DnaA immuno-blots are shown for UJ199 (Gluc 16 h) and NA1000 cells that were starved for carbon for the times indicated. NA1000 cells starved for carbon for 120 minutes were returned to carbon-containing media (+C) to identify that loss of DnaA is specific to carbon.

Figure 8: The effects of CtrA on DnaA and the converse.

A. Anti-DnaA immuno-blots for CtrA Ts when grown for the length of time indicated at non-permissive temperature (42°C) are shown. DnaA protein is not lost due to the global stressed induced by CtrA loss.

B. β- galactosidase assays were performed to measure *ctrA* promoter transcription in the presence or absence of DnaA. The left panel shows Miller Units plotted over time. The right panel shows total β-galactosidase activity plotted over time.

C. Anti-McpA and anti-CtrA immuno-blots were performed on GM2471 synchronized swarmer cells in the absence (right panel) of presence (left panel) of *dnaA*.

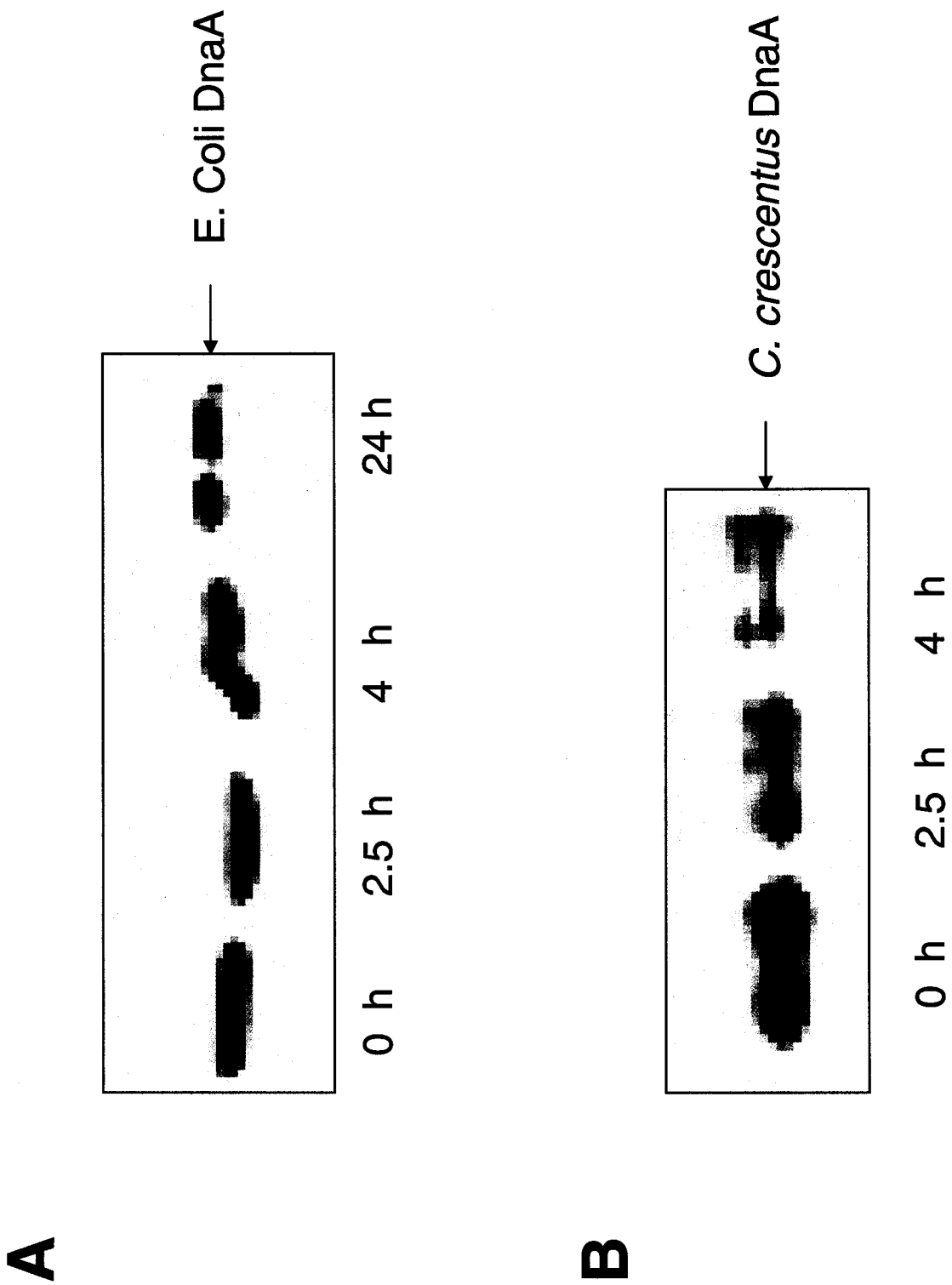
Figure 9: The upper band from anti-DnaA IPs is GroEL.

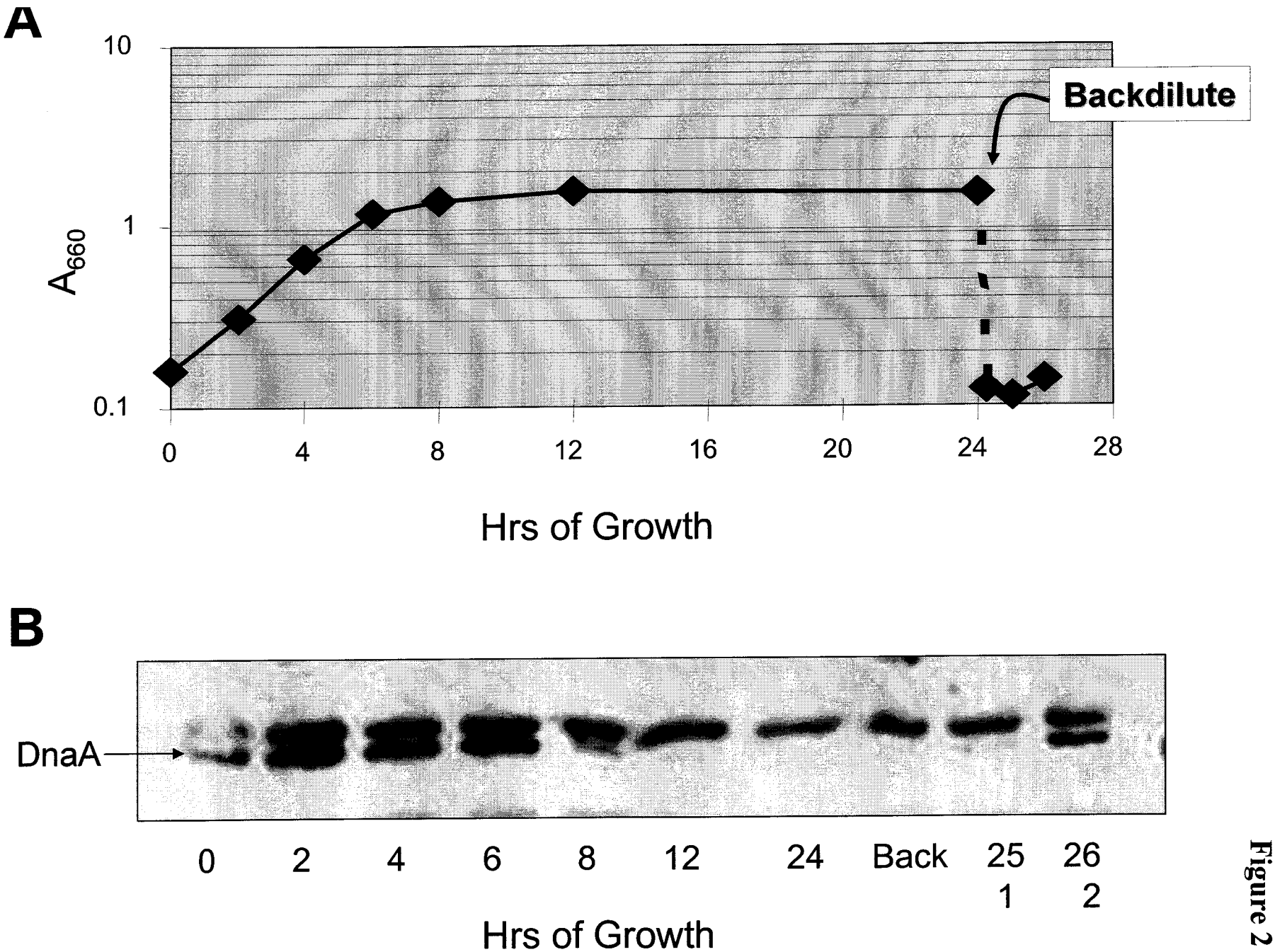
A. Anti-DnaA and anti-GroEL IPs on NA1000 cells that were [^{35}S]-methionine pulse labeled and chased for the times indicated are shown. The upper bands from both anti-DnaA and anti-GroEL IPs are the same molecular weight whereas the lower bands are not.

B. Cell-cycle synthesis pattern of anti-GroEL and anti-DnaA IPs are shown. Anti-flagelling IPs mark the cell-cycle. The time after swarmer cells were resuspended and allowed to grow is indicated. The Upper bands have the same cell-cycle synthesis pattern.

C. SG300 and NA1000 were grown in M2X and then shifted to M2G. Each sample was [^{35}S]-methionine pulse-labelled and immunoprecipitated with anti-DnaA and GroEL antibodies. GroEL synthesis by SG300 is turned off in M2G and the upper band synthesis from anti-DnaA IPs is turned off in SG300 in M2G as well. Therefore, GroEL is the upper band detected in anti-DnaA IPs.

Figure 1




A
10
1
0.1
A_{660}
Backdilute
0
4
8
12
16
20
24
28
Hrs of Growth
B
DnaA
0
2
4
6
8
12
24
Back
25
1
26
2
Hrs of Growth

269

Figure 2

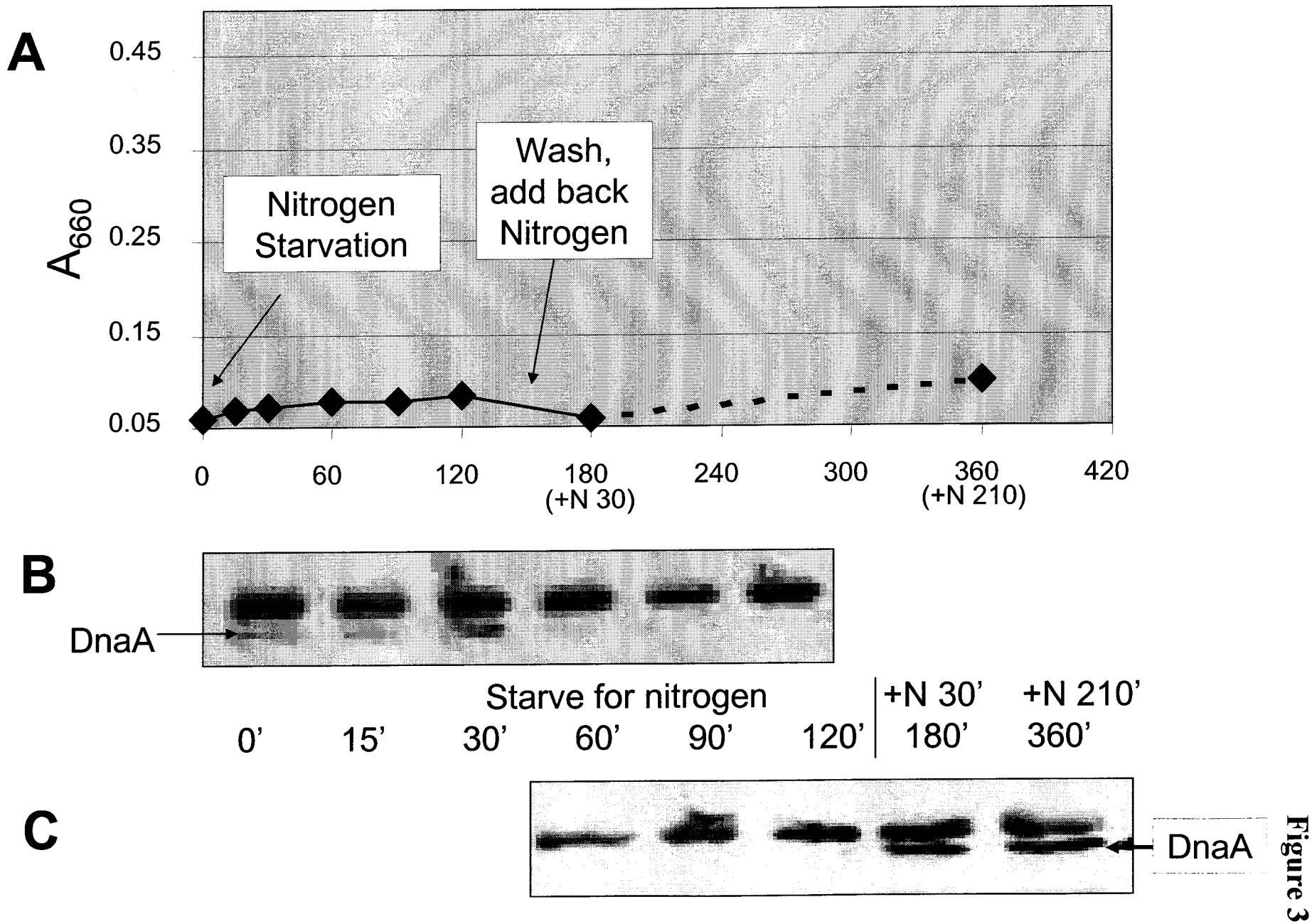
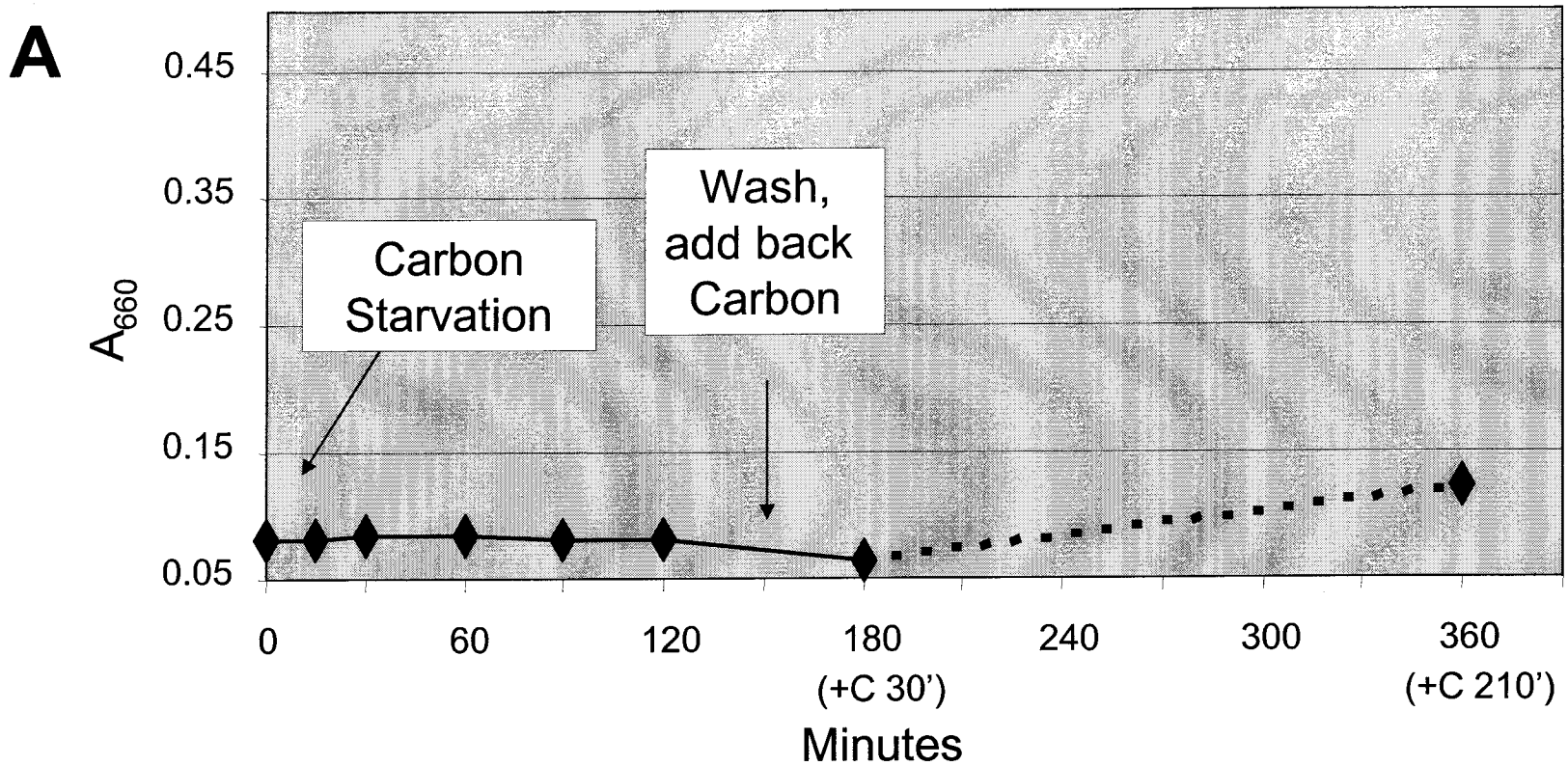

A
0.45
0.35
0.25
0.15
0.05
A_{660}
Nitrogen Starvation
Wash, add back Nitrogen
0
60
120
180 (+N 30)
240
300
360 (+N 210)
420
B
DnaA
Starve for nitrogen
+N 30'
+N 210'
0'
15'
30'
60'
90'
120'
180'
360'
C
DnaA

Figure 3


A
0.45
0.35
0.25
0.15
0.05
A_{660}
Carbon Starvation
Wash, add back Carbon
0
60
120
180
(+C 30')
240
300
360
(+C 210')
Minutes

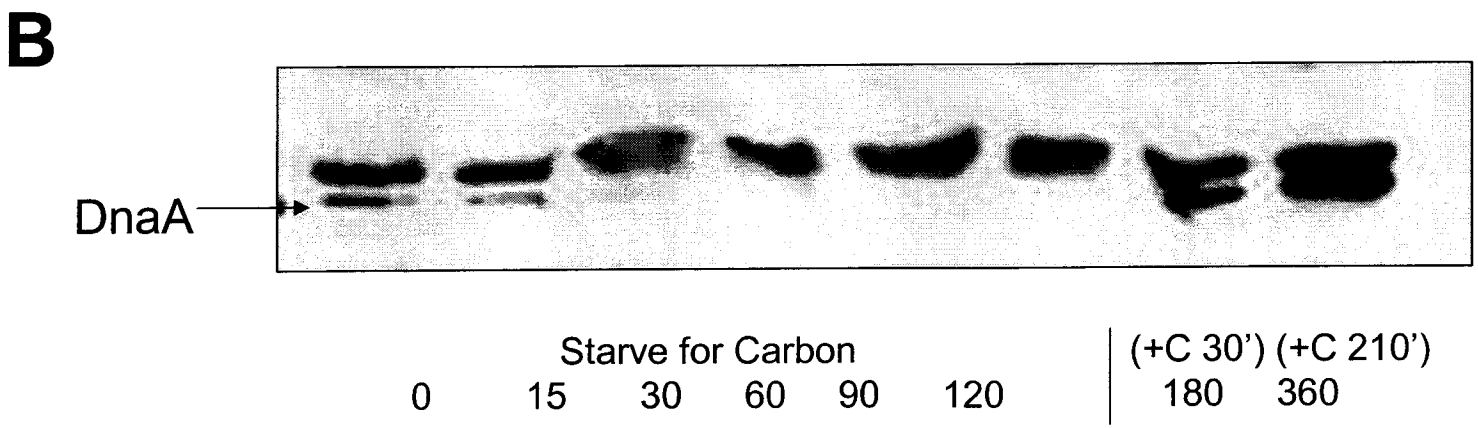
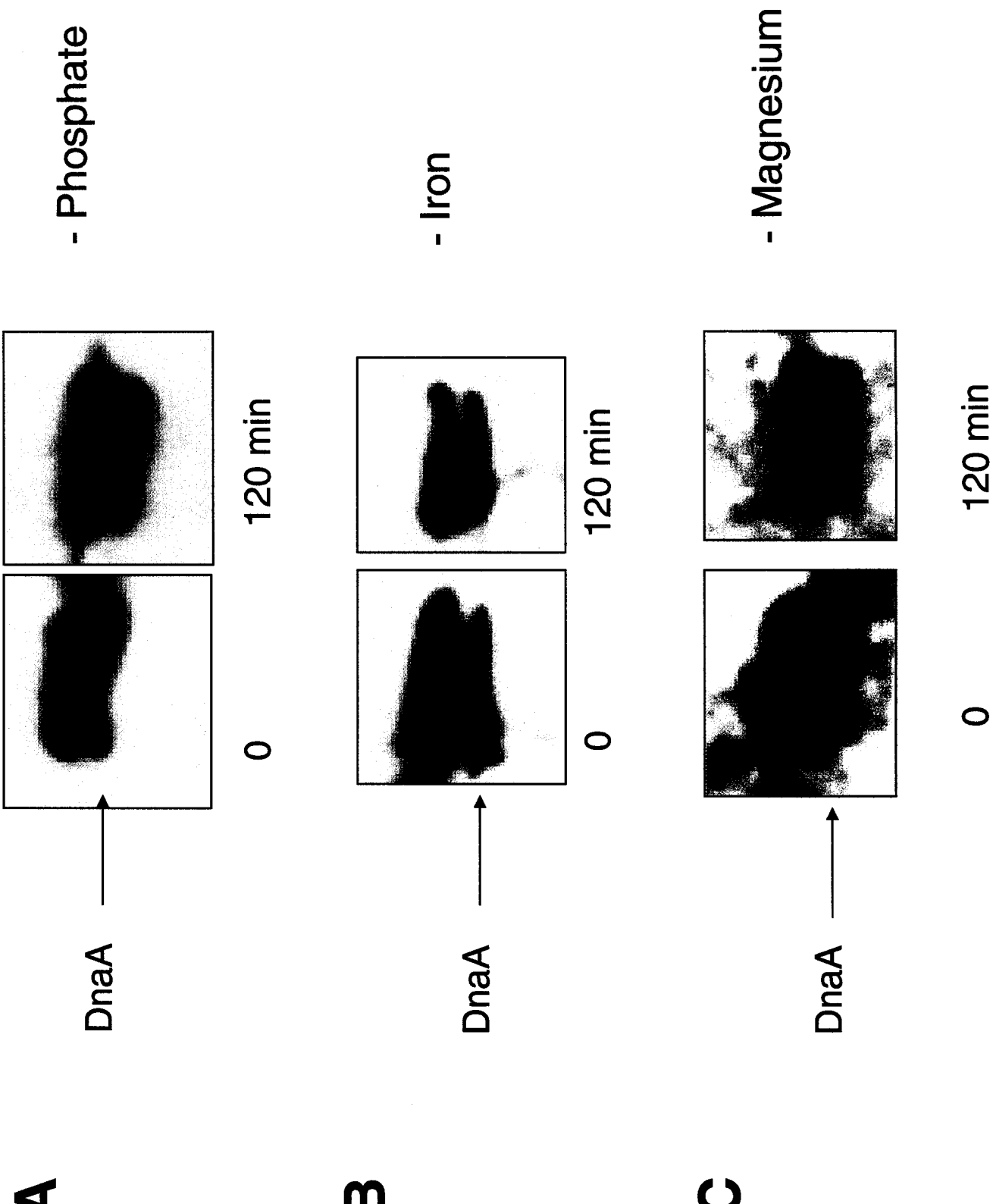

B
DnaA
Starve for Carbon
0
15
30
60
90
120
(+C 30')
180
(+C 210')
360

Figure 4

Figure 5


A
- Phosphate
DnaA
0
120 min
B
- Iron
DnaA
0
120 min
C
- Magnesium
DnaA
0
120 min

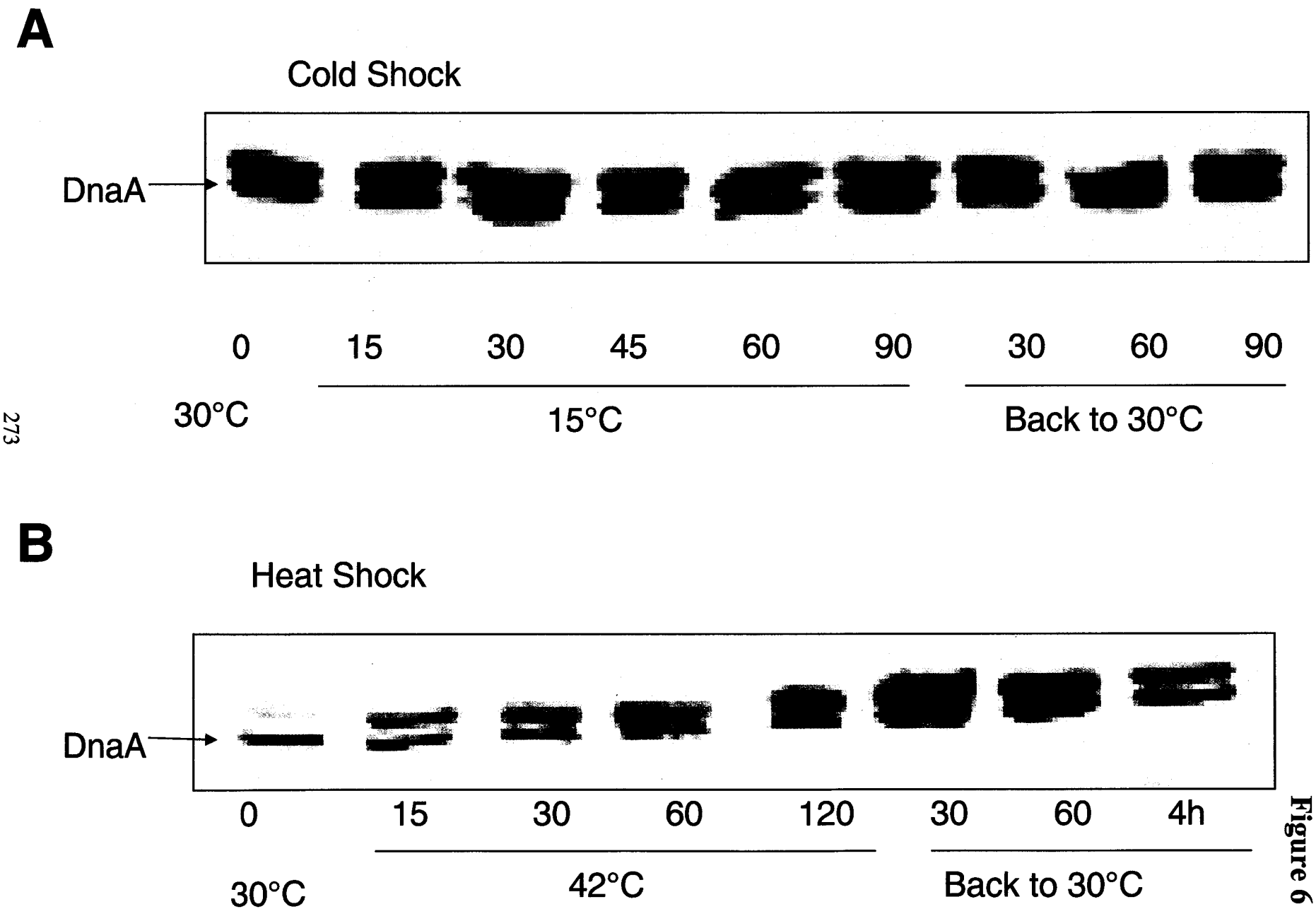

A
Cold Shock
DnaA
0
15
30
45
60
90
30
60
90
30°C
15°C
Back to 30°C
B
Heat Shock
DnaA
0
15
30
60
120
30
60
4h
30°C
42°C
Back to 30°C

Figure 6

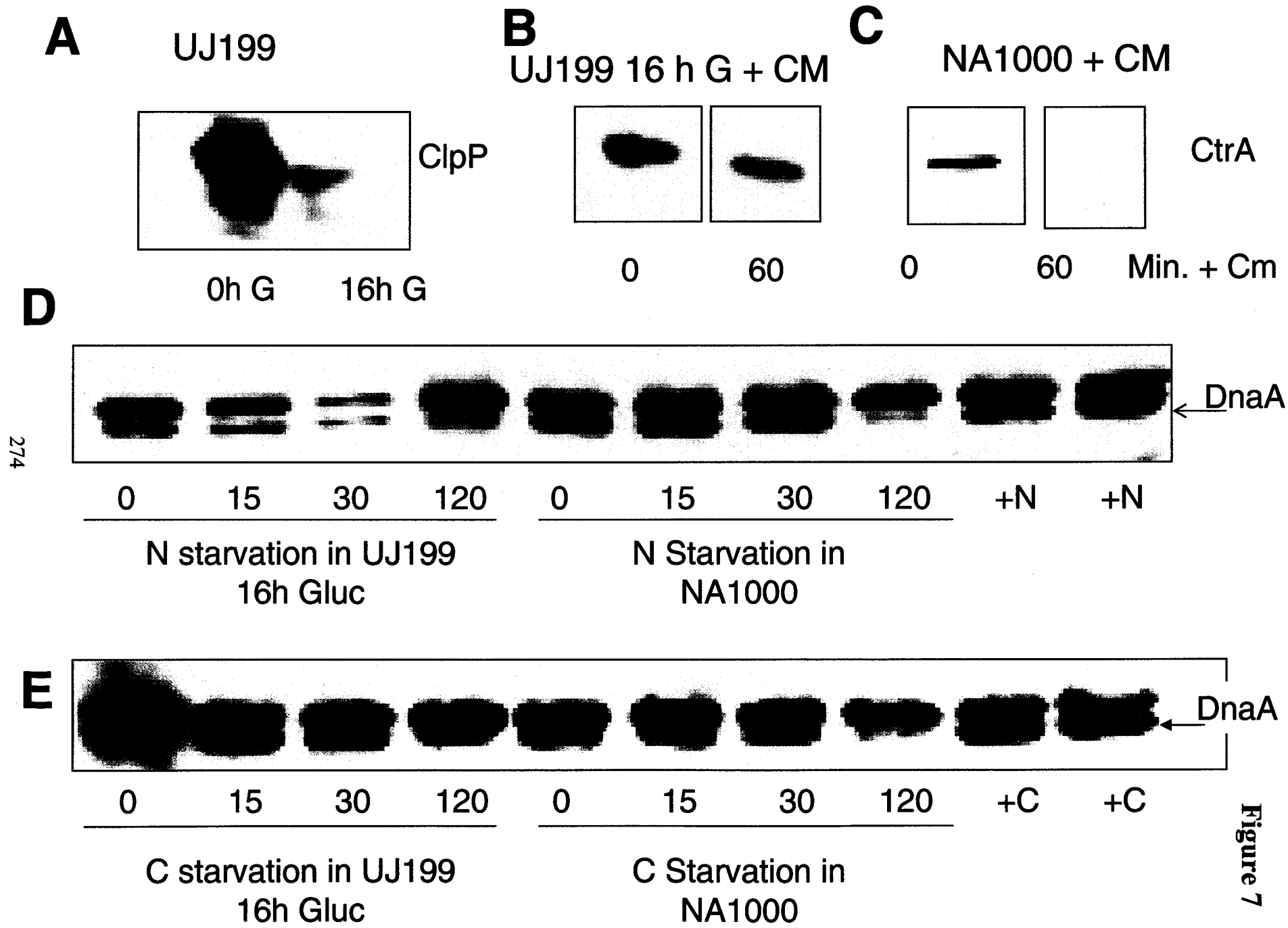
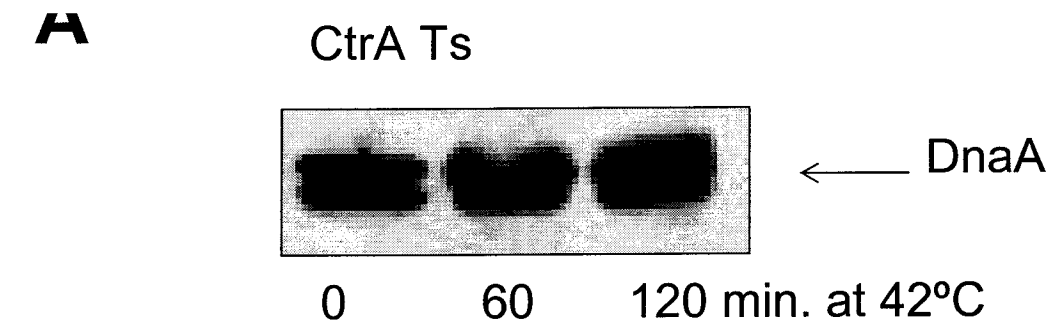

A
UJ199
ClpP
0h G
16h G
B
UJ199 16 h G + CM
0
60
C
NA1000 + CM
CtrA
0
60
Min. + Cm
D
DnaA
0
15
30
120
0
15
30
120
+N
+N
N starvation in UJ199
16h Gluc
N Starvation in
NA1000
E
DnaA
0
15
30
120
0
15
30
120
+C
+C
C starvation in UJ199
16h Gluc
C Starvation in
NA1000

Figure 7


A
CtrA Ts
DnaA
0
60
120 min. at 42ºC

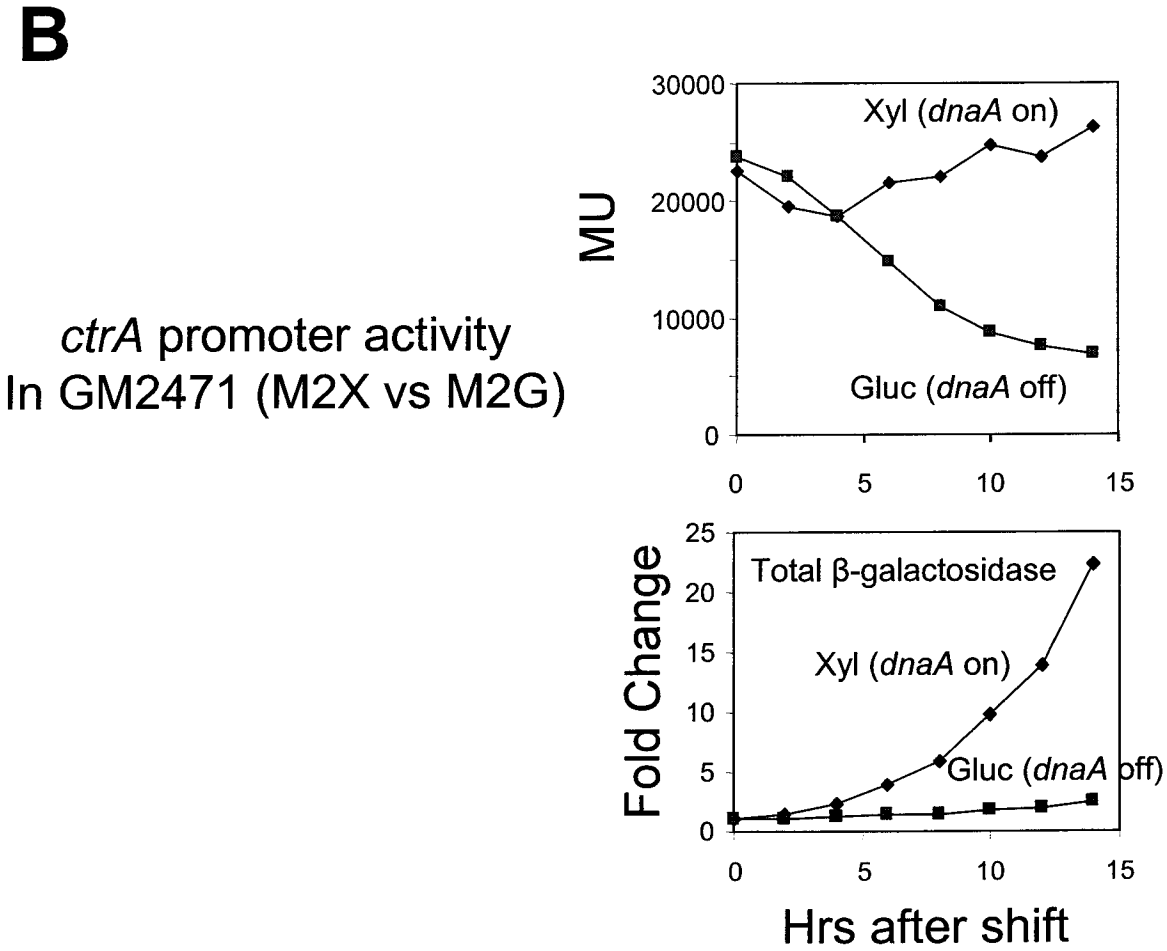
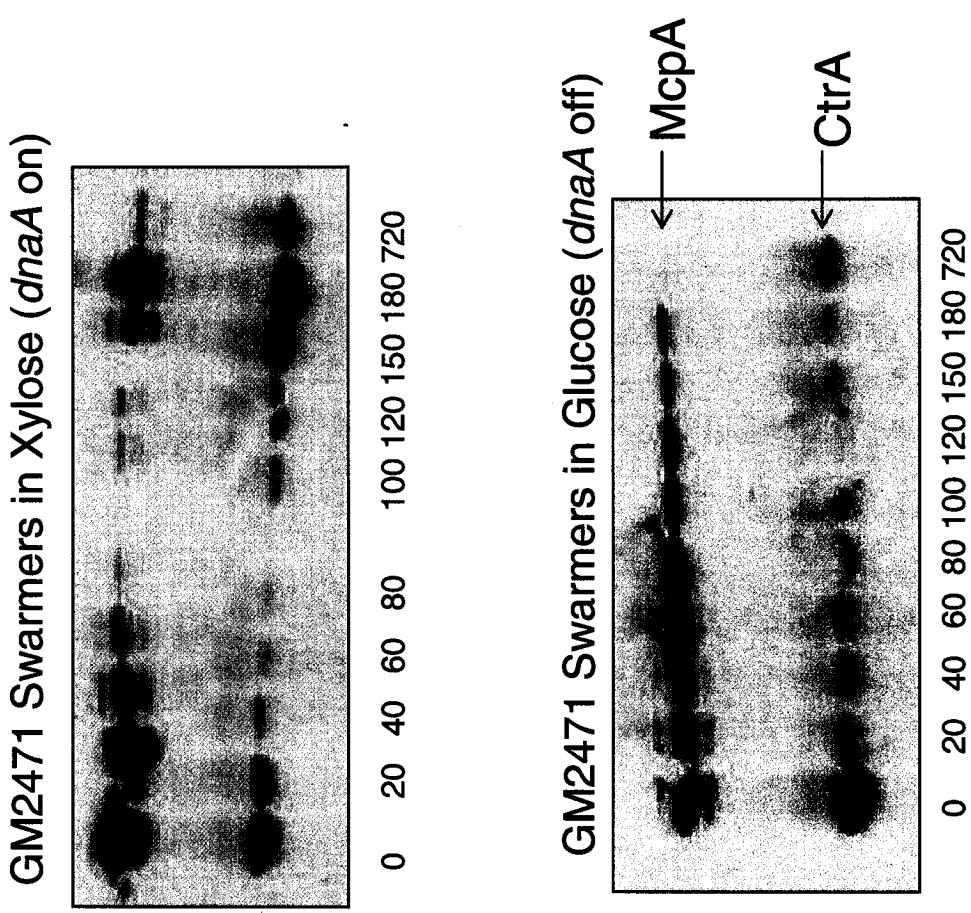

B
ctrA promoter activity
In GM2471 (M2X vs M2G)
MU
30000
20000
10000
0
Xyl (dnaA on)
Gluc (dnaA off)
0
5
10
15
Fold Change
25
20
15
10
5
0
Total β-galactosidase
Xyl (dnaA on)
Gluc (dnaA off)
0
5
10
15
Hrs after shift

Figure8

Figure8


C
GM2471 Swarmers in Xylose (dnaA on)
0 20 40 60 80 100 120 150 180 720
GM2471 Swarmers in Glucose (dnaA off)
McpA
CtrA
0 20 40 60 80 100 120 150 180 720

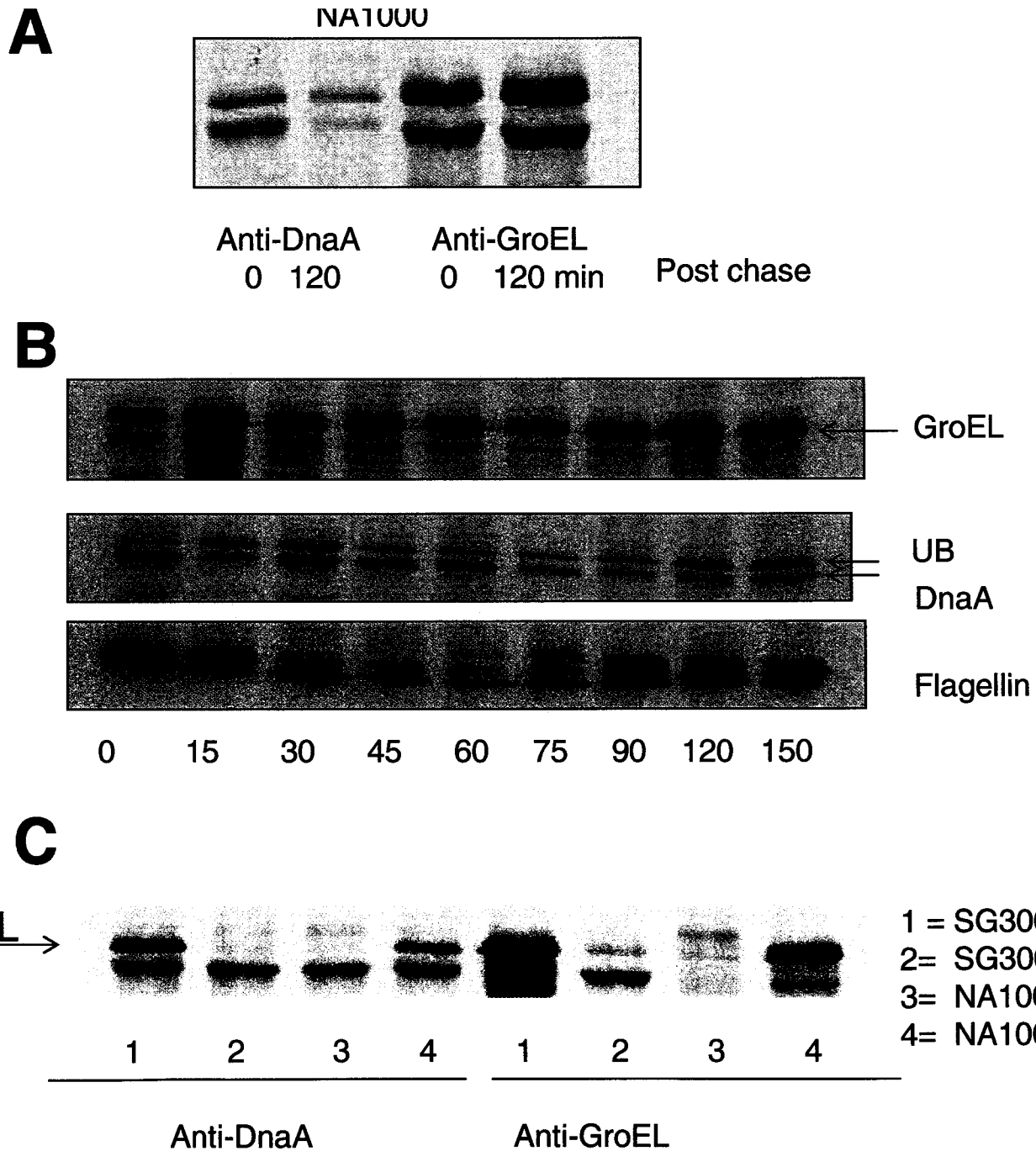

A
NA1000
Anti-DnaA
0 120
Anti-GroEL
0 120 min
Post chase
B
GroEL
UB
DnaA
Flagellin
0 15 30 45 60 75 90 120 150
C
GroEL
1 = SG300 PxylX::GroEL M2X > X
2= SG300 PxylX::GroEL M2X > G
3= NA1000 from SG M2G > X
4= NA1000 from SG M2G > G
1 2 3 4 1 2 3 4
Anti-DnaA
Anti-GroEL

Figure 9

CHAPTER 5: CONCLUSIONS AND FUTURE DIRECTIONS

This thesis conclusively demonstrate that DnaA is an absolutely essential replication initiator in *C. crescentus* (Chapter 2) (Gorbatyuk and Marczynski 2001). Although DnaA is believed to be a replication initiator in all bacteria, before our results, this was only conclusively demonstrated in *E. coli* and *B. subtilis*. The conclusive identification of DnaA as the replication initiator also excludes an alternative hypothesis for replication control in *C. crescentus* based upon CtrA protein directed control of Ps promoter transcription in *Cori* (Marczynski et al. 1995; Quon et al. 1998). This hypothesis predicts that in stalked cells, the absence of CtrA will lead to a de-repression of a promoter within *Cori* that could then prime replication (Marczynski et al. 1995). Our results from transcriptional reporter analysis (Chapter 2) do not rule out the possibility that Ps transcription promotes replication, but Ps transcription on its own cannot control replication, and replication initiation absolutely requires DnaA. This implies that DnaA is a major target of regulatory mechanisms.

Further studies should address how DnaA and CtrA co-ordinate their activities as an initiator and repressor, respectively, of *Cori* replication throughout the cell-cycle. Since replication initiation is coupled to swarmer to stalked cell differentiation, this co-ordination in *Cori* may prove to be a key activity in the decision to remain as swarmer cells or to replicate the chromosome and differentiate into stalked cells. It will also become important to characterize the DnaA binding sites in *Cori* and to see how DnaA binding in the origin influences CtrA binding.

In addition to characterizing the role of DnaA as the replication initiator in *C. crescentus*, our data characterized several other interesting physiological consequences

from DnaA loss. For example, non-midpoint pinching or constrictions in filamentous cells resulted from DnaA loss. This observation was followed up by a separate study that used fluorescent microscopy to localize the FtsZ ring that forms during cell-division in our genetic *dnaA* depletion strain (Quardokus and Brun 2002). Their results identified that FtsZ rings did not localize properly to the midpoint in response to *dnaA* block (Quardokus and Brun 2002). This suggests that DnaA acts in a checkpoint control for proper midpoint localization of cell-division proteins. It would be very interesting to identify the mechanism by which DnaA mediates this checkpoint control.

Another physiological consequence of DnaA depletion is the loss of viability in *C. crescentus* (Chapter 2) (Gorbatyuk and Marczynski 2001). This result was surprising since DnaA loss should not cause strand breakage that occurs for example during replication elongation block, since DnaA is an initiation protein. The mechanisms that contribute to this viability loss could be an interesting area of focus in the future.

In this thesis, we document that *C. crescentus* DnaA is an unstable protein (Chapter 3). This is the first demonstration of an unstable DnaA homologue. *C. crescentus* DnaA has a cell-cycle degradation pattern that is unique from any other cell-cycle degraded protein in *C. crescentus*. This suggests a novel mechanism for targeting DnaA proteolysis. Indeed, we excluded the two conventional chaperones, ClpA and ClpX. We argued in Chapter 3 that this degradation ensures that mostly newly synthesized DnaA (*de novo* synthesized DnaA) would contribute to replication initiation in newly divided *C. crescentus* cells. It is very important in the future to create a stable version (a non-proteolysed) of the *C. crescentus* DnaA protein and to assess its function within the *C. crescentus* cell-cycle. Phylogenetic studies will help to identify the

importance of the degradation property of the *C. crescentus* DnaA protein. For example, our data in chapter 4 confirms that *E. coli* DnaA is a stable protein. It would be interesting in the future to test the ability of the *E. coli* DnaA protein to complement *C. crescentus* DnaA depleted cells and *vice versa*. In addition, phylogenetic studies extended to other alpha-purple bacteria will help to address whether this instability and therefore this regulatory mechanism for DnaA is conserved amongst this diverse family of bacteria.

The degradation of DnaA in *C. crescentus* appears to be an adaptation to specific poor growth conditions that are detrimental for growth. In Chapter 3 and Chapter 4 of this thesis, we identified nutritional cues that contribute to DnaA degradation. Therefore, DnaA degradation in *C. crescentus* is a regulatory adaptation to specifically limiting nutritional conditions in addition to cell-cycle cues. Nitrogen starvation and carbon starvation are nutritional cues that contribute to DnaA loss. It would be interesting in the future to identify molecular messengers that are induced under these starvation conditions that trigger DnaA loss. It has been demonstrated that ppGpp is induced during nitrogen starvation in *C. crescentus* (Chiaverotti et al. 1981), its possible role in triggering DnaA degradation should be explored.

In chapter 4 of this thesis, I show that DnaA is also degraded upon entry into stationary phase, but returns during active growth and is present throughout the cell-cycle. This is a contrast to *E. coli* DnaA that accumulates in stationary phase (Ali et al. 1999). In the future, experiments aimed at identifying whether the means of degrading DnaA in stationary phase is cell-density specific or if it is specific for nutrient and

oxygen limitation in this stage of growth. In addition, it will be interesting to observe whether DnaA degradation in stationary phase is directed by ClpP or not.

In this thesis we also show that DnaA is degraded by the ClpP protease. Since ClpP is primarily believed to degrade proteins with the help of a chaperone, it is important to identify the chaperone or specificity factor that recognizes DnaA and targets it for ClpP degradation. Our data in Chapter 3 ruled out the typical ClpP chaperones in DnaA degradation. Whether ClpP can degrade proteins non-processively with the help of other chaperones has been suggested through work in *E. coli* (Kandror et al. 1999), but it would be highly speculative to suggest that ClpP pairs with other (non-Clp) chaperones to degrade DnaA. The identity of the chaperone in DnaA degradation should be an important area of focus in the future.

The SsrA tagging mechanism for degradation is important for proper cell-cycle progression in *C. crescentus* (Keiler and Shapiro 2003) and has been suggested to target replication specific proteins that are involved in swarmer to stalk transition. In addition, SsrA targeting for degradation could be a mechanism for targeting oligomerizing proteins (like Mu repressor) through "trans-targetting" especially under starvation conditions (O'Handley and Nakai 2002). It would be interesting in the future to address whether DnaA could be a candidate for SsrA tagged degradation and, if so, to test how SsrA tagging would occur in *C. crescentus* DnaA.

In the *C. crescentus* sequenced genome, an *hda* homologue was identified (W. Spencer and G.T. Marczyski, unpublished data) and the *C. crescentus* DnaA protein sequence reveals that it contains conserved Walker A and Walker B motifs that are involved in ATP binding and hydrolysis in *E. coli*. This circumstantial evidence suggests

that ATP hydrolysis through RIDA may be a mechanism for regulating DnaA activity in *C. crescentus* like it is in *E. coli* (Boye et al. 2000). It would be interesting to characterize the ATP and ADP bound states of DnaA throughout the cell-cycle and to see whether RIDA is a mechanism of DnaA regulation in *C. crescentus*. Moreover, future experiments aimed at monitoring how RIDA, in conjuction with DnaA proteolysis, serves to regulate DnaA activity throughout the cell-cycle will be extremely important in understanding the regulation of replication initiation in *C. crescentus*.

Although DnaA appears to be ubiquitous amongst species, there is merit in studying its role and regulation in diverse bacterial species that occupy assorted environmental niches and must therefore employ unique strategies to allow growth at appropriate times and to limit growth under unfavorable conditions. In *C. crescentus* there is a variation on the theme, as DnaA is targeted for proteolysis throughout its cell-cycle presumably as a means to allow replication to occur within the precise cell-cycle time, and DnaA is also degraded under specific nutritional stresses. Further phylogenetic analysis should provide even more variations onto what allows replication initiation to be such a tightly regulated process not just in bacteria, but in all species.

References

1. Ali, Azam T., Iwata, A., Nishimura, A., Ueda, S., and Ishihama, A. 1999. Growth phase-dependent variation in protein composition of the Escherichia coli nucleoid. *J. Bacteriol.* **181**: 6361-6370.

2. Boye, E., Lobner-Olesen, A., and Skarstad, K. 2000. Limiting DNA replication to once and only once. *EMBO Rep.* **1**: 479-483.

3. Chiaverotti, T. A., Parker, G., Gallant, J., and Agabian, N. 1981. Conditions that trigger guanosine tetraphosphate accumulation in *Caulobacter crescentus*. *J. Bacteriol.* **145**: 1463-1465.

4. Gorbatyuk, B. and Marczynski, G. T. 2001. Physiological consequences of blocked *Caulobacter crescentus dnaA* expression, an essential DNA replication gene. *Mol. Microbiol.* **40**: 485-497.

5. Kandror, O., Sherman, M., and Goldberg, A. 1999. Rapid degradation of an abnormal protein in Escherichia coli proceeds through repeated cycles of association with GroEL. *Journal of Biological Chemistry* **274**: 37743-37749.

6. Keiler, K. C. and Shapiro, L. 2003. TmRNA is required for correct timing of DNA replication in Caulobacter crescentus. *J. Bacteriol.* **185**: 573-580.

7. Marczynski, G. T., Lentine, K., and Shapiro, L. 1995. A developmentally regulated chromosomal origin of replication uses essential transcription elements. *Genes Dev.* **9**: 1543-1557.

8. O'Handley, D. and Nakai, H. 2002. Derepression of bacteriophage mu transposition functions by truncated forms of the immunity repressor. *J. Mol. Biol.* **322**: 311-324.

9. Quardokus, E. M. and Brun, Y. V. 2002. DNA replication initiation is required for mid-cell positioning of FtsZ rings in Caulobacter crescentus. *Mol. Microbiol.* **45**: 605-616.

10. Quon, K. C., Yang, B., Domian, I. J., Shapiro, L., and Marczynski, G. T. 1998. Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin. *Proc. Natl. Acad. Sci. USA* **95**: 120-125.

APPENDIX 1: RESEARCH COMPLIANCE CERTIFICATES

ii) the protocol for decontaminating spills

The spilled cultures are absorbed in paper towels, placed in biohazard bags, then autoclaved.

The contaminated area is disinfected, e.g. washed with Javex, then soap & hot water.

The washing materials are segregated, placed in biohazard bags, then autoclaved.

Does the protocol present conditions (e.g. handling of large volumes or high concentrations of pathogens) which could increase the hazards of the infectious agent(s)?

No pathogens are grown. These bacterial cultures are all contained in standard sterile laboratory flasks.

Do the specific procedures to be employed involving genetically engineered organisms have a history of safe use?

Yes, these bacteria have a greater than 30 year history of safe laboratory use.
They were among the first organisms to be genetically engineered.

What precautions are being taken to reduce production of infectious droplets and aerosols?

Flamed inoculation loops are quenched in sterile agar prior to contact with these bacteria.
All flasks and test tubes are sealed or covered, except at the times of sterile manipulation.

List the biological safety cabinets to be used.

required

Building	Room No.	Manufacturer	Model No.	Serial No.	Date Certified

APPENDIX 2: REPRINT OF CHAPTER 2 PUBLISHED IN THE JOURNAL "MOLECULAR MICROBIOLOGY"

Molecular Microbiology (2001) **40**(2), 485–497

Physiological consequences of blocked *Caulobacter crescentus dnaA* expression, an essential DNA replication gene

Boris Gorbatyuk and Gregory T. Marczynski*
Department of Microbiology and Immunology, McGill University, 3775 University Street, Montreal, Quebec, Canada H3A 2B4.

Summary

***Caulobacter crescentus* chromosome replication is precisely coupled to a developmental cell cycle. Like most eubacteria, *C. crescentus* has a DnaA homologue that is presumed to initiate chromosome replication. However, the *C. crescentus* replication origin (*Cori*) lacks perfect consensus *Escherichia coli* DnaA boxes. Instead, the *Cori* strong transcription promoter (Ps) may regulate chromosome replication through the CtrA cell cycle response regulator. We therefore created a conditional *dnaA C. crescentus* strain. Blocking *dnaA* expression immediately decreased DNA synthesis, which stopped after approximately one doubling period. Fluorescent flow cytometry confirmed that DNA synthesis is blocked at the initiation stage. Cell division also stopped, but not swarmer to stalked cell differentiation. All cells became stalked cells that grew as long filaments. Therefore, general transcription and protein synthesis continued, whereas DNA synthesis stopped. However, transcription was selectively blocked from the flagellar *fliQ* and *fliL* and methyltransferase *ccrM* promoters, which require CtrA and are blocked by different DNA synthesis inhibitors. Interestingly, transcription from *Cori* Ps continued unaltered. Therefore, Ps transcription is not sufficient for chromosome replication. Approximately 6–8 h after blocked *dnaA* expression, cells lost viability exponentially. Coincidentally, β-galactosidase was induced from one transcription reporter, suggesting an altered physiology. We conclude that *C. crescentus* DnaA is essential for chromosome replication initiation, and perhaps also has a wider role in cell homeostasis.**

Introduction

The DnaA protein is essential for chromosome replication initiation in *Escherichia coli* (for recent reviews, see Skarstad and Boye, 1994; Messer and Weigel, 1996; Kaguni, 1997). *E. coli* DnaA binds four DnaA boxes within the *E. coli* origin of replication, *oriC*, and mediates open complex formation by making secondary contacts with three 13-mer motifs within an AT-rich region. DnaA also recruits DnaB helicase to the open complex, where it unwinds the origin and commits the chromosome for bidirectional replication (Bramhill and Kornberg, 1988; Baker and Wickner, 1992).

Although *dnaA* sequence homologies have been identified in many eubacteria (Skarstad and Boye, 1994), aside from *E. coli*, DnaA's role in replication control has been demonstrated genetically only in *Bacillus subtilis* (Moriya *et al.*, 1990). DnaA homologues have been found in *Mycoplasma* spp. (Fraser *et al.*, 1995; Seto *et al.*, 1997), *Streptococcus pneumoniae* (Gasc *et al.*, 1998), *Rhizobium meliloti* (Margolin *et al.*, 1995), *Rickettsia prowazeki* (Waite *et al.*, 1998), *Caulobacter crescentus* (Zweiger and Shapiro, 1994), *Mycobacterium tuberculosis* (Rajagopalan *et al.*, 1995a), *Mycobacterium smegmatis* (Rajagopalan *et al.*, 1995b) and the cyanobacteria, *Synechocystis* sp. (Richter and Messer, 1995). It is assumed that DnaA is universally essential to initiate bacterial chromosome replication; however, genetic disruption studies demonstrated that *dnaA* is not essential for *Synechocystis* sp. and its circadian cell cycle control (Richter *et al.*, 1998).

We wanted to test the assumption that DnaA is essential for chromosome replication in the phylogenetically distant Gram-negative, α-purple bacterium, *C. crescentus*. *C. crescentus* provides an exceptional model for studying the bacterial cell cycle. *C. crescentus* divides asymmetrically to yield a motile, flagella-bearing swarmer cell and a sessile stalked cell (for recent reviews, see Brun *et al.*, 1994; Shapiro and Losick, 1997; Hung *et al.*, 2000). Swarmer cells repress chromosome replication (Quon *et al.*, 1998) and, in order for replication to proceed, they must shed their flagella and differentiate into stalk cells (Degnen and Newton, 1972a; Marczynski *et al.*, 1990). *C. crescentus* has one circular chromosome, and bidirectional chromosome replication (Dingwall and Shapiro, 1989) occurs once and only once per cell cycle (Marczynski, 1999).

The *C. crescentus* replication origin (*Cori*) uniquely supports autonomous *Cori* plasmid replication in the

Accepted 12 February, 2001. *For correspondence. E-mail gmarczynski@nexus.microimm.mcgill.ca; Tel. (+1) 514 398 3917; Fax (+1) 514 398 7052.

stalked cell type (Marczynski and Shapiro, 1992). Several unique regulatory elements are present in *Cori*. There are five 9-mer GTTAA-N7-TTAA motifs (Marczynski and Shapiro, 1992) that are bound by the CtrA response regulator (Quon *et al.*, 1998; Siam and Marczynski, 2000). CtrA represses chromosome replication in swarmer cells (Quon *et al.*, 1998), and its degradation is thought to allow chromosome replication in stalk cells (Domian *et al.*, 1997). Several promoters have also been identified in *Cori, and* transcription from the strong promoter (Ps) is believed to stimulate replication, as transcription from Ps is required for *Cori* plasmid replication and only occurs in stalk cells (Marczynski *et al.*, 1995). Phosphorylated CtrA binds co-operatively to binding sites 'a' and 'b' of Ps in *Cori*. This presumably represses Ps transcription and consequent chromosome replication in swarmer cells (Siam and Marczynski, 2000).

Unlike the *B. subtilis and E. coli oriC*, as well as other bacterial replication origins that contain DnaA boxes that match the proposed consensus sequence, the *C. crescentus Cori* has no DnaA boxes that match the consensus perfectly (Marczynski and Shapiro, 1992). However, point mutations in one potential DnaA box, which matches 8/9 bp of the TTATCCACA *E. coli* consensus, abolish *Cori* plasmid replication (Marczynski and Shapiro, 1992). This suggests a role for DnaA in *C. crescentus* replication control but, considering the lack of consensus DnaA boxes as well as other unique sequence features, this role may be significantly different.

Here, we report the physiological consequences of blocked *dnaA* expression on cell division and chromosome replication. We also studied the transcription of replication-dependent promoters and *Cori* promoters in response to blocked *dnaA* expression. We propose that constant transcription and translation of DnaA in *C. crescentus* is required for normal cell cycle progression.

Results

DnaA is an essential gene for C. crescentus

The chromosome *dnaA* gene cannot be disrupted except when complemented by a plasmid *dnaA* gene. The *dnaA* gene was subcloned from a cosmid DNA spanning the entire replication origin (*Cori*) region (Fig. 1A). To disrupt the *dnaA* gene, the indicated *Xba*I to *Bam*HI *dnaA* fragment was ligated into plasmid pGM1360. This *Xba*I to *Bam*HI fragment contained the entire *dnaA* gene, its promoter, as well as downstream sequences into the *alkB* gene that points in the opposite orientation (Fig. 1A). To create the *dnaA*::Ω disruption plasmid (pGM1531), the omega cassette, encoding spectinomycin and streptomycin resistance genes (from pHP45), was ligated between two unique *Xho*I sites and therefore disrupted and removed most of the *dnaA* coding sequences (Fig. 1A). This *dnaA*::Ω plasmid was conjugated with the synchronizable mutant *C. crescentus* strain NA1000, a derivative of wild-type CB15. Southern blot analysis demonstrated that the resulting kanamycin (Km)-resistant colonies acquired pGM1531 by homologous recombination at the *dnaA* gene (Fig. 1B; data not shown). These cells also acquired spectinomycin and streptomycin resistance from the marked *dnaA*::Ω gene and sucrose sensitivity from the plasmid-encoded *sacB* gene.

To test if the intact *dnaA* gene could be exchanged by the *dnaA*::Ω allele, we selected for a second homologous exchange that would remove integrated pGM1531 from the chromosome (Fig. 1B). These cells were plated on 3% sucrose, and surviving colonies were scored for antibiotic resistance. No spectinomycin- and streptomycin-resistant colonies were obtained (*dnaA*::Ω, Fig. 1B), unless this strain also contained the intact *dnaA* gene on autonomously replicating pGM1522 (Fig. 1C). This implied allelic replacement (*dnaA*::Ω) was confirmed by Southern blot analysis (data not shown). The *dnaA*::Ω cells also acquired stable tetracycline (Tc) resistance from pGM1522 (Fig. 1C), which is otherwise easily lost after subculture in PYE media without tetracycline. As pGM1522 cannot be lost from the *dnaA*::Ω cells when it provides the only copy of an intact *dnaA* gene, this also argues that *dnaA* is essential. Therefore, the natural *dnaA* gene can be disrupted only when another *dnaA* gene is present.

Conditional dnaA *expression also demonstrates that it is essential*

We used the conditional *xylX* promoter (P_{xylX}) and placed *dnaA* expression under both transcriptional and translational control of the *xylX* operon. The transcription of P_{xylX} is induced by xylose and repressed by glucose (Meisenzahl *et al.*, 1997). A 5′ *Eco*RI site and a 3′ *Spe*I site were introduced into *dnaA* by a polymerase chain reaction (PCR) that amplified *dnaA* with oligonucleotide primers containing these sites (see *Experimental procedures*), and cloned into the *Eco*RI–*Spe*I sites of the kanamycin (*nptII*)-resistant plasmid pNPT228XNE (Table 1). This created a translational fusion of *dnaA* with the first six amino acids of the first gene of the *xylX* operon, pGM2195 (Fig. 2A). Plasmid pGM2195 was integrated into the *xylX* locus of a wild-type strain, NA1000, to create a strain, GM2409, with an asymmetric *xylX* gene duplication in the chromosome (Fig. 2A), which was confirmed by Southern blot hybridization (data not shown). The wild-type *dnaA* gene at the wild-type locus was converted to the *dnaA*::Ω disruption by φCr30 transduction from strain GM2446 (Fig. 1C) into strain GM2409 (Fig. 2A). The resulting strain, GM2471 (Fig. 2B), was selected for spectinomycin and streptomycin resistance. GM2471 was sensitive to

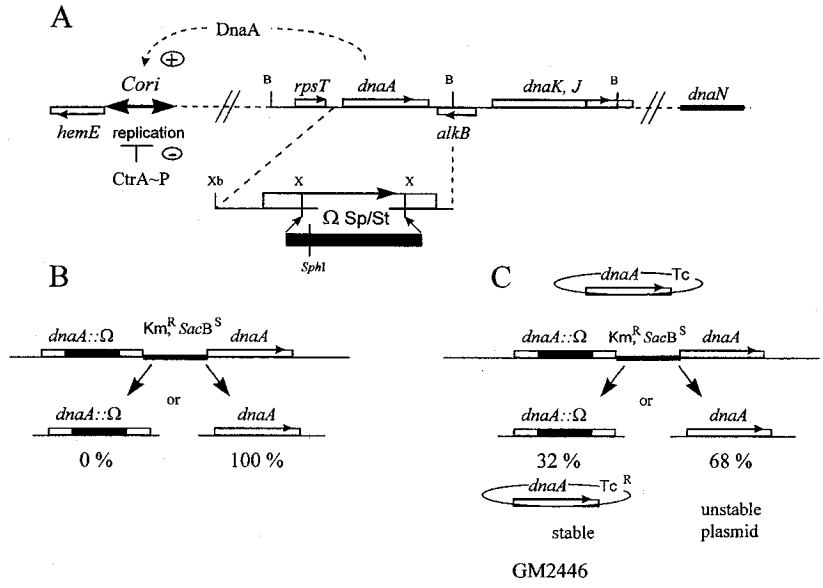


Fig. 1. Genetic disruptions at the *C. crescentus dnaA* locus.
A. The *C. crescentus dnaA* gene and its relationship to the replication origin (*Cori*), flanking genes and cloned DNA. This chromosome map is based on published data (Marczynski and Shapiro, 1992; Zweiger and Shapiro, 1994; Colombi and Gomes, 1999). The dotted line shows *dnaA* sequences cloned into pGM1360 and used to create the *dnaA*::Ω disruption in pGM1380 and subsequently pGM1531, as described in the text.
B. Plasmid pGM1531 (*dnaA*::Ω) integrated at the wild-type *dnaA* locus and frequencies of alternative (left or right side) plasmid excisions after 3% sucrose counterselection and scoring 100 colonies for the Ω-cassette (100 μg ml^{-1} spectinomycin) antibiotic resistance.
C. Strain GM2446 created by *trans*-complementation of the *dnaA*::Ω chromosome disruption. Integrated plasmid pGM1531 (*dnaA*::Ω at the wild type *dnaA* locus), as before, and replicating plasmid pGM1522 (wild-type *dnaA* gene). Frequencies of alternative pGM1531 excisions after 3% sucrose counterselection and scoring 100 colonies for the Ω-cassette (100 μg ml^{-1} spectinomycin) antibiotic resistance.

tetracycline and non-viable when xylose was absent from PYE media or substituted by glucose. Occasionally, tetracycline resistance, as a result of *dnaA* plasmid pGM1522, was co-transduced with chromosome *dnaA*::Ω. These cells survived on glucose PYE media without xylose but did not lose tetracycline resistance. Therefore, GM2471 cells require xylose to grow and express the essential *dnaA* gene. Single-copy P_{xylX} expression of *dnaA* complements the *dnaA*::Ω disruption apparently as effectively as multicopy plasmid expression of *dnaA*.

Blocked dnaA *expression allows growth but blocks cell division*

To block *dnaA* expression, *C. crescentus* strain GM2471 (*dnaA*::Ω, P*xylX*::*dnaA*) was grown in minimal (M2) media containing xylose as its sole carbon source (M2X), washed repetitively in M2 media lacking sugar, back-diluted to a low optical density (OD) and allowed to proceed growth in either M2G (glucose) or M2X (xylose) media. Cells shifted back to M2X media grew exponentially (Fig. 3A) with a growth rate comparable to wild-type NA1000 cells. However, cells shifted to M2G media entered a linear growth phase indicative of blocked cell division (Fig. 3). Control wild-type NA1000 cells shifted from xylose to glucose grew exponentially and maintained normal cell division (data not shown). Darkfield microscopy confirmed that M2G cells progressively filament as stalked cells (Fig. 4A–C). Some cells within this filamentous population developed a constriction near one cell pole. This constriction was also visible in some late GM2471 M2G cells (Fig. 4C), although cells were never able to complete cell division. When shifted to M2G, these GM2471 cells maintained a normal morphology up to 3 h after the shift, but subsequently failed to divide. Instead, their cell length increased progressively. However, washed cells returned to M2X maintained a typically wild-type morphology, for example at 10.5 h after the shift (Fig. 4D). When synchronous swarmer populations of GM2471 were shifted to M2G, they all differentiated into stalked cells that also grew as stalked filaments. However, the duration of the swarmer to stalk transition was ≈30 min longer than that of parallel swarmer cells shifted to M2X (data not shown).

Blocked dnaA *expression selectively and rapidly blocks DNA replication*

We examined whether filamentous GM2471 cells synthesize DNA. GM2471 cells were shifted to M2G or M2X media for 7 h and diluted to an OD of 0.1. [α-^{32}P]-dCTP

Table 1. Bacterial strains and plasmids.

Strain/plasmid	Genotype or description	Sources
E. coli		
S17-1	*E. coli* 294::RP4-2(Tc::Mu)(Km::Tn*7*)	Simon *et al.* (1983)
C. crescentus		
GM1609	NA1000 Δ*bla*	Marczynski *et al.* (1995)
GM2409	pGM2195 in *xylX* locus of NA1000, Km	This work
GM2415	pGM1531 in *dnaA* locus of NA1000 right cross-over, Sp/St	This work
GM2446	*dnaA*::Ω in chromosome from GM2415 after sucrose, pGM1522 complement, Sp/St, Tc	This work
GM2471	GM2446 φCr30 lysate transduced into GM2409Km, Sp/St	This work
NA1000	Synchronizable mutant of wild-type CB15	Evinger and Agabian (1977)
Plasmids		
pCS91	pRKlac290 P*rsaA*::*lacZ* transcriptional fusion, Tc	C. Stephens
pCS98	pRKlac290 P*flgF*::*gus*, P*fliL*::*lacZ*, Tc	C. Stephens
pGM384	Cosmid I, *Cori* DNA region, Tc	Marczynski and Shapiro (1992)
pGM915	pRKlac290 *lacZ*, Tc	Marczynski *et al.* (1995)
pGM976	pRKlac290 *Cori BamHI* to *Hin*dIII Ps::*lacZ*	Siam and Marczynski (2000)
pGM1052	pRKlac290 *Cori PstI* to *BglII*, Pw::*lacZ*	Marczynski *et al.* (1995)
pGM1054	pRKlac290 *Cori BamHI* to *EcoRI*, P3::*lacZ*	Marczynski *et al.* (1995)
pGM1181	pSK(+)-*dnaA*, *Bam*HI from pGM384	This work
pGM1360	pUC BM21-*dnaA*, *Xba*I–*Hin*dIII from pGM1181	This work
pGM1380	pUC BM21-*dnaA*::Ω, from pGM1360	This work
pGM1522	pRK404-*dnaA*, *Bam*HI from pGM1360	This work
pGM1531	pNPTS138-*dnaA*::Ω, from pGM1380	This work
pGM2195	pNPT228XNE-*dnaA*, PCR *Eco*RI–*Spe*I from pGM1181	This work
pGZ22	pRKlac290 P*ccrM*::*lacZ*, Tc	C. Stephens
pHP45	Sp/St antibiotic cassette, Ap	Prentki and Krisch (1984)
pNPTS138	Integrative, Km (nptI), *sac*B, *ori*T	M. R. K Alley
pNPT228XNE	Integrative, Km (nptII) *xylX* locus	U. Jenal
pRK404	Broad-host-range RK2 based, Tc	Ditta *et al.* (1985)
pSK(+)	pBluescript II, cloning vector, Ap	Stratagene
pUC BM21	Cloning vector, Ap	Boehringer Mannheim
pWZ162	pRKlac290 P*fliQ*::*lacZ*, Tc	Zhuang and Shapiro (1995)

was added, and [^{32}P]-DNA synthesis was measured during a 2.5 min time course (Fig. 5A). Whereas the M2X culture demonstrated vigorous [α-^{32}P]-dCTP incorporation into DNA, the parallel M2G culture showed only a baseline level of [α-^{32}P]-dCTP incorporation (Fig. 5A). As the M2G culture OD continues to increase after 7 h of shift (Fig. 3) because of continued growth (Fig. 4A–C), this indicates that general metabolism, including RNA and protein synthesis, persists while DNA synthesis is arrested.

We next examined how rapidly GM2471 glucose cultures arrested DNA synthesis. Cultures were sampled from 0 to 8 h after the shift from xylose to glucose, normalized for cell number to an OD of 0.1 and pulse labelled for 90 s with [α-^{32}P]-dCTP. This method provided a linear incorporation of [α-^{32}P]-dCTP that reflects the

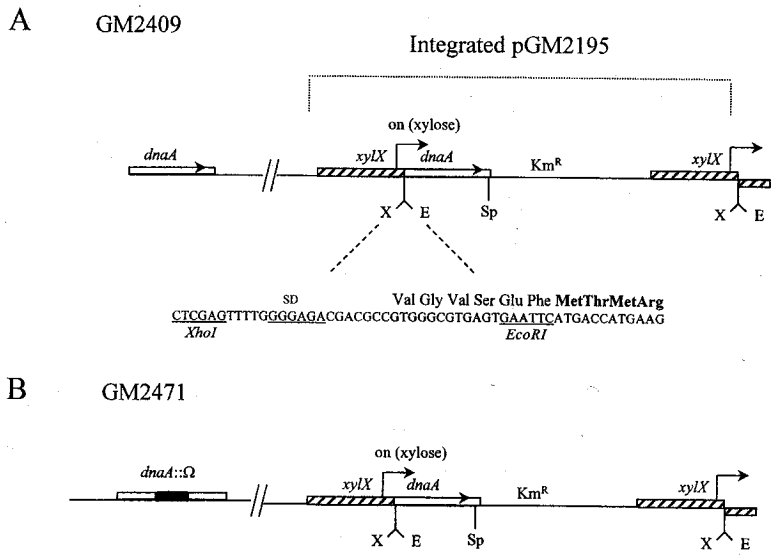


Fig. 2. Formation of a strain that conditionally expresses *dnaA*.
A. pGM2195 was constructed by PCR amplifying *dnaA*, as described in *Experimental procedures*, such that an *Eco*RI site was created at the 5′ end of the gene, directly upstream of the first methionine codon. This allowed for a transcriptional and translational fusion with the first six amino acids of the *xylX* operon as shown. pGM2195 is drawn integrated at the NA1000 *xylX* locus (Southern data not shown) to create strain GM2409.
B. In order to create strain GM2471, the *dnaA*::Ω disruption was transduced from GM2446 (Fig. 1C) by φCr30 transducing phage into the wild-type *dnaA* locus of GM2409. Km-/Sp-/Strp-resistant and Tc-sensitive colonies were selected for growth on PYE 0.3% xylose plates.

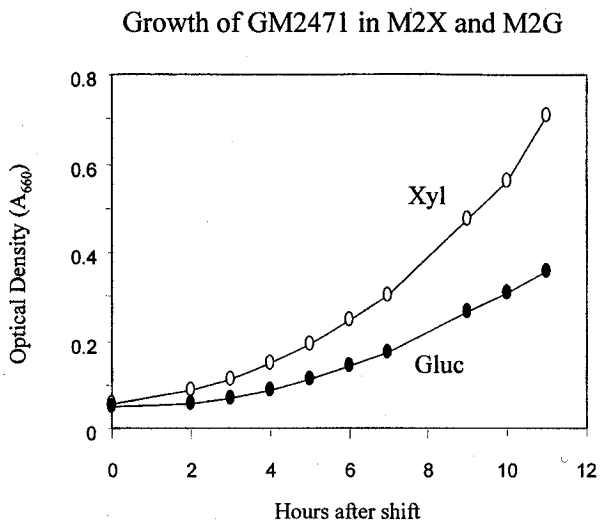


Fig. 3. Growth of GM2471 after blocked *dnaA* expression. M2X (0.3% xylose) cultures were shifted back to either M2X (open circles) or M2G (0.2% glucose) (closed circles). Subsequent growth was measured by absorbance at 660 nm and followed by microscopy (Fig. 4).

DNA synthetic rate (Fig. 5A; data not shown). In the control shift, GM2471 M2X cells continued vigorous DNA synthesis (Fig. 5B, Xyl). However, GM2471 M2G cells progressively decreased DNA synthesis and completely arrested DNA synthesis between 1.5 and 2 h after the shift (Fig. 4B, Gluc).

C. crescentus *cells blocked in* dnaA *expression accumulate one chromosome*

We examined whether the DNA synthetic block described in Fig. 5B is specific for replication initiation. GM2471 cells were grown in filtered M2X media overnight and shifted to M2G and M2X. Cells were sampled at various time points after the shift, treated with chloramphenicol for 3 h, fixed in 70% ethanol, stained with chromomycin A3 and analysed by fluorescent flow cytometry as described in *Experimental procedures* (Fig. 6) (Winzeler and Shapiro, 1995).

GM2471 shifted to M2X for 0, 4 and 6 h maintained populations of cells that had both one and two chromosomes (Fig. 6, 0 Xyl, 4 Xyl, 6 Xyl). As cells were treated with chloramphenicol, all cells in this mixed population that initiated DNA replication were able to complete replication, but were unable consequently to reinitiate replication. Thus, all cells that had the ability to initiate replication have exactly twice as much DNA content as cells that do not have the ability to initiate replication and are represented by the second major peak in Fig. 6 (0 Xyl, 4 Xyl and 6 Xyl). All cells grown in M2X that did not initiate replication (swarmer cells) are represented by the first major peak in Fig. 6 (0 Xyl, 4 Xyl and 6 Xyl).

However, although GM2471 cells shifted to M2G originally had relatively equal proportions of cells with both one and two chromosomes after chloramphenicol treatment (Fig. 6, 0 Gluc), they accumulated only one chromosome at 4 h and 6 h after shift to M2G (Fig. 6, 4 Gluc, 6 Gluc). As most cells blocked in *dnaA* expression accumulated one chromosome, these cells cannot initiate chromosome replication. Therefore, blocked *dnaA* expression selectively blocks replication initiation.

Blocked dnaA *expression is bactericidal*

GM2471 cells were likewise shifted to M2X and M2G, sampled periodically and plated on PYE media supplemented with xylose to reinduce DnaA. Whereas the M2X culture increased in cell numbers and plating efficiency, the M2G culture exhibited constant cell numbers and, subsequently, decreasing plating efficiency. Specifically, from 0 to 6 h after the shift to M2G, viable cell numbers remained constant, but decreased exponentially between 6 and 14 h (Fig. 7). A 10-fold drop in viable cell numbers is observed within 10 h after the shift, and a 100-fold drop is observed within 14 h. Although OD is increasing at a linear rate (Fig. 3), as a result of increased cell length, the number of cells that can form colonies decreases significantly. This suggests that the longer M2G cells grow without DNA synthesis, the less likely they are to survive.

Effect of blocked dnaA *expression on* Cori *promoters*

Transcription from the *C. crescentus* replication origin

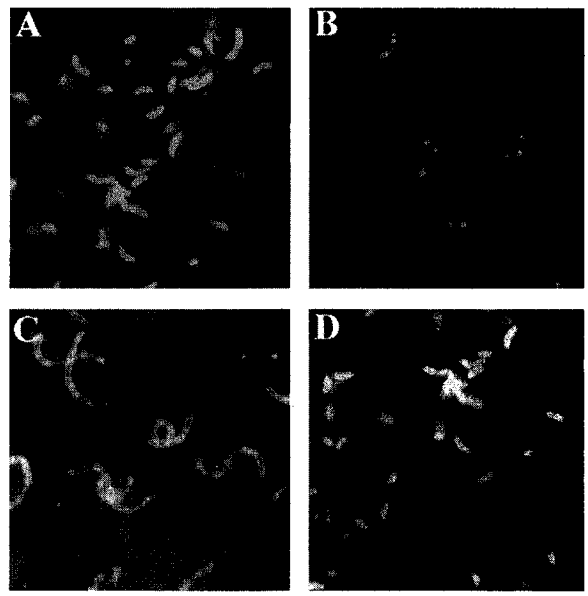


Fig. 4. Morphology of GM2471 shifted to M2G or M2X. Darkfield micrographs at 1000× magnification are shown.
A–C. GM2471 shifted to M2G for 3, 7 and 10.5 h respectively.
D. GM2471 shifted back to M2X for 10.5 h.

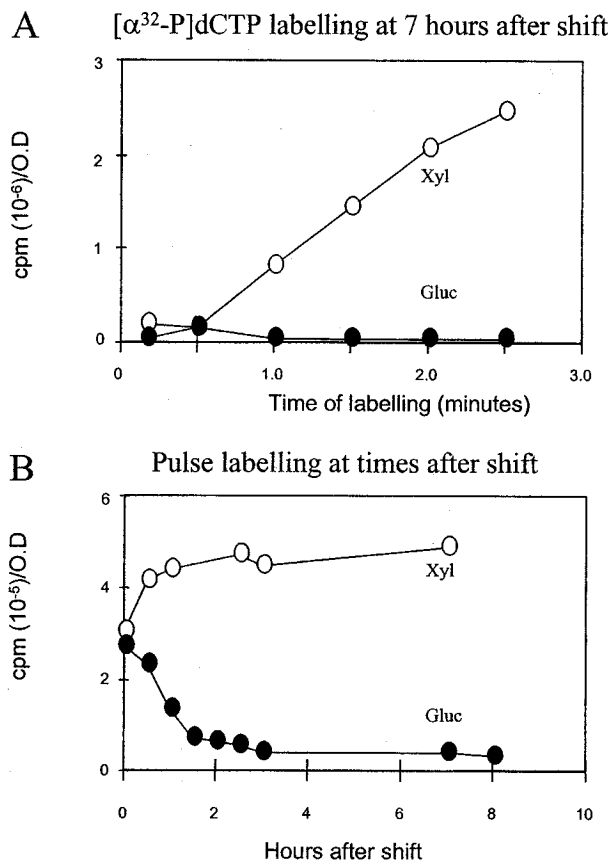


Fig. 5. GM2471 shifted to M2G quickly arrests DNA synthesis.
A. GM2471 cells were shifted to M2X (open circles) or M2G (closed circles) for 7 h, normalized for cell number to an OD (A_{660}) of 0.1 and labelled with [α^{32}-P]-dCTP for 10, 30, 60, 90, 120 and 150 s. DNA synthesis was measured as described in *Experimental procedures*.
B. GM2471 cells shifted to M2G and M2X were sampled periodically, normalized for cell number to an OD (A_{660}) of 0.1 and pulse labelled with [α-^{32}P]-dCTP for 90 s. As sampled cells were normalized to an OD of 0.1, we express c.p.m. as c.p.m./OD.

(*Cori*) promoters may regulate chromosome replication (Marczynski *et al.*, 1995). We therefore studied three promoters within *Cori*: a CtrA-regulated strong promoter (Ps) that transcribes towards the *hemE* gene; a constitutive weak promoter (Pw) that transcribes the *hemE* gene; and a divergent promoter (P3) (R. Siam and G. T. Marczynski, unpublished) that may overlap an essential DnaA box (Marczynski and Shapiro, 1992) (Fig. 8A). Transcription from Ps apparently yields an untranslated RNA that is restricted to the nascent stalked cells and may play a key role in replication initiation (Marczynski *et al.*, 1995). We introduced pRK290-derived plasmids containing the Ps (pGM1631), Pw (pGM1629) or P3 (pGM1630) promoters (Fig. 8A) fused to a *lacZ* reporter into strain GM2471. After shifting the cultures from M2X to M2G or M2X as described above, we sampled the cells periodically and measured β-galactosidase activity. Promoter activity was calculated and expressed in both conventional Miller units (activity per cell mass) and total enzyme activity present in the culture.

Transcription from the constitutive Pw promoter, as expected, followed cell growth when shifted to M2X or M2G (Fig. 8B). However, the P3 promoter showed a selective drop in transcription, as measured by Miller units, when shifted to M2G. As total enzyme activity increased, the P3 promoter was not off, but its transcription decreased relative to the cell mass (Fig. 8C). Interestingly, transcription from Ps remained strong and unchanged after the shift to M2G, and its transcription was very active even 12 h after the shift (Fig. 8D).

Blocked dnaA *expression effects transcription of DNA synthesis-dependent promoters*

Transcription from the *fliL* promoter (Stephens and Shapiro, 1993), the *fliQ* promoter (Dingwall *et al.*, 1992) and the *ccrM* promoter is selectively downregulated when DNA replication is blocked by hydroxyurea. We wanted to investigate whether these promoters would also be specifically downregulated in response to blocked *dnaA* expression. We therefore introduced pRK290-derived plasmids, containing the *fliQ* (pGM1574), *fliL* (pGM1576) or *ccrM* (pGM1612) promoters fused to a *lacZ* transcription reporter, into strain GM2471, shifted these cells from M2X to M2G and measured β-galactosidase activity as described above.

Transcription from the *fliL* promoter stopped rapidly after the M2X to M2G shift. Total enzyme activity did not increase during growth under these conditions, indicating that the *fliL* promoter was completely turned off (Fig. 9A). The *ccrM* promoter showed the same response (Fig. 9B). The *fliQ* promoter, like the *fliL* and the *ccrM* promoter, halted transcription after the M2X to M2G shift; however, its activity increased dramatically after 8 h of growth (Fig. 9C). The constitutive *rsaA* promoter is not sensitive to treatment with hydroxyurea (Stephens and Shapiro, 1993) and served as a control promoter not influenced by chromosome replication. The *rsaA* promoter (pGM1575) showed an insignificant change in Miller units in cultures shifted to M2G, because the total β-galactosidase activity tracked the increased cell mass (Fig. 9D).

Discussion

Genetic and biochemical experiments have clearly established that DnaA is an initiator of DNA replication in *E. coli* and *B. subtilis* but, excluding our present study, direct genetic experiments have not been extended to other bacteria. However, the presence of *dnaA* in many eubacteria, coupled with some recent biochemical analysis, suggests conserved mechanisms. For example, the

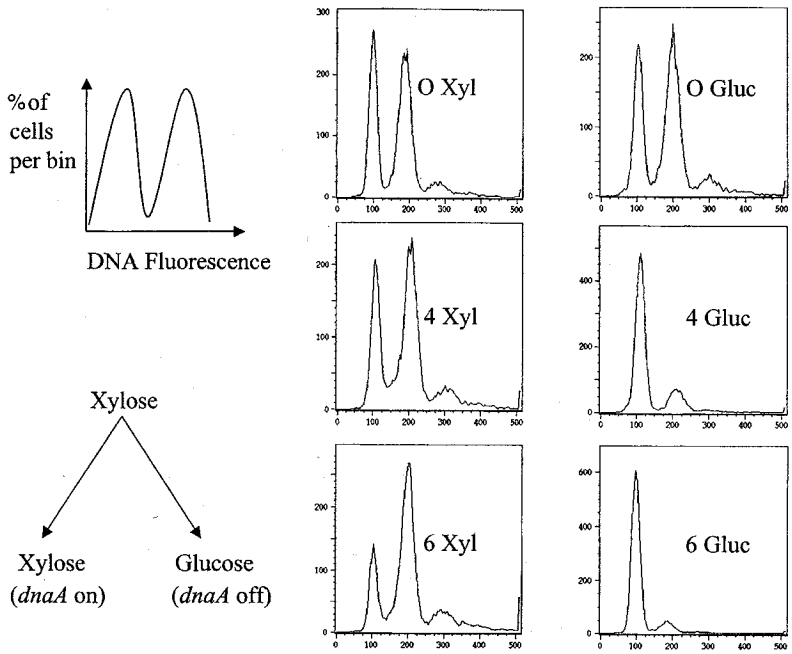


Fig. 6. GM2471 shifted to M2G accumulate only one chromosome. Flow cytometry was performed on GM2471 cells shifted to M2G and M2X. Cells were grown overnight in M2X and shifted to M2X and M2G for 0, 4 and 6 h, treated with chloramphenicol for 3 h and stained with chromomycin A3. Approximately 10 000 cells from each sampling were segregated into discrete bins according to chromomycin A3 DNA fluorescence intensity and are shown as density plots (0 Xyl, 4 Xyl, 6 Xyl, 0 Gluc, 4 Gluc, 6 Gluc).

Streptomyces lividans DnaA protein binds DnaA boxes within the *S. lividans oriC* (Jakimowicz *et al.*, 1998), and the strongest binding occurs between DnaA and the TTGTCCACA DnaA box consensus that is only 1 bp away from the *E. coli oriC* consensus TTATCCACA (Schaper and Messer, 1995). Although binding to consensus DnaA boxes suggests that the *S. lividans* DnaA performs the same function as the *E. coli* DnaA, it has yet to be demonstrated that the *S. lividans* DnaA mediates unwinding of *oriC* (Jakimowicz *et al.*, 1998). Also, in *M. smegmatis*, mutations of DnaA boxes severely impaired *M. smegmatis oriC* plasmid replication (Qin *et al.*, 1997). Point mutations in a near-consensus DnaA box (TGATCCACA) suggested that the *C. crescentus* DnaA protein is also essential for the replication of a uniquely organized origin, *Cori* (Marczynski and Shapiro, 1992). Furthermore, transcription of the *C. crescentus dnaA* by an artificial Ptac promoter appears to stimulate chromosome replication (Zweiger and Shapiro, 1994), although whether DnaA plays an essential role for *C. crescentus* replication was not critically tested.

Therefore, we created a *C. crescentus* strain GM2471, whose *dnaA* expression requires xylose. We demonstrated that the *C. crescentus dnaA* is essential and that blocking *dnaA* expression causes rapid loss of DNA synthesis, an accumulation of cells with only one chromosome, loss of transcription from replication-dependent *fliL* and *ccrM* promoters and eventual loss of viability. Interestingly, *Cori* strong promoter (Ps) transcription remains active, whereas DNA synthesis stops. These results are summarized in Fig. 10.

Pulse-labelling experiments demonstrated that DNA synthesis drops within 30 min after GM2471 cells are shifted from xylose to glucose, and DNA synthesis ceases within one generation time (Fig. 5B). These kinetics imply completion without the initiation of new chromosome replication, which is necessary for entry into a new cell cycle. As well, fluorescent flow cytometry data (Fig. 6) reveal that the *dnaA* block is specific for replication initiation. Microscopic observations of synchronous GM2471 cells with blocked *dnaA* expression reveal that

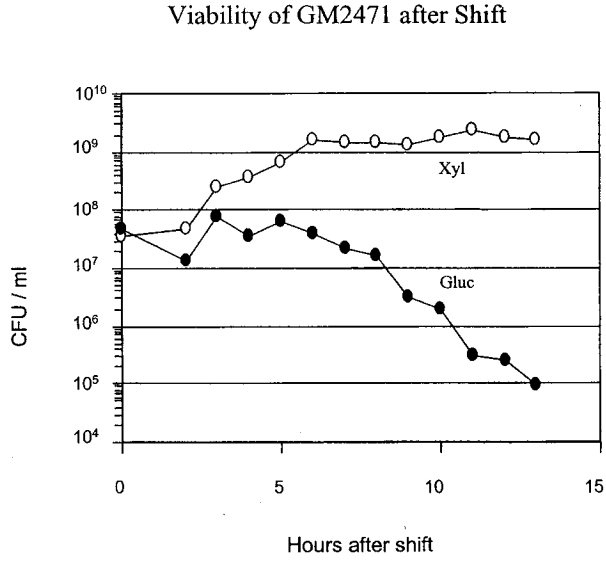


Fig. 7. GM2471 cells were shifted to M2G (closed circles) and M2X (open circles) and periodically sampled as described in *Experimental procedures*. The number of viable cells is presented as colony-forming units (cfu) ml^{-1} culture.

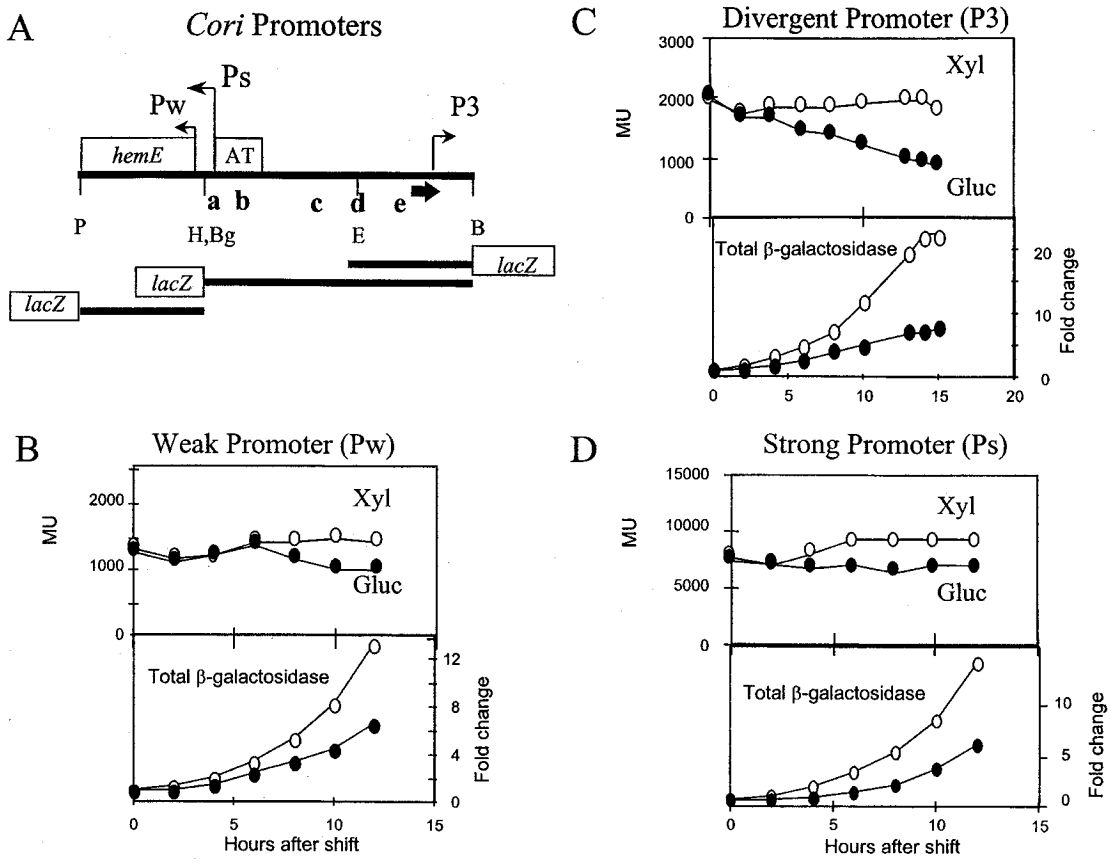


Fig. 8. Transcription from *Cori* promoters in response to blocked *dnaA* expression.
A. *Cori* promoters (Ps, Pw, P3) are shown in relation to CtrA binding sites (a, b, c, d, e), an essential DnaA box (filled arrowhead), the *hemE* gene and an AT-rich region. Restriction sites *Bgl*II (Bg), *Hin*dIII (H), *Eco*RI (E), *Pst*I (P) and *Bam*HI (B) are shown. Plasmid *lacZ* reporters were constructed for Ps (pGM976), Pw (pGM1052) and P3 (pGM1054) by digestion with the appropriate enzymes and cloning the fragments into pGM915 (Table 1).
B–D. pGM1052 (Pw), pGM1054 (P3) or pGM976 (Ps) were conjugated into GM2471, and cultures were shifted to either M2G (closed circles) or M2X (open circles), sampled and assayed for β-galactosidase activity. The values are plotted in Miller units (MU) and fold change in total enzyme activity.

cells start to filament as stalked cells (Fig. 4; data not shown). This filamentous phenotype is consistent with previous observations that DNA synthesis mutants filament as unpinched stalked cells (Osley and Newton, 1977). The appearance of constrictions near the cell pole in some of the cells in the filamentous M2G population suggests that a block in *dnaA* expression inhibits cell division at both the initiation and the progression stages. However, this point deserves further attention that is beyond the scope of this study. The *C. crescentus* cell cycle is conceptually organized into two pathways, a DNA synthesis pathway coupled to a cell division pathway (reviewed by Ohta *et al.*, 2000), in which DNA synthetic mutants fail to enter the cell division pathway. It remains unclear how the chromosome replication pathway communicates with the *C. crescentus* cell division pathway. In *E. coli*, the SOS response protein, SulA, directly interacts with FtsZ and consequently blocks Z-ring formation at the site of septation (Mukherjee *et al.*, 1998; Trusca *et al.*, 1998).

Analysis of *Cori* promoters revealed that strong promoter (Ps) transcription was ongoing in response to blocked *dnaA* expression (Fig. 7D). Transcription from Ps is required for *Cori* plasmid replication, and transcription from Ps only occurs in stalk cells (Marczynski *et al.*, 1995). CtrA-mediated repression of Ps transcription is believed to control replication initiation (Quon *et al.*, 1998; Siam and Marczynski, 2000). The fact that Ps transcription was ongoing when cells clearly stopped DNA replication indicates that, if Ps plays a role in replication control, replication still requires DnaA.

It is unclear how Pw and P3 are involved in replication control. The Pw promoter is transcribed throughout the cell cycle (Marczynski *et al.*, 1995) and was unaffected by blocked *dnaA* expression (Fig. 7B). The P3 promoter may overlap an essential DnaA box, and P3 transcription

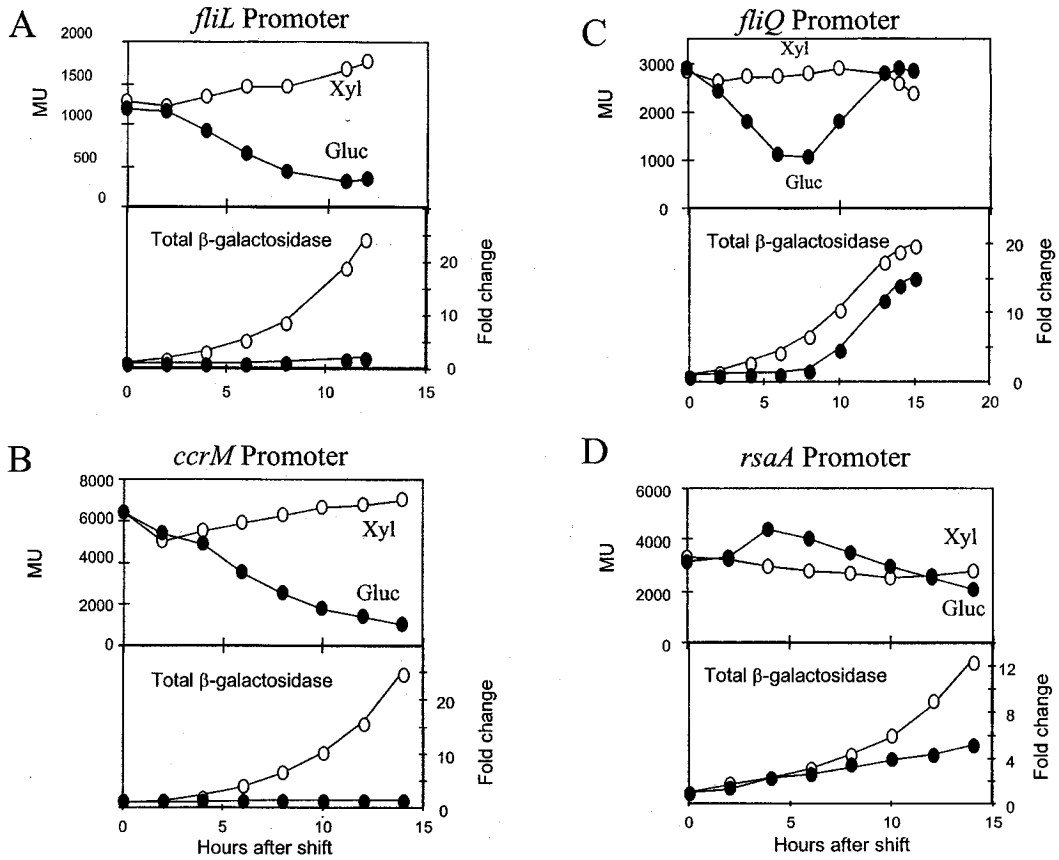


Fig. 9. Transcription from replication-dependent and control promoters.
A–C. Replication-dependent promoter reporters pCS98 (P*fliL*::*lacZ*), pGZ22 (P*ccrM*::*lacZ*) and pWZ162 (P*fliQ*::*lacZ*).
D. Control promoter reporter pCS91 (P*rsaA*::*lacZ*).Each reporter was conjugated into GM2471. Cultures were shifted to either M2G (closed circles) or M2X (open circles), sampled periodically and assayed for β-galactosidase activity. The values are plotted in Miller units (MU) and fold change in total enzyme activity.

decreased (Fig. 7C) relative to Pw, Ps as well as other control promoters (Figs 7 and 8).

The mechanism by which DnaA mediates replication initiation in *C. crescentus* is unclear. In *E. coli*, DnaA binds to four DnaA boxes within the origin on a negatively supercoiled template and serially melts three AT-rich tandem repeats forming an open complex, thereby setting the stage for replication initiation (reviewed by Bramhill and Kornberg, 1988; Messer and Weigel, 1996). An essential DnaA box was apparently identified in *Cori*, in which point mutations abolished plasmid replication (Marczynski and Shapiro, 1992). Moreover, several hypothetical DnaA boxes are also present inside *Cori*, but they share even less sequence similarity with *E. coli* DnaA boxes (Marczynski and Shapiro, 1992). It remains to be tested whether *C. crescentus* DnaA protein binds to the hypothetical *C. crescentus* DnaA boxes.

Several cell cycle-regulated promoters are selectively sensitive to blocked DNA replication in the presence of hydroxyurea or at the non-permissive temperature for the *dnaC* elongation mutant (Ohta *et al.*, 1990). This is true for the *ccrM* methyltransferase promoter (Stephens *et al.*, 1995), the *fliL* (Stephens and Shapiro, 1993) and *fliQ* (Dingwall *et al.*, 1992) class II flagellar promoters (for a review on flagellum biosynthesis in *C. crescentus*, see Gober and England, 2000). All three replication-sensitive promoters are regulated by the CtrA response regulator, which in turn is cell cycle regulated (Quon *et al.*, 1996; Domian *et al.*, 1997). The observation that flagellin synthesis is coupled to DNA synthesis has been reported previously (Osley and Newton, 1977) and is now attributed to a dependence on the expression of genes early in the flagellar hierarchy (Dingwall *et al.*, 1992). The *fliL* gene product is essential for flagellum function but not assembly (Jenal *et al.*, 1994), and the *fliQ* gene product is believed to participate in flagellar-specific protein export (Zhuang and Shapiro, 1995).

We examined how the *ccrM*, *fliL* and *fliQ* promoters respond to blocked *dnaA* expression because, in principle, this treatment should block the initiation step of chromosome replication, whereas hydroxyurea treatment and growth at non-permissive temperature in the *dnaC* Ts

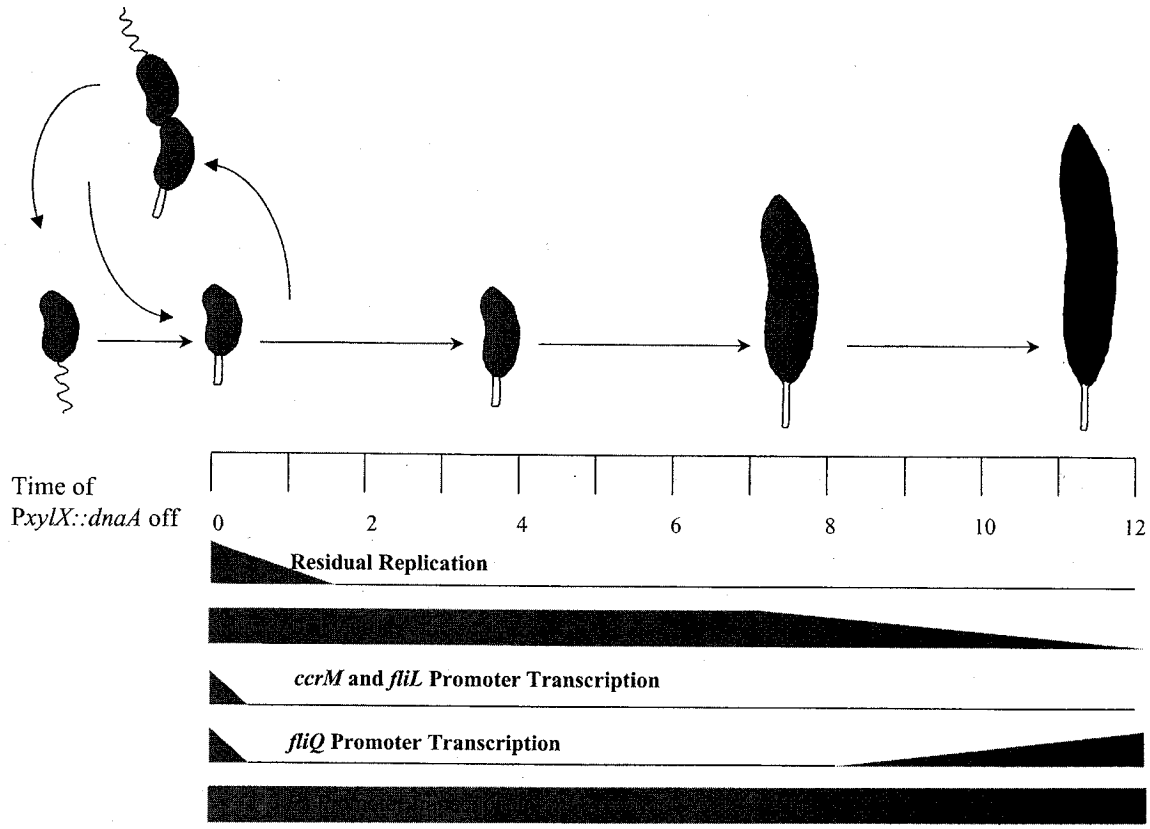


Fig. 10. Summary of physiological consequences of blocked *dnaA* expression.

mutation block the elongation step and may damage the DNA. Here, we show that the *fliL and ccrM* promoters are indeed sensitive to replication initiation blocks by blocked *dnaA* expression (Fig. 8A and B). Initially, the *fliQ* promoter construct demonstrated the same replication-sensitive response as the *fliL and ccrM* reporters; however, transcription from this reporter became induced at ≈8 h after the shift (Fig. 8C). This induction coincided with the drop in cell viability (Fig. 6 and 9). It is unclear whether the *fliQ* promoter itself or a different promoter in the reporter construct induces transcription at this late period after blocked *dnaA* expression. However, these events clearly mark an altered cell physiology.

The loss of cell viability in response to blocked *dnaA* expression is a puzzling result. Loss of *E. coli* cell viability was reported for temperature-sensitive *E. coli dnaA* (CRT46) after shift to non-permissive temperature, 40°C (Hirota *et al.*, 1968). If DnaA's only function is to bind DnaA boxes and then melt the AT-rich region, then there are no covalent modifications or DNA strand breakage that could conspicuously cause cell death. Therefore, in principle, *dnaA* initiation mutants should be different from, for example, DNA polymerase mutants whose inactivation may leave nicked or broken DNA. Treatment with increasing concentrations of hydroxyurea and mitomycin C, two drugs that inhibit DNA synthesis but allow for cell growth and elongation, also decreased cell viability (Degnen and Newton, 1972b), and these treatments presumably damaged the DNA. We also observed a rapid loss in viability of *C. crescentus dnaC* temperature-sensitive DNA synthesis mutants (Osley and Newton, 1977; Ohta *et al.*, 1990) when shifted to non-permissive temperature (data not shown). Also, DNA fragmentation correlates with a drop in viability in the *rfc2* replication elongation mutant in the budding yeast, *S. cerevisiae* (Noskov *et al.*, 1998).

Direct loss of replication initiation may not be sufficient to lose viability, as cells that stopped chromosome replication by 2 h after the xylose to glucose shift remained viable up to 8 h. So, what is the difference between cells at 2 h and those at 8 h? The cells are filamenting and increasing in length, so they could increase past a threshold size. The repercussions of getting too big may be that, even if the cells are able to resynthesize DnaA and initiate chromosome replication, they may be unable to segregate the newly replicated chromosome into the swarmer cell compartment because the distance may be too long for chromosome partitioning. Changes in cell membrane permeability have also been reported for *dnaA* mutants in *E. coli* (Wegrzyn *et al.*,

1999), and this may provide another possible explanation for the loss of viability.

In summary, we demonstrated that *C. crescentus dnaA* is absolutely required for chromosome replication. However, it is unclear and deserving of further study just how DnaA-mediated replication co-ordinates with cell cycle cues to exercise tight and precise replication control.

Experimental procedures

Bacterial strains and growth conditions

All bacterial strains and plasmids used in this study are shown in Table 1. *C. crescentus* strains were grown in PYE complex (Poindexter, 1964) or M2G (0.2% glucose) or M2X (0.3% xylose) minimal media (Ely and Johnson, 1977). To shift unsynchronized GM2471 cells, fresh overnight cultures grown at 30°C in M2X were washed three times with M2 media with no sugar supplement and then seeded in either M2G or M2X media to a final OD (A_{660}) of 0.05–0.1 and allowed to grow at 30°C. We used 0.3% xylose in both PYE and M2 media to induce *dnaA* in GM2471, as it has been shown previously to stimulate maximal induction of P_{xylX} (Meisenzahl *et al.*, 1997). Plasmids were mobilized to *C. crescentus* from *E. coli* S17-1 by conjugation (Simon *et al.*, 1983). To ascertain the total number of viable cells, growing GM2471 cultures shifted to M2G or M2X were serially diluted in M2 medium without sugar and plated on PYE plates supplemented with 0.3% xylose.

Transcription analysis

β-Galactosidase assays were performed as described previously (Miller, 1972) on cultures of *C. crescentus* strain GM2471 containing pRK290 *lacZ* reporter plasmids (Table 1) shifted to M2G or M2X as described above.

Microscopic techniques

C. crescentus strains were analysed by darkfield light microscopy using a Leitz Dialux microscope at a magnification of 1000×. Bacterial samples were immobilized on glass slides by mixing cells quickly with 1% low-melt agarose that was kept molten at 35°C.

DNA manipulations and DNA radiolabelling

Standard cloning (Sambrook *et al.*, 1989) and PCR protocols (Dieffenbach and Dveksler, 1995) were used in this study. To create the *dnaA*::Ω disruption in pGM1380 and pGM1531 (Fig. 1A), pGM1360 (Table 1) was cut with *Xho*I, made blunt ended with T4 DNA polymerase and ligated with the *Bam*HI-cut antibiotic cassette from pHP45, similarly made blunt ended. PCR was used to introduce an *Eco*RI site to the 5′ end of *dnaA* directly upstream of the first coding ATG to create a translational fusion with the *xylX* gene (pGM2195; Fig. 2A). An *Spe*I site was introduced into the 3′ end via PCR amplification of template pGM1181. The 5′ primer containing the desired mutation was DNAA-UP (5′-CAG GAA TTC ATG ACC ATG AAG GGC GGG GT). The 3′ primer containing the desired mutation was DNAA-DOWN (5′-GTC ACT AGT CCT GTC TCC AGA ACG ACC CT).

Generalized transduction was performed essentially as described previously (Ely, 1991). Phage lysates were UV irradiated by exposure to short-wave UV light from a FischerBiotech FBUVLS-80 UV hand lamp (Fischer Scientific) for 1–3 min. Transduction with bacteriophage φCr30 was used for introducing the *dnaA*::Ω disruption from phage lysates grown on GM2446 (Fig. 1C) into GM2409 (Fig. 2A) to create strain GM2471 (Fig. 2B).

Replication was assayed by DNA radiolabelling with [α-^{32}P]-dCTP, as described previously (Marczynski and Shapiro, 1992), except that labelled dCTP (3000 Ci mmol^{-1}) was used instead of dGTP. Before radiolabelling, cells shifted to M2X and M2G and sampled at various time points were normalized to an OD (A_{660}) of 0.1.

Flow cytometry

All cultures analysed using flow cytometry were grown in filtered M2G or M2X media at 30°C. All cells were sampled between an A_{660} of 0.05 and 0.2 and treated with 25 μg ml^{-1} chloramphenicol for 3 h at 30°C. All samples were washed in ice-cold water before fixation in order to avoid cell clumping. Samples were fixed by the addition of ice-cold ethanol to a final concentration of 70%. Cells were concentrated and stained with 10 μg ml^{-1} chromomycin A3 as described previously (Winzeler and Shapiro, 1995). Approximately 10 000 cells from each sampling were segregated into discrete bins according to chromomycin A3 DNA fluorescence intensity. Cells were analysed on a Becton Dickinson FACStar Plus machine equipped with an argon ion laser at the Stanford University Shared FACS Facility. Excitation was at 458 nm, and fluorescence was measured at 495 nm. FACS data were analysed using the FLOWJO analytical program (Tree Star).

Acknowledgements

We thank Drs M. R. K. Alley, U. Jenal, C. Stephens and Y. Brun for providing strains and plasmids, Dr E. C. S. Chan and R. McLaughlin for kind help with darkfield microscopy, Ann Reisenauer and members of the Stanford University Shared FACS facility for invaluable help with flow cytometry, S. L Chen for helpful discussion and critical reading of the manuscript, and members of the Marczynski laboratory and referees for critical reading of the manuscript. This work was supported by the Medical Research Council of Canada (MRC) grant MT-13453 and an MRC Scholarship Award 273-46 to G.T.M.

References

Baker, T.A., and Wickner, S.H. (1992) Genetics and enzymology of DNA replication in *Escherichia coli*. *Annu Rev Genet* **26**: 447–477.

Bramhill, D., and Kornberg, A. (1988) A model for initiation at origins of DNA replication. *Cell* **54**: 915–918.

Brun, Y.V., Marczynski, G., and Shapiro, L. (1994) The expression of asymmetry during *Caulobacter* cell differentiation. *Annu Rev Biochem* **63**: 419–450.

Colombi, D., and Gomes, S.L. (1999) An *alkB* gene homologue is differentially transcribed during the *Caulobacter crescentus* cell cycle. *J Bacteriol* **179**: 3139–3145.

Degnen, S.T., and Newton, A. (1972a) Chromosome replication during development in *Caulobacter crescentus*. *J Mol Biol* **116**: 671–680.

Degnen, S.T., and Newton, A. (1972b) Dependence of cell division on the completion of chromosome replication in *Caulobacter crescentus*. *J Bacteriol* **110**: 852–856.

Dieffenbach, C.W., and Dveksler, G.S. (1995) *PCR Primer: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Dingwall, A., and Shapiro, L. (1989) Rate, origin, and bidirectionality of *Caulobacter* chromosome replication as determined by pulsed-field gel electrophoresis. *Proc Natl Acad Sci USA* **86**: 119–123.

Dingwall, A., Zhuang, W., Quon, K., and Shapiro, L. (1992) Expression of an early gene in the flagellar regulatory hierarchy is sensitive to an interruption in DNA replication. *J Bacteriol* **174**: 1760–1768.

Ditta, G., Schmidthauser, T., Yakobson, E., Lu, P., Liang, X.W., Finlay, D.R., *et al.* (1985) Plasmids related to the broad host range vector, pRK290, useful for gene cloning and for monitoring gene expression. *Plasmid* **13**: 149–153.

Domian, I.J., Quon, K.C., and Shapiro, L. (1997) Cell type-specific phosphorylation and proteolysis of a transcriptional regulator controls the G1-to-S transition in a bacterial cell cycle. *Cell* **90**: 415–424.

Ely, B. (1991) Genetics of *Caulobacter crescentus*. *Methods Enzymol* **204**: 372–384.

Ely, B., and Johnson, R.C. (1977) Generalized transduction in *Caulobacter crescentus*. *Genetics* **87**: 391–399.

Evinger, M., and Agabian, N. (1977) Envelope-associated nucleoid from *Caulobacter crescentus* stalked and swarmer cells. *J Bacteriol* **132**: 294–301.

Fraser, C.M., Gocayne, J.D., White, O., Adams, M.D., Clayton, R.A., Fleischmann, R.D., *et al.* (1995) The minimal gene complement of *Mycoplasma genitalium*. *Science* **270**: 397–403.

Gasc, A.M., Giammarinaro, P., Richter, S., and Sicard, M. (1998) Organization around the dnaA gene of *Streptococcus pneumoniae*. *Microbiology* **144**: 433–439.

Gober, J.W., and Englund, J.C. (2000) Regulation of flagellum biosynthesis and motility in *Caulobacter*. In *Prokaryotic Development.* Brun, Y.V., and Shimkets, L.J. (eds). Washington, DC: American Society for Microbiology Press, pp. 319–339.

Hirota, Y., Jacob, F., Ryter, A., Buttin, G., and Nakai, T. (1968) On the process of cellular division in *Escherichia coli* I. Asymmetrical cell division and production of deoxyribonucleic acid-less bacteria. *J Mol Biol* **35**: 175–192.

Hung, D., McAdam, H., and Shapiro, L. (2000) Regulation of the *Caulobacter* cell cycle. In *Prokaryotic Development.* Brun, Y.V., and Shimkets, L.J. (eds). Washington, DC: American Society for Microbiology Press, pp. 361–378.

Jakimowicz, D., Majka, J., Messer, W., Speck, C., Fernandez, M., Martin, M.C., *et al.* (1998) Structural elements of the *Streptomyces oriC* region and their interactions with the DnaA protein. *Microbiology* **144**: 1281–1290.

Jenal, U., White, J., and Shapiro, L. (1994) *Caulobacter* flagellar function, but not assembly, requires FliL, a non-polarly localized membrane protein present in all cell types. *J Mol Biol* **243**: 227–244.

Kaguni, J.M. (1997) Escherichia coli DnaA protein: the replication initiator. *Mol Cells* **7**: 145–157.

Marczynski, G.T. (1999) Chromosome methylation and measurement of faithful, once and only once per cell cycle chromosome replication in *Caulobacter crescentus*. *J Bacteriol* **181**: 1984–1993.

Marczynski, G.T., and Shapiro, L. (1992) Cell-cycle control of a cloned chromosome origin of replication from *Caulobacter crescentus*. *J Mol Biol* **226**: 959–977.

Marczynski, G.T., Dingwall, A., and Shapiro, L. (1990) Plasmid and chromosomal DNA replication and partitioning during the *Caulobacter crescentus* cell-cycle. *J Mol Biol* **212**: 709–722.

Marczynski, G.T., Lentine, K., and Shapiro, L. (1995) A developmentally regulated chromosomal origin of replication uses essential transcription elements. *Genes Dev* **9**: 1543–1557.

Margolin, W., Bramhill, D., and Long, S.R. (1995) The *dnaA* gene of *Rhizobium meliloti* lies within unusual gene arrangement. *J Bacteriol* **177**: 2892–2900.

Meisenzahl, A.C., Shapiro, L., and Jenal, U. (1997) Isolation and characterization of a xylose-dependent promoter from *Caulobacter crescentus*. *J Bacteriol* **179**: 592–600.

Messer, W., and Weigel, C. (1996) Initiation of chromosome replication. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology.* Neidhardt, F.C., Ingraham, J.L., Low, K.B., Magasanik, B., Schaechter, M., and Umbarger, H.E. (eds). Washington, DC: American Society for Microbiology Press, pp. 1555–1577.

Miller, J.H. (1972) *Experiments in Molecular Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Moriya, S., Kato, K., Yoshikawa, H., and Ogasawara, N. (1990) Isolation of a *dnaA* mutants of *Bacillus subtilis* defective in initiation of replication: amount of DnaA protein determines cells' initiation potential. *EMBO J* **9**: 2905–2910.

Mukherjee, A., Cao, C., and Lutkenhaus, J. (1998) Inhibition of FtsZ polymerization by SulA, an inhibitor of septation in *Escherichia coli*. *Proc Natl Acad Sci USA* **95**: 2885–2890.

Noskov, V.N., Araki, H., and Sugino, A. (1998) The RFC2 gene, encoding the third-largest subunit of the replication factor C complex, is required for an S-phase checkpoint in *Saccharomyces cerevisiae*. *Mol Cell Biol* **18**: 4914–4923.

Ohta, N., Masurekar, M., and Newton, A. (1990) Cloning and cell cycle-dependent expression of DNA replication gene *dnaC* from *Caulobacter crescentus*. *J Bacteriol* **172**: 7027–7034.

Ohta, N., Grebe, T.W., and Newton, A. (2000) Signal Transduction and cell cycle checkpoints in developmental regulation of *Caulobacter*. In *Prokaryotic Development.* Brun, Y.V., and Shimkets, L.J. (eds). Washington, DC: American Society for Microbiology Press, pp. 341–359.

Osley, M.-A., and Newton, A. (1977) Mutational analysis of developmental control in *Caulobacter crescentus*. *Proc Natl Acad Sci USA* **74**: 124–128.

Poindexter, J.S. (1964) Biological properties and classification of the *Caulobacter* group. *Bacteriol Rev* **28**: 231–295.

Prentki, H., and Krisch, H.M. (1984) *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* **29**: 303–313.

Qin, M.-H., Madiraju, M.V.V.S., Zachariah, S., and Rajagopalan, M. (1997) Characterization of the *oriC* region of *Mycobacterium smegmatis*. *J Bacteriol* **179**: 6311–6317.

Quon, K.C., Marczynski, G.T., and Shapiro, L. (1996) Cell cycle control by an essential bacterial two-component signal transduction protein. *Cell* **84**: 83–93.

Quon, K.C., Yang, B., Domian, I.J., Shapiro, L., and Marczynski, G.T. (1998) Negative control of bacterial DNA replication by a cell cycle regulatory protein that binds at the chromosome origin. *Proc Natl Acad Sci USA* **95**: 120–125.

Rajagopalan, M., Qin, M.-H., Steingrube, A., Nash, D.R., Wallace, R.J., Jr, and Madiraju, M.V.V.S. (1995a) Amplification and cloning of the *Mycobacterium tuberculosis dnaA* gene. *Gene* **163**: 75–79.

Rajagopalan, M., Qin, M.-H., Nash, D.R., and Madijaru, M.V.V.S. (1995b) *Mycobacterium smegmatis dnaA* region and autonomous plasmid replication activity. *J Bacteriol* **177**: 6527–6535.

Richter, S., and Messer, W. (1995) Genetic structure of the *dnaA* region of the cyanobacterium *Synechocystis* sp. strain PCC6803. *J Bacteriol* **177**: 4245–4251.

Richter, S., Hagemann, M., and Messer, W. (1998) Transcriptional analysis and mutation of a *dnaA*-like gene in Synechocystis sp. strain PCC 6803. *J Bacteriol* **180**: 4946–4949.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schaper, S., and Messer, W. (1995) Interaction of the initiator protein DnaA of *Escherichia coli* with its DNA targets. *J Biol Chem* **270**: 17622–17626.

Seto, S., Murata, S., and Miyata, M. (1997) Characterization of dnaA gene expression in *Mycoplasma capricolum*. *FEMS Microbiol Lett* **150**: 239–247.

Shapiro, L., and Losick, R. (1997) Protein localization and cell fate in bacterial. *Science* **276**: 712–718.

Siam, R., and Marczynski, G.T. (2000) Cell cycle regulator phosphorylation stimulates two distinct modes of binding at a chromosome replication origin. *EMBO J* **19**: 1138–1147.

Simon, R., Prieffer, U., and Puhler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram-negative bacteria. *Biotechnology* **1**: 784–790.

Skarstad, K., and Boye, E. (1994) The initiator protein DnaA: evolution, properties and function. *Biochim Biophys Acta* **1217**: 111–130.

Stephens, C.M., and Shapiro, L. (1993) An unusual promoter controls cell-cycle regulation and dependence on DNA replication of the *Caulobacter* fliLM early flagellar operon. *Mol Microbiol* **9**: 1169–1679.

Stephens, C.M., Zweiger, G., and Shapiro, L. (1995) Coordinate cell cycle control of a *Caulobacter* DNA methyltransferase and the flagellar genetic hierarchy. *J Bacteriol* **177**: 1662–1669.

Trusca, D., Scott, S., and Bramhill, D. (1998) Bacterial SOS checkpoint protein SulA inhibits polymerization of purified FtsZ cell division protein. *J Bacteriol* **180**: 3946–3953.

Waite, R.T., Shaw, E.I., Winkler, H.H., and Wood, D.O. (1998) Isolation and characterization of the *dnaA* gene of *Rickettsia prowazekii*. *Acta Virol* **42**: 95–101.

Wegrzyn, A., Wrobel, B., and Wegrzyn, G. (1999) Altered biological properties of cell membranes in *Escherichia coli dnaA* and *seqA* mutants. *Mol Gen Genet* **261**: 762–769.

Winzeler, E., and Shapiro, L. (1995) Use of flow cytometry to identify a *Caulobacter* 4.5S RNA temperature-sensitive mutant defective in the cell cycle. *J Mol Biol* **251**: 346–365.

Zhuang, W.Y., and Shapiro, L. (1995) *Caulobacter* FliQ and FliR membrane proteins, required for flagellar biogenesis and cell division, belong to a family of virulence factor export proteins. *J Bacteriol* **177**: 343–356.

Zweiger, G., and Shapiro, L. (1994) Expression of *Caulobacter dnaA* as a function of the cell cycle. *J Bacteriol* **176**: 401–408.