Investigating the genetic linkage between chromosomal replication and cell division in *Caulobacter crescentus*

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Abstract

Caulobacter crescentus is a well-established model for studying the bacterial cell cycle, a complex process where the stages of growth, chromosome replication, and cell division often overlap, highlighting the presence of a highly coordinated regulatory network that remains partially understood. To search for novel regulators of the cell cycle, specifically of chromosomal replication, we developed a novel molecular screen to isolate dipM-like mutants. DipM is an endopeptidase and a cell division protein that was implicated in the coordination of DNA replication and cell division by our group. We believe that by generating Caulobacter mutants and selecting dipM-like phenotypes, it is possible to target defects in the pathway that regulate the progression from chromosomal replication to cell wall division, where DipM seems to be a key player. Out of nearly a hundred mutants, three *dipM*-like mutants were selected for genome sequencing, MUT1, MUT2, and MUT3. Bioinformatics analysis of these mutants allowed the identification of six gene candidates that could be linked to the regulation of the bacterial cell cycle, specifically chromosomal replication. We further investigated MUT1 and MUT3 by complementing them with the wildtype (WT) counterparts of the mutated genes to test for WT phenotype restoration. Our findings suggest that the identified gene candidates contribute to the cell cycle progression, specifically to chromosomal replication, by maintaining protein homeostasis. We speculate that DipM works with other regulatory proteins to sense and react to disturbances in protein homeostasis. Overall, our findings provide evidence for the effectiveness of our genetic screening technique and its capacity to detect cell cycle regulators coordinating between chromosome replication and cell wall division.

Sommaire

Étude du lien génétique entre la réplication chromosomique et la division cellulaire chez Caulobacter crescentus

Caulobacter crescentus est un modèle bien établi pour étudier le cycle cellulaire bactérien: un processus complexe où les étapes de croissance, de réplication chromosomique et de division cellulaire se chevauchent souvent, soulignant la présence d'un réseau de régulation hautement coordonné qui ne demeure que partiellement discerné. Pour chercher de nouveaux régulateurs du cycle cellulaire, en particulier de la réplication chromosomique, nous avons développé un nouveau crible moléculaire pour isoler des mutants de type dipM. DipM est une endopeptidase et protéine de division cellulaire qui a été impliquée dans la coordination de la réplication de l'ADN et de la division cellulaire par notre groupe. Nous pensons qu'en générant des mutants de Caulobacter et en sélectionnant des phénotypes de type dipM, il est possible de cibler les défauts de la voie qui régule la progression de la réplication chromosomique jusqu'à la division de la paroi cellulaire, où DipM semble être un acteur clé. Sur près d'une centaine de mutants, trois mutants de type dipM ont été sélectionnés pour le séquençage du génome : MUT1, MUT2 et MUT3. L'analyse bio-informatique de ces mutants a permis l'identification de six gènes candidats pouvant être liés à la régulation du cycle cellulaire bactérien, en particulier la réplication chromosomique. Nous avons d'avantage étudié MUT1, MUT2 et MUT3 en les agrémentant avec leurs homologues de type sauvage afin de tester la restauration du phénotype sauvage. Nos résultats suggèrent que les gènes candidats identifiés contribuent à la progression du cycle cellulaire et plus particulièrement à la réplication chromosomique en maintenant l'homéostasie des protéines. Nous présumons que

DipM fonctionne avec d’autres protéines régulatrices pour détecter et réagir aux perturbations de l’homéostasie de protéines. Dans l’ensemble, nos résultats fournissent des preuves démontrant l'efficacité de notre technique de dépistage génétique et sa capacité à détecter les régulateurs du cycle cellulaire qui coordonnent la réplication des chromosomes et la division de la paroi cellulaire.

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Preface

This thesis was written in accordance with McGill University's "Guidelines for Thesis Preparation".

All work towards this thesis was performed under the supervision of Dr. Gregory Marczynski, who was involved throughout the project from concept formation to experimental design/analysis to thesis feedback. The candidate performed majority of experiments, data analysis, and wrote this thesis. Dr. Marczynski repeated the complementation experiments and provided the images used in figures 5 and 6. Project idea and design was led by Dr. Marczynski. Experiments were designed by Duha AlAwad with Dr. Marczynski's input. With the candidate's assistance, Sophia Stegman and Hannah Dou, the undergraduate students of McGill's Microbiology and Immunology department, performed mutagenesis experiments and generated MUT1, MUT2, and MUT3. French translation for the abstract presented in this thesis was completed by Fio Vialard. Dr. Patrick Lypaczewski taught me how to analyze the sequencing data, provided valuable input regarding bioinformatics data analysis, and confirmed the results I produced

List of abbreviations

Amp	Ampicillin
Cori	Caulobacter's origin of replication
dipM	Division and polarity-related metallopeptidase
gDNA	Genomic DNA
GTP	Triphosphate guanosine 5'-triphosphate
Km	Kanamycin
LB	Luria Bertani / Lysogeny Broth
Nx	Nalidixic acid
oriC	Origin of replication
pDNA	Plasmid DNA
PNS	Plasmid non-supporting
Pol III	Polymerase III
PYE	Peptone-Yeast Extract
SAP	Shrimp Alkaline Phosphatase
Spec	Spectinomycin
Strep	Streptomycin
Tet	Tetracycline
tmRNA	Transfer-messenger RNA
TS	Temperature sensitivity
UV	Ultraviolet

WGS	Whole-genome sequencing
WT	Wildtype
DUE	DNA unwinding element
RC	Rolling circle
SMC	Structural maintenance of chromosome

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Background and Literation Review

1.1 Introduction

Bacteria are involved in maintaining the ecosystem as we know it [1], they are important players in human health [2-4], they may hold the key to solving the calamitous issue of pollution [5-7], and their biological functions can be harnessed in an industrial context to benefit humankind in a variety of fields [8-11]. Despite being such an essential component of life, a harmful subset of bacteria is causing one of the world's greatest threats to global health, the antibiotic resistance crisis [12]. Most antibiotics used target classical metabolic pathways in bacteria like RNA, DNA, protein synthesis, or cell wall synthesis [13]. With the emergence of new pathogens that are resistant to currently used antibiotics, it is essential to find new antibiotics with novel targets in these metabolic pathways. Hence, it is crucial that we explore bacterial biology to have a complete understanding of how these microorganisms grow and propagate and what factors regulate these processes.

Bacterial DNA replication and cell division has received considerable attention lately as potential target mechanisms for antibiotic therapy due to the presence of proteins essential to bacterial survival that do not exist in eukaryotic cells [14, 15]. In early attempts to understand bacterial replication, Cooper and Helmstetter proposed a model describing the division cycle of the gram-negative *E. coli*, where they suggested genomic DNA replication must be completed prior to cell division [16]. These findings laid the groundwork for our understanding of the bacterial cell cycle, which comprises the stages of; cell growth, DNA replication, and cell division [17].

1.2 Bacterial cell cycle

A newly formed bacterial cells must undergo important steps before cell division may take place to produce two daughter cells. These include, but are not limited to, DNA replication, chromosome segregation, and doubling in size while precisely situating its division machinery. For a division to be successful, these duties must be synchronized both spatially and temporally [18].

Historically, the bacterial cell cycle has been divided into three stages or periods known as B, C, and D (Figure 1). For simplicity purposes, we will mark the B period as the start of the cell cycle, which begins with the birth of the cell and end as chromosome replication is initiated. The B period starts at the onset of replication initiation. This period marks the time required for replication. Lastly, starting prior to the completion of chromosome segregation, cell wall division begins marking the start of the D period [19]. Despite the presence of distinct cell cycle stages in bacteria, they are generally overlapping and lack clear checkpoints that must be passed for the cycle to progress, unlike their eukaryotic counterparts [20]. Nonetheless, lack of firm checkpoints does not corroborate the long-standing belief that bacteria lack sophisticated developmental control mechanisms due to their simplicity compared to eukaryotic cells [21]. It is now clear that bacteria utilize complex spatiotemporal regulatory pathways to execute developmental, morphological, and functional programs as needed [22]. In fact, fast-growing bacteria can have up to 8 replication rounds happening simultaneously as proposed by Cooper and Helmstetter [16] after Yoshikawa *et al.* discovered the “multifork replication” in Bacillus subtilis [23]. The overlap in bacterial cell cycle stages provides a survival advantage where bacteria can speed up their growth rates to adapt to rapid fluctuations in their environments

[17]. This reflects the complexity of the regulation of the bacterial cell cycle as well as the intricacy of coordination between essential physiological processes such as growth and propagation.

The cell cycle events are influenced by the bacterial growth rate and environmental factors, including the availability of nutrients and surrounding temperature [19], further adding to the complexity of the system. Based on the Cooper and Helmstetter model, growth, DNA replication, and division are coupled processes, where replication termination is the triggering event for cell division [17]. However, opposing evidence suggests that cell division is not coupled to DNA replication but rather parallel and occurs at a definitive cell size [24]. Recent studies supported the Cooper and Helmstetter model [25, 26], while others provided evidence proposing an "adder" model, where a specific volume must be added to the cell, which then triggers cell division [27, 28]. A new study explains the discrepancy regarding the coupling of replication and division as a result of growth rate [29]. Investigators report that in slow-growing bacteria, replication and division are coupled. However, in fast-growing bacteria, division is independent of replication. While another view, arguably one that can be generalized to all bacteria, presents cell division as a process controlled by either: 1) chromosome-related events (e.g., end of segregation) or 2) Interdivision events (e.g., septum formation) [30]. Whichever of these two events is completed last will determine the onset of division [31].

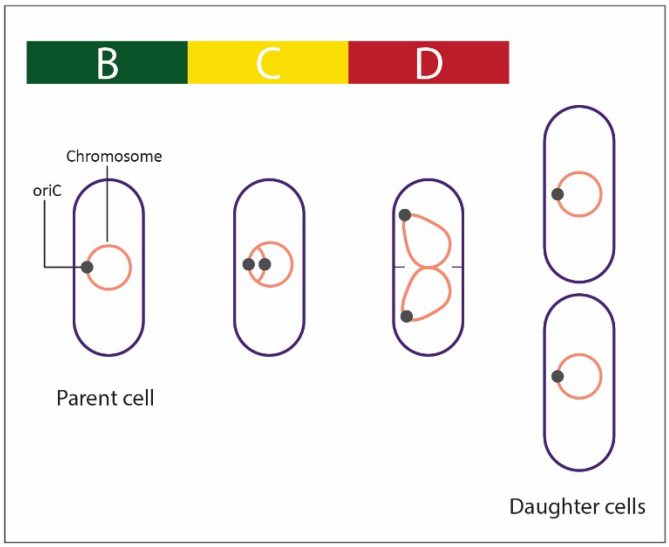

Figure 1: The bacterial cell cycle.

The B period starts at cell birth after completion of the last division cycle and continues through cell growth. The C period beings once chromosome replication is initiated and includes most of chromosome segregation. Period D begins at the end of chromosome segregation as the cell wall septum is formed. The D period concludes with the full cell division resulting in two daughter cells.

1.2.1 Bacterial chromosome replication

DNA replication is a key event in the bacterial cell cycle. It is an incredibly complex process that occurs by the coordinated action of various enzymes to catalyze the steps of DNA replication, like DNA unwinding and the production of two sister DNA strands. To avoid any excessive energy loss and to guarantee that DNA is faithfully and completely duplicated only once each cell-division cycle, this process must be extremely precise and accurately timed [32]. Most bacterial genomes consist of one chromosome. However, some bacteria have two or more chromosomes [33]. Similarly, some species of bacteria have linear chromosomes like the gram-positive bacterium *Streptomyces coelicolor* [33].

Most of our understanding of the replication process is a result of intensive studies done on *E. coli* [34]. Unlike eukaryotes, the bidirectional replication of the bacterial chromosome starts at a single unique location known as the origin of replication (*oriC*) (Figure 1) [19]. The *oriC* contains multiple unique sequences (usually 9 base pairs in length) known as DnaA boxes. The

naming comes after the binding affinity of the replication initiator DnaA to these regions on *oriC*. DnaA was the first protein identified as a cause of the famous "temperature-sensitive" phenotype in *E. coli*. Which in turn established the use of temperature sensitivity as a mutation detector in bacterial cellular processes, including replication [35].

DnaA is the first member of the initiation complex, a nucleoprotein complex that drives the start of chromosome replication. The activity of DnaA is regulated by ATP binding and hydrolysis. The ATP-bound form is the active form required for initiation by oligomerization at *oriC*. Replication is initiated when DnaA binds to DnaA-boxes at *oriC*, leading to the separation of the DNA strands at the AT-rich DNA unwinding element (DUE), which makes room for DnaB to bind [36]. DnaB is the helicase responsible for the unwinding of the DNA double helix that allows the replication complex to move along the DNA strand as replication progresses. DnaC, the "helicase loader," assists in the delivery of DnaB to *oriC* and dissociates afterward to allow the activity of DnaB [37].

The initiation complex is part of a bigger protein complex known as the replisome, which is the molecular machinery responsible for faithful DNA replication in bacteria. The structure of the replisome is most studied in *E. coil* and -to a lesser extent- *Bacillus subtilis* [38]. For simplicity, only the replisome of *E. coli* will be further discussed. A detailed review contrasting the replisomes of *E. coli* and *B. subtilis* is available here [38]. Once the initiation complex is loaded onto *oriC*, DnaG, a primase, associates with DnaB and promotes the dissociation of DnaC leading to the assembly of the two replisomes to direct bidirectional replication [39]. DnaG continuously synthesizes RNA primers for the lagging strand and primes the leading strand once.

The central replication unit directly responsible for copying the DNA is polymerase III (Pol III). Pol III is a holoenzyme made of 10 different proteins that assemble into three functionally distinct units, and together, they work to synthesize and elongate the new DNA strand. Lastly, DNA synthesis is terminated at loci opposite to the *oriC* known as Ter sites. A protein called Tus recognizes Ter sites and halt the activity of the replisome, leading to the disassembly of the replisome and marking the completion of chromosome replication [40].

1.2.2 Bacterial cell wall division

Cell wall division in bacteria is carried by a division machinery known as the divisome [41]. Recruitment of the division proteins beings at the division septum as the tubulin homologue [42] and well conserved primary division protein FtsZ polymerizes, forming the Z ring [43] and initiating division assembly which later drives the constriction process that separates the two daughter cells [44]. Spontaneous polymerization of FtsZ occurs in the presence of guanosine 5'-triphosphate (GTP) [45]. FtsZ polymerization is a highly dynamic process with constant polymer synthesis and hydrolysis [44], eventually leading to the formation of the Z ring. The divisome proteins are recruited sequentially after Z ring formation in a two-step manner, with early arrival proteins working to stabilize the Z ring, followed by the recruitment of functional late divisome proteins [46-48]. Early divisome proteins include FtsA [49] and ZipA (unique to *E. coli* [50]), which are essential in anchoring FtsZ (and the Z ring) to the cell membrane. FtsA (or ZipA in *E. coli*) stabilize the FtsZ polymers as they join forming protofilaments [51] which are sometimes referred to as the proto-ring [52].

Several systems exist to correctly position the Z ring along the division septum [44, 53]. In *E. coli* and *B. subtilis*, a group of proteins collectively known as the Min system inhibits the incorrect localization of FtsZ polymers [54-56]. The Min system is an example of a negative spatial regulator of the Z ring localization that functions by inhibiting the polymerization of the Z ring at the cell poles and permitting it only midcell [53]. Another group of regulators known as positive spatial regulators localize and trigger the assembly of the Z ring directly at midcell and are found in several bacterial species [57-59].

Assembly of the proto-ring is essential for the recruitment of late phase of divisome proteins [46, 47], most of which function in chromosome segregation and peptidoglycan remodeling [53]. It was generally believed that the Z ring is the main force of constriction during cell division, especially after Osawa *et al.* demonstrated that FtsZ contained within liposomes polymerized forming Z rings that curved the liposomal membrane inwards [60]. However, recent studies are shifting the focus to peptidoglycan synthesis as the powerhouse behind constriction [61]. Hence, the constrictive capacity of FtsZ is now believed to be a result of the treadmilling dynamic of the Z ring, which guides the distribution of peptidoglycan synthases as they move around the division plane [62]. Treadmilling refers to the dynamic motion of the Z ring where new polymers are added at one end and depolymerize at the opposite end, creating the rotating movement of the ring [63]. Overall, current consensus is tilted toward peptidoglycan synthesis providing the constrictive force, while the role of FtsZ in constriction and peptidoglycan distribution differs between bacterial species [64]. In an attempt to explain these differences, an interesting report by McCausland *et al.* suggests that Z ring treadmilling guides the localization of peptidoglycan synthases through Brownian ratchet mechanism [65].

1.3 Bacterial plasmid replication

Aside from chromosomal DNA, bacteria carry extrachromosomal DNA elements called plasmids. Plasmids are found in many species in the three domains *Archaea, Eukaryota,* and *Bacteria [66]*. Unlike chromosomal replication, DNA plasmids are not essential for the host's survival. Yet, plasmids frequently contain genes that enable survival in adverse conditions, such as those containing antibiotics or toxins. Plasmids are advantageously free to be lost or altered through mutation, offering a genetic locus where genes could evolve faster than in the chromosome [67]. Plasmids enable horizontal gene transfer by their ability to spread from one host to another by conjugation, transformation, or transduction. Plasmids' distinctive properties make them unique components of the genome that can respond to selective pressure as seen by the recent expansion of microorganisms carrying antibiotic resistance genes.

Replication in plasmids starts from a specific region known as the origin of vegetative replication *(oriV).* Three main replication mechanisms for circular plasmids are known, theta, strand displacement, and rolling circle (RC) replication. Theta type replication is similar to chromosomal replication in that the leading and lagging strands are replicated with a discontinuous lagging strand synthesis. [68]. In this type of replication, double stranded DNA unwinds at the origin of replication, creating a replication bubble, that when viewed from above the structure resembles the Greek letter θ. Majority of the early investigations on plasmid replication were done on *E. coli* plasmids that were all found to replicate by theta type replication [69]. Most plasmids that replicate by theta replication need a protein called Rep

initiator that is encoded by the plasmid. Some theta replicons also require the host DNA polymerase I in the initial stages of leading strand synthesis.

During strand displacement replication, only one strand is replicated at a time. This type of replication produces a single strand of DNA, which is then duplicated to create double-stranded DNA. Strand displacement replication is linked with plasmids from the IncQ family that functions in a broad range of bacterial hosts [70]. To initiate DNA replication, IncQ plasmids encode three essential initiation proteins.

Plasmids that utilize the RC replication are small and range from 1.3 to 10 kb. They are mainly found in gram-positive bacteria, although they have been recently described in gram-negative bacteria and some archaea [71]. In RC DNA replication, an initiator Rep protein encoded by the plasmid nicks one of the parental DNA strands in the double-stranded DNA at the double-stranded origin (*dso*) [72]. This results in a 3' -OH end that is elongated using unnicked strand as a template. Replication continues around the circular plasmid, displacing the 5' end nicked strand. The displacement of the nicked strand is catalyzed by a host-encoded helicase called PcrA in the presence of the plasmid replication initiation proteins. The displaced DNA is made double-stranded by a series of Okazaki fragments and then it circularizes [72].

Some bacterial species have linear plasmids. Linear plasmids are found in both gram-positive and gram-negative bacteria. They belong to one of two types, those with a hairpin at each end and those with a protein covalently bound at the 5'end [73]. The replication of linear hairpin plasmids occurs through concatemeric intermediates, and the replication of linear

plasmids with covalently bonded proteins occurs by a protein-priming mechanism [74]. Linear plasmids are reviewed in detail by Hinnebusch *et al.* [75].

1.4 Caulobacter crescentus

Caulobacter crescentus is a dimorphic gram-negative aquatic a-proteobacterium that thrives in low nutrient environments [76]. The dimorphic cell cycle of *C. crescentus* begins as the parent cell divides asymmetrically, yielding two distinct cells (Figure 2). Stalked cells are sessile and replication-competent, while swarmer cells are flagellated, pileated, and replication-incompetent. Swarmer cells explore their environment until they differentiate into replication-competent stalked cells. During differentiation, swarmer cells anchor themselves to a surface and produce a remarkably strong polysaccharide adhesin, the holdfast. Swarmer cells then lose their flagellum and replace it with a cell wall and membrane protrusion known as the "stalk" [77].

E. coli and *B. subtilis* have both been studied extensively and provided significant insight into the biology of other bacteria. However, much remains unknown in the immensely diverse bacterial world, and more organisms need to be studied to fill the knowledge gaps. *C. crescentus* is a great example of a model organism. The asymmetric and elaborate cell cycle of *C. crescentus* drew a lot of attention around the 1970s [78]. Ever since, many research laboratories dedicated their effort to uncover the details of the different mechanisms employed by this organism to survive and reproduce. Namely, Dr. Lucy Shapiro's lab pioneered research efforts on *C. crescentus* and greatly aided our understanding of the organism [78].

Moreover, *C. crescentus* has another unique characteristic, synchronization, where swarmer cells can be isolated and studied separately as they undergo life cycle events, making it possible to biochemically or genetically study cell cycle in synchronized cultures [79].

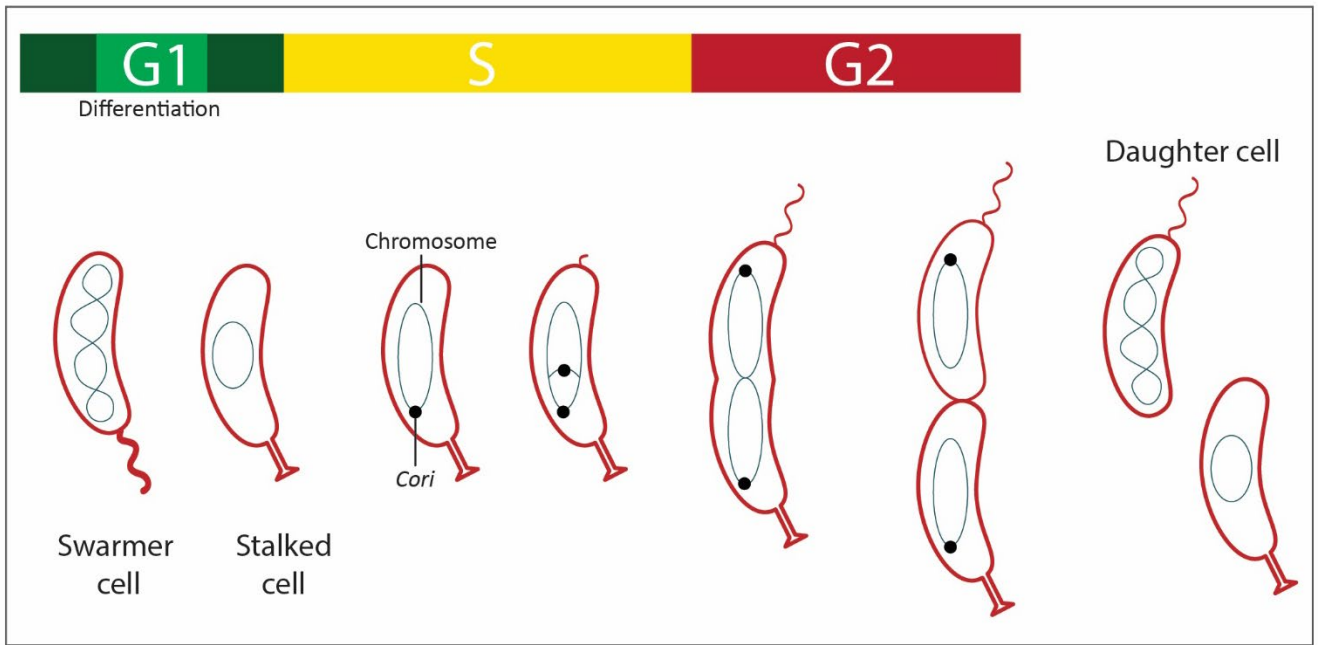

Figure 2: The cell cycle of Caulobacter crescentus

The B period (commonly referred to as G1 in *C. crescentus*) starts at cell birth after completion of the last division cycle and continues through cell growth. The C period (commonly referred to as S in *C. crescentus*) beings once chromosome replication is initiated and includes most of chromosome segregation. Flagellum biosynthesis begins mid-C period (S phase). Period D (commonly referred to as G2 in *C. crescentus*) begins at the end of chromosome segregation as the cell wall septum is formed. The D period concludes with the full cell division resulting in two daughter cells.

1.4.1 Cell cycle regulation in Caulobacter crescentus

The dimorphism and synchronization of *C. crescentus* allowed for the intensive investigation of signaling pathways underlying asymmetric division and cell cycle progression,

revealing the tight regulatory mechanisms underlying these processes [78]. These signaling pathways are thought to form functional cellular modules that complete specific tasks [80]. Lasker *et al.* further expanded the concept of modules, and cellular functions were attributed to different modules that completed these functions [81]. The modules are complex and integrative and can receive input from several other modules. Additionally, they are able to sense and respond to spatial and temporal signals. A detailed review of the modules and their function can be found here [81].

The master transcriptional regulators of the cell cycle: CtrA, GcrA, DnaA, SciP, MucR1, MucR2, and CcrM, constitute a cyclical genetic circuit that is responsible for majority of the crucial cell-cycle events in *C. crescentus* [81]. CtrA (cell cycle transcriptional regulator) is the core response regulator in a two-component signal transduction system that is involved in the regulation of many cell cycle events, including chromosome replication [82]. CtrA acts as a transcription factor [82] and a repressor of chromosome replication [83]. GcrA is a transcription regulator that activates the *ctrA* promoter and is negatively regulated by CtrA [84]. DnaA is the direct replication initiator that also functions in regulating transcription of cell cycle genes including CcrM [85] and GcrA [86]. SciP is yet another regulatory protein that inhibits the activity of CtrA by direct binding [87] or by blocking the transcription of ctrA or CtrA activated genes [88]. The paralogs MucR1 and MucR2 are ancestral zinc-finger transcription factors that represses *sciP* and CtrA activated genes, thereby preventing replication and allowing swarmer cells to maintain a non-replicative status [89]. CcrM is a DNA methyltransferase [90] that provides an added layer of regulation of *ctrA*'s promoter as it methylates GANTC sites on

hemimethylated DNA [91], making *ctrA*'s P1 promoter inaccessible, thereby negatively controlling *ctrA* expression [92].

1.4.1.1 Polar asymmetry in Caulobacter crescentus

Perhaps one of the major events dictating the outcome of asymmetric cell division is polar organization, where the internal organization at the "old" pole differs from that of the "new" pole [93, 94]. Since bacterial cells lack sophisticated organelles that carry specific functions, they depend on protein localization, where "anchor" proteins position themselves in target location and recruit functional proteins that work together towards a specific task, for example, cell wall division [95]. Protein localization was also described as one of the events contributing to the spatial architecture and asymmetry in *C. crescentus* [96]. Furthermore, it was recently shown that regulatory proteins (PopA in the presented study) equally distributed throughout the predivisional cell performed different functions depending on the polar end they interact with [97]. Generally, developmental regulators that determine polar differentiation are PleC, which is the major determinant of the flagellar pole architecture, and DivJ, the protein essential for polar differentiation of the flagellar pole in swarmer cells to a stalked pole [21]. Both proteins are members of a larger "asymmetry determination module" that drives polar differentiation by activating or degrading CtrA.

1.4.1.2 Replication initiation in Caulobacter crescentus

Like many bacteria, *C. crescentus* possesses a single chromosomal origin of replication (Cori) that is the target for the regulation of chromosomal replication [98]. *C. crescentus*

initiates DNA replication only once per cell cycle [99]. Swarmer cells initiate replication as they differentiate to stalked cells, while stalked cells initiate a new round of replication as cell division is nearing completion [100]. During replication, Cori is anchored to the stalked pole of the predivisional [101], and as the new chromosome is formed it is pulled towards the opposite pole of the cell (the pole that will form the flagellum) [102]. Plasmids containing Cori were shown to replicate at a similar time to the chromosome during the cell cycle implying that the elements of temporal regulation of replication are encompassed within Cori [100]. Chromosomal replication in *C. crescentus* is tightly controlled by the function of two master regulators, DnaA and CtrA. In addition to regulating the transcription of approximately 100 cell cycle associated promoters [103], CtrA is a negative regulator of replication that executes its function after being phosphorylated by recognizing and directly binding to five specific sites in Cori, thus, physically preventing the binding of DnaA [83].

Like the *oriC* of E. coil and other bacteria, Cori contains multiple DnaA boxes [104]. However, these DnaA boxes have unique features. First, the affinity of DnaA to Cori's DnaA boxes is weaker than that seen in *E. coli*. Second, Cori has two types of DnaA boxes, classified based on the binding affinity of DnaA. They are the moderate affinity G-boxes (two) and the weak affinity W-box (five) [104]. Once DnaA successfully binds to and initiates replication, the replisome (similar to the of *E. coli*) assembles and DNA is replicated bidirectionally [105]. As the new DNA strand is being synthesized, the replisome is pushed away from Cori and towards the opposite pole as it extrudes the new DNA strand until the replisome dissociates at mid predivisional cell after replication completion [105].

Chromosome replication is regulated by the controlled synthesis and degradation of DnaA and CtrA [106]. Their levels oscillate throughout the cell cycle by various redundant regulatory processes, with CtrA being most abundant in swarmer cells [82] and DnaA most abundant in stalked cells [86]. Temporal regulation of DnaA synthesis is achieved by several mechanisms, including trans-translation. Trans-translation is the primary mechanism used by bacteria to rescue stalled ribosomes and degrade their nascent dysfunctional polypeptides [107]. Trans-translation was shown to be necessary for the appropriate timing of *dnaA* expression [108]. Additionally, DnaA proteolysis is another method used to maintain correct levels of DnaA [109].

1.4.1.3 Chromosome segregation in Caulobacter crescentus

Chromosomal segregation is a highly regulated process in *C. crescentus.* Several proteins govern this process, including the ParABS system and structural maintenance of the chromosome (SMC) complex. A loss in any of these proteins inhibits cell division which ensures that daughter cells only inherit a full set of *Caulobacter* genome.

A DNA region close to C*ori* and resembles a centromere is found in most bacteria, including *C. crescentus* and is called *parS* [110]. It is the first DNA locus to be segregated following chromosome replication, and it exists at one of the cell's poles known as the "old pole" [111, 112]. The process of chromosome segregation starts when a DNA-binding protein called ParB nucleates on *parS* and then recruits additional ParB molecules to form a network of protein-DNA complexes [113]. Although the complete functions of this complex are not yet known, it seems to be a requirement for faithful chromosome segregation evident by the death or

increased number of anucleate cells in *parB* defective mutants that are capable of nucleation but not spreading [114-116].

The ATPase activity of another protein, ParA is activated by the ParB-DNA nucleoprotein complex creating a ParA-ATP gradient that propels the original-proximal region of the chromosome to the opposite pole of the cell [117, 118]. This movement reorients the entire chromosome to the opposite pole [119]. In this process, ParA produces dimers that strongly bind DNA, and ParB stimulates ATP hydrolysis releasing ParA-ADP monomers [118]. Additionally, ParB promotes the individualization of replicated chromosomes by recruiting structural maintenance of chromosome (SMC) complex, which reduces DNA entanglement [120].

The movement of the chromosome to its destination is guided by the two polar proteins, PopZ and TipN. PopZ tethers one of the sister chromosomes at the old pole by directly binding ParB [121, 122]. While ParB-DNA complex is moving to the new pole, PopZ accumulates at that pole and captures the moving complex [121, 122]. The shift in PopZ localization from unipolar to bipolar is a crucial step in coordinating the initiation of chromosome segregation with the FtsZ ring [123]. Anchoring ParB-DNA complex at the two poles by PopZ stabilizes MipZ (an inhibitor of FtsZ polymerization), which supports FtsZ assembly close to midcell where the activity of MipZ is at its lowest [123, 124]. However, TipN serves as a birthmark identifying the new pole as it localizes there, directing the placement of new-pole markers like the flagellum [125]. Like PopZ, TipN also influences the localization of ParA and impacts the movement of ParB-DNA complex [126].

1.4.1.4 Cell division in Caulobacter crescentus

Like E. coil and many other bacteria, FtsZ is the main cell division protein that directs the divisome assembly and cell wall division in *C. crescentus* [127]. Although FtsA is necessary for the correct functioning of the divisome, it is recruited to the division plan after Z ring formation, unlike in *E. coli*, where it is essential for anchoring FtsZ [128]. Instead, in *C. crescentus,* FzlC tethers FtsZ to the membrane as the Z ring forms [128]. Over a dozen proteins with functions in Z ring anchoring and peptidoglycan synthesis, remodeling, and elongation are recruited to the site of division [129]. One of those peptidoglycan remodeling proteins is DipM [130], a LytM endopeptidase that is critical for the cell wall invagination in *C. crescentus* [131]. Studies Priya Patel unexpectedly implicated DipM in chromosome replication, as described further below.

As with other processes, *C. crescentus* utilizes elaborate mechanisms to coordinate cell wall division with other cell cycle stages like replication that make use of the bacterium's master regulators [132]. For instance, CtrA represses the transcription of early arriving FtsZ (*ftsz*) while simultaneously repressing DNA replication [133]. Additionally, stalled DNA replication was shown to inactivate CtrA and subsequently late arriving divisome proteins FtsQ & FtsA (*ftsQA*), delaying cell wall division [134]. Additionally, *ftsZ* transcription is also regulated epigenetically by the master regulators CcrM and GcrA [132], where the methylation of the *ftsZ* promoter by CcrM is necessary for its transcription [135] and allows for site recognition by GcrA, which then recruits Pol III [136].

C. crescentus does not use the Min system to control the localization FtsZ at the division site. Instead, it uses MipZ, another essential protein that coordinates DNA segregation by

interacting with ParB and simultaneously preventing the polymerization of FtsZ at the cell poles and permitting it only at midcell, where MipZ concentrations are at their lowest [124]. MipZ is also under a similar cell cycle-dependent control. The same regulatory circuit involving CcrM and GcrM and is involved in controlling the transcription of *ftsZ* also regulates the transcription of *mipZ* [135, 136].

1.4.2 Genomics of Caulobacter crescentus

To truly understand the physiological processes dictating the morphological fate of *C. crescentus*, it is necessary to examine the genetic makeup of this bacterium. In 1977, Bert Ely and Reid C. Johnson took the first step towards revealing the genetic makeup of *C. crescentus* by testing the transduction capacity of two bacteriophages, Cr30 and Cr35, on the bacterium [137, 138]. Soon after, Barret *et al.* constructed the first genetic map of *C. crescentus*. Then in 2001, the complete 4 million bps genome of *C. crescentus* CB15 was sequenced and annotated [139]. Genetic studies of *C. crescentus* were further facilitated by the introduction of a set of plasmids and expression vectors by the Shapiro lab [140], which remains as valuable tools used until today. Most recently, a CRISPR interference system for *C. crescentus* was developed by the Laub lab [141], marking another valuable addition to the existing toolbox that will allow the navigation of previously uncharted territories.

Genomic studies of *C. crescentus* are constantly reshaping our understanding. In fact, even before the genome sequencing of *C. crescentus* was completed, preliminary sequencing data was used to discover the presence of a tmRNA system in α-proteobacteria, negating the previous belief that a tmRNA system does not exist in α-proteobacteria [142]. Moreover,

genome studies comparing DNA repair genes in *C. crescentus* and *E. coli* shed light on the possible range of mechanisms utilized by bacteria to repair their DNA [143]. Additionally, a comparative study was performed on the two commonly studied *C. crescentus* strains, CB15 and its derivative NA1000 [144]. From an evolutionary angle, this study revealed that the differences seen between the two strains was a result of five polymorphisms. From here, it is apparent that genomic studies of *C. crescentus* are an important venue to explore while studying this microorganism.

1.5 Plasmid replication and maintenance in Caulobacter crescentus

1.5.1 The repABC operon

There is extensive research on chromosome dynamics in *C. crescentus*, yet studies investigating plasmid replication and maintenance in this bacterium are limited. The majority of Caulobacter genomes that have been sequenced and uploaded to the GenBank database lack plasmids, however, some plasmids have been found, including those in the CB4, K31, FWC26, and FWC2 strains [145].

A recent study investigating *C. crescentus* plasmids have identified a total of nine different plasmids from 70 *Caulobacter* genomes (available on Genbank before October 2020) in 6 strains [145]. The identified plasmids are all large or medium sized plasmids and there are no small sized plasmids reported in *Caulobacter* to date. All identified plasmids had the *repABC* genes common to α-proteobacteria except for K31p2 plasmid which had *repA* and *repB* genes only [145].

The *RepABC* is an operon that contains at least three genes *repA, repB, and repC* that code for proteins involved in DNA replication and plasmid partitioning [146]. RepA and RepB are proteins encoded by *repA* and *repB* respectively and are essential for plasmid segregation while RepC encoded by *repC* is essential for the initiation of plasmid replication [146]. The proteins RepA and RepB belong to the families of ParA and ParB, partitioning proteins that regulate partitioning in low copy number plasmids and in chromosomes [147].The two proteins partake in partitioning and in the negative transcriptional regulation of their own operons [148]. Mutations in either *repA* or *repB* genes result in a reduced plasmid copy number [149].

RepC type proteins are exclusively found in alpha-proteobacteria and they function by binding to the origin of replication that is located within the *repC* sequence [146]. The origins of replication that need RepC do not contain iterons, which are repeated sequences that are present in the origins of many types of plasmids and operate as binding sites for replication initiators [150]. Plasmids containing initiator proteins that bind iterons usually recruit host-DnaA following binding creating a replication bubble which recruit host DNA helicase that is bound to the loading factor (in *E. coli* these are DnaB and DnaC) [151]. There are no DnaA-binding motifs in repC that correspond to the alphaproteobacterial consensus sequence [152]. However, it is possible that RepC accomplishes DnaB-DnaC recruitment, either directly or by recruiting DnaA [35].

1.5.2 RK2 plasmid

RK2 plasmid is a broad-host range plasmid that belongs to the incP1α incompatibility group and it is a theta type replicating plasmid [153]. It is unique in its ability to replicate stably

in a wide range of gram-negative bacteria [154]. RK2 requires host initiator proteins and the plasmid encoded TrfA protein to initiate DNA replication at the *oriV.* Host initiators required by RK2 differ based on the species of bacteria. In *E. coli* DnaA is required for initiation while in other species like *P. aueroginosa* a DnaA-independent mechanism for plasmid replication initiation is used [155, 156]. The TrfA protein encoded by the plasmid is generated in two forms, a 33 kDa (TrfA-33) form and a 44 kDa (trfA-44) form [157, 158]. Both forms recognize and bind to iterons at *oriV* [159].

C. crescentus is a host of the RK2 plasmid. An early study cells using quantitative dot plot hybridization to study RK2 plasmid partitioning in *C. crescentus* showed that the plasmids partitioned equally in daughter cells [160]. However, a recent study reported that in *C. crescentus* RK2 exhibits an asymmetrical distribution of plasmid foci in the stalked and swarmer compartments prior to cell division resulting in an incorrect distribution of plasmids in progeny [161]. The distribution of plasmid foci within the cell was observed using fluorescent microscopy. Asymmetrical plasmid distribution in daughter cells could explain the lower stability of RK2 plasmid in *C. crescentus* compared to its' stability in *Pseudomonas* and *E. coli* [162, 163].

To add, in *C. crescentus* RK2 TrfA binds *oriV* during the G1 phase (Figure 2) but plasmid replication mainly occurs in the S phase of the bacterial cell cycle owing to higher availability of host replication proteins [161]. Wegrzyn *et al.* also report RK2 replication initiation in *C. crescentus* to be DnaA-independent evidenced by the ability of plasmids lacking DnaA-box to replicate in the presence of TrfA-44. However, stability tests of RK2 derivate encoding only TrfA-33 suggested that a DnaA-dependent plasmid replication is also possible in *C. crescentus* [161].

Introduction, rationale, and research objectives

The Marczynski lab is one of the many labs worldwide that provided many answers about the bacterial cell cycle coordination, specifically in the context of replication, by utilizing the model bacterium *C. crescentus*. In this thesis, we focus on developing a new method to discover a new class of regulatory proteins coordinating chromosomal replication and cell wall division. To find such novel regulators, we modified our original molecular screening technique that was designed to select for mutants that upregulate transcription at Cori.

The original molecular technique employed reporter *C. crescentus* strains that were developed in-house by inserting a neo reporter gene downstream from Cori's strong transcription promoter. Mutant strains with increased activity at Cori thus display higher resistance to neomycin and kanamycin. Mutants that survive selection on high and otherwise lethal kanamycin concentrations are also tested for growth at higher temperatures. An early study demonstrated how studying temperature-sensitive (TS) mutants can answer questions regarding the coordination between the cell cycle and other developmental events in *C. crescentus* [164]. Similarly, we used temperature-sensitivity, along with other parameters to develop the molecular screen. Positive TS tests where mutant strains struggle or fail to grow at 37 °C points at mutations residing in protein-coding genes. Applying this technique led our group to unexpectedly identify a point mutation in *dipM* [165].

According to previous studies, dipM is a periplasmic cell wall remodeling endopeptidase that plays a role in cell division [130, 166]. Work from our lab has linked dipM to DNA replication coordination [165]. These findings inspired our group to utilize the observations

collected about dipM mutants and use them to update our molecular-genetic screen. *dipM* mutants had two consistent phenotypes: 1) they were temperature sensitive, and 2) they could not support plasmid replication. Due to DipM's role in cell division and novel implication in replication, we hypothesised that DipM in the periplasmic space communicates with Cori in the cytoplasm by a pathway of intermediate components. Pathway mutations should have similar *dipM* phenotypes. Accordingly, we included tests for plasmid maintenance in the molecular screen and added a WT *dipM* gene to the reporter strain to account for functional loss of DipM and prevent the isolation of *dipM* mutants. These modifications allowed the detection of other proteins that may work in conjunction with DipM to coordinate between DNA replication and cell division.

To test our hypothesis, we pursued the following aims:

1) Generate mutants with altered chromosome replication and plasmid replication.

2) Identify the mutations found in aim 1 by genome sequencing and gene library complementation

3) Confirm the mutations found in aim 2 and determine the effectiveness of the new screen

Methods

Bacterial strains and growth conditions

Bacterial cells were cultured on peptone-yeast extract (PYE) agar plates with or without antibiotics and incubated at 28 ^{o}C for at least 48 hours prior to each experiment. Plasmids were propagated in *E. coli* S17-1 and were cultured in Luria Bertani (LB) agar plates. Liquid cultures were incubated at normal growth permissive temperatures for *C. crescentus* (28 ^{o}C) or *E. coli* (37^{o}C) for 24hrs at 200 rpm. Antibiotic concentrations in agar plates were as follows (PYE, LB): kanamycin (km 20 µL/mL, 50 µL/mL), Streptomycin (Strep 1.5 µL/mL, 30 µL/mL), Chloramphenicol (Cm 1.5 µL/mL, 15 µL/mL), Nalidixic acid (Nx 20 µL/mL, NA), Ampicillin (Amp 40 µL/mL, 100 µL/mL), and tetracycline (Tet 2 µL/mL, 15 µL/mL). Half the concentrations used to prepare respective agar plates were used when culturing in liquid media.

Bacterial mutagenesis and screening

C. crescentus mutants were prepared through spontaneous mutagenesis or ultraviolet (UV) mutagenesis. During spontaneous mutagenesis, reporter strains were grown in the same PYE culture media for seven days. During UV mutagenesis, 1 mL of overnight liquid culture was subjected to shortwave (254 nm) UV radiation for 20, 40, or 80 seconds. Cells were recovered by the addition of 2 mL of fresh PYE media followed by an incubation period of 2 hrs at 28^{o}C at 200 rpm. Cells were then collected in 1.5 mL eppendorfs by centrifugation and resuspended in 200 µL of fresh PYE media. To screen for mutants of interest, 100 µL of bacteria were spread on high PYE-Km plates at concentrations of: 200 µg/mL, 250 µg/mL, and 300 µg/mL. Bacterial spreads were incubated at 28^{o}C for 3-5 days. After which, replica printing was used to

subculture highly kanamycin-resistant colonies on PYE plates, PYE-Nx plates, and PYE-Cm plates for an additional 3-5 days. All candidate mutant strains were streaked for isolated colonies on PYE plates. These pure cultures were then retested on the PYE-Nx and PYE-Cm plates. A single colony from each mutant candidate was grown in a PYE liquid media prior to storing them at -80^{o}C. To store cells, 900 µL of overnight liquid cultures were mixed with 100 µL DMSO (10%) in 1.5mL cryogenic tubes and stored at -80^{o}C. Lastly, mutants were tested for temperature sensitivity by simultaneously incubating duplicate cultures at 28^{o}C and 37^{o}C and observing for growth defects at higher temperatures.

Bacterial transformation

Conjugation

Bacterial strains were transformed with desired vectors and plasmids by conjugation with S17-1 donor *E. coli* strains. Equal volumes of recipient *C. crescentus* strain and donor *E. coli* strain were mixed in a PYE plate and incubated for 18-24 hours at 28^{o}C. After which, the cell mixture was spread on selection plates containing two antibiotics: Nalidixic acid or Ampicillin (to kill *E. coli*) and a plasmid selection antibiotic. Selection plates were incubated at 28^{o}C for 3-5 days.

Electroporation

Electroporation was used to transform competent *E. coil* DH5α. All materials used for this experiment were chilled on ice, including plasmid DNA (pDNA), competent cells, and electroporation cuvettes. To transform *E. coli*, 10 ng (preferably in only 1 µL) of pDNA resuspended in distilled H_2O or low EDTA (0.1 mM) TE buffer was added to 50 µL of

concentrated competent cells and mixed by pipetting. The entire volume is transferred to a 2 mm electroporation cuvette that was wiped and completely dried from water and condensation. The cuvette is placed in the electroporation apparatus and the cells are pulsed with 2.7 kV. To recover cells, 1 mL of SOC media (super optimal broth) was quickly added to the electroporated cells, and the entire volume was transferred to a 15 mL tube that was incubated at 37°C in the shaker for 1 hr. Cells were then diluted at 1:100, 100 µL were spread on appropriate LB selection plates, and cultures were incubated at 37°C for 24-48 hrs.

Preparation of electroporation competent DH5α cell

Electroporation competent *E. coli* DH5α cells were used to create a *C. crescentus* gene library. Cells were prepared for transformation by first growing a 5 mL *E. coli* culture in LB media overnight. Next, bacteria were sub-cultured in 150 mL LB media at 37°C with shaking until the culture's OD was between 0.5-0.6 (requires approximately 3 hrs). Bacteria were then cooled down quickly by swirling the culture in an ice bath and concentrated by spinning the culture in 50 mL conical tubes at 4°C at 4000 RPM for 20 minutes. Culture media was decanted, and cells were washed twice with prechilled 2% glycerol and once with prechilled 10% glycerol. Cells were then resuspended in the small volume left after the last wash. 50 µL of concentrated cells are then aliquoted into prelabeled and prechilled eppendorf tubes, flash-frozen in a -80°C 100% ethanol bath and stored at -80°C.

DNA extraction

Cell lysis – Genomic DNA preparation

5 mL PYE cultures of WT *C. crescentus* (#3637) grown overnight were pelleted and resuspended in 200 µL EDTA-saline (0.01 M, 0.15 M, pH 8) solution. To lyse the cells and denature RNA, 100 µL of lysozyme (10mg/mL) solution and 7 µL of RNase A were added to the resuspended cells and incubated for 1 hr at 37^{o}C. Bacterial proteins were denatured by adding 80 µL of 20% SDS, vortexing, and the subsequent 10-minute at 65^{o}C incubation. 200 µL of 5 M sodium chloride were then added to the lysate followed by thorough mixing (vortex) for 15 seconds. The lysate was spun at maximum speed for 1 minute and phenol/chloroform extraction was performed.

Cell lysis – Plasmid DNA

Cells were pelleted in a similar manner to the cells lysed for genomic DNA (gDNA) extraction. The pellet was resuspended in 100 µL GET (50 mM glucose, 10 mM EDTA, and 25 mM Tris-HCl, pH 8.0) solution followed by the addition of 200 µL lysis buffer (1% SDS in 0.2 M NaOH), gentle mixing by inversion, the addition of 200 µL 10 M ammonium acetate, and a final step of gentle inversion. Cell lysate was spun for 15 minutes at maximum centrifugation speed and the supernatant was collected and transferred to fresh 1.5 mL eppendorf tubes. Lastly, phenol/chloroform extraction was performed to collect pDNA.

DNA extraction and alcohol precipitation

Genomic and pDNA were extracted using phenol/chloroform extraction. 200 µL of phenol:chloroform:isoamyl (25:24:1) was added to the cell lysates and vortexed for 15 seconds. Extraction lysate was spun at maximum centrifugation speed for 15 minutes at room temperature. The aqueous phase (top layer) was carefully collected and transferred to fresh 1.5

mL eppendorf tubes. If remnants of a white pellet were observed in the organic phase (bottom layer), phenol/chloroform extraction is repeated. To precipitate DNA from the aqueous solution, 1/10 volumes of 3 M sodium acetate and 2.5 volumes of 100% prechilled ethanol were added to the eppendorf and the solutions were incubated at -20^{o}C overnight. DNA was then pelleted by centrifugation at maximum speed for 15 minutes at 4^{o}C. Supernatant was decanted and 500 µL of prechilled 75% ethanol was added and the eppendorf was shaken gently to wash the DNA pellet. Supernatant was spun for 5 minutes at 4^{o}C, and the washing steps are repeated twice. Finally, the pallet is allowed to air dry for 60-90 minutes and is then resuspended in the desired volume of low TE (10 mM Tris, 0.1 mM EDTA) buffer.

DNA quantification

Nanodrop 2000 (Thermo scientific, USA) was used to measure DNA quantity and purity. Additionally, to verify gDNA quantity prior to genome sequencing and ensure absence of contamination by UV absorbing material, gel images obtained after agarose gel electrophoresis of gDNA samples were processed using imageJ [167]. DNA concentration was determined using imageJ by measuring the intensity of the bands on the gel and comparing them to the GeneRuler 1 Kb Plus DNA ladder (Thermo Scientific, USA) reference. Both methods gave similar final concentrations of DNA with a difference ranging between 10 – 20 ng. Lastly, all samples were diluted in low TE buffer to a concentration of 50 ng/µL.

Gel electrophoresis

1% agarose gel was prepared by dissolving 1 gram of agarose powder in 100 mL 1X Tris-Borate-EDTA (TBE) buffer (by microwaving). 5 µl of 10 mg/mL Ethidium bromide was added to

the dissolved agarose solution and mixed well. The solution was poured into the gel cast, a comb was applied, and the gel was allowed to solidify. After solidification, the gel was placed in an electroporation chamber that was filled with 1 L of 1X TBE buffer and appropriately connected to a power source. The gel was run for 1.5 hrs at a voltage of 97 V and images were taken of the gel using a Gel-Doc (Bio-Rad, USA).

Whole Genome Sequencing (WGS)

Whole-genome sequencing services were provided by the University of Guelph's Advanced Analysis Center [168]. In short, illumina DNA libraries were prepared by fragmenting gDNA and tagging the fragments with adapter sequences as described by illumina [169]. Prior to sequencing, clusters are created by amplifying each DNA fragment using the bridging polymerase chain reaction (BPCR) [170]. Finally, paired-end DNA sequencing with a coverage of 100x was performed (illumina MiSeq) and illumina reads were analyzed using the bioinformatic pipeline described below (Figure 3).

Bioinformatic analysis

Short sequencing reads generated from illumina sequencing were used to reconstruct bacterial genomes. MobaXterm is a remote computing tool that was used in conjunction with Compute Canada to perform tasks requiring high computing efficiency. *C. crescentus* NA1000 reference genome was downloaded from GenBank's assembly library under the identifier ASM2200v1 [171]. Initially, the *C. crescentus* reference genome was indexed using Burrows-Wheeler Aligner (bwa) software package to facilitate computation during analysis. Using the same bwa package, the short reads were then aligned to the reference genome. Further post-

alignment analysis was done using samtools to generate BAM files that are manageable in size and allow for sorting, ordering, and viewing of aligned reads. Variant calling to identify single nucleotide polymorphisms (SNPs) and mutations was performed using Varscan and the newly reconstructed bacterial genomes were annotated in Galaxy using SnpEff Build & SnpEff Eff. Last, mutations were ranked based on the quality of the reads and the effect of the mutation on the protein. Gene mutations with the most severe effects are selected for further analysis.

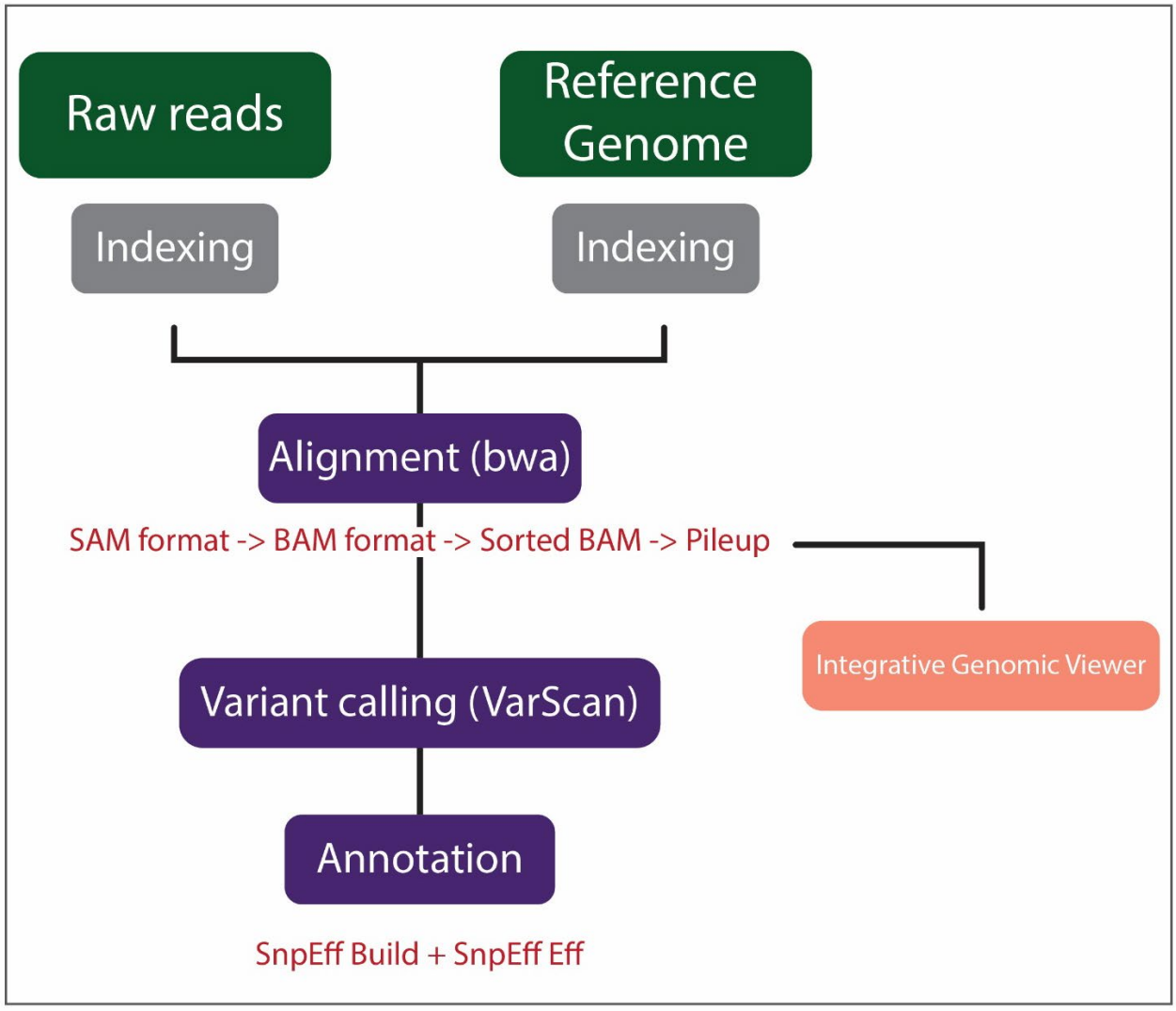

Figure 3: Bioinformatics pipeline used to analyze gDNA data.

Illumina sequencing reads and the reference genome were indexed prior to the analysis. BWA & SAM tools were used to align the reads to the reference genome. Aligned and sorted genomes can be visualized using Integrative Genomic Viewer (IGV) software. Lastly, variants are identified (Varscan) and annotated (SnpEff Eff).

WT Caulobacter crescentus gene library construction

Attempts were made to construct an in-house *C. crescentus* gene library using inserts from WT *C. crescentus* genome (Figure 4). Briefly, gDNA was extracted from WT *C. crescentus* (#3637) and digested using SalI (NEB, USA) or XhoI (NEB, USA) to prepare genomic inserts. pBXMCS-6-Cm-R vectors were linearized using SalI and treated with Shrimp Alkaline Phosphatase (SAP) to prevent self-ligation. gDNA inserts were mixed with linear pBXMCS-6-Cm-R vectors in the presence of ligase (ABclonal, USA) and incubated at RT for 5 minutes as per the manufacturer's instruction. pBXMCS-6-Cm-R vectors containing *C. crescentus* genome inserts were used to transform *E. coli* DH5-α through electroporation. To evaluate the quality of the gene library created, transformed DH5-α colonies were cultured in liquid LB-Cm media overnight. Single colonies were then cultured in liquid LB-Cm media overnight and their plasmid content were extracted, digested, and visualized through gel electrophoresis.

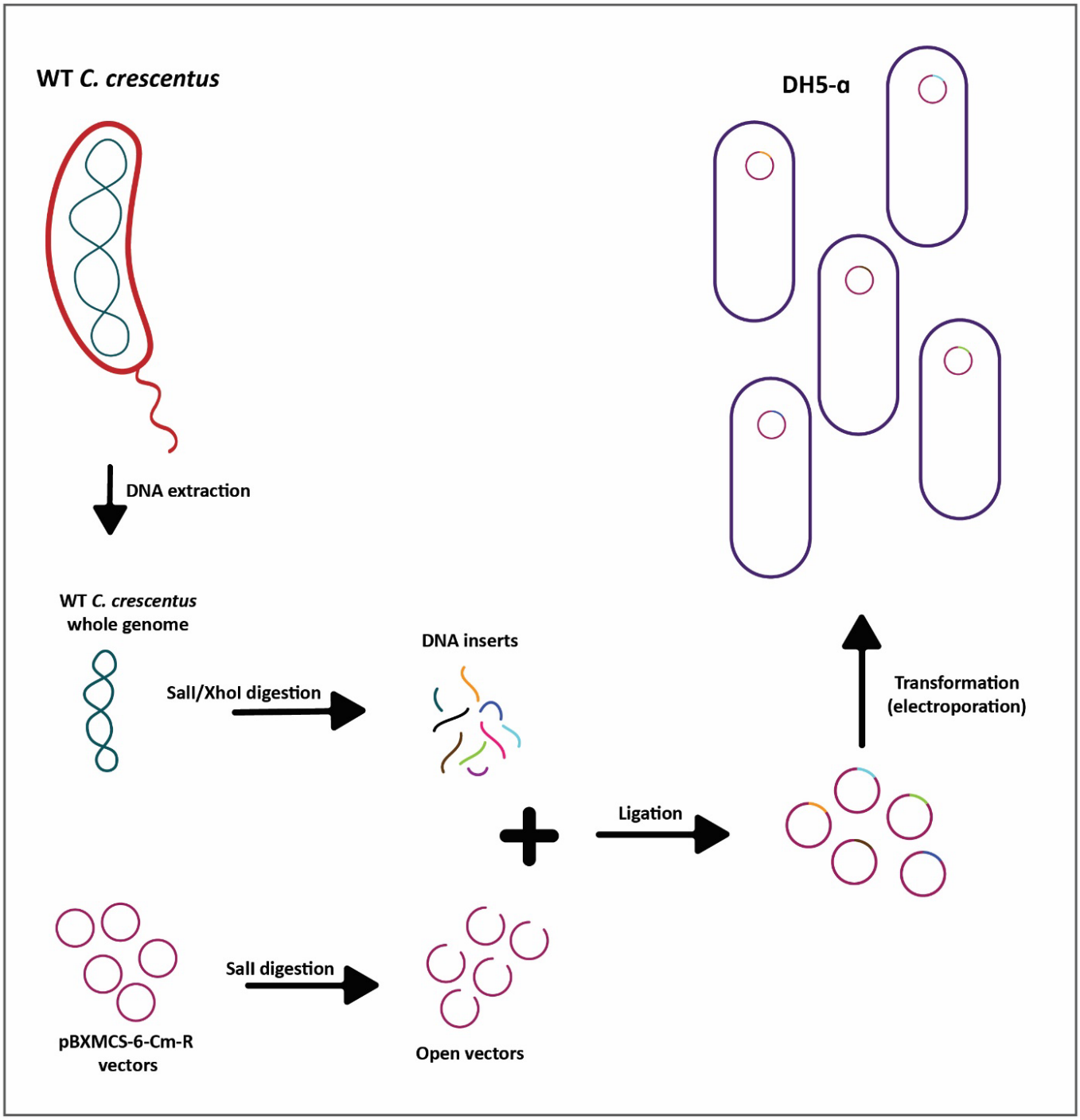

Figure 4: Construction of an in-house *C. crescentus* library.

WT C. crescentus gDNA is used to create DNA inserts that are then ligated into pBXMCS-6-Cm-R vectors. The recombinant DNA constructs are used to transform E. coli DH5-α cells through electrophoresis.

Strain	**Genetic background**	**Source**
#3637	Wildtype *C. crescentus* strain NA1000	Marczynski lab
#3636	Cori Ps::neo(+) strain with a neo gene without a promoter integrated downstream of Cori's strong promoter in the same direction	Marczynski lab
#4901	Strain #3636 transformed with a pBXMCS-6-dipM-CM-R plasmid	This study
#4907	Strain #3636 transformed with integrating plasmid dnaN-pYFPC-1 (NdeI-EcoRI) - Strep/Spec-R	This study

#4910	Mutant strain derived from strain 4901 by spontaneous mutagenesis	This study
#4915	Mutant strain derived from strain 4907 by UV mutagenesis	This study
#4926	Mutant strain derived from strain 4901 by UV mutagenesis	This study
Plasmids		
pBXMCS-6-Cm-R (#4717)	High copy replicating plasmid derived from pBBR1MCS. Chloramphenicol resistant.	Marczynski Lab
pBXMCS-6-dipM-Cm-R (#4709)	pBXMCS-6-Cm-R plasmid containing a WT dipM gene. Chloramphenicol resistant.	Marczynski Lab
dnaN-YFP (NdeI-EcoRI) (#4859)	WT dnaN gene fused with yellow fluorescent protein – Spec/Strep resistant.	Reyes Lab

pKK838 (ssrA/tmRNA) (#4927)	High copy pJS14 derivative (pBBR based) with complete ssrA and promoter. Chloramphenicol resistant.	Keiler Lab
A13 cosmid (#2466)	pRK290LacZ derived cosmid containing DnaJ. Tetracycline resistant.	Marczynski Lab
pLAFR-5 vector (#2470)	pLAFR-5 vector. Tetracycline resistant.	Marczynski Lab

Table 1: Strains and plasmids used in this study

Results

Generation and selection of mutants of interest

To find novel mutations that resembled *dipM* mutations, we used the following reporter strains: Strain #3637 is WT *C. crescentus* strain NA1000. Strain #3636 contains a *neo* gene reporter at Cori's strong promoter and detects increased initiation activity at Cori. Strain #4901 is a plasmid non-supporting (PNS) reporter derived from #3636 and contains plasmid pBXMCS-6-dipM-Cm-R (#4709). Strain #4907 was derived from #3636 by replacing WT *dnaN* with *dnaN-YFP* using integrating vector dnaN-PYFPC-1 (#4859) and introducing replicating plasmid pBXMCS-6-dipM-Cm-R (#4709).

Mutants were generated through either spontaneous or UV mutagenesis, with the majority resulting from UV manipulations. Preliminary selection of highly kanamycin-resistant colonies resulted in the isolation of approximately 100 mutants. Kanamycin resistance is an indirect indicator of increased activity at Cori, suggesting that all mutants isolated have higher levels of chromosome replication initiation, hitting at an alteration in replication regulation. In spontaneous mutagenesis, the culture is grown to saturation, and cells are allowed to accumulate mutations for two to three days. This technique increased the number of colonies detected on PYE-Km plates after mutagenesis. Replica printing was used to test surviving kanamycin-resistant colonies by printing them on PYE-Nx plates to test for *dipM*-like cell pole defects, and PYE-Cm plates to test for PNS phenotypes. Previously, we reported that *dipM* mutants displayed higher sensitivity to nalidixic acid by altering the cell pole localization protein TipN [165]. Therefore, we incorporated a test for nalidixic acid resistance. Mutants of interest

were narrowed down by selecting chloramphenicol-sensitive mutants. Mutants that lose their ability to maintain plasmids also lose the chloramphenicol resistance gene (*cat*) encoded in the pBXMCS-6-dipM-Cm-R plasmid that was originally present in the reporter strains (#4901 & #4907) prior to mutagenesis. To confirm PNR phenotypes are true phenotypes and not a result of spontaneous plasmid loss, mutants were transformed with pBXMCS-6-dipM-Cm-R or pBXMCS-6-Cm-R plasmids by conjugating them with *E. coli* S17-1 stains #4717 and #4709 respectively. Only mutants that displayed either complete or partial loss of their ability to maintain plasmids, by failing or struggling to grow on PYE-Cm plates, were selected for further analysis.

This screening process ultimately led to the selection of three mutants of interest, MUT1, MUT2, and MUT3 (Table 2). MUT1 failed to grow on PYE-Cm plates even after conjugation with *E. coli* S17-1 stains #4717 and #4709. Indicating two major observations: 1) MUT1 cannot maintain pBBR-1 plasmids, and 2) WT *dipM* does not reverse this defect. MUT2 on the other hand weakly grew on PYE-Cm plates, despite conjugation with *E. coli* S17-1 stains #4717 and #4709. MUT2 also seemed to have had a cell division defect -that is not shown here- where cells failed to divide resulting in long filamentous morphology under the microscope. MUT2 was also strongly temperature-sensitive, completely failing to grow at 37 ^{o}C. Similar to MUT1, MUT3 failed to grow on PYE-Cm plates after conjugation with *E. coli* S17-1 stains #4717 and #4709. Therefore, leading to the same conclusion that MUT3 cannot maintain pBBR-1 plasmids, and WT *dipM* does not reverse this defect.

Strain	Kanamycin resistance	Support Plasmid replication	Chloramphenicol sensitive	Temperature -sensitive	Morphology
4901 (WT)	Moderate	Yes	No	No	WT
4910 (MUT1)	High	No	Yes	No	WT
4915 (MUT2)	High	Partially	Weak	Yes	Filamentous
4926 (MUT3)	High	No	Yes	No	Larger, wider cells

Table 2: Properties and phenotypes of mutants selected for further analysis

Identification of gene mutations in mutants of interest

Whole-genome sequencing (WGS) and bioinformatic analysis led to the identification of a total of six mutations across MUT1, MUT2, and MUT3 (table 4). After the final VCF file was generated by following the workflow discussed in Figure 3, variants were filtered based on their Genotype quality (GQ), allele frequency (FREQ), and the impact effect of the variant. All mutants shown in table 3 have the maximum GQ value of 255, an FREQ level of 100%, and an impact effect of “modifier” (in MUT1), “moderate” (in MUT3) or “high” (in MUT2). See table 3 for more information about impact effects.

Impact	Meaning
High	The disruptive impact of the variant on the protein is predicted to be high, likely rendering it dysfunctional.
Moderate	The variant is unlikely to disrupt protein function but may interfere with its effectiveness.
Modifier	It is difficult to predict the disruptive effect of the variant since it is in a non-coding gene. OR the algorithm could not detect evidence of impact.

Table 3: Types of variant impact and their meanings. ***Adapted from [172]***

MUT1 has a base substitution mutation in the acceptor RNA component of *Caulobacter's* two-piece tmRNA that is encoded by the *ssrA* gene. MUT2 had a frameshift mutation in an uncharacterized LPS biosynthesis protein. MUT3 had four missense mutations in DnaJ, SpoT, Aminobenzoyl-glutamate utilization protein B, and major facilitator superfamily transporter. To narrow down the selection of gene mutations to study further, we took a closer look at the known functions of the mutated genes. Based on their published connection to the cell cycle progression, we selected *ssrA* in MUT1 and *dnaJ* in MUT3 for further investigation.

Variant	Gene ID	Gene name	Mutation	Gene Function
MUT1	CCNA_R0049	ssrA	n.2338560G>A	Codes for tmRNA in *C. crescentus*. tmRNA releases stalled ribosomes and

				degrades their incomplete products [142]
MUT2	CCNA_00667 (used to be CC_0631)	lipopolysaccharide biosynthesis protein (wbqL)	p.Val118fs	Poorly characterized capsular polysaccharide biosynthesis protein that affects curvature by altering the function of crescentin [173].
MUT3	CCNA_00011	*dnaJ*	p.Phe260Cys	Codes for chaperon/heat-shock protein DnaJ which functions in protein maintenance and refolding [174]
MUT3	CCNA_01622	*spoT*	p.Ser485Ala	Codes for SpoT, a (p)ppGpp synthase/hydrolase with an essential function during nutrient (carbon) starvation [175]

MUT3	CCNA_01625 (used to be CC_1556)	Aminobenzoyl-glutamate utilization protein B	p.Thr492Ala	An Amidohydrolase, part of the M20 Proteinases family [176]
MUT3	CCNA_03165 (used to be CC_3069)	Major facilitator family transporter	p.Phe266Cys	An integral transmembrane transporter [176]

Table 4: Gene mutations present in MUT1, MUT2, and MUT3

Genetic complementation and restoration of WT phenotypes in mutant C. crescentus strains

To investigate the causal relationship between mutations in *ssrA* and *dnaJ* and the failure of mutants *to* maintain plasmids, genetic complementation of MUT1 and MUT3 was performed using WT *ssrA* and *dnaJ* genes respectively carried on replicating plasmids. MUT1 restored its capacity to maintain plasmids when complemented with WT *ssrA* (carried on the pBBR replicon pKK838) as evident by growth on selective media (Figure 5). This was seen as the MUT1 was able to survive and grow on PYE-Cm agar plates after complementation with WT *ssrA* (#4927, pKK838) (Figure 5A), but not after WT *dipM* (#4709) control complementation (Figure 5D).

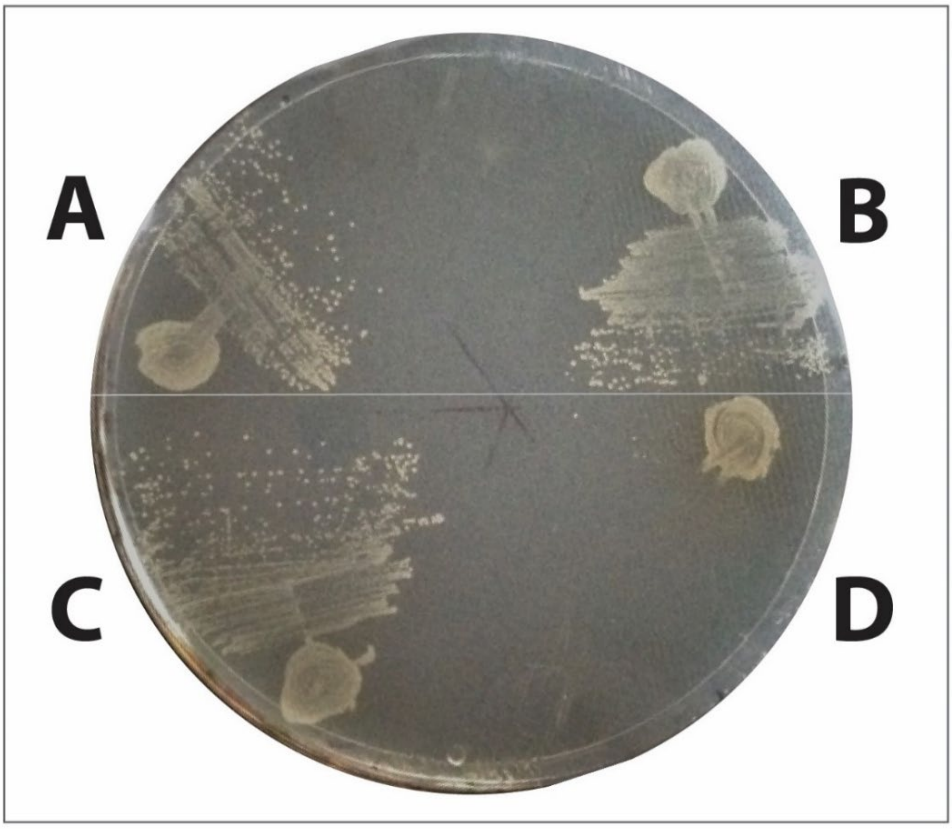

Figure 5: Restoration of plasmid maintenance in MUT1 after receiving WT ssrA gene

A MUT1 after receiving WT ssrA (#4927)**. B** WT C. crescentus (#3637) after receiving WT ssrA (#4927). **C** WT C. crescentus (#3637) after receiving pBXMCS-6-dipM-Cm-R (#4709). **D** MUT1 after receiving pBXMCS-6-dipM-Cm-R (#4709). Growth on selective PYE-Cm agar plates indicates recipient *C. crescentus* stains successfully accepted plasmid from donor *E. coli.* Growth failure indicates failure of recipient cells to accept or maintain plasmids. **Note:** Heavy circular streaks on the plate are heavy growth of donor E. coli and recipient C. crescentus, however, as the heavy growth is spread, only C. crescentus cells that received the plasmid can form single colonies.

A double conjugation experiment was performed on MUT3. pRK290LacZ-tet cosmid used to complement MUT3 contains a stretch of DNA that spans twelve different genes including *dnaJ*. Initially, when MUT3 was complemented with an empty pLAFR-5 plasmid, it

failed to maintain it and pBXMCS-6-Cm-R plasmid added simultaneously. However, after pRK290LacZ-tet cosmid complementation (carrying *dnaJ*), MUT3 restored its capacity to maintain pBXMCS-6-Cm-R plasmids and survive on PYE-Cm-Tet plates (Figure 6).

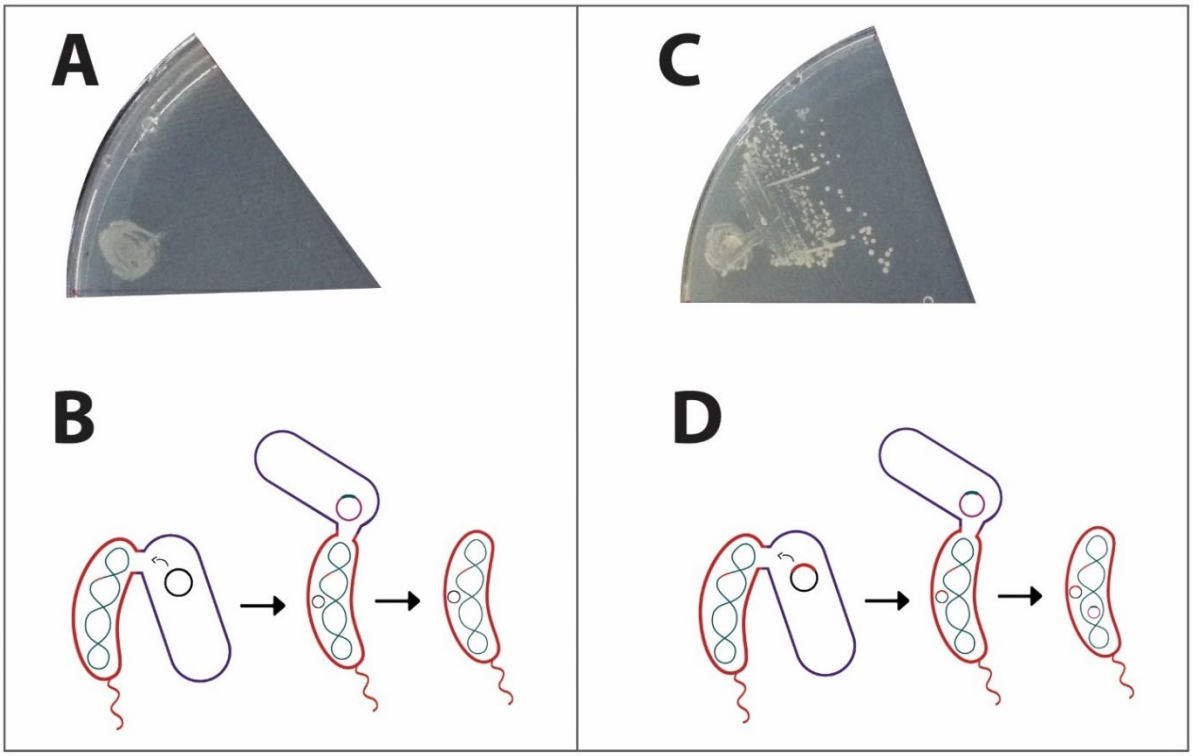

Figure 6: Restoration of plasmid maintenance in MUT3 after a double donor test.

A MUT3 after receiving an empty plasmid vector and pBXMCS-6-Cm-R plasmid. **B** Graphical demonstration of the double donor test in A. **C** MUT3 after receiving pRK290LacZ-tet cosmid and pBXMCS-6-Cm-R plasmid (both plasmids must be maintained for colonies to grow). **D** Graphical demonstration of the double donor test in C. Growth on selective PYE-Cm-Tet agar plates indicates recipient *C. crescentus* stains successfully accepted plasmid from donor *E. coli.* Growth failure indicates failure of recipient cells to accept or maintain plasmids. **Note**: Both donor E. coli strains were mixed with recipient C. crescentus strains simultaneously during conjugation, however, for simplicity purposes, the illustration shows plasmid transfer as a two-step process instead of a single double conjugation.

WT Caulobacter crescentus gene library

To efficiently and rapidly identify mutations in large numbers of C. crescentus mutants, we attempted to create a gene library containing the entire CB15 genome on replication plasmids. However, pilot experiments to create an in-house *C. crescentus* gene library did not achieve desirable results. WT gDNA and pDNA were extracted, quantified, and digested using SalI or XhoI (Fig 3, Table 5). Due to the large size of WT *C. crescentus* gDNA (~ 4M bp) it traveled a very short distance not down the agarose gel (Fig 3A, lane 1). The linear pDNA is approximately 6 kbps in size (Fig 3A, lane 2). Genomic inserts were prepared by digesting gDNA with SalI (Fig 3A, lane 3) or XhoI (Fig 3A, lane 4). SalI digestion produced smaller DNA fragments ranging in size 10 kbps and 1 kbps. While XhoI resulted in large DNA fragments with sizes between 20 kbps and 5kbps. gDNA inserts and linearized plasmid were ligated and yielded vectors of an equivocal difference in sizes. All recombinant plasmids in lanes 3-9 appear to be larger in size (~ 5.5 kbps) when compared to linearized plasmid, which is estimated to have a size of 5 kbps (Figure 7B, lanes 4-9). Similar results were obtained when this experiment was repeated. All plasmid material, including linearized plasmid, generated in this experiment was used for *E. coli* transformation.

The newly constructed recombinant plasmids were used to transform *E. coli* DH5-α with a transformation efficiency of $2x10^7$. Transformed DH5-α cells gained chloramphenicol resistance after accepting the pBXMCS-6-Cm-R plasmid and were able to grow on LB-Cm plates (Figure 7C). To confirm the presence of pBXMCS-6-Cm-R plasmid and test for the presence of DNA inserts, pDNA was recovered from 5 different colonies from each of the 9 transformation plates, resulting in 45 different plasmid extracts. All plasmids were then digested with SalI or

NcoI and visualized using gel electrophoresis. NcoI was used here since XhoI cut sites have been destroyed when XhoI inserts were ligated to SalI linearized plasmid.

Representative gel image depicting the results obtained from 10 different extraction from two different transformation conditions is shown in Figure 7D. Lanes 1 through 6 are plasmids extracted from 5 different colonies from the same transformation plate, while lanes 7 through 12 were also extracted from 5 different colonies, but from a different transformation plate (Table 5). The first transformation plate (representing the first six lanes, excluding the ladder) contained the previously prepared XhoI inserts in a pBXMCS-6-Cm-R vector (Fig 3B, lane 7). While the second transformation plate (representing lanes 7-12) was the positive transformation control plate.

Lanes 2 and 7 represented uncut coiled plasmids that travel slower than linearized plasmids. After plasmid digestion, linearized plasmids extracted from the recombinant plasmids did not have equal sizes despite being from the same plate. Moreover, control plasmids (lanes 8-12) also lacked uniformity in plasmid sizes. Additionally, inserted fragments did not appear on the gel in any of the SalI digested recombinant plasmids, contrary to our expectations. Overall, gene library results were inconclusive.

Figure 7A	
Lane	Description
1	*C. crescentus* WT genome extracted from strain #3637
2	pBXMCS-6-Cm-R plasmid extracted from *E. coli* S17-1 (#4717) and treated with SalI
3	Genomic WT *C. crescentus* genome digest with SalI

4	Genomic WT *C. crescentus* genome digest with XhoI
Figure 7B	
Lane	Description
1	Undigested pBXMCS-6-Cm-R plasmid
2	Linear pBXMCS-6-Cm-R plasmid
3	Linear pBXMCS-6-Cm-R plasmid + ligase
4	Linear pBXMCS-6-Cm-R plasmid + SalI + ligase
5	Linear pBXMCS-6-Cm-R plasmid + SalI inserts diluted 1:10 + ligase
6	Linear pBXMCS-6-Cm-R plasmid + SalI inserts diluted 1:100 + ligase
7	Linear pBXMCS-6-Cm-R plasmid + XhoI inserts + ligase
8	Linear pBXMCS-6-Cm-R plasmid + XhoI inserts diluted 1:10 + ligase
9	Linear pBXMCS-6-Cm-R plasmid + XhoI inserts diluted 1:100 + ligase
Figure 7D	
Lane	Description
1	Undigested pBXMCS-6-Cm-R plasmid + XhoI from plate #1, colony #1
2	Linear pBXMCS-6-Cm-R plasmid + XhoI cut with NcoI from plate #1, colony #1
3	Linear pBXMCS-6-Cm-R plasmid + XhoI cut with NcoI from plate #1, colony #2
4	Linear pBXMCS-6-Cm-R plasmid + XhoI cut with NcoI from plate #1, colony #3
5	Linear pBXMCS-6-Cm-R plasmid + XhoI cut with NcoI from plate #1, colony #4
6	Linear pBXMCS-6-Cm-R plasmid + XhoI cut with NcoI from plate #1, colony #5
7	Undigested pBXMCS-6-Cm-R plasmid from plate #2, colony #1

8	Linear pBXMCS-6-Cm-R plasmid cut with SalI from plate #2, colony #1
9	Linear pBXMCS-6-Cm-R plasmid cut with SalI from plate #2, colony #2
10	Linear pBXMCS-6-Cm-R plasmid cut with SalI from plate #2, colony #3
11	Linear pBXMCS-6-Cm-R plasmid cut with SalI from plate #2, colony #4
12	Linear pBXMCS-6-Cm-R plasmid cut with SalI from plate #2, colony #5

Table 5: Numbers key for gel pictures in figure 7A, 7B, and 7D

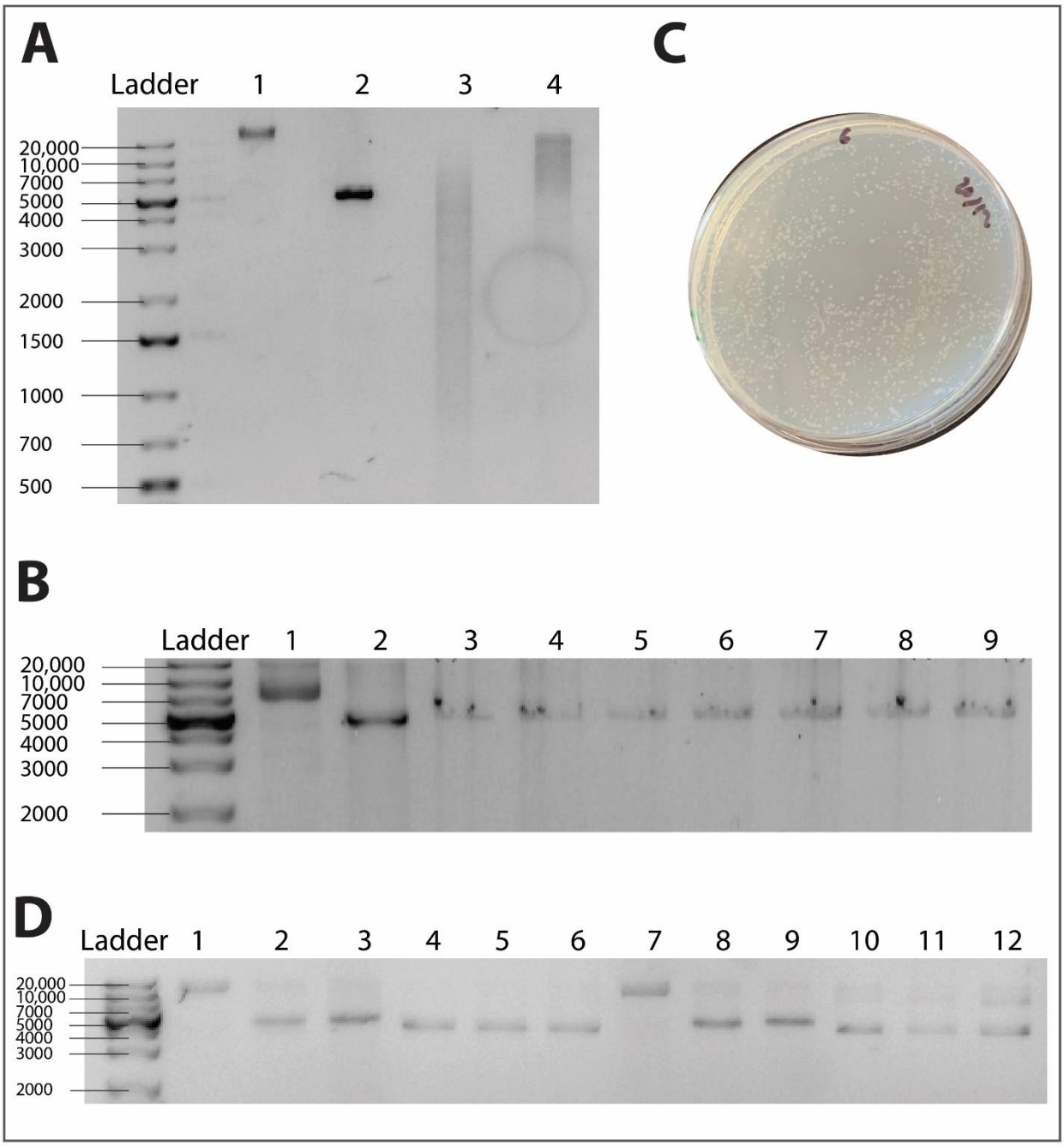

Figure 7: Construction of an in-house WT *C. crescentus* gene library

A WT C. crescentus gDNA extraction (lane 1) and enzymatic digestion of pBXMCS-6-Cm-R plasmid and gDNA (lanes 2-4). **B** gDNA inserts ligated into pBXMCS-6-Cm-R vectors (lanes 3-9). **C** A representative transformation plate. **D** Reconstructed vectors recovered from transformed colonies and enzymatically digested. See table 5 for number key.

Discussion

The overlap between cell cycle phases in bacteria and the intricate regulatory control modules governing cellular functions in *C. crescentus* is complex. Many proteins involved in cell cycle regulation have been functionally characterized, but some, like DipM, remain to be fully characterized. Throughout this thesis, we are hoping to set the groundwork for future efforts that can reveal components of the pathway that coordinates chromosome replication and cell division.

DipM is a non-canonical endopeptidase that has evolved interactions, through its LytM domain, with other proteins. More particularly, DipM and other LytM proteins regulate so called "autolysins" and thereby regulate other hydrolytic proteins that determine cell division and peptidoglycan cell shape. These established regulatory roles support the hypothesis that DipM can interact with additional proteins that might also lead to the regulation of chromosome replication. We obtained preliminary evidence supporting the involvement of the endopeptidase DipM in coordinating replication and division in *C. crescentus*, therefore, we based the new molecular screen around DipM.

Here, we tested the capacity of a new molecular screening technique to detect genes involved in coordinating DNA replication and cell wall division. We detected mutations in proteins involved in regulating the replication initiator DnaA (explained further below). However, the number of mutants investigated here was insufficient to formulate a possible pathway linking replication to cell wall division.

Our lab generated approximately 100 *C. crescentus* mutants that were screened for three phenotypic changes: kanamycin resistance, plasmid maintenance, and temperature sensitivity. All mutants were strongly resistant to kanamycin, reflecting high transcription at *Cori*. However, most mutants were able to maintain plasmids, leading to their elimination. Of all the mutants, only three made the final cut and were selected for further analysis. They were renamed as MUT1, MUT2, and MUT3. All three mutants were plasmid non-supporting (PNS), although MUT2 showed partial or weak plasmid support, making it an interesting candidate to pursue as it may provide answers to how a PNS phenotype arises. Of all the mutants that satisfied the first two conditions, only MUT2 was TS and demonstrated filamentous growth under the light microscope.

Although plasmid replication mechanisms are different from chromosomal replication mechanisms, both processes share replication or segregation factors. As a result, a defect in the regulation of plasmid replication may suggest a possible defect in the regulation of chromosomal replication. Additionally, it is unclear if the plasmid replicon type affects the ability of *C. crescentus* mutants to maintain them. In previous studies, our lab reported that DipM mutants could not support the maintenance of plasmids from four different families: pBBR-1, pIncP, pIncQ, and pCori [165]. However, mutants tested here were only tested for the ability to maintain pBBR-1 replicon. Nonetheless, future studies must be done to determine if MUT1, MUT2, and MUT3 can support other types of plasmid replicons.

Strong kanamycin resistance is suggestive of upregulation in replication initiation. However, despite this upregulation, mutant strains did not show comparable differences in their growth rates (data not shown). This is an interesting observation since it hints that despite

the high levels of activity at *Cori*, hence increased replication initiation, *C. crescentus* maintains a somewhat regular growth rate. Possibly due to the tight regulation exhibited in *C. crescentus* that uses multiple pathways to target the replication initiator DnaA.

WGS revealed single nucleotide polymorphisms (SNPs) in the *ssrA* gene in MUT1 and in *dnaJ*, *spoT*, Aminobenzyol-glutamate utilization protein B, and Major facilitator family transporter in MUT3. MUT2 had a frameshift mutation in the LPS biosynthesis protein.

Like *dipM* mutants, MUT1 was consistently unable to support plasmid replication (PNS). In this mutant, we detected a SNP in the *ssrA* gene, which codes for tmRNA. After complementing MUT1 with WT *ssrA*, the mutant restored its ability to maintain pBBR plasmids. In bacteria, tmRNA releases stalled ribosomes and tags their incomplete polypeptides for degradation [177]. Interestingly, in *C. crescentus*, the functional tmRNA is actually a two-piece tmRNA [142]. The process of releasing stalled ribosomes has been termed trans-translation and was shown to be a conserved quality control process in bacteria [107]. Keiler *et al.* showed that *ssrA* (tmRNA) is essential for the correct timing of DNA replication in *C. crescentus* [178]. Additionally, in *C. crescentus*, trans-translation has been shown to be necessary for the correct timing of DNA initiation by regulating the transcription of *dnaA*, however, the exact mechanism of this regulation is not clear [108]. It is possible that due to the *ssrA* mutation in position 60, which falls in the periphery of the T-arm of the two-piece tmRNA (as illustrated in [142]), the functional capacity of mutant tmRNA decreased, affecting trans-translation, which influences many proteins in the cell. Thereby, an uncharacterized regulator that may have drastic effects on plasmid replication, but none on chromosomal replication, may have been indirectly affected by a mutation in *ssrA*.

MUT2 showed a weak PNR phenotype and had a detrimental frameshift mutation in a poorly characterized lipopolysaccharide (LPS) synthesis protein encoded by *wbqL* [173]. This protein affects cell curvature by undermining the function of the cell curvature protein crescentin. The exact mechanism is unclear, however, dysfunctional WbqL results in O-polysaccharide (S-layer) shedding, which in turn prevents crescentin from associating with the cell envelope, leading to curvature loss. Additionally, *wbqL* mutants overexpressing crescentin displayed a filamentous growth under the microscope and blocked cell wall division [173]. Similarly, filamentous growth was indeed observed in MUT2. Nonetheless, crescentin is a non-essential protein that does not affect cell division [179] unless overproduced. It is possible that cell division was defective in MUT2 due to the debilitating mutation in WbqL that may have resulted in the accumulation of crescentin disturbing division. Additionally, WbqL may have a role in cell wall division and an indirect effect on replication, since it was the only significant mutation defected in MUT2 (which had high kanamycin resistance, indirectly suggesting higher activity at *Cori*). This is exciting because if WbqL is part of the division machinery, it is possible that it coordinates with periplasmic DipM to complete cell wall division. This hypothesis needs to be further investigated before any conclusions can be made, however, it hints that we are heading in a promising direction, where a possible pathway linking division to chromosome replication lies. The next step would be to test the complementation of MUT2 with a WT *wbqL* gene and observe for PNS phenotype restoration and reversal of TS growth. Regardless, further characterization of WbqL and its role in the regulation of division and replication is necessary.

MUT3 had several mutations, making it a challenging task to determine which are involved in the PNR phenotypes. Additionally, the cosmid used to complement MUT3 had a

DNA segment that span twelve genes including *dnaA*, *dnaJ*, and *dnaK*. As a result, we can not determine if the PNS phenotype is due to a single mutation or more, or whether an additional dose of DnaA or DnaK ameliorated the effect of the gene mutations. The pRK290LacZ-tet cosmid is an RK2 replicon belonging to the IncP incompatibility group. The cosmid was sufficient to restore plasmid replication of pBBR replicon and was able to support RK2 replicon. DnaJ is a heat shock and a chaperon protein that is part of the *dnaKJ* operon, which is responsible for maintaining protein homeostasis [180]. Jonas *et al.* showed the role of Lon proteases in halting chromosome replication initiation in *C. crescentus* under proteotoxic conditions by triggering the degradation of DnaA as a response to the lack of DnaKJ [181]. We speculate that a deficiency in DnaJ may have resulted in the accumulation of misfolded proteins, thereby activating Lon proteases and limiting the supply of DnaA. Hence, similar to the case in MUT1, *C. crescentus* favors chromosomal replication over plasmid replication, leading to the PNR phenotype. On the other hand, the PNR phenotype might have been a result of two, three, or all mutations in MUT3. SpoT for example, was shown to limit DNA initiation in *C. crescentus* by triggering the proteolysis of DnaA during carbon starvation [175]. Aminobenzoyl-glutamate utilization protein B is an uncharacterized M20_dimer domain-containing protein with a hydrolase activity [182]. While the major facilitator family transporter is another uncharacterized transmembrane transporter protein [183]. Cloning the mutations in these gene and testing their effect on PNR either alone or in combination will allow the determination of the cause of PNR in MUT3 and if these different proteins interact to result in the PNS phenotype.

It is possible that DipM (and other regulators in the same system, which may include WbqL) work together to sense the robustness of cellular processes, specifically proteolysis. DnaJ and SpoT have both been shown to trigger DNA proteolysis in response to internal (proteotoxicity [181]) or external (carbon starvation [109]) stressors, respectively. Additionally, trans-translation was also shown to be necessary for the temporal transcription of *dnaA* and correct timing of replication initiation [108, 178]. Moreover, DipM, WbqL and major facilitator family transporter may all be part of transmission of sensory messages from cell wall division back to the replication machinery (Figure 8).

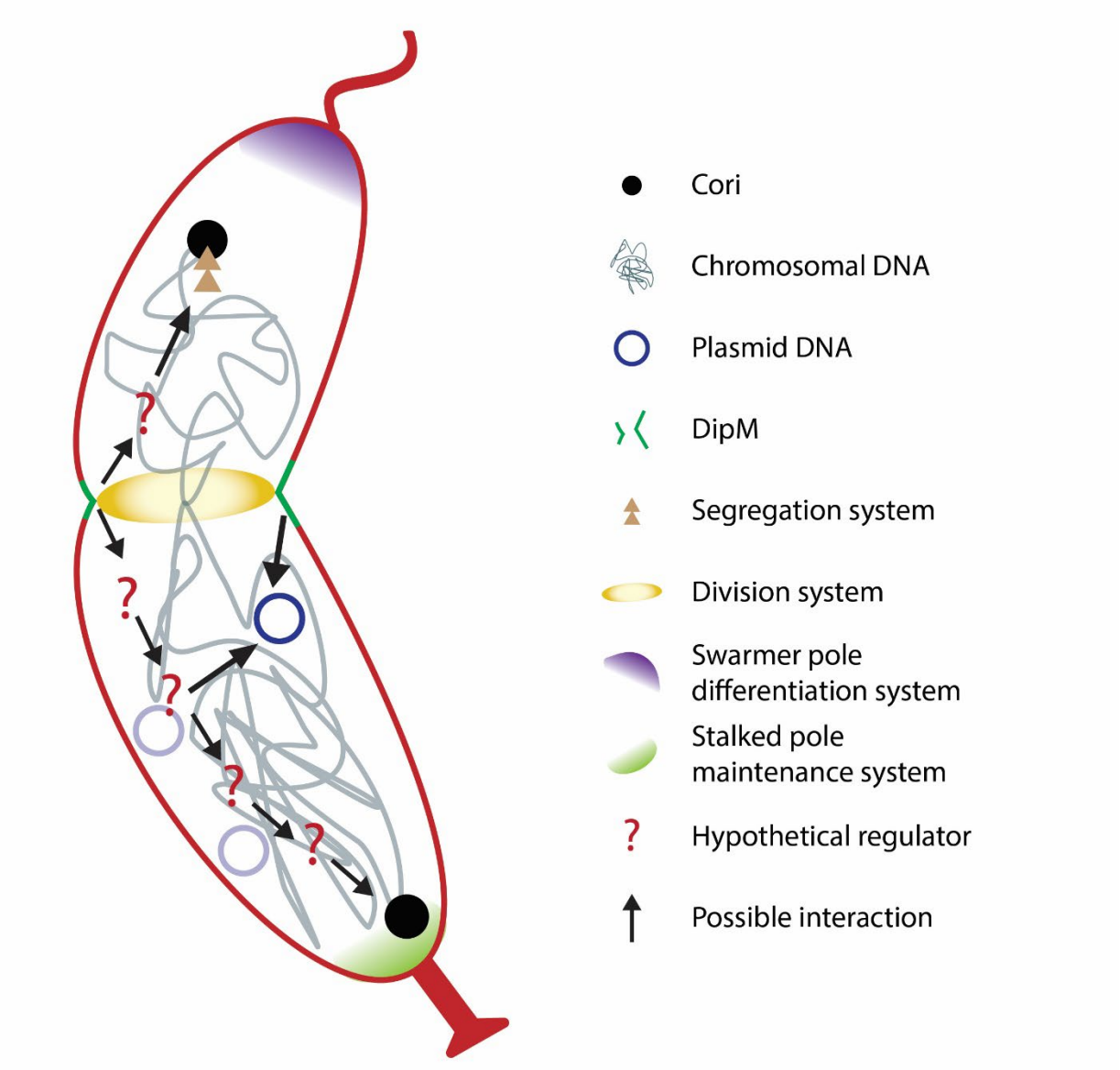

Figure 8: Hypothetical interactions of DipM with cellular regulatory systems.

Our genetic screen is constructed to isolate mutants that work with DipM to regulate replication and division. The pathway used by DipM and the interacting regulators are unknow. However, it is possible that DipM and associated proteins sense changes protein homeostasis and respond by transmitting an up-regulatory message to the replication machinery.

Conclusion and future direction

All in all, we identified and tested mutations that affected the cell cycle progression, namely *ssrA* in MUT1 and *dnaJ* in MUT3. However, other mutations identified here may also be involved in the crosstalk between division and chromosome replication. We also provided evidence for the potency of the new genetic screen and the broad range of replication-division specific regulators it can detect. Understanding the cues, responses, and signaling pathways that regulate the cell cycle is crucial for antibiotic development strategies. By looking into how chromosome replication and cell division are coordinated at the gene level, we can infer regulatory networks and provide a foundation for further protein analysis studies.

Nevertheless, much work remains to be done. Initially, it is necessary to clone all the mutations found in this study and test the reproducibility of mutated phenotypes. Additionally, to increase the scale of the screening technique, we attempted to construct a WT *C. crescentus* gene library to be used to readily identify mutated genes. By conjugating donor *E. coli* containing WT genes with mutated *C. crescentus* and watching for WT phenotype restoration, we can isolate and sequence the plasmids, and thereby the WT genes, that restored the WT phenotype in (hence successfully complemented) *C. crescentus*. Our library remains to be a work in progress. However, it will allow the rapid identification of mutated genes without the need for full genome sequencing, which requires a lot of resources and has a steep learning curve. Finally, additional studies on plasmid replication in mutated *C. crescentus* and the possible mechanisms by which it is linked to chromosomal replication and DipM must be investigated further.

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