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TRANSCRIPTIONAL REGULATION OF *SINORHIZOBIUM MELILOTI* CELL  
CYCLE-RELATED GENES IN THE  $\Delta$ *CBRA* MUTANT AND ROOT NODULES OF  
*MEDICAGO SATIVA*

A Thesis Presented

by

COREY S. HAZEKAMP

Submitted to the office of Graduate Studies,  
University of Massachusetts, Boston,  
In fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2014

Biological Science Program

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## ABSTRACT

# TRANSCRIPTIONAL REGULATION OF *SINORHIZOBIUM MELILOTI* CELL CYCLE-RELATED GENES IN THE $\Delta$ *CBRA* MUTANT AND ROOT NODULES OF *MEDICAGO SATIVA*

August 2014

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Directed by Assistant Professor Katherine E. Gibson

*Sinorhizobium meliloti* is a Gram-negative alphaproteobacterium and nitrogen-fixing symbiont, which undergoes a novel cell cycle modification during its' host-microbe interaction. I intend to monitor the transcriptional regulation of cell cycle-related genes during free-loving growth, in addition to monitoring their expression during symbiosis. Using genes known to be regulated by CtrA in *C. crescentus* or predicted to be regulated by CtrA in *S. meliloti*, I aim to show how certain cell cycle genes are regulated in *S. meliloti*. In *C. crescentus*, CtrA acts as a transcription factor that is active when phosphorylated and inactive when not phosphorylated. In *S. meliloti*, CbrA is a histidine kinase that ultimately inhibits CtrA phosphorylation. Using a  $\Delta$ *cbrA* null mutant, which leads to increased levels of CtrA in *S. meliloti*, and the  $\beta$ -glucuronidase

(GUS) reporter gene, I can monitor the expression levels of target genes that are potentially regulated by CbrA and CtrA. Promoter regions, transcription start sites, and translation start sites of target genes have been cloned into the plasmid pVO155 upstream of the GUS gene. I measured the GUS enzymatic activity using the 4-methylumbelliferyl-beta-D-glucuronide (MUG) substrate. Additionally, after infecting *Medicago sativa* seedlings with these fusion strains, I used a different GUS substrate to test for the presence of target gene expression in root nodules. Results thus far have shown some target genes with large differences in expression coinciding with the absence of *cbrA* and increased CtrA levels while some target genes show only slight differences, if any at all. Tracking the expression location and patterns of target genes in root nodules has shown that some genes are expressed ubiquitously throughout the nodule while other genes are expressed in specific locations. These results are significant because no one has looked at genes regulated by CbrA or CtrA in *S. meliloti*, which is more applicable to host-microbe interactions than *C. crescentus*, especially since *Agrobacterium tumefaciens* and *Brucella abortus* both have a CbrA homologue. Additionally, I will provide critical insight into the molecular biology of the *S. meliloti* host-microbe interaction.

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CHAPTER I  
INTRODUCTION

**Rhizobium symbiosis**

Soil dwelling rhizobial bacteria have co-evolved with certain leguminous plants to form a symbiotic relationship. In this relationship, the rhizobial bacteria invade the host plant and live inside plant-made root nodules. Once living inside the nodules, rhizobia adapt and gain the ability to perform symbiotic nitrogen fixation. These relationships are species-specific and will only ensue in nitrogen-limited soils. Therefore, it is critical for the plant to promote this relationship so that it can gain fitness in otherwise nutrient-deficient environments.

The signaling events and survival mechanisms utilized by rhizobial cells within its' host provides a powerful model to investigate chronic intracellular infection. This model is especially useful since many of the requirements for host colonization are shared among related bacteria. Recent advances in genomic sequencing abilities have also aided in further characterizing the molecular determinants involved in this relationship. Thus, the study of this symbiotic interaction will provide critical insight that

will be useful in gaining a greater understanding of intracellular infection of humans by virulent microbes.

### **Signaling and host specificity**

Bacteria within the *Rhizobiales* order have the ability to sense flavonoids exuded by the *Fabaceae* plant family in nitrogen-limited soil environments (**Figure 1A**). The *Fabaceae* family of leguminous plants includes genus such as *Medicago* (alfalfa), *Melilotus* (sweet clover), and *Trigonella* (fenugreek). These legumes are able to secrete a diverse cocktail of flavonoids and isoflavonoids from their roots (Perret, Staehelin et al. 2000). Flavonoid derivatives of 2-phenyl-1,4-benzopyrone are specifically produced by these leguminous plants and are likely the first signals exchanged between *Rhizobium* and *Fabacea* (Perret, Staehelin et al. 2000). Rhizobia are able to directly sense these flavonoids through their NodD receptor, which is a DNA-binding protein that induces the expression of nodulation (*nod*) genes (Barnett and Fisher 2006).

The host range of each rhizobium species is determined by the ability of its' endogenous NodD to bind a certain flavonoid or variety of flavonoids (Perret, Staehelin et al. 2000). Interactions with flavonoids produced by plants that are not authentic hosts to the *Rhizobium* species have been shown to inhibit expression of *nod* genes (Peck, Fisher et al. 2006). The amount and types of flavonoids produced in the rhizosphere can be detected by rhizobia, however it is difficult to determine precisely which flavonoid is

compatible with a particular rhizobium species. It is thought that that certain mixtures might determine compatibility rather than a single independent flavonoid (Gibson, Kobayashi et al. 2008). The *Rhizobiaceae* family includes bacterial species with both limited (recognizing only one or a few flavonoids) and wide (recognizing many different flavonoids) ranges of host-specificity. For example, *Rhizobium etli* is capable of inducing nodulation in only one type of legume, *Phaseolus* (bean), whereas *Sinorhizobium fredii* NGR234 can live symbiotically with 232 legumes (Pueppke and Broughton 1999).

Once the NodD receptor of a rhizobium species has sensed its' compatible flavonoid or mixture of flavonoids, it induces the catalysis of nodulation factors (NF), which are secreted by the rhizobium species into the rhizosphere (**Figure 1B**) (Capela, Carrere et al. 2005). These Nod factors are composed of lipochito-oligosaccharides and are produced by the enzymes encoded by *nod* genes, which are under transcriptional regulation by NodD (Barnett and Fisher 2006). Therefore, the connection between legume and rhizobium is heavily dependent on the interaction between exuded flavonoids and NodD responses. The bacterial NFs that are produced as a response to NodD sensing compatible flavonoids are required for nodulation and successful host infection by rhizobia. Perception of NFs by the host plant stimulates a series of complex developmental responses from the plant that results in the formation of a root hair structure called a nodule, within which the bacteria are housed (Oldroyd and Downie 2006).

In our research we study the alfalfa species *Medicago sativa* and *Sinorhizobium* species *Sinorhizobium meliloti*. *S. meliloti* has a relatively limited host range compared to

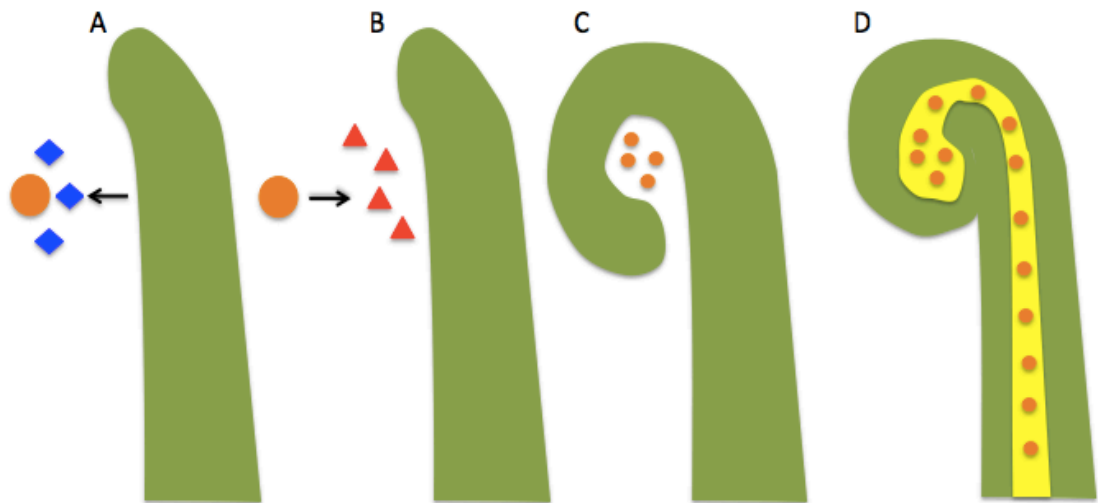
other rhizobia, which has been attributed to its' recognition of specific flavonoids (Perret, Staehelin et al. 2000). *S. meliloti* can induce nodulation with three genus of *Fabaceae* – *Medicago* (alfalfa), *Melilotus* (sweetclover), and *Trigonella* (fenugreek) (Gibson, Kobayashi et al. 2008). The extremely precise species-specific sensing mechanism between a eukaryote and prokaryote is an exquisite example of a symbiotic relationship that has evolved over millions of years. This relationship provides a strong model for studying the invasion of a eukaryotic host by a prokaryotic symbiont.

### **Nod factors and nodulation**

Once communication has commenced between legume and rhizobium through flavonoids, NFs serve as a mechanism by which rhizobium species can respond to its' targeted host. Perception of NF by legume roots elicits multiple responses that are essential for successful nodulation to occur, just like the interaction between flavonoids and NodD (Oldroyd and Downie 2006). Similar to flavonoids produced by the host, the type of NF produced by the microbe can vary greatly. The *nodABC* gene cluster encodes proteins which synthesize the core structure of NFs. Different *Rhizobium* species contain additional *nod* genes that chemically modify NFs, and producing many types of NFs is common among *Rhizobium* species (Perret, Staehelin et al. 2000). The primary response of the plant root to NF perception is root hair curling (**Figure 1C**). Root hair curling is dependent on fluctuations in intracellular calcium levels and alterations to the

cytoskeleton, both of which are induced as a response to NF sensing (Timmers, Auriac et al. 1999, Cardenas, Thomas-Oates et al. 2003, Sieberer, Timmers et al. 2005). This process results in an engulfed rhizobium cell that is attached to the root hair as it curls (**Figure 1D**).

Once the host-microbe interaction successfully completes the signaling process through flavonoids and NF, the development of the infection thread (IT) begins. This stage of the relationship is absolutely critical – the rhizobia must be internalized before they can begin to fix nitrogen (Perret, Staehelin et al. 2000). The ultimate goal of the IT is to allow infection of rhizobia cells into the root cortex of the host (**Figure 1D**). To successfully promote IT formation in its' host, *Rhizobium* species must produce exopolysaccharides (EPS), such as succinoglycan and galactoglucan (Glazebrook and Walker 1989, Pellock, Cheng et al. 2000).



**Figure 1:** (A) A rhizobium cell (orange circle) senses flavonoids (blue diamonds) exuded by a leguminous plant species from its roots into the rhizosphere. (B) After flavonoids have been sensed by a rhizobium species, the response is the production of nodulation factors (red triangles), which are meant as a mechanism to communicate with its' host. (C) Once both the rhizobium cell and host plant have perceived the appropriate signals, the



response is the capture of rhizobium cells by the root through root hair curling. (D) Once the root has curled over and trapped rhizobium cells, the rhizobium cells will be engulfed into the developing infection thread (yellow). The infection thread will continue to grow and penetrate the underlying cell layers of the host root.

With sufficient production of NFs and EPSs by the rhizobia, cells are trapped in the curled root hair, and development begins. New membrane synthesis occurs at the tip of the newly formed IT and is thought to be the result of cellular polarity (Gage 2004). Within the new IT, the plant produces reactive oxygen species (ROS), which act as antimicrobial agents against unwanted invaders (Vandenbosch, Bradley et al. 1989, Santos, Herouart et al. 2001, Rathbun, Naldrett et al. 2002). To successfully reach the inner plant cortex and fix nitrogen, the rhizobia species must first continuously induce IT formation through each successive cell layer of the plant root (Jones, Kobayashi et al. 2007). The infection thread grows and develops through the plant nodule until it reaches the inner plant cortex (**Figures 1D and 2**). Once the IT reaches the inner plant cortex, the plant is required to envelope each individual cell into its' own "symbiosome," through endocytosis (Brewin 2004). Only then can the cell differentiate into a nitrogen-fixing bacteroid.

### **Evasion of the host immune system**

*S. meliloti* is a powerful model organism for a variety of reasons. One of the most compelling reasons to use *S. meliloti* as a model organism is to elucidate the mechanisms

that underlie its' host-microbe relationship. *S. meliloti* is closely related to the mammalian pathogen *Brucella abortus* and the plant pathogen *Agrobacterium tumefaciens*, which cause brucellosis in mammals or crown galls in plants, respectively. *S. meliloti* shares a number of homologous proteins with both *B. abortus* and *A. tumefaciens* that are essential for successful host invasion. One of the largest obstacles for the bacteria within these host-microbe interactions is escaping the immune system of the host. Once this challenge is overcome, the microbe can more easily proliferate within its' host.

The invasion of *Medicago* root nodules by *S. meliloti* induces the production of reactive oxygen species (ROS), which is a common plant response to invading pathogens (Abramovitch, Anderson et al. 2006). ROS have been detected in both the IT and the infected host cells of developing nodules (Santos, Herouart et al. 2001, Rubio, James et al. 2004). ROS play a large role in most plant defenses against invaders; however, this oxidative burst does not kill rhizobium cells within the IT or the symbiosome. This is intriguing since free-living rhizobia have been shown to be more vulnerable to ROS than other rhizosphere-associated prokaryotes (Ohwada, Shirakawa et al. 1999). Since ROS do not kill rhizobia during invasion, it is thought that they are instead involved as a signaling system between rhizobia and their host.

Various genes required for EPS biosynthesis are essential to a successful invasion of *M. sativa* by *S. meliloti*. It is possible that the combination of EPS, NF, and LPS produced by the microbe interact with the ROS produced by the host to create a feedback response. (Shaw and Long 2003, Scheidle, Gross et al. 2005). EPS are a type of

polysaccharide commonly used by Gram-negative bacteria for protection from the extracellular environment. More specific to *S. meliloti*, succinoglycan is the type of EPS that is most often associated with symbiosis. Succinoglycan is a polymer of an octasaccharide repeating unit modified with acetyl, succinyl and pyruvyl substituents (Reinhold, Chan et al. 1994, Jones, Kobayashi et al. 2007). The coupling of succinoglycan with galactoglucan contributes to the induction of infection thread development upon invasion into *Medicago* plants (Glazebrook and Walker 1989, Pellock, Cheng et al. 2000). As an EPS, succinoglycan potentially serves various roles for *S. meliloti*. Similar to other polysaccharides, succinoglycan protects *S. meliloti* from stress and more specifically the stressful environment of its' host and the IT (D'Haese, Glushka et al. 2004). A low molecular weight succinoglycan may be more effective at promoting symbiosis versus high molecular weight succinoglycan; therefore, it is thought that this EPS might function as a signaling molecule (Gibson, Kobayashi et al. 2008). Furthermore, it is thought that succinoglycan contributes to species-specific invasion in addition to NFs (Simsek, Ojanen-Reuhs et al. 2007). Therefore, succinoglycan may play both a traditional protective role in addition to a signaling role, each of which are required for successful invasion of roots by *S. meliloti*. This putative contribution to the evasion of the host immune system through a protective and signaling role remains an outstanding question.

## Comparisons of intracellular host-microbe interactions

*S. meliloti* shares similarities in the genetic requirements and mechanisms by which it establishes its' host-microbe interaction with other symbiotic bacteria as well as pathogens. Intracellular symbionts that invade the cells of insects and mammals undergo an analogous process with the same end results – intracellular invasion of a host organism. For example, in the *S. meliloti*/*M. truncatula* model system, *S. meliloti* cells in the IT are engulfed within symbiosomes and enter the host cells where they establish an intracellular infection (**Figure 2**). The pea aphid is an insect that provides a model for studying host-microbe interactions and intracellular infection within insects. The aphids' endosymbionts, *Buchnera aphidicola* and *Rickettsiella*, undergo a process analogous to *S. meliloti* and reside within the aphid cells in bacteriomes (Kondorosi, Mergaert et al. 2013). Certain host cells within *Medicago* nodules eventually become highly polyploidy after full development as do their nitrogen-fixing bacteroids, and this is also the case for bacteriomes.

Another parallel between these two host-microbe systems is the secreted peptides, which induces the filamentous and polyploidy phenotypes of the endosymbiont. *Medicago* secretes “Nodule-specific Cysteine-Rich” peptides (NCR), which induce nitrogen-fixing *S. meliloti* cells, and pea aphids use an analogous peptide, bacteriocyte-specific cysteine-rich peptides (BCR) to promote its' symbiotic relationship (Kondorosi, Mergaert et al. 2013). These similarities demonstrate that the mechanism by which *S. meliloti* engages in its' host-microbe interaction and intracellular infection are not novel

but rather a shared mechanism with other distantly related bacteria. It has recently been shown *in vitro* that NCRs do in fact have an effect on the cell cycle of *S. meliloti* cells (Penterman, Abo et al. 2014). It would be interesting to test for the same effect on pea aphid endosymbionts with BCRs, and whether NCRs and BCRs are similar enough to be interchangeable.

Alphaproteobacteria more closely related to *S. meliloti* also share analogous molecular mechanisms to establish their pathogenic host relationship. Just like *S. meliloti*, the production of LPS in *Brucella* is critical for successful invasion of their mammalian host. Even though these two alphaproteobacteria do not share the same genes for producing LPS, both were found ineffective at invasion when the genes encoding their LPS core were mutated (Jones, Kobayashi et al. 2007). Additionally,  $\beta$ -glucans are critical for the surface interactions that both species use to gain access to their host (Dickstein, Bisseling et al. 1988, Arellano-Reynoso, Lapaque et al. 2005).

CbrA is a cell cycle regulator and sensor histidine-kinase that is required for an effective symbiosis between *S. meliloti* and *M. sativa*, and is shared between several alphaproteobacteria, including PdhS in *B. abortus* (Sadowski, Wilson et al. 2013). However, in *B. abortus*, PdhS is essential and cannot be tested as a requirement for infection (Hallez, Mignolet et al. 2007). *Agrobacterium tumefaciens* is another closely related alphaproteobacterium that encodes a *pdhS1* gene within its' genome. Insight into how CbrA influences cell cycle regulation will therefore contribute to the understanding of how PdhS might be regulating the cell cycle in *B. abortus* and *A. tumefaciens*.

## **Bacteroid development and function**

### **Intracellular infection**

A unique characteristic that makes *S. meliloti* so important and interesting to study is the transition between its' two distinct types of cell cycles. The novel cell cycle, discussed later, is only seen within *S. meliloti* cells that have invaded its host. After free-living rhizosphere-associated *S. meliloti* have successfully communicated with its' host and have been engulfed into the symbiosome, the *S. meliloti* cell must adopt a novel cell cycle to maintain this relationship and fix nitrogen.

The intracellular infection of rhizobia species within *Medicago* species begins when the bacterium travel to the base of the infection thread where they escape into the host cell and are thought to undergo an endocytosis-like process (Roth and Stacey 1989). The IT mediates this process as it passes through the root hair and into the cortical root cells. Once the IT has reached the cortical root cells, it grows through them into the nodule primordium (Perret, Staehelin et al. 2000, Gage 2004). Rhizobia are released into the nodule primordium, but need protective barriers in order to sustain their intracellular persistence (**Figure 2A**). The result of this endocytosis-like process is the bacterial cell being enclosed by a plasma membrane, creating what is known as the symbiosome (Brewin 2004). At this point the rhizobia have breached the plant cells and are protected

from the cytosol by a membrane known as the peribacteroid membrane (Perret, Staehelin et al. 2000). This marks the initiation of an intracellular infection and enables the differentiation of the “wild type” rhizobium cells into bacteroid cells. Bacteroids are filamentous cells with a high quantity of greater than 1N DNA.

### **Endoreduplication**

Once rhizobia cells have reached the nodule primordium and are residing within their symbiosome, more communication between the bacteria and eukaryotic host ensue. At this point the host plant, *Medicago* in this case, is in complete control of the survival of the resident microbe. The host creates a microaerobic environment within the symbiosome and mediates nutrient exchange with the microbe. An important distinction between *S. meliloti* and other rhizobium species is the type of nodule that develops following a successful invasion. Even though the type of nodule produced is a result of the plant host, it is still important to differentiate between the two primary types: determinate and indeterminate. Determinate nodules lack a persistent meristem, which allows the invasive microbes to proliferate, differentiate, and senesce synchronously (Mergaert, Uchiumi et al. 2006). This is distinctly different from legumes that produce indeterminate meristems, which have a persistent meristem and allows constant invasion of new bacteria so that different zones within the nodules represent successive developmental stages of infection, differentiation, and senescence (**Figure 9A**).

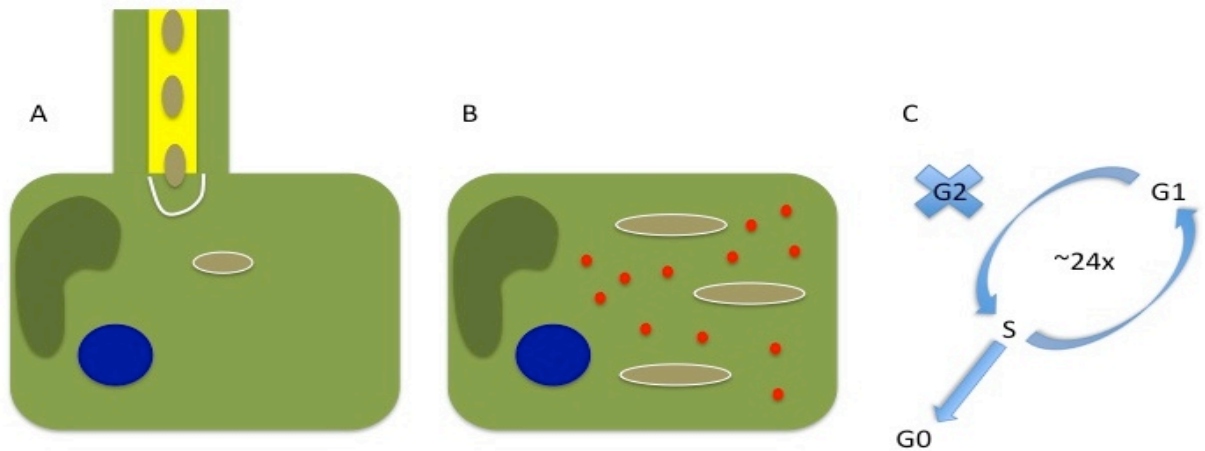
Indeterminate nodules will continuously grow through the apical meristem in contrast to the determinant nodules, which grow to a certain size and stop. Within the indeterminate *Medicago* nodules, a bacterial cell will divide in association with its' symbiosome membrane before differentiating into a bacteroid. This is distinct from bacteria residing in determinant nodules, which divide independent of the symbiosome (Prell and Poole 2006).

The different zones within indeterminate nodules are critical to understanding cell cycle regulation in *S. meliloti* during symbiosis since they utilize distinctly different regulation of their cell cycle at different stages of nodule development. Specifically, in the infection thread near the apical meristem (**Figure 9A**), *S. meliloti* are thought to utilize the same cell cycle program seen in free-living bacteria. In contrast, within the nitrogen-fixing zone, cells undergo endoreduplication before exiting the cell cycle into G<sub>0</sub> phase (**Figure 9A, Figure 2C**).

During a normal cell cycle of *S. meliloti*, the cell will exit S phase after DNA replication is completed and enter G<sub>2</sub> phase, initiating division into two daughter cells with equal DNA content. However, during endoreduplication, *S. meliloti* will exit S phase and reenter G<sub>1</sub> phase, skipping G<sub>2</sub> phase (Gibson, Kobayashi et al. 2008) (**Figure 2C**). This leads to repeated rounds of genome replication and an increase in cell wall content resulting in a filamentous cell with an increased amount of DNA. Once endoreduplication is complete, the cell is considered to be terminally differentiated into a bacteroid, which is non-reproductive and lacks the ability to revert back to a free-living cell cycle program.



The purpose of endoreduplication is still unclear and the mechanisms required to adopt this novel cell cycle program is still under investigation. It is thought that the increase in DNA and cell size is necessary to meet the high metabolic requirement for proper nitrogen fixation (Galitski, Saldanha et al. 1999, Mergaert, Uchiumi et al. 2006), but its role in promoting symbiosis remains unclear. It was recently shown that, despite the antimicrobial characteristics of NCRs, they induce this terminal differentiation of rhizobia into bacteroids (Van de Velde, Zehirov et al. 2010, Wang, Griffiths et al. 2010). In the presence of sub-lethal levels of NCRs, free-living *S. meliloti* cells will undergo the same process of endoreduplication, replicating their genome up to 24N, through a unique and not yet understood adaptation to its' cell cycle (**Figure 2C**). Perhaps endoreduplication leads to efficient nitrogen fixation or contributes to protection within the intracellular environment of the host.



**Figure 2:** (A) As rhizobium cells reach the base of the infection thread and enter the cells of its' host, they undergo endocytosis and become engulfed into a symbiosome. (B) Once invasion of the host cells is complete, the host produces nodule-specific cysteine-rich peptides (red circles) that stimulate the rhizobium cells to undergo endoreduplication.

(C) During endoreduplication, rhizobium cells exit S phase, bypass G2 phase completely and reinitiate G1 phase. Eventually, after 4-5 rounds of genome replication to create ~24N genome complement, they will exit the cell cycle and enter into what is known as the G0 phase.

## Nitrogen fixation

*S. meliloti* is perhaps most well known for its ability to perform symbiotic nitrogen fixation (SNF). This process is thought to be exclusive to microbes and entails converting atmospheric dinitrogen, which is inert to eukaryotes, into readily available ammonia. This process is critical to agriculture as nitrogen can be limiting in soil environments. Having the ability to gain a nitrogen source from atmospheric dinitrogen confers great fitness advantages to eukaryotes. Therefore, it should not be a surprise to think that eukaryotic hosts favored the evolution of the symbiotic relationship with rhizobia species. The gene classes that are generally associated with SNF are *nod*, *nif*, and *fix* genes. I have already introduced and discussed the purpose and function of *nod* genes and will not revisit them here. The *nif* genes, which specifically encode the nitrogenase enzyme, and *fix* genes, which are important for SNF, generally reside in distinct clusters within the bacterial genome and tend to be species-specific (Fischer 1994). In *S. meliloti*, clusters containing the *nif* and *fix* genes both reside on an extremely large plasmid known as pSymA or megaplasmid 1 (David, Domergue et al. 1987). Certain *nif* genes such as *nifS*, and *fdxN* have been shown to be required for SNF whereas the function of other *nif* genes such as *nifB* and *nifW* are not yet known (Fischer 1994).

The *fix* gene class is a little more straight forward, where a mutation to the *fixABCX* or *fixGHI* genetic clusters will abolish SNF (Earl, Ronson et al. 1987). In *S. meliloti*, mutations to any of the *fixNOQP* genes will induce defective nitrogen fixation (Fischer 1994).

## **Two-component systems**

### **Purpose and mechanisms**

Bacteria are required to adapt to constantly changing environments so they can proliferate in nutrient rich, nutrient poor, or highly competitive environments. For bacteria to sense what type of environment they are in, they must sense extra-cellular stimuli. Additionally, to utilize energy and resources at the correct time while conserving energy at other times, bacteria must maintain strict control over their biological functions. To accomplish these two goals, bacteria use two-component systems (TSCs), which are abundant throughout the prokaryote kingdom and in their simplest form comprise a sensor histidine kinase (HK) and a cognate response regulator (RR) (**Figure 3**). The purpose of the TCS is to sense extracellular stimuli and elicit a specific physiological response. The HK component of the TCS is typically a transmembrane protein with an extracellular sensor domain (Mascher, Helmann et al. 2006). The second component of

the TCS is the RR, which receives input from the HK and responds by triggering a downstream response, which is usually transcriptional regulation (Mascher, Helmann et al. 2006, Gao and Stock 2009).

The mechanism by which a TCS functions is through an autophosphorylation event on the HK in response to a specific stimulus. HKs comprise a C-terminal catalytic domain (CA) that will bind ATP and phosphorylate a histidine residue on a dimeric helical HK domain (Casino, Rubio et al. 2010). This phosphoryl group is then transferred to an aspartic acid residue on the receiver domain (REC) of its cognate RR. After the RR is phosphorylated by the HK, the RR will then regulate a specific physiological response, often by promoting or suppressing gene expression (Gao and Stock 2009, Casino, Rubio et al. 2010).

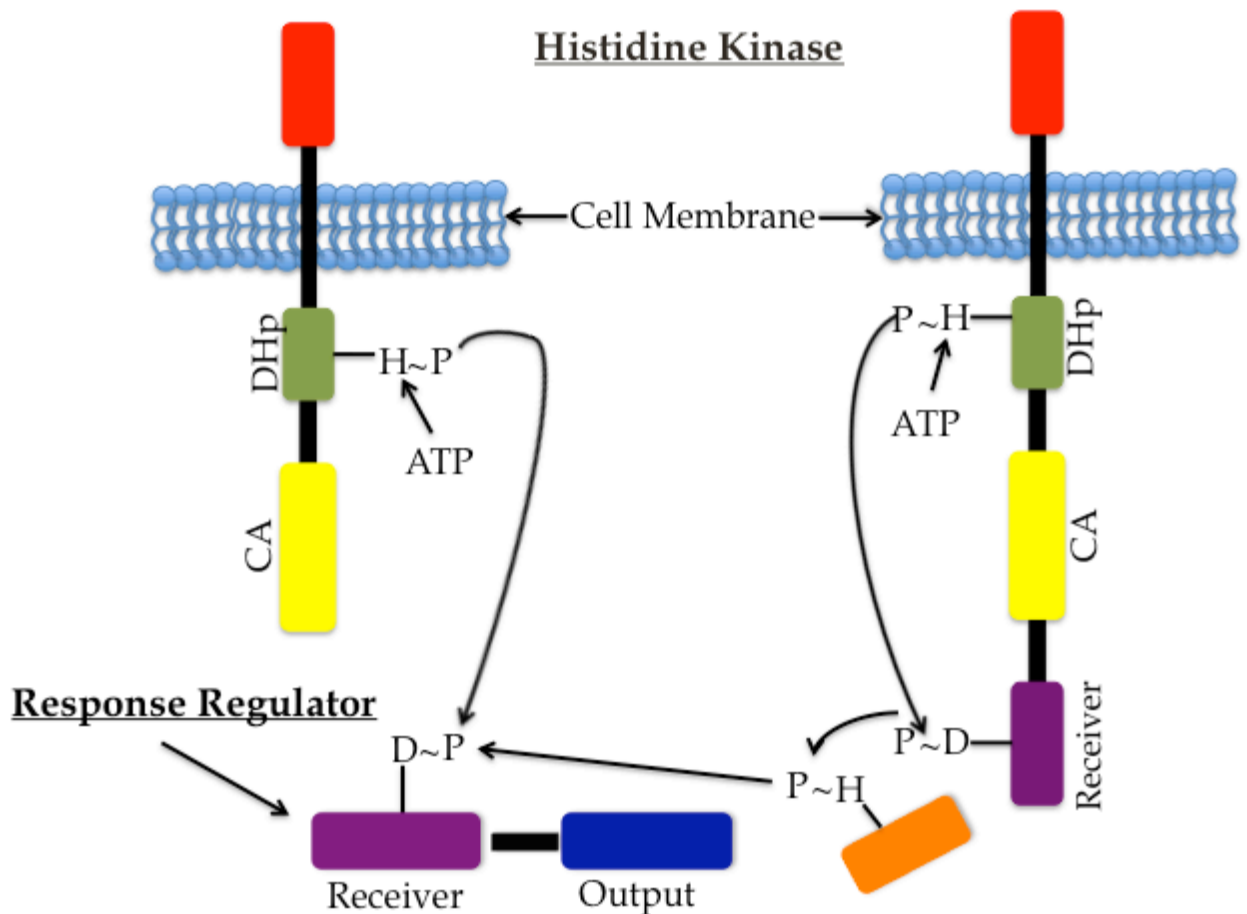
TCSs are typically linear signal transduction pathways that can be altered and modified in certain ways. The HK of the TCS not only autophosphorylates itself and then phosphorylates its' specific RR, but it can also act as a phosphatase for its RR (Casino, Rubio et al. 2010). Variants of TCSs are known as phosphorelays (Laub and Goulian 2007). In a phosphorelay, such as the one in *S. meliloti* or *C. crescentus* that will be discussed later, a hybrid histidine kinase senses specific stimuli, autophosphorylates, and then transfers the phosphoryl group to a response regulator-like receiver domain that resides within an intermolecular region. Next, the phosphoryl group is transferred to a histidine phosphotransferase (HPT) which phosphorylates a specific RR (Laub and Goulian 2007) (**Figure 3**). This raises the question of how TCSs are so specific and avoid promiscuous cross talk between multiple TCSs in the same cell. Answering the question

of how these cells maintain specificity within their TCSs to prevent promiscuity is currently a major focus of research. It is a promising research focus because in nearly every sequenced bacterial genome, there are multiple HKs and RRs, with some having up to 200 pairs (Laub and Goulian 2007). However, these TCSs have not been found in the animal kingdom and show promise as targets for antibacterials (Gotoh, Eguchi et al. 2010).

### **Two-component system specificity**

Although TCSs have been extensively studied, the high fidelity of TCSs is still being investigated. The concern for cross talk is due to conservation at the genetic level between HKs and RRs. Cross talk as defined by (Laub and Goulian 2007) is the communication between two pathways that, if eliminated, would leave two distinct, and intact functioning pathways. The majority of *in-vivo* experiments observed cross-talk only after genetic perturbations were induced; however, there are examples of cross talk occurring in wild type cells under stressful conditions (Laub and Goulian 2007). Despite the few examples of cross talk, there are three mechanisms we know that bacteria use to ensure specific signal transduction: molecular recognition, phosphatase activity, and substrate competition. Molecular recognition provides a strong means by which a TCS can ensure that it will induce the response it intended due to its stimulus. Not only can a HK preferentially select for its' specific cognate RR over other potential targets through molecular recognition but there is evidence that HKs phosphorylate this RR with strong kinetic preference (Skerker, Prasol et al. 2005). The predetermined molecular recognition

is driven by small sets of amino acids in each protein. Initially, the amino acids that are required for the recognition of a RR by its HK were identified through computational analysis but have been validated through protein-protein interactions as well (Skerker, Perchuk et al. 2008, Weigt, White et al. 2009, Capra, Perchuk et al. 2010). Additionally, a



**Figure 3: (Left)** A basic two-component system (TCS) is comprised of a histidine kinase (HK) and a response regulator (RR). The HK will autophosphorylate upon stimulation by an extracellular factor and then phosphorylate its' response regulator to achieve a molecular response. The autophosphorylation occurs on the dimerization and histidine phosphotransferase (DHp) and is catalyzed by the catalytic and ATPase (CA) domain that is also responsible for binding ATP. **(Right)** A phosphoryl TCS is also common, where the HK autophosphorylates and then transfers the phosphoryl group to an aspartic acid residue on its' own receiver. The receiver domain phosphorylates a histidine phosphotransferase that uses the phosphoryl group to phosphorylate a RR.

RR by its HK were identified through computational analysis but have been validated through protein-protein interactions as well (Skerker, Perchuk et al. 2008, Weigt, White et al. 2009, Capra, Perchuk et al. 2010). Additionally, a RR can be rewired to be phosphorylated with high specificity by a non-cognate HK through modification of the specific amino acid groups that the non-cognate HK preferentially binds (Bell, Porter et al. 2010, Capra, Perchuk et al. 2010).

Bifunctional HKs, containing both kinase and phosphatase activity, are more effective at preventing cross talk than monofunctional HKs, which contain either kinase or phosphatase activity (Alves and Savageau 2003, Laub and Goulian 2007). Bifunctional HKs serve two primary purposes: 1) To dephosphorylate the RR after the extracellular stimulus that was initially received has subsided or been abolished (Huynh and Stewart 2011), and 2) To dephosphorylate its' RR after being phosphorylated by a different HK (McCleary, Stock et al. 1993, Klein, Shulla et al. 2007, Boll and Hendrixson 2011). Competition between RRs for phosphorylation by HKs may prevent cross talk and cells may control this by limiting the amount of HK present. Lower quantities of HKs have been shown to result in less cross talk (Batchelor and Goulian 2003) whereas over-expression of HKs show increased crosstalk (Ninfa, Ninfa et al. 1988).

### **Cell cycle control in *Caulobacter crescentus***

Two-component signaling is essential for a wide range of life styles throughout the prokaryotic kingdom. Since there are so many different TCSs that provide specific functions, I will focus on a particular TCS in *C. crescentus*. *C. crescentus* is a model organism that has been extensively studied to understand asymmetric daughter cell fate and precise cell cycle regulation. Through these investigations, a TCS has been found that is responsible for cell cycle regulation in *C. crescentus* and is conserved throughout some alphaproteobacteria. Therefore, for the relevance of this thesis, I will provide an overview of this TCS, known as the DivK TCS signal transduction pathway or just “DivK pathway,” and the proteins that are involved in providing temporal and spatial control of the cell cycle. The DivK pathway is currently under investigation within *S. meliloti*, *B. abortus*, and *A. tumefaciens*, therefore I will first focus on what is known in *C. crescentus*.

When *C. crescentus* divides, the result is two distinct asymmetric daughter cells, one with a stalk, known as a stalked cell, and the other with both pili and a single flagellum, known as a swarmer cell. Once cell division is complete, the stalked cell will immediately reinitiate chromosome replication while the swarmer cells are inhibited from this initiation event, as it is born into G1 phase (Purcell, Boutte et al. 2008). Therefore, *C. crescentus* provides a model that demonstrates how prokaryotes are complex and highly organized in their cellular mechanisms. More specifically, *C. crescentus* is ideal for studying how the cell cycle is regulated such that one daughter cell immediately begins its’ cell cycle again whereas the other is restricted from doing so. The swarmer cell will



eventually shed its' flagellum and pili, grow a stalk, exit from G1 phase, and initiate chromosome replication and cell division. The primary proteins that are involved in this tight cellular regulation are CtrA, GcrA, and DnaA.

DnaA is a transcription factor that is required for chromosomal replication initiation and shares redundant regulation of multiple genes required for cell cycle progression with CtrA (Gorbatyuk and Marczynski 2001). The DNA-binding protein DnaA is required for S phase cells to initiate DNA replication by recruiting the replisome complex containing DNA polymerase to the chromosome origin. However, the cell will only initiate chromosome replication once to ensure one replication event per cell cycle, leaving both daughter cells with one copy of the genome. To accomplish this, DnaA upregulates expression of GcrA, which also functions as a transcription factor to repress *dnaA* expression so that DnaA does not reinitiate chromosomal replication prematurely. This is coupled with the upregulation of the DNA-binding protein CtrA, which blocks DnaA binding to the origin of replication. The end result is a guaranteed one DNA replication event per cell cycle.

CtrA is an essential response regulator that binds DNA to regulate DNA replication initiation and gene transcription. CtrA activity is controlled through the DivK pathway and is considered active when phosphorylated. When CtrA is active, it will bind DNA at the origin of replication and block DnaA from initiating chromosome replication (Quon, Yang et al. 1998). Swarmer cells are blocked at G1 phase and not allowed to enter into S phase due to this CtrA activity. During the transition of swarmer cell to stalked cell, CtrA activity is inhibited and thereby allows DNA replication to be initiated. CtrA is

activated again in late stalked cells when it functions as a transcription factor to regulate genes involved in cell division and swarmer cell fate (Quon, Marczynski et al. 1996, Biondi, Reisinger et al. 2006).

GcrA is not a HK or RR, but still plays a primary role in regulating the cell cycle in *C. crescentus*. GcrA is upregulated as CtrA is being down regulated during the G1 to S phase transition and has been shown to indirectly activate *ctrA* in later in stalked cells (Holtzendorff, Hung et al. 2004). Additionally, *gcrA* is negatively regulated by phosphorylated CtrA and positively regulated by DnaA (Purcell, Boutte et al. 2008). More specifically, GcrA down-regulates the transcriptions of DnaA and affects the transcription of many genes other than *ctrA* (Holtzendorff, Hung et al. 2004). The out of phase oscillation of GcrA with CtrA and DnaA is coordinated with the progression of different phases of the cell cycle and therefore is implemented in the regulatory system *C. crescentus* utilizes. Contradictory to the redundancy seen between CtrA and DnaA, GcrA and DnaA provide a transcriptional feedback system where DnaA positively regulates *gcrA* and GcrA negatively regulates *dnaA* while positively regulating *ctrA*. To complete the feedback loop, CtrA acts as a transcription factor to negatively regulate *dnaA* (Purcell, Boutte et al. 2008).

## Conserved DivK pathway

### **Conserved global regulator CtrA**

Throughout the alphaproteobacteria class of prokaryotes there is an abundance of species that divide asymmetrically. Within this sub-group of alphaproteobacteria that divide asymmetrically, there is diversity among phenotypes and functions that are associated with the development of asymmetric daughter cells. For a cell to divide into two distinct daughter cells, it requires a precise regulatory network to ensure that each cell is equipped to fulfill its' intended function. The benefit of dividing asymmetrically for certain alphaproteobacteria is still unknown. Perhaps asymmetric cell division was an acquired trait at an early time during the evolution of the genera or families within alphaproteobacteria. It may have held certain advantages at the time, which since have been lost or adapted. For example, the *C. crescentus* immobile stalked cell will immediately begin to divide again creating a larger population while the swarmer cell waits until nutrient levels are ideal before dividing again. However, *S. meliloti* divides asymmetrically into two distinct daughter cells of different sizes, which both seem to be motile. There is still no definitive answer for why *S. meliloti* divides asymmetrically or what the benefit might be.

There is one common theme amongst asymmetrically dividing alphaproteobacteria. The essential response regulator CtrA is generally required for

successful cell cycle progression and asymmetric cell division. CtrA seems to consistently be a transcription factor that is highly conserved, essential for viability, and directly or indirectly involved in regulating the majority of genes involved in the cell cycle. For the purpose of brevity and the objective of this Thesis, I will primarily focus this discussion of CtrA in *C. crescentus* and *S. meliloti*.

In *C. crescentus* swarmer cells, which arrest in G1 phase, CtrA is abundant and phosphorylated (Domian, Quon et al. 1997). When CtrA is phosphorylated it is considered active, and when it is active it binds to the origin of replication and blocks DnaA from binding, thereby inhibiting replication initiation (Quon, Yang et al. 1998, Bastedo and Marczyński 2009). Additionally, phosphorylated CtrA transcriptionally regulates ~100 genes, most of which are involved in the cell cycle (Laub, McAdams et al. 2000, Laub, Chen et al. 2002). Somewhere between a quarter and half the genes known to participate in the cell cycle are thought to be directly or indirectly regulated by CtrA, either positively or negatively (D'Ari 2001).

*C. crescentus* tightly controls cell cycle progression by regulating the levels of phosphorylated CtrA in addition to maintaining specific levels of CtrA through degradation. In G1 phase swarmer cells, CtrA is stable and phosphorylated, and thereby inhibits DNA replication initiation. In S phase stalked cells, CtrA is dephosphorylated and degraded (Domian, Quon et al. 1997). After replication initiation is allowed, as a result of CtrA being inactivated, CtrA is then synthesized *de novo* and phosphorylated so that it can regulate expression of its' target genes (Tsokos and Laub 2012). The transcription of *ctrA* is therefore also cell cycle-dependent and CtrA autoregulates *ctrA*

expression, providing a feedback loop (Domian, Reisenauer et al. 1999). The transition from G1 into S phase as well as the asymmetric division that *C. crescentus* utilizes to produce a G1 phase swarmer cell and an S phase stalked cell is ultimately dependent on two HKs, DivK and CckA, which are discussed at length in the next section (**Figure 4**).

Although CtrA has not been as extensively studied in *S. meliloti* as it has in *C. crescentus*, much progress has been made over the past decade in revealing the role of CtrA in the regulation of its' cell cycle. Despite the limitations of cell cycle regulation research within *S. meliloti* due to the lack of a synchronization method, predictive models have emerged and new advances show how CtrA regulates the cell cycle within *S. meliloti*. Research has shown that in contrast to *C. crescentus* regulation, CtrA does not bind the origin of replication in *S. meliloti*. As a guide to potential CtrA binding sites, Schluter *et al.* mapped transcription start sites (TSS) within *S. meliloti* to provide a platform for more experiments (Schluter, Reinkensmeier et al. 2013). Similar to CtrA self-regulation in *C. crescentus*, they found five putative CtrA binding sites upstream of the *ctrA* open reading frame (ORF) in *S. meliloti*. Position specific scoring matrices (PSSM) were used to identify additional CtrA binding sites within *S. meliloti* upstream of known ORFs, and this was compared to the TSS that they had previously mapped. They found putative CtrA binding sites upstream of TSS belonging to cell cycle genes such as *minC*, *chpT*, *rcdA*, *pleC*, *ftsE*, and *rpoD*, in addition to others. These results indicate that CtrA could bind to and transcriptionally regulate these genes in a cell cycle-dependent manner, as has been seen in other alphaproteobacteria such as *C. crescentus* or *B. abortus* (Bellefontaine, Pierreux et al. 2002, Cheng, Sibley et al. 2007, Brilli, Fondi et al. 2010).

In a more recent study, populations of *S. meliloti* were synchronized by nutrient deprivation and position weight matrices were used with the 16 nucleotide CtrA-binding motif to identify *S. meliloti* cell cycle genes that might be regulated by CtrA (De Nisco, Abo et al. 2014). De Nisco et al. identified putative CtrA-binding motifs in the promoter regions of *minC*, *chpT*, *pleC*, *ftsK*, and others that were mapped by Shluter et al. (2013). To the surprise of De Nisco et al. (2014), they found a high amount of divergence in the *S. meliloti* CtrA regulon when compared to the *C. crescentus* CtrA regulon. Only eight genes were shared between these two species. This led to a comparison between the *S. meliloti* CtrA regulon and a sampling of the CtrA regulons within alphaproteobacteria that are more closely related to *S. meliloti*, such as *B. abortus* and *A. tumefaciens*. The CtrA regulon was much more conserved in 11 alphaproteobacteria species more closely related to *S. meliloti* than *C. crescentus*. The DNA methylase, *ccrM*, a crucial component of chromosomal replication, did not have a CtrA-binding motif in *S. meliloti*. This was another surprise because in *C. crescentus*, CtrA regulates *ccrM* in a cell-cycle dependent manner (Wright, Stephens et al. 1997, Brill, Fondi et al. 2010). De Nisco et al. (2014) showed that *ccrM* is cell cycle regulated, but without a CtrA upstream regulatory region.

These results are intriguing because they suggest that there may be a deviation in the role that CtrA plays between *C. crescentus* and *S. meliloti*. After predicting that CtrA regulation is distinct between *S. meliloti* and *C. crescentus*, the next step will be to test for direct regulation of predicted CtrA targets. The notion that the direct regulation of genes involved in *C. crescentus* cell cycle by CtrA is different or absent in *S. meliloti* suggests that there is another protein or response regulator that is in direct control of

these genes. Perhaps the symbiotic lifestyle that *S. meliloti*, *B. abortus*, and *A. tumefaciens* have evolved is also associated with modification to their cell cycle regulation.

### **Histidine kinases of DivK pathway**

Molecular control of the cell cycle within *C. crescentus* is modulated primarily through the DivK pathway. The DivK pathway consists of TCS histidine kinases (HK), phosphatases, and phosphorelays, as well as transcription factors and proteases. It is through this pathway that tight molecular regulation has been shown to control CtrA activity and the cell cycle. There is a great amount of overlap between the DivK pathway in *C. crescentus* and the DivK pathway in *S. meliloti*. I will focus my discussion on the DivK pathway in *C. crescentus*, which has been extensively studied, and point out differences in the *S. meliloti* DivK pathway, which is just starting to be dissected.

PleC and DivJ are both transmembrane HKs that act in opposite manners on DivK. PleC has kinase activity and is able to phosphorylate DivK *in vitro*, however it is the sole phosphatase of DivK *in vivo*. Therefore, it is thought that DivJ is the kinase that acts on DivK *in vivo* (Wheeler and Shapiro 1999, Matroule, Lam et al. 2004). In *C. crescentus*, DivJ phosphorylates DivK in newly born S phase stalked cells and during the transition from G1 to S phase in swarmer cells, leading to dephosphorylation and degradation of CtrA and subsequent DNA replication initiation. In contrast, PleC

dephosphorylates DivK in newly born swarmer cells and during the transition from S to G2 phase, which ultimately allows phosphorylation of CtrA and prevents replication re-initiation.

In *S. meliloti* and other alphaproteobacteria, there are additional DivJ and PleC orthologs that influence the DivK pathway and cell cycle regulation. CbrA and CbrB are DivJ orthologs found in *S. meliloti*. Deletion of *cbrA* results in defective cell cycle, motility and symbiosis phenotypes (Gibson, Campbell et al. 2006, Gibson, Barnett et al. 2007, Sadowski, Wilson et al. 2013). This novel histidine kinase acts on the DivK pathway in a manner similar to DivJ to regulate CtrA activity (Gibson, Campbell et al. 2006, Sadowski, Wilson et al. 2013). CbrA homologs that are thought to act through the DivK pathway were also found in *A. tumefaciens* (PdhS1) and in *B. abortus*, (PdhS). Since all three of these alphaproteobacteria are host-associated species, it may be that these novel DivJ orthologs are involved in their symbiotic life style. Whereas CbrA is not essential in *S. meliloti*, PdhS is essential in *B. abortus* and shows branching filamentous growth and decreased CtrA levels when overexpressed (Bellefontaine, Pierreux et al. 2002). It seems that PdhS in *B. abortus* and PdhS2 in *A. tumefaciens* share the likely role of acting as a kinase to DivK whereas PdhS1 is thought to act as a phosphatase to DivK in *A. tumefaciens* (Bellefontaine, Pierreux et al. 2002, Hallez, Bellefontaine et al. 2004).

In *C. crescentus*, DivK regulates CtrA activity indirectly through its effects on CckA, an essential hybrid histidine kinase that controls both the phosphorylation and proteolysis of CtrA (Jacobs, Domian et al. 1999). When DivK is dephosphorylated by PleC during G1 phase, CckA is able to phosphorylate itself. In contrast, when DivK is



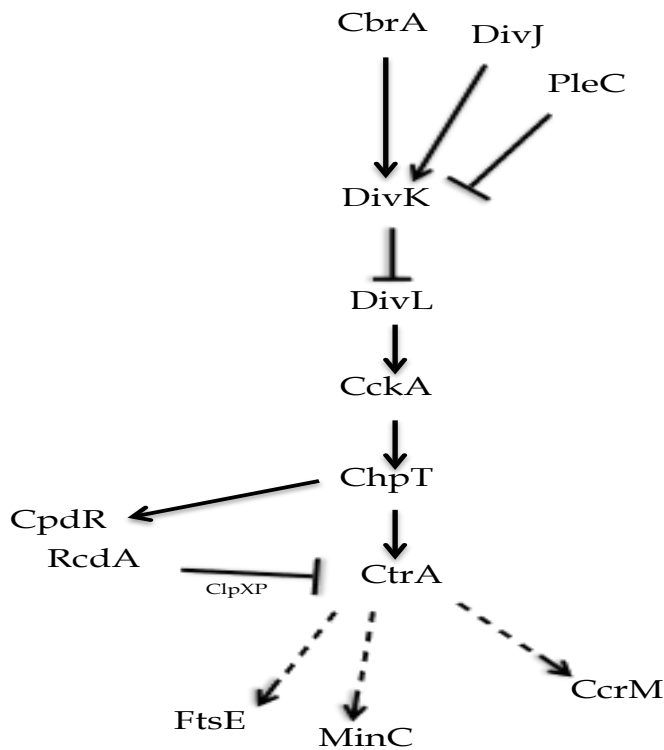
phosphorylated by DivJ during S phase, it inhibits CckA autophosphorylation. CckA is a membrane bound histidine kinase that lacks a periplasmic sensing domain but does have a receiver domain, which is used to relay a phosphate to a histidine phosphotransferase (Hpt) protein, ChpT (Biondi, Reisinger et al. 2006). ChpT will then continue the phosphorelay by phosphorylating two targets: CtrA, activating it for DNA binding, and CpdR, which is required for CtrA degradation by the ClpXP protease.

G1 phase, with its high level of CtrA activity, is maintained through a combination of CtrA and CpdR phosphorylation by ChpT. When phosphorylated, CpdR is not active in targeting CtrA for degradation by ClpXP. Thus, CckA kinase activity in G1 phase allows transfer of its phosphoryl group to CtrA and CpdR through ChpT, activating CtrA and inhibiting CtrA degradation through CpdR phosphorylation (**Figure 4**). In contrast, during the transition from G1 into S phase, CckA is inactivated and this prevents the phosphorylation of CtrA and CpdR, which leads to CtrA deactivation through a combination of dephosphorylation and proteolysis. Once CtrA is deactivated, DNA replication initiation is allowed to proceed.

ClpXP is an ATP-dependent protease that degrades CtrA in a CpdR-dependent manner (Jenal 2004, Curtis and Brun 2010). Another protein that is required for CtrA proteolysis is RcdA. In contrast to the stabilization of CtrA in *rcdA* mutants, *in vitro* analysis shows that ClpXP alone can degrade CtrA and that RcdA has no effect on the rate of proteolysis (Chien, Grant et al. 2007). This suggests that RcdA is an adaptor protein and still leaves the function of RcdA *in vivo* unknown. PopA is a protein found in *C. crescentus* and that is absent from the *S. meliloti* genome, and is required for CtrA

degradation (Duerig, Abel et al. 2009). This is another example of how the DivK pathway is plastic between organisms and may differ slightly depending on the lifestyle of the organism.

The primary function of DivL, a histidine kinase with unusual characteristics, stumped experts for a long time. Instead of the conserved histidine that becomes phosphorylated, DivL has a tyrosine residue (Wu, Ohta et al. 1999). It was recently found that DivL is required for activation of CtrA since *divL* mutants lead to decreased levels of phosphorylated CtrA (Tsokos, Perchuk et al. 2011). This suggests that DivL modulates the activation and phosphorylation of CtrA. The same study found that instead of directly



**Figure 4. Model of DivK pathway in *S. meliloti*.** Through DivL, DivJ/PleC/DivK regulates CckA/ChpT and ultimately CtrA. Additionally, the novel histidine kinase CbrA regulates CtrA through DivK. Arrows indicate kinase activity. Dashed arrows indicate regulation of CtrA targets that has not yet been shown.

regulating the activation of CtrA through phosphorylation, DivL is required to localize CckA. CckA localization is required for CtrA regulation, and in *divL* mutants, CckA does not localize properly (Tsokos, Perchuk et al. 2011). Therefore, one function of DivL is to bind and localize CckA. This suggests that CckA localization is required for autophosphorylation, however, this is not the case. Instead, CckA activity depends on whether or not DivK is phosphorylated (Hecht, Lane et al. 1995, Cabantous, Guillet et al. 2002, Guillet, Ohta et al. 2002). Dephosphorylation of DivK allows CckA kinase activation in a DivL-dependent manner (Tsokos, Perchuk et al. 2011). Phosphorylated DivK binds to DivL and inhibits CckA; but when DivK is not phosphorylated, DivL promotes CckA activity (Tsokos, Perchuk et al. 2011). Thus, through a complex TCS network, the HKs DivJ and PleC are able to mediate cell cycle control over CtrA phosphorylation and degradation through DivK and thereby regulate the G1 to S phase transition as well as asymmetric daughter cell fate.

### **CtrA targeted genes: function and implication for the cell cycle**

I have discussed the general mechanism by which CtrA is cell cycle regulated in alphaproteobacteria and will now delve into the genes known to be transcriptionally regulated by CtrA, some of the genes thought to be regulated by CtrA, and their significance. CtrA is as a transcription factor and has been shown to either directly or

indirectly regulate the transcription of certain genes in *C. crescentus*. Work in *S. meliloti* has shown hypothetical relationships between CtrA and numerous targets based on TSS. For my thesis, I chose to focus on genes that function as general cell cycle factors involved in either chromosome replication or cell division. Additionally, I chose to examine putative CtrA targets that are likely components of the DivK pathway, previously discussed, that may provide feedback regulation within the cell cycle. Here I will introduce the genes I examined in this thesis and any complimentary genes required for their function. I will only discuss genes not already introduced that are likely regulated by CtrA and implicated in the cell cycle.

I found two genes useful for investigating the role of cell cycle regulators in cell division, FtsE and MinC. MinC works with MinD and MinE to form a MinCDE complex. Together MinCDE restricts FtsZ at polar sites in cell of *Escherichia coli* to ensure that FtsZ forms a Z-ring at the correct midcell location, allowing for successful cellular division into two viable daughter cells (Errington, Daniel et al. 2003, Cheng, Sibley et al. 2007). *S. meliloti* carries a single copy of *minCDE* on the pSymB megaplasmid of its' genome (Finan, Weidner et al. 2001, Galibert, Finan et al. 2001). The location of the *min* genes, on the megaplasmid, suggests that they may provide a dispensable function, which is supported by the results showing their non-essentiality for viability (Cheng, Sibley et al. 2007). In *E. coli*, deletion of *minCDE* produces a mini-cell phenotype that indicates an aberration in cell division, which is not seen in *S. meliloti* (Errington, Daniel et al. 2003). There seems to be a system in *S. meliloti* that compensates for or is redundant to the *min* system in localizing the septum for correct

cell division. Cheng et al. (2007) looked at  $\Delta minCDE$  mutants in both free-living cells and nodules and found that, in addition to being expressed in free-living cells, the *minCDE* genes are expressed in nodules. However, the *min* system is also dispensable for symbiosis. A previous transcriptome analysis showed that *fts* genes are down regulated in bacteroids (Barnett, Toman et al. 2004), which is consistent with the lack of a requirement for *min* genes and the absence of cell division in bacteroids. Typically the *min* genes will work to prevent the *fts* genes from catalyzing cytokinesis in the wrong location of the cell. Additionally, *C. crescentus* lacks *min* homologues, which supports the notion that *S. meliloti* picked up the Min system on the SymA megaplasmid but may not need it for cell division (Figge, Easter et al. 2003, Margolin 2003, Cheng, Sibley et al. 2007).

The exact function of FtsE is still somewhat ambiguous despite progress in elucidating its role and how it functions in prokaryote cells. FtsZ is primarily implicated in cell division, as it is the first known component of the prokaryotic cell division apparatus to localize to the site of cellular division (Bi and Lutkenhaus 1991, Addinall, Bi et al. 1996). Once FtsZ is in the proper location for cell division it will form a Z-ring, providing a scaffold that recruits FtsA, ZipA, FtsE, FtsX, FtsK, FtsQ, FtsL, FtsB, TfsW, FtsI, FtsN, and AmiC (Goehring and Beckwith 2005, Margolin 2005, Pichoff and Lutkenhaus 2005).

Previous studies have shown that FtsE acts alongside FtsX as an ATP-binding cassette transporter, where FtsE acts as the ACB component with FtsX in the cytoplasmic membrane (Gill and Salmond 1987, de Leeuw, Graham et al. 1999). This

FtsEX complex has been shown to localize to the Z-ring (Schmidt, Peterson et al. 2004). Depletion of FtsE results in inhibition of cell division and growth (Corbin, Wang et al. 2007), suggesting that FtsE is an integral component of the divisome in *E. coli*. Another report showed that FtsE interacts directly with FtsZ, providing additional evidence that it is an important cell division protein (Corbin, Wang et al. 2007). There is still much work to be done to fully elucidate FtsE function. Therefore, it provides an ideal target to understand cell division and regulation with *S. meliloti* since it has an FtsE homologue that is predicted to have a CtrA regulatory box (Schluter, Reinkensmeier et al. 2013).

The primary function for DNA methylation in prokaryotes is DNA restriction-modification, which allows the cell to differentiate between self and foreign DNA (Wilson 1988, Bickle and Kruger 1993). However, in *C. crescentus* and *S. meliloti* the CcrM DNA methyltransferase has a different function. CcrM has been found to be essential in both *C. crescentus* and *S. meliloti* and functions to methylate the adenine residue in the sequence GANTC (Zweiger, Marczyński et al. 1994). I used the DNA methyltransferase, CcrM, to investigate the regulation of chromosome replication. Overexpression in either organism causes defective cell division and over-initiation of DNA replication, resulting in aberrant cell morphology (Wright, Stephens et al. 1997). Wright et al. (1997) also showed that CcrM is so well conserved between *S. meliloti* and *C. crescentus* that it is interchangeable between the two species.

## Research goals

The observation that  $\Delta cbrA$  leads to a filamentous, branched, and swollen morphology suggests that there may be a disruption to the cell division machinery in these mutants. Additionally, Sadowski et al. (2013) found the  $\Delta cbrA$  mutant has ploidy defects as indicated by an increase in  $<1N$  and  $>2N$  chromosome content. These results led to my hypothesis that genes involved in cell division and chromosome replication are misregulated in the absence of *cbrA*. My first research goal was to determine how expression of cell division and chromosome replication genes are regulated in  $\Delta cbrA$ . In addition to the observations mentioned above, there is no known CbrA DNA binding domain. Therefore, if CbrA is contributing to the regulation of cell division or chromosome replication, it is doing so through the DivK pathway. I hypothesize that the regulatory proteins in the DivK pathway are also misregulated in the absence of *cbrA*. Therefore, my second research goal is to determine how the expression regulatory genes in the DivK pathway are altered in  $\Delta cbrA$ .

Using the  $\beta$ -glucuronidase (GUS) reporter system to track transcriptional regulation in free-living *S. meliloti* enabled the investigation of target gene expression in free-living and symbiotic cells. My final research goal was to provide qualitative evidence showing the location of gene expression during nodule development. The genes I used to track cell division and chromosome replication might be utilized during bacteroid formation and nitrogen fixation. Additionally, the regulatory genes in the DivK pathway might also contribute to bacteroid formation and nitrogen fixation through the

regulation of cell division and chromosome replication genes. Large pink nodules have all stages of nodule development and allowed me to investigate if these genes were involved in nodule invasion, colonization, and nitrogen-fixation simultaneously.



## CHAPTER II

### METHODS

#### General techniques

##### **Culture and genetic techniques**

*Sinorhizobium meliloti* cultures were grown in LB medium supplemented with 2.5 mM each of CaCl<sub>2</sub> and MgSO<sub>4</sub> (LB/MC) at 30°C unless otherwise specified. *Escherichia coli* strains were grown in LB medium at 37°C unless otherwise specified. When required, logarithmic phase cultures of *S. meliloti* were obtained by diluting overnight cultures to an OD<sub>600</sub> of 0.1 and allowing cells to achieve an OD<sub>600</sub> of between 0.5 and 0.8. Relative succinoglycan production was observed by growing *S. meliloti* strains on LB/MC agar supplemented with 0.02% calcofluor (CF), and 10 mM HEPES (pH 7.4).

Strains were constructed through triparental mating, which entails transferring plasmids engineered in *E. coli* strain DH5 $\alpha$  into either the wild type (WT) laboratory Rm1021 strain of *S. meliloti* or the  $\Delta cbrA::cat$  laboratory strain CSS6000 with *E. coli* MT616 as the helper strain. Where appropriate, antibiotics were added to growth medium at the following concentrations: chloramphenicol (20  $\mu\text{g ml}^{-1}$ ), kanamycin (50  $\mu\text{g ml}^{-1}$ ), neomycin (200  $\mu\text{g ml}^{-1}$ ), and streptomycin (2  $\text{mg ml}^{-1}$ ).

### **Construction and verification of GUS transcription fusion strains**

Eight genes were chosen to represent cell cycle regulation in *S. meliloti* (**Table 2**). The promoter region, transcriptional start sites (TSS) and predicted CtrA binding sites upstream of each gene was cloned from Polymerase Chain Reaction (PCR) products, which were sequenced for verification, into the pVO155 plasmid (Oke and Long 1999). The pVO155 vector is a suicide plasmid with a promoter-less  $\beta$ -glucuronidase (GUS) open-reading frame (ORF) on it. Each PCR product was inserted into the pVO155 plasmid using unique restriction digest enzyme cut sites, upstream of the GUS ORF (**Table 3**). These clones (GUS fusions) were then mated into WT strain Rm1021 and  $\Delta cbrA$  strain CSS6000 using triparental mating described previously. The ATG translation start codon was also included in the construction of these plasmids. In general, approximately 800-1000 bps upstream of the ATG start site and 100-200bps downstream of the ATG start site were cloned upstream of the promoter-less GUS ORF.

Each GUS fusion that was mated into WT strain Rm1021 was verified using T880RXh and G743FBm primers to amplify the *cbrA* PAS domain (**Table 1, Figure 5A**). The GUS fusions mated into  $\Delta cbrA$  strain CSS6000 were verified using the cat 1 and cbra dn primers to amplify the chloramphenicol resistant cassette used to replace *cbrA* (**Table 1, Figure 5B**).

The doubling time of each GUS fusion strain was monitored and quantified. Strains were grown overnight, in triplicate, in LB/MC medium and subcultured to an OD<sub>600</sub> of 0.1. Subsequent OD<sub>600</sub> measurements were taken at 2 and 4 hours. Additionally, relative succinoglycan production was observed by growing *S. meliloti* strains on LB/MC agar supplemented with 0.02% calcofluor (CF) and 10 mM HEPES (pH7.4).

### **Quantification of $\beta$ -glucuronidase (GUS) expression**

#### **GUS assays**

GUS fusions strains were assayed with 4-Nitrophenyl  $\beta$ -D-glucopyranoside in order to quantify the amount of GUS enzyme being produced in each strain. Log phase cultures of each strain were obtained in triplicate and pelleted by spinning each culture at full speed for 5 minutes. The supernatant was removed from the pellet and the pellet was re-suspended in a Cell Lysis Buffer (374  $\mu$ L of B-PER<sup>TM</sup> Bacterial Protein Extraction

Reagent, 50 uL Protease and Phosphatase Inhibitor Cocktail, 1 uL of 34 mg/mL chloramphenicol (prepared in methanol), and 6 uL of 10 mg/ml lysozyme), and allowed to lyse for 5 minutes on ice, creating cell lysates. In a separate 2 mL eppendorf tube, 200 uL from each cell lysate was added to 800 uL of GUS Assay Buffer (50 mM NaPO<sub>4</sub> pH7, 1 mM EDTA, .07% β-mercaptoethanol, and 1.25 mM 4-Nitrophenyl β-D-glucopyranoside substrate). Reactions were stopped by adding 100 uL of sample reaction to 800 uL of .4 M Na<sub>2</sub>CO<sub>3</sub>. Measurements were then taken in a spectrophotometer at an absorbance of 415nm.

### **Western Blots**

Exponential cultures were centrifuged at 4 °C for 10 min at 5000 x g. Cell pellets were re-suspended in 2x Laemmli loading buffer and boiled for 5 min. The volume of sample loaded was normalized to OD<sub>600</sub>, subjected to 4-20% SDS-PAGE with TRIS running buffer (250 mM Tris Base, 1.92 M Glycine, 1% SDS) at a constant 100 V for 100 min, and then transferred onto a low fluorescence PVDF membrane with Tris-glycine transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at a constant 100 V for 1 h. The membrane was probed with anti-beta Glucuronidase (GUS) antibodies (1:200 or 1:500 dilution in Licor blocking buffer and 0.2% Tween) for 16 h at 4 °C, and subsequently probed with donkey anti-rabbit IRDye 800 CW (1:20,000 dilution in Licor blocking buffer with 0.2% Tween and 0.1% SDS). Cross-reacting proteins were

visualized with a Licor Odyssey CLx Infrared Imaging system and images were quantified with the Image Study Software.

### **MUG assays**

Log phase cultures were normalized to an  $OD_{600}$  of 0.5 at a final volume of 200  $\mu\text{L}$  to control for cell density. Cultures were then spun for 5 min at full speed, supernatant was decanted, and pellets were re-suspended in 800  $\mu\text{L}$  of Lysis Buffer (.05 M  $\text{NaPO}_4$  buffer, .07% beta-mercaptoethanol, .01 M EDTA, 0.10% Sarcosyl, 0.1% Triton). In a 96-well Dynex Fluorolux HB black flat bottom microplate, 160  $\mu\text{L}$  of each cell lysate was added to its' own individual well. A dilution series of 4-methyumbelliferone (4MU), the fluorescent product of this assay, was also included in each plate from 2  $\mu\text{M}$  to 10  $\mu\text{M}$  in increments of 2  $\mu\text{M}$  4MU. The GUS substrate, 4-methylumbelliferyl-beta-D-glucuronide (MUG), was added in a volume of 40  $\mu\text{L}$  (.005 M MUG ) to make a final concentration of 0.001 M (1 mM) of MUG. The plate was assayed at an excitation of 355nm with absorbance read at 405nm using a POLARstar Omega BMG LABTECH fluorescent plate reader. BMG LABTECH Omega firmware version 1.2 software was used to control the machine and settings. A gain of 600 was used to generate the arbitrary fluorescent units for each reaction. The optimized protocol used the arbitrary fluorescent unit read at minute 30 to determine GUS enzymatic activity. BMG LABTECH Mars Data Analysis Software version 1.2 was used to analyze the data after readings were taken. The slope

from the 4MU dilution was used to provide the slope and y-intercept to calculate GUS enzymatic activity from the arbitrary fluorescent unit read at minute 30. The equation ( $x = (y-b/m)/30$ ), derived from  $y = mx + b$ , was used where y was the arbitrary fluorescent unit from the GUS fusion sample, giving us the corresponding x, or 4MU amount produced, divided by 30 (minutes) resulting in the 4MU/minute produced, and thus enzyme activity units.

### **Tracking gene expression in nodules**

#### **Nodules assays**

Gene expression during symbiosis was assayed by inoculating *Medicago sativa* (alfalfa) plants with wild type and  $\Delta cbrA$  strains on buffered NOD medium (BNM) agar (Ehrhardt, Atkinson et al. 1992). Bacteria were grown into log phase, diluted to an  $OD_{600}$  of 0.10 at a final volume of 1.0 ml, washed and subsequently re-suspended in 1.0 ml half-strength BNM (1/2 x BNM) before being inoculated directly onto the plants' roots. As a negative control, plants were inoculated with WT strain Rm1021. At 21 and 35 days post-inoculation, plant root nodules were harvested for histochemical assays.

Nodules were sliced in half and added to an eppendorf tube containing 50  $\mu$ L of Nodule Assay Buffer (0.1 M  $NaPO_4$  buffer, 0.1% SDS, and 0.001 M 5-Bromo-4-chloro-

3-indolyl-beta-D-glucuronide cyclohexylammonium (X-GUS)). Tubes were then placed in a vacuum-sealed chamber for 90 minutes. After 90 minutes, nodules were washed with 250  $\mu$ L of 0.1 M NaPO<sub>4</sub> Buffer for 5 minutes, three times each. Nodules were then placed onto a slide with a minimum amount of NaPO<sub>4</sub> Buffer and the slide cover was fixed using nail polish. Slides were prepared using a Leica Zoom 2000 microscope. Images of nodules were taken using an Olympus BX60 microscope equipped with a SPOT Xplorer camera. SPOT software was used to analyze images.

### **Allelic replacement**

*Caulobacter crescentus* strain YB1804 was used to amplify, through PCR, the temperature-sensitive *dnaE* allele using primers CcDnaE OLD Fwd and CcDnaE OLD Rev (Table 1). *S. meliloti dnaE* upstream (5') and downstream (3') flanking regions were amplified using primer pairs Sm5 Fwd/Sm5 OLD Rev and Sm3 OLD Fwd/Sm3F flank Rev, respectively. The YB1804 *dnaE* gene was ligated to the two *S. meliloti* flanking regions, in their respectable position, through overlap-extension PCR. The resulting construct included the *S. meliloti dnaE* upstream region, YB1804 *dnaE*<sup>ts</sup>, and the *S. meliloti* downstream region. The 5' end of the *S. meliloti dnaE* upstream flank and the 3' end of the *S. meliloti dnaE* downstream flank were constructed with *EcoRI* restriction digest cut sites. These cut sites were used to ligate this ~4.5kb construct into the ~3.5 suicide plasmid pK18*mobsacB* yielding the plasmid, pK18CSH10 (Table 1). The

pK18*mobsacB* vector is non-replicative in *S. meliloti* and carries the *sacB* gene, which confers lethality when grown on sucrose (Schafer et al. 1994). Transformations were used to transfer the plasmid carrying the *S. meliloti* flanks and YB1804 *dnaE* into *E. coli* strain DH5 $\alpha$  (Kushner 1978). After successful cloning into DH5 $\alpha$ , the *S. meliloti* upstream, YB1804 *dnaE*<sup>ts</sup>, and *S. meliloti* downstream regions were all sequenced. The sequence showed no unintentional mutations in the YB1804 *dnaE*, and included the T to A point mutation, which should cause the V to E amino acid change (Lo, van Der Schalie et al. 2004). Tri-parental matings were used to conjugate pK18CSH10 into wild type strain Rm1021. Homologous recombination with just YB804 *dnaE* in pK18*mobsacB* was unsuccessful. However, when the *S. meliloti* flanking regions were included in the construct added to pK18*mobsacB*, homologous recombination into the *S. meliloti* genome proved successful. Successful recombination was indicated by the conferral of Neomycin resistance to *S. meliloti*. PCR primers were used to verify the presence of YB1804 *dnaE* within *S. meliloti* using the primers CcDnaE OLD Fwd and CcDnaE OLD Rev, with positive control, YB1804 genomic prep, and negative control *S. meliloti* WT strain 1021 genomic prep.



**Table 1. Strains and reagents used in this study**

<u>Strains</u>	Relevant Characteristics	Reference or Source
MT616	E. coli MM294; pRK600 Cmr	T. Finan
DH5 $\alpha$	E. coli <i>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 <math>\Delta(lacZYA-argG)</math></i>	BRL Corp
Rm10210	SU47 Smr	F. Ausubel
CSS6000	Rm1021 <i>ΔcbrA::cat</i>	C. Sadowski
CSH1001	Rm1021 <i>cbrA::pVO155</i>	This study
CSH6001	CSS6000 <i>cbrA::pVO155</i>	This study
CSH1002	Rm1021 <i>ccrM::pVO155</i>	This study
CSH6002	CSS6000 <i>ccrM::pVO155</i>	This study
CSH1003	Rm1021 <i>cpdR1::pVO155</i>	This study
CSH6003	CSS6000 <i>cpdR1::pVO155</i>	This study
CSH1004	Rm1021 <i>ctrA::pVO155</i>	This study
CSH6004	CSS6000 <i>ctrA::pVO155</i>	This study
CSH1005	Rm 1021 <i>ftsE::pVO155</i>	This study
CSH6005	CSS6000 <i>ftsE::pVO155</i>	This study
CSH1006	Rm 1021 <i>minC::pVO155</i>	This study
CSH6006	CSS6000 <i>minC::pVO155</i>	This study
CSH1007	Rm 1021 <i>pleC::pVO155</i>	This study
CSH6007	CSS6000 <i>pleC::pVO155</i>	This study
CSH1008	Rm 1021 <i>redA::pVO155</i>	This study
CSH6008	CSS6000 <i>redA::pVO155</i>	This study
CSH2031	Rm1021 YB1804dnaE::pK18 <i>mobsacB</i>	This study
<u>Plasmid</u>		
pVO155	Suicide plasmid with promoter-less $\beta$ -glucuronidase	Oke et al. 1999
pK18 <i>mobsacB</i>	Allelic exchange plasmid	Schafer et al. 1994
<u>Primers</u>		
cbrA fusion fwd	AATCCTAGGAATTCACATACGGTCACTCACAC	This study
cbrA fusion rev	CCGCTCGAGCGGCGATGAAATCGTAGACCGAGGT	This study
CcrM fwd	AATCCTAGGAATACCCATGAGGCTTTCGAGCAT	This study
CcrM rev	CCGCTCGAGCGGTCTCCCTTGATGATGTGTC	This study
cpdR1 fusion fwd	AATCCTAGGAATCAGTCGATGAGGATCGACAT	This study
cpdR1 fusion rev	GTCTAGACCATGTCGTTGTCGCTCTCG	This study
ctrA fusions fwd	AATCCTAGGAATTGCATCTTCAGCTCGCGAAT	This study
ctrA fusions rev	CCGCTCGAGCGGCATGAGCTCGATGCTCTGAG	This study
FtsE Fwd	AATCCTAGGAATTGTCGTTGAACACCGGATAG	This study
FtsE Rev	CCGCTCGAGCGGCGGAATATCGAATGTCAGGTCC	This study
minC Fwd	AATCCTAGGAATACCACAAGCTATAGTTGTCG	This study
minC fusion rev	ACTAGTTCAACTGTTCCGGTCGATC	This study

plecfusions fwd	AATCCTAGGAATTCTCCTATCTCGTCTATGCC	This study
plec fusions rev	TGCTAGACTGAGATCGCCACGATGAGAAGA	This study
rcdafusion fwd	AATCCTAGGAATTCTCCTATCTCGTCTATGCC	This study
rcdafusions rev	CCGCTCGAGCGGAGGGCCATCGAGATAGCTTG	This study
CcDnaE OLD Fwd	AGAATCGGAGTGCTCATGTGCGACGCGGA	This study
CcDnaE OLD Rev	TGCTGCATCTCTGTAACGTCTTCCAGCA	This study
Sm 3 OLD Fwd	TGCTGGAAGACGTTACAGAGATGCAGCA	This study
Sm 3 Flank Rev*	CCGGAATTCCGGCACCCTGAATGGCGTATTG	This study
Sm5 Fwd*	CCGGAATTCCGGACTGAAATAGTCCGGC	This study
Sm 5 OLD Rev	TCCGCGTCCGACATGGCACTCCGATTCT	This study
DnaE1490	GTGTCGATGTTATCCATCGG	This study
DnaE1950	AACGTCTCAGCCTGTCTGGA	This study
T880RXh	ATTATTACTCGAGTTAGGTGATGTCGCGGATGACCGCGCA	Sadowski et al. 2013
G743FBm	AATTTATGGATCCGGCGCAAGCGAAGCGGAGAAG	Sadowski et al. 2013
cat 1	AACTCACCCAGGGATTGGCT	Sadowski et al. 2013
cbra dn	CAACATTCCTTACAGCATCG	Sadowski et al. 2013

**Table 2. List of endonucleases used to clone each strain**

Strain	Restriction Endonuclease Used for Cloning	Relevant Characteristics
CSH1001	AvrII, XhoI	Rm1021 <i>cbrA</i> ::pVO155
CSH6001	AvrII, XhoI	CSS6000 <i>cbrA</i> ::pVO155
CSH1002	AvrII, XhoI	Rm1021 <i>ccrM</i> ::pVO155
CSH6002	AvrII, XhoI	CSS6000 <i>ccrM</i> ::pVO155
CSH1003	AvrII, XbaI	Rm1021 <i>cpdR1</i> ::pVO155
CSH6003	AvrII, XbaI	CSS6000 <i>cpdR1</i> ::pVO155
CSH1004	AvrII, XhoI	Rm1021 <i>ctrA</i> ::pVO155
CSH6004	AvrII, XhoI	CSS6000 <i>ctrA</i> ::pVO155
CSH1005	AvrII, XhoI	Rm1021 <i>ftsE</i> ::pVO155
CSH6005	AvrII, XhoI	CSS6000 <i>ftsE</i> ::pVO155
CSH1006	AvrII, SpeI	Rm1021 <i>minC</i> ::pVO155
CSH6006	AvrII, SpeI	CSS6000 <i>minC</i> ::pVO155
CSH1007	AvrII, XbaI	Rm1021 <i>pleC</i> ::pVO155
CSH6007	AvrII, XbaI	CSS6000 <i>pleC</i> ::pVO155
CSH1008	AvrII, XhoI	Rm1021 <i>redA</i> ::pVO155
CSH6008	AvrII, XhoI	CSS6000 <i>redA</i> ::pVO155
CSH2031	EcoRI	Rm1021 YB1804 <i>dnaE</i> ::pK18 <i>mobsacB</i>

## CHAPTER III

### RESULTS

#### GUS activity in free-living *S. meliloti*

##### **Construction of $\beta$ -glucuronidase (GUS) plasmids**

Previous data showed that  $\Delta cbrA$  leads to severe defects in the *S. meliloti* cell cycle and symbiosis, and that CbrA represses CtrA levels through DivK (**Figure 3**) (Sadowski, Wilson et al. 2013). Therefore, my goal is to test whether CbrA affects other regulatory genes in the DivK pathway as well cell cycle effector genes targeted by this pathway. I hypothesize that the cell cycle and symbiosis defects seen in  $\Delta cbrA$  are a result of excess CtrA levels and I therefore chose to track target gene expression at the transcriptional level since CtrA is a transcription factor in *C. crescentus*. The identification of genes whose expression is altered in the  $\Delta cbrA$  mutant will help provide

a molecular explanation for its observed cell cycle defects, as well as identify putative CtrA regulatory targets.

A plasmid utilizing the  $\beta$ -glucuronidase (GUS) reporter system, pVO155, was used to track transcriptional regulation of cell cycle genes. GUS activity was used as a way to assay transcriptional regulation of specific genes chosen due to their known function in *S. meliloti* or known function in other bacteria (**Table 3**). The genes were also chosen based on the predication that they belong to the CtrA regulon based on their promoter region containing a hypothetical CtrA binding motif (Barnett, Hung et al. 2001, Schluter, Reinkensmeier et al. 2013, De Nisco, Abo et al. 2014).

**Table 3. Genes targeted for transcriptional analysis**

<b>Target Gene</b>	<b>Function/Role</b>
<i>cbrA</i>	Histidine Kinase/Required for symbiosis
<i>ccrM</i>	DNA methylase
<i>cpdR1</i>	Response Regulator/CtrA degradation
<i>ctrA</i>	Transcription factor
<i>ftsE</i>	Cell wall hydrolysis/Cell division
<i>minC</i>	Negative regulator/Inhibitor of septation
<i>rcdA</i>	Response Regulator/CtrA degradation
<i>pleC</i>	Histidine Kinase/Phosphatase to DivK

### **Verification of GUS fusion strains**

The slow growth and excess production of succinoglycan by the  $\Delta cbrA$  mutant are phenotypes that likely result from increased CtrA levels (Sadowski, Wilson et al.

2013). Initial attempts to analyze GUS expression in the  $\Delta cbrA$  mutant led to the observation that some of the fusions allowed faster growth and no longer displayed excess succinoglycan production compared to  $\Delta cbrA$  without the fusion. In particular, the  $\Delta cbrA cbrA::GUS$  strain displayed the exopolysaccharide phenotypes of WT  $cbrA::GUS$  (**Figure 7**). Therefore, I performed the following tests to verify the  $cbrA$  allele present in each fusion strain.

To verify that each fusion plasmid was integrated into either  $\Delta cbrA$  or WT, PCR was used to amplify either  $\Delta cbrA::cat$  or the  $cbrA$  PAS domain showing the absence or presence of  $cbrA$  in the genome, respectively (**Figure 5**). Additionally, growth assays were performed using the GUS fusion strains: WT  $cbrA::GUS$  (CSH1001),  $\Delta cbrA cbrA::GUS$  (CSH6001), WT  $ctrA::GUS$  (CSH1004),  $\Delta cbrA ctrA::GUS$  (CSH6004), WT  $rcdA::GUS$  (CSH 1008), and  $\Delta cbrA rcdA::GUS$  (CSH6008) to test for suppression of growth phenotypes typically seen in  $\Delta cbrA$  strains. The  $cbrA::GUS$  strains do not double as fast as WT, however, they do double faster than  $\Delta cbrA$  (**Figure 6**).

### Quantification of GUS activity

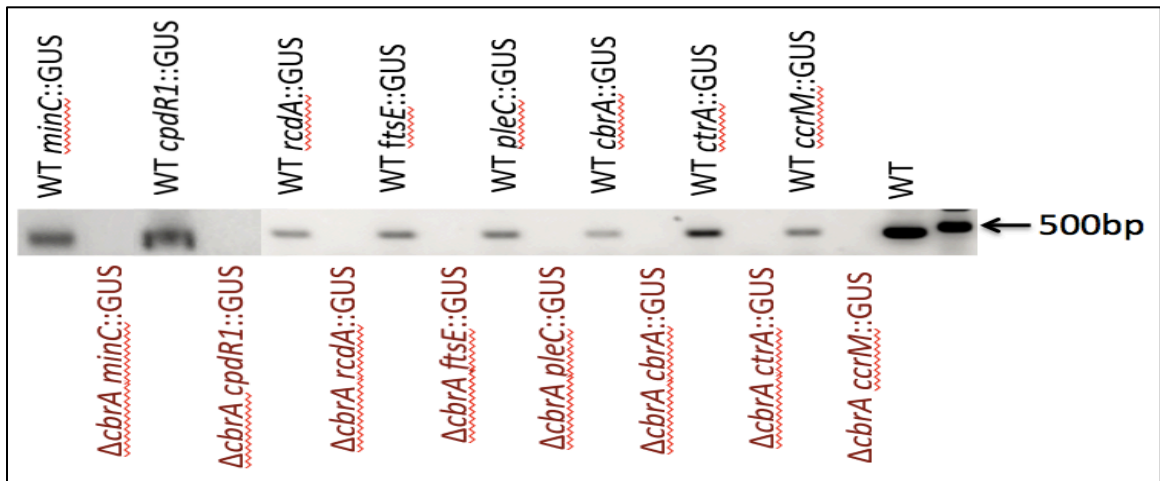
I hypothesize that increased levels of CtrA caused by the absence of  $cbrA$  leads to altered expression of genes with functions specific to cell cycle progression. My goal is to provide novel data showing how cell cycle effector and regulatory genes are affected in free-living *S. meliloti* when  $cbrA$  is absent and CtrA levels are higher than normal.

Using the GUS reporter system to track gene expression allowed me to quantitatively analyze the expression of specific genes in the absence of *cbrA*.

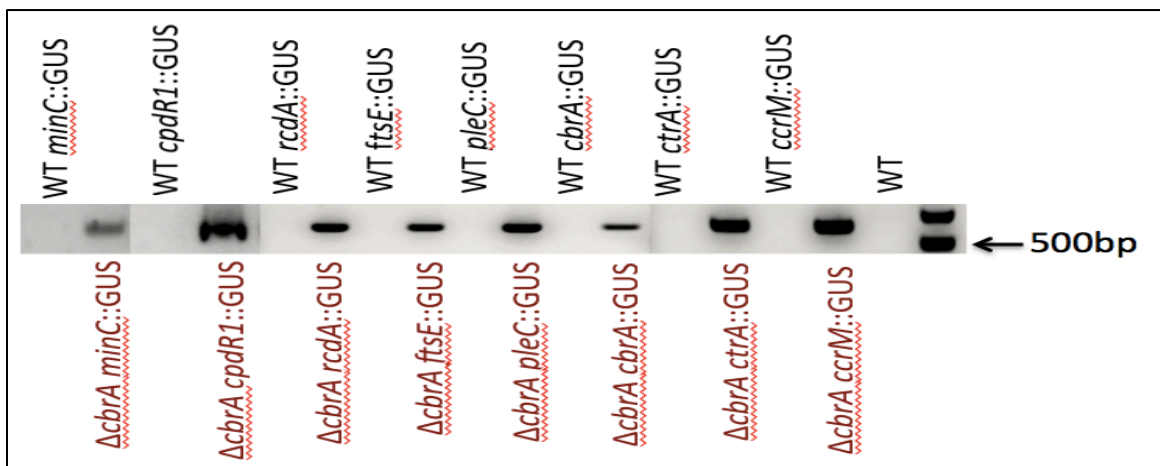
Initial attempts to quantify the amount of GUS being produced in each strain using GUS assays and Western Blots proved unsuccessful. GUS assays were performed as previously described, with the exception of using 4-Nitrophenyl  $\beta$ -D-gluco-pyranoside as a substrate rather than p-nitrophenyl- $\beta$ -D-glucuronide (Jefferson, Burgess et al. 1986). This method proved to be not useful for our strains since WT and  $\Delta$ *cbrA* showed similar, if not more, activity than the GUS fusion strains. The WT (Rm1021) and  $\Delta$ *cbrA* (CSS6000) strains are negative controls due to the lack of a GUS fusion and are meant to represent background activity. It seems there is an endogenous enzyme produced by *S. meliloti* that will hydrolyze the 4-Nitrophenyl  $\beta$ -D-gluco-pyranoside substrate.

Therefore, I turned to using Western Blotting to quantify the amount of GUS protein being produced in each strain. Anti-GUS was obtained from Abcam and Western blots were analyzed using the Kodak 4000R Image Station camera. These Western blots showed promise as a tool for measuring GUS levels as there was not extensive background and a distinct band was identified at the correct size for GUS that was specific to the fusion strains and not observed in WT negative controls. However, once we changed to the Licor Oddysey Clx Infrared Imaging system, and changed the solutions and protocol, the background found in each blot dramatically increased. We were not able to identify a distinguishable band at the intended size for the GUS protein that did not show in the WT negative control. The anti-GUS from Abcam seemed to be incompatible with the solutions used for the Licor Oddysey imaging system.

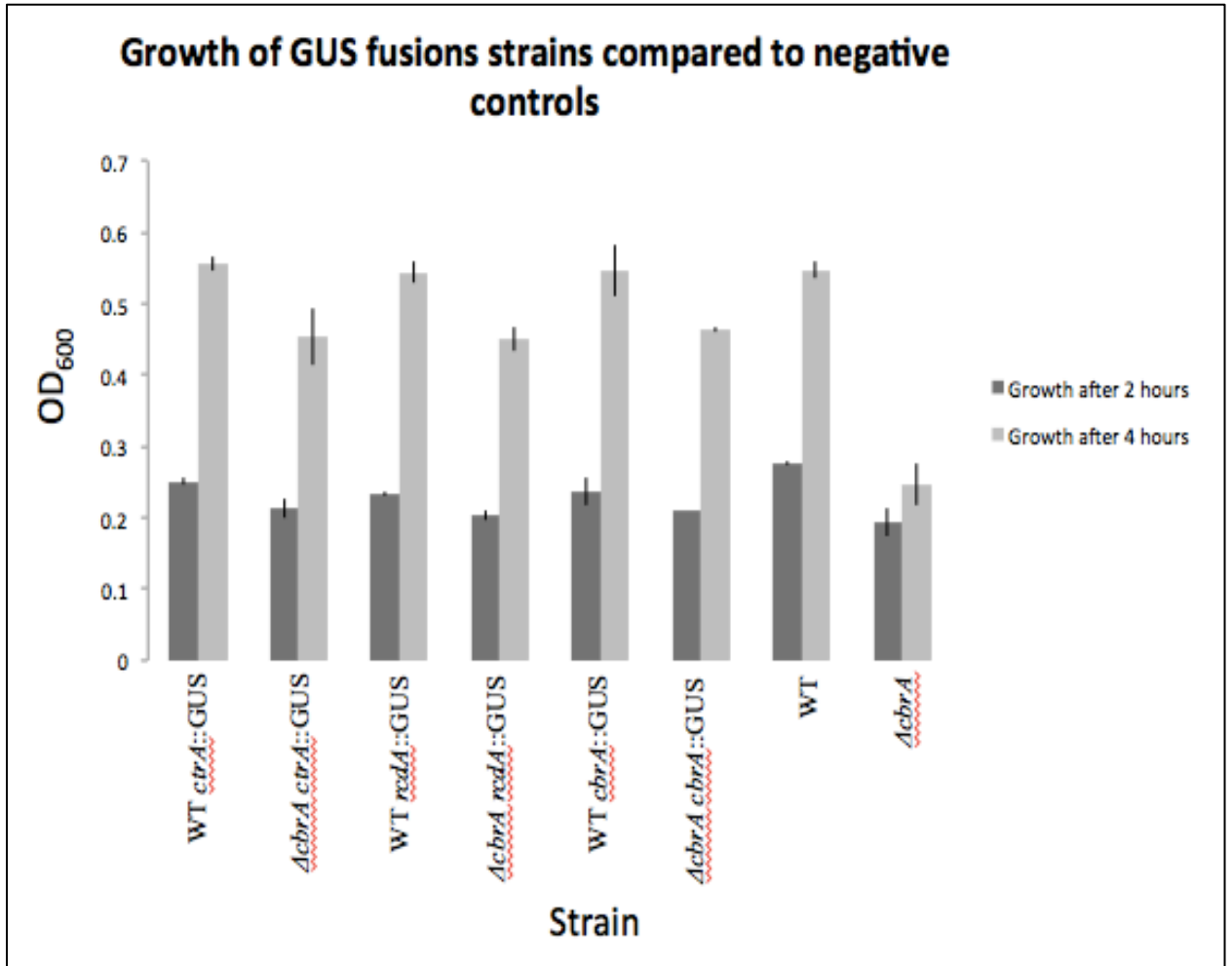
A



B



**Figure 5. Confirmation of the *cbrA* allele present in each gene fusion strain.** (A) Amplification of the *cbrA* PAS domain using T880R<sub>xh</sub> and G743FB<sub>m</sub> primers, indicating presence of the *cbrA* gene. (B) Amplification of  $\Delta cbrA::cat$  using *cat* 1 and *cbrA* dn primers, indicating the absence of the *cbrA* ORF and the presence of the antibiotic resistance cassette used to replace *cbrA*. Strain names listed in black indicated WT GUS fusion strains and strain names listed in red indicate  $\Delta cbrA$  GUS fusion strains.



**Figure 6. Growth of GUS fusion strains compared to negative controls at 2 and 4 hours.** Growth rates of some of the GUS fusions were measured and compared to the isogenic WT (Rm1021) or  $\Delta cbrA$  (CSS6000) background. Overnight cultures were subcultured to an OD<sup>600</sup> of 0.1 and measured every two hours. Data is reported as the average of triplicates.  $\Delta cbrA$  *ctrA*::GUS,  $\Delta cbrA$  *rcdA*::GUS,  $\Delta cbrA$  *cbrA*::GUS grow faster than the  $\Delta cbrA$  strain with no GUS plasmid but not as fast as WT with no GUS plasmid.  $\Delta cbrA$  *cpdR1*::GUS,  $\Delta cbrA$  *minC*::GUS, and  $\Delta cbrA$  *pleC*::GUS also grow faster than  $\Delta cbrA$  with no GUS plasmid (data not shown).  $\Delta cbrA$  *ftsE*::GUS and  $\Delta cbrA$  *ccrM*::GUS do grow as slow or even slower than  $\Delta cbrA$  with no GUS plasmid (data not shown).





**Figure 7. Analysis of succinoglycan production.** Calcofluor was used to assay the amount of succinoglycan produced by each GUS fusion strain. Only one gene fusion resulted in the suppression of the  $\Delta cbrA$  phenotype of excess succinoglycan synthesis. **(Left top)**  $\Delta cbrA cbrA::GUS$  does not produce more succinoglycan than **(Left bottom)** WT  $cbrA::GUS$ , whereas **(Right top)**  $\Delta cbrA minC::GUS$  produces a distinguishably excess amount of succinoglycan compared to **(Right bottom)** WT  $minC::GUS$ .

The next strategy was to use the 4-methylumbelliferyl-beta-D-glucuronide (MUG) substrate hydrolyzed by the GUS enzyme into 4-methylumbelliferone (4MU), which is excited at 355nm and emits a fluorescent signal at 405nm. To make sure that MUG is not metabolized into 4MU in WT, I lysed overnight cultures of WT *S. meliloti* and several GUS fusion strains, added MUG substrate, and checked for fluorescence using a handheld 355nm UV light. This verified that, even if left overnight, the mixture of WT cells and MUG substrate never produced fluorescent 4MU, but that the fusion strains did. Therefore, I optimized a protocol combining components from several previously described MUG assay protocols to measure GUS enzymatic activity in *S. meliloti*.

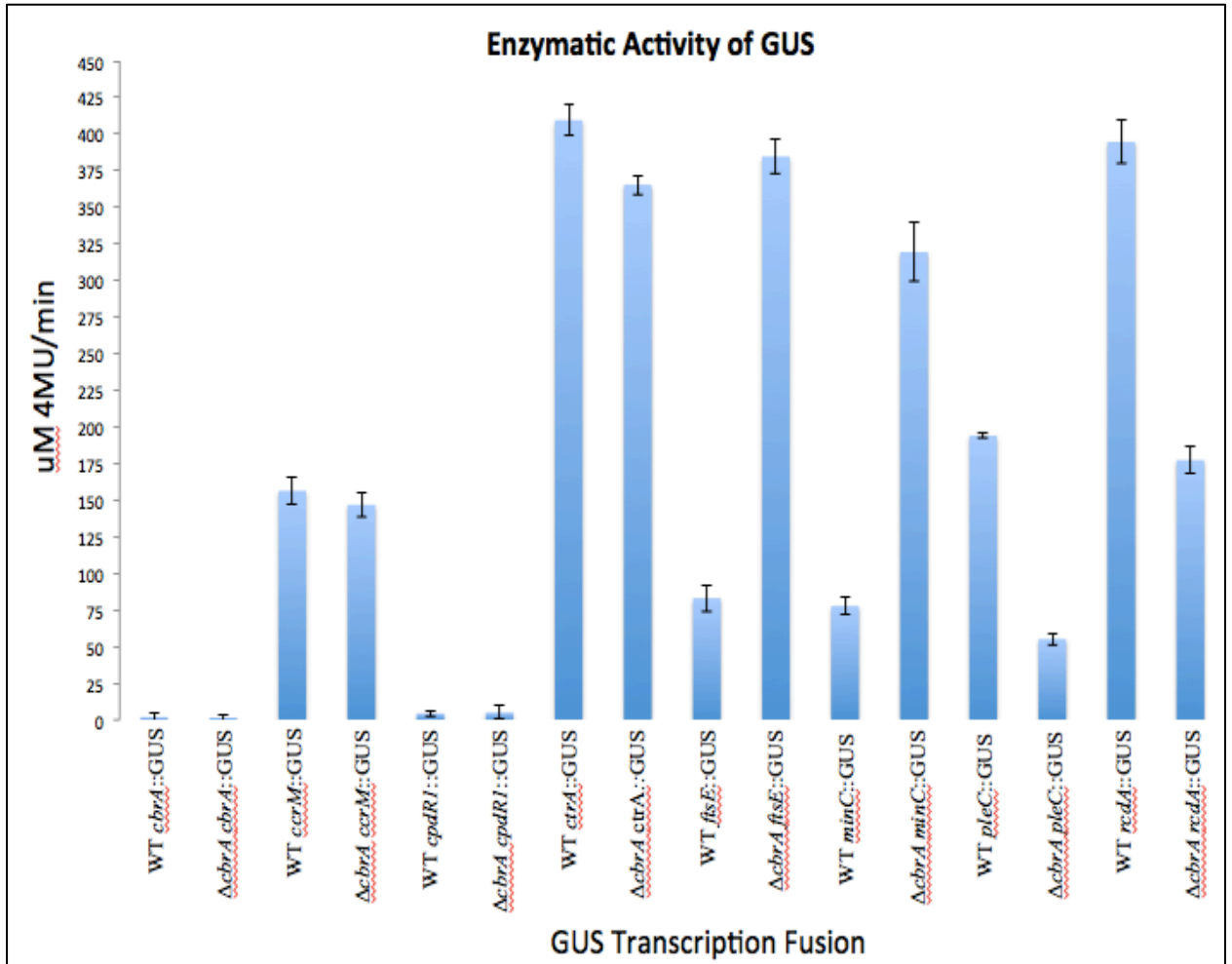
The novel histidine kinase *cbrA* and response regulator *cpdRI* are transcribed at extremely low levels compared to all of the other target genes. In addition to being expressed at significantly lower levels than every other target gene, there was no statistical difference in GUS activity between WT and  $\Delta cbrA$ . After 30 minutes, these two fusions are almost indistinguishable from the background arbitrary fluorescent units (FU) read in the negative controls, WT and  $\Delta cbrA$  with no GUS fusion plasmid. However, if the reaction is left to run overnight, there becomes an increased amount of FU produced by the *cpdRI* fusion strain compared to the negative controls (**Figure 8**). This suggests that, even though there is no statistical difference between these fusions in WT and  $\Delta cbrA$ , they are indeed expressed but at very low levels and in a CbrA-independent manner.

The essential DNA methylase, *ccrM*, is expressed at moderate levels in both WT and  $\Delta cbrA$  (**Figure 7**). However, the level of *ccrM* expression in WT versus  $\Delta cbrA$  is not significantly different.

The *pleC*, *rcdA*, and *ctrA* GUS fusions are each expressed at significantly lower levels in  $\Delta cbrA$  than in WT, whereas the *ftsE* and *minC* GUS fusions are both expressed at significantly higher levels in  $\Delta cbrA$  than in WT (**Figure 7**). Two-tailed unequal variance T-Tests were used to determine statistical significance between the WT::GUS and  $\Delta cbrA$ ::GUS strains (**Table 4**).

### Gus expression in *Medicago sativa* nodules

In addition to testing for misregulation of specific genes in  $\Delta cbrA$ , I also aimed to investigate which of the cell cycle target genes are expressed during the symbiotic relationship between *S. meliloti* and *M. sativa*. Using the WT *geneX::GUS* fusion strains, I can track where within root nodules these genes are expressed during a normal symbiosis and try to temporally analyze them as well. I predict that genes involved in chromosome replication will be expressed in the infection thread and bacteroid differentiation zone, but not the nitrogen-fixing zone and senescent zone (**Figure 9A**). Presumably, cells in the infection thread should be dividing like wild type and cells in the bacteroid differentiation zone should be going through endoreduplication, which will require the expression of genes involved in chromosome replication. In contrast, cells in the nitrogen-fixing and senescent zones are not replicating, thus diminishing any need for the expression of genes required for chromosome replication. Additionally, I predict that genes required for cell division will be expressed in the infection thread but not in the bacteroid differentiation, nitrogen-fixing, or senescent zones (**Figure 9A**). In the bacteroid differentiation zone, during endoreduplication, the cells will be growing into a filamentous morphology and not dividing. Therefore, genes used to build The GUS fusion strains that were assayed as free-living cells were also used to assay gene expression within root nodules. *M. sativa* (alfalfa) was inoculated with each GUS fusion strain and allowed to grow for up to five weeks. Histochemical GUS assays



**Figure 8.  $\beta$ -glucuronidase (GUS) enzymatic activity in each of the GUS fusion strains.** The arbitrary fluorescence unit measured for each strain at 30 minutes was used as the Y variable in the slope equation of a standard curve created with 4MU, to find the corresponding X value, or 4MU amount. The data reported in this graph is the micromole ( $\mu$ M) amount of 4MU hydrolyzed from MUG by GUS per minute. The equation used was  $(Y-B (Y\text{-intercept of standard curve}) / M (\text{slope of standard curve}) = X (\text{amount of 4MU})) / 30$  to produce 4MU produced per minute.

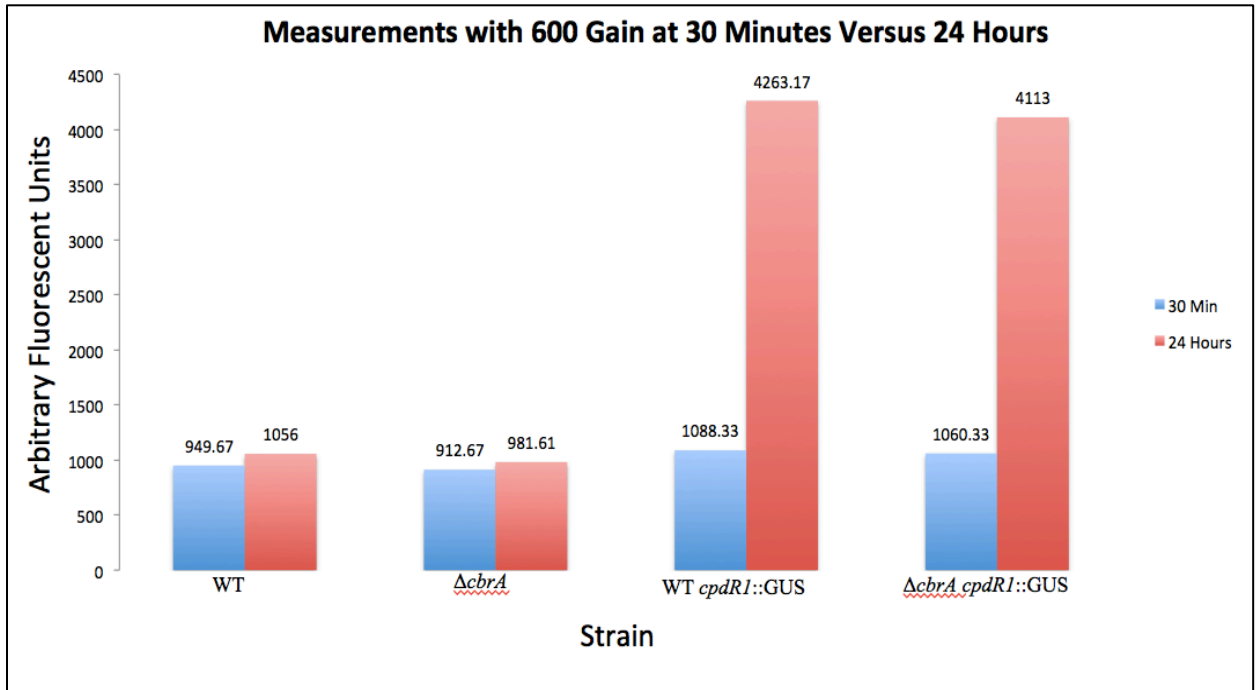
were performed on nodules at 3 and 5 weeks to determine the spatial and temporal expression of target genes. There are four distinct developmental zones within a nodule (Figure 9A).

**Table 4. P Value for each WT::GUS fusion versus  $\Delta cbrA$ ::GUS Fusion**

<b>Fusion</b>	<b>P Value</b>
<i>cbrA</i> ::GUS	0.8645
<i>ccrM</i> ::GUS	0.2508
<i>cpdR1</i> ::GUS	0.8467
<i>ctrA</i> ::GUS	0.001867
<i>ftsE</i> ::GUS	6.6569E-06
<i>minC</i> ::GUS	0.00143
<i>pleC</i> ::GUS	0.00006246
<i>rcdA</i> ::GUS	0.00008407

Stained nodules were examined under a microscope to identify where within the nodule these genes were expressed and if expression changed over the time of the symbiosis assay. As in the free-living assay, there was little to no expression of *cpdR1* or *cbrA* in nodules (**Figure 9A**). One pattern observed during these assays was an unexpected expression restricted to the periphery of the nodules, starting in the infection thread and continuing down to the senescent zone, without expression in the middle of the nodule (**Figure 9D**). The target genes *ctrA* and *ftsE* are expressed ubiquitously throughout the entire nodule after three weeks (**Figure 9B**) and tend to slightly shift to a more localized expression within the infection thread zone, periphery, and senescent zone of the nodules after five weeks (**Figures 9D and 10**). This peripheral expression pattern was observed primarily with *pleC* and *rcdA* GUS fusions. Expression of *pleC* and *rcdA*

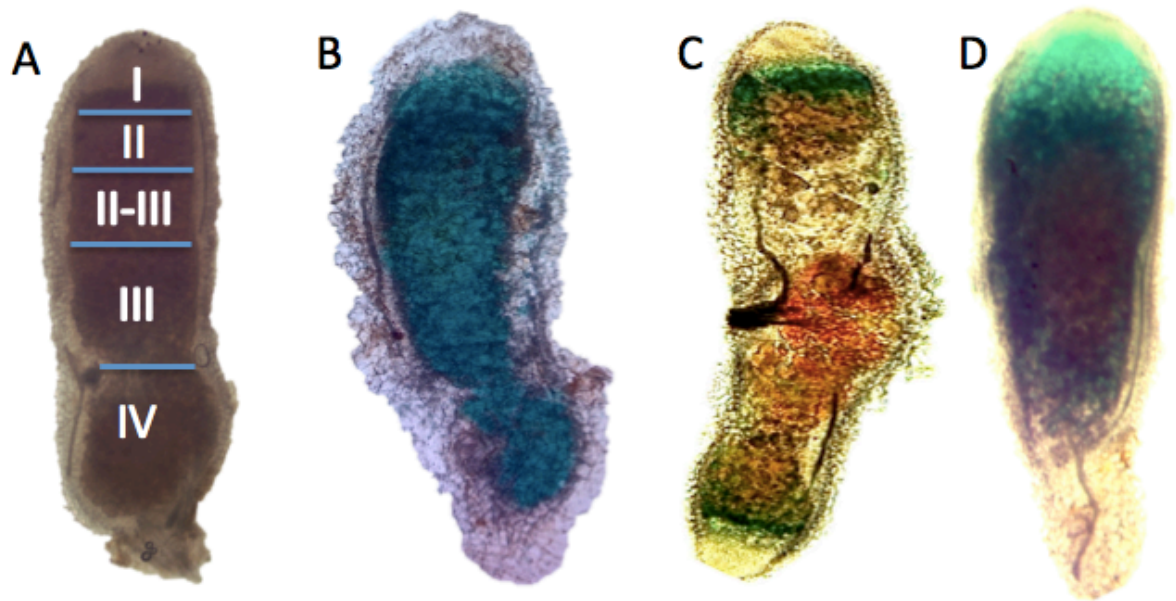
was evenly split between ubiquitous expression (**Figure 9B**) and the peripheral pattern (**Figure 9D**) at three weeks, with the majority of nodules showing the peripheral expression pattern after five weeks (**Figure 10**).



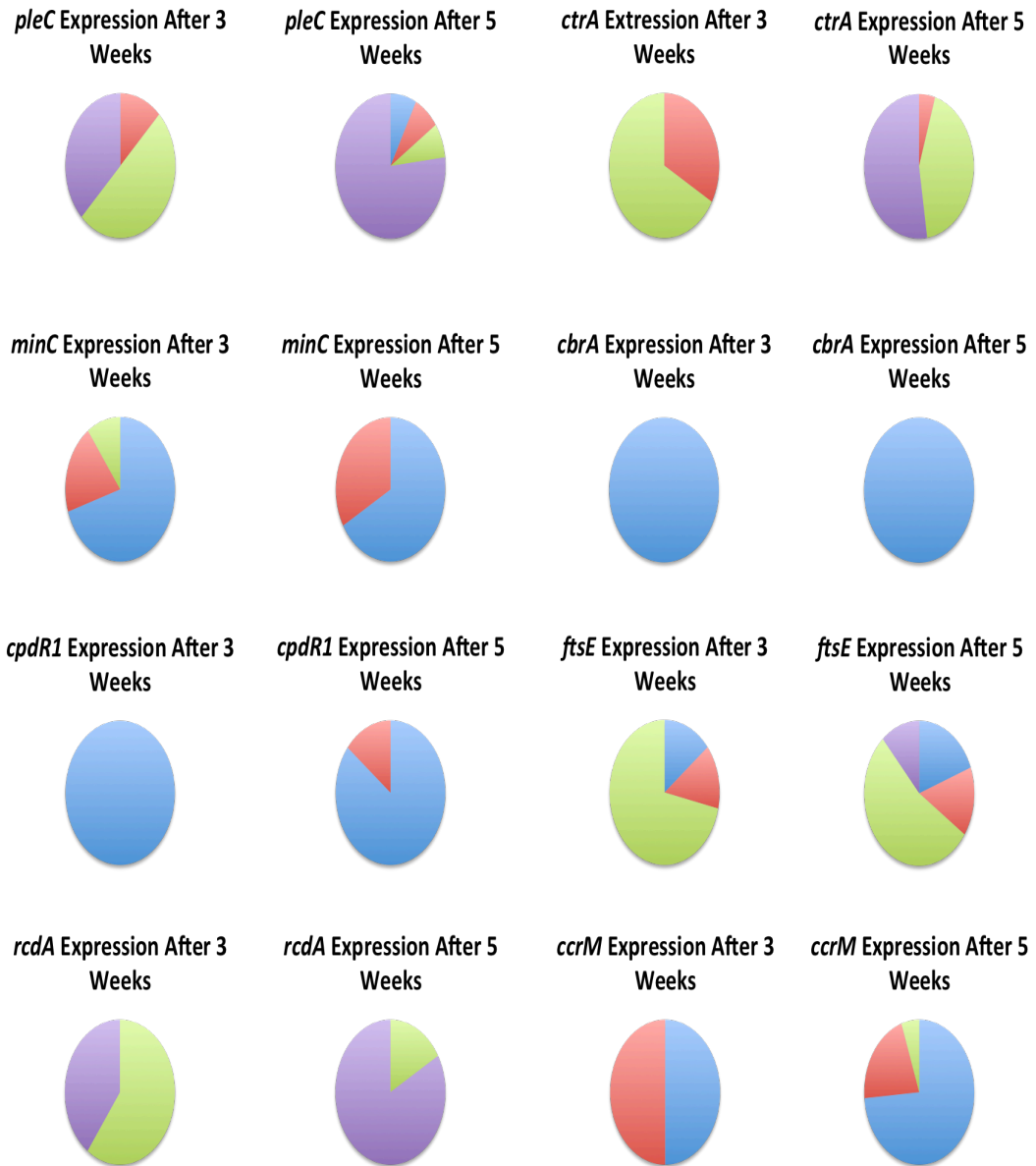
**Figure 9. GUS enzymatic activity of *cpdR1::GUS* versus negative controls.** Two single time points are used to show that *cpdR1* is expressed in *cpdR1::GUS* strains. The first reading was taken at 30 minutes and the second at 24 hours. The enzymatic activity of WT *cpdR1::GUS* and  $\Delta cbrA$  *cpdR1::GUS* is such that there is no significant difference versus the WT or  $\Delta cbrA$  negative controls at 30 minutes. After 24 hours however, WT *cpdR1::GUS* was statistically different from WT with a p-value of  $2.52 \times 10^{-14}$  and  $\Delta cbrA$  *cpdR1::GUS* was statistically different from  $\Delta cbrA$  with a p-value of  $2.77 \times 10^{-14}$ .

The last two fusions, *minC* and *ccrM*, were found to be expressed mostly in the infection thread at three weeks (**Figure 9C**), with the majority of nodules observed after 5 weeks showing no expression of these genes (**Figure 10**). CcrM, which is involved in DNA methylation and representing chromosomal replication, showed no expression

outside of the infection thread whereas FtsE, usually implicated in cell division and cell wall hydrolysis, was expressed everywhere in the nodule. CtrA was expressed everywhere in younger nodules and then restricted to the periphery of the nodules in older nodules.



**Figure 10. GUS expression in *Medicago sativa* root nodules.** The nodules shown above are representative examples of different bacterial GUS expression patterns seen within root nodules of *M. sativa*. (A) WT nodule showing no GUS expression (negative control) is used to illustrate approximate zones within the nodule: I is the apical meristem, II is the infection thread zone (IT), II-III is a mid-zone with bacteroid differentiation between the IT and nitrogen fixing zone, III is the nitrogen fixing zone and IV is the senescent zone. (B) Nodules expressing blue GUS expression ubiquitously. (C) Blue GUS expression restricted to the IT. (D) A novel GUS expression pattern in the IT, the periphery of the nodule, and in the senescent zone but not in the nitrogen-fixing zone.



**Figure 11. Expression patterns of GUS fusion strain in *Medicago sativa* root nodules.** Pie charts represent the location of GUS expression seen within nodules for each GUS fusion strain. **Purple** indicates expression seen in the IT and periphery of the nodules, as seen in Figure 10D, **Red** indicates expression in the IT as seen in figure 10C, **Green** indicates expression seen everywhere in the nodules, and **Blue** indicates no expression seen at all. Nodules were assayed at three weeks post-inoculation and five weeks post inoculation. Each pie chart represents the 8-10 nodules used in each assay.



**Synchronization of *S. meliloti* using the YB1804 temperature sensitive *dnaE* allele**

Further understanding how *S. meliloti* cell division and chromosomal replication is regulated will require in-depth analysis of each cell cycle phase, G<sub>1</sub>, S, and G<sub>2</sub>. However, this is not achievable without synchronizing a population of *S. meliloti* cells, allowing for the temporal analysis of each cell cycle phase. My goal is to create a temperature sensitive strain of *S. meliloti*, which will allow G<sub>1</sub> phase synchronize at a restrictive temperature but wild-type growth at the permissive temperature.

A successful mating of the plasmid containing the YB1804 temperature sensitive *dnaE* allele into *S. meliloti* resulted in neomycin resistant (Nm<sup>r</sup>) *S. meliloti*. Two Nm<sup>r</sup> *S. meliloti* strains, DnaE10 and DnaE12, were obtained to proceed with sucrose selection. DnaE 12 was randomly chosen to undergo sucrose selection, using both 5% and 10% sucrose in LB/MC. I obtained about 25 Nm resistant strains out of 1000 plated on both Nm and Sm after sucrose selection for both the 5% and 10% sucrose selections. Therefore, I had about 50 strains out of 2000 that seemed to excise the pK18*mobsacB* plasmid out of their genome. To test temperature sensitivity, I struck each strain onto two LB/MC plates and incubated one in 30°C and the other in 37°C. None of these strains were temperature sensitive and grew at identical rates at both 30°C and 37°C. Additionally, I used PCR to test for the presence of the *S. meliloti dnaE* or the YB1804 *dnaE*. The YB1804 *dnaE* was not detected in any of the Nm resistant strains.

## CHAPTER IV

### DISCUSSION

#### **Misregulation of cell cycle genes in $\Delta cbrA$ free-living *S. meliloti***

The goal of this project is to provide additional insight into how CbrA contributes to the regulation of the cell cycle in *S. meliloti*. We knew that CbrA acts through DivK to regulate CtrA protein levels and that loss of CbrA leads to distinctly aberrant changes in free-living growth, motility and symbiosis. Previous results showed that in the  $\Delta cbrA$  mutant there is an overall decrease in 1N and 2N genome compliments when analyzed by fluorescence flow cytometry (Sadowski, Wilson et al. 2013). There is a three-to fourfold increase of <1N genome content in addition to a distinct increase of 2N< content in  $\Delta cbrA$  compared to WT. This observation, in addition to data showing that CtrA does not regulate DNA replication rate *per se* but may regulate cell division led to the conclusion that  $\Delta cbrA$  mutants undergo filamentation as a result of defects in septum localization or cell division. I therefore hypothesized that  $\Delta cbrA$  leads to the misregulation of genes

involved in cell division. The genes used to represent two specific components of the cell cycle in *S. meliloti*, cell division and chromosomal replication, were *ftsE*, *minC*, and *ccrM*. Given that CbrA functions through the DivK pathway to regulate CtrA activity, I further hypothesized that  $\Delta cbrA$  also leads to the misregulation of different histidine kinases (HK) or response regulators (RR) within the pathway.

The two genes specifically representing cell division, *ftsE*, and *minC*, were both expressed at significantly higher levels in  $\Delta cbrA$  than in WT. The Min system works to inhibit FtsZ from assembling the Z-ring, the prokaryote homolog of tubulin, at the poles of the dividing cell to assure proper cell division. FtsE couples with FtsX to form FtsEX and acts as an ATP-cassette, recruiting specific enzymes to contribute to cell division. Alteration of the cell division machinery was expected in  $\Delta cbrA$ , since the cells exhibit swelling, branching, and filamentous morphology.

The Min system in *S. meliloti* includes the *minC*, *minD* and *minE* genes. A  $\Delta minCDE$  mutant showed no distinct change in growth rate or viability when compared to WT (Cheng, Sibley et al. 2007). When *minC* is overexpressed independent of *minD* and *minE* the viability of *S. meliloti* is not compromised but morphology was slightly altered showing filamentous, swollen and branched cells found along with WT cells (Cheng, Sibley et al. 2007). However, the overexpression of *minC minD* and *minC minD minE* led to a drastic cell viability decrease compared to WT and also increased filamentous, swollen, and branched morphology. Transcription initiation of the min operon is 174 nucleotides upstream of *minC* so the transcription of *minD* and *minE* is dependent on the promoting region upstream of *minC* (Cheng, Sibley et al. 2007).

I hypothesize that overexpression of *minCDE* in  $\Delta cbrA$  contributes to cell division defects and the filamentous morphology that is characteristic of  $\Delta cbrA$ . The MinCDE system negatively regulates septum formation. The result of increased MinCDE levels should lead to lower levels of septum formation, which would decrease the efficiency of Z-ring formation and could potentially be fatal. The results presented here suggest that CbrA does contribute to cell cycle gene regulation and more specifically to the proper expression of cell division genes.

FtsE is involved in cellular division and is thought to be associated with FtsZ, which is directly responsible for building the constrictive Z-ring apparatus at the site of cellular division (Addinall, Bi et al. 1996, Pichoff and Lutkenhaus 2005). FtsE has been shown to associate with FtsX to form an FtsEX complex, which is implicated in cell division. Recent reports show that FtsEX recruits the EnvC amidase and contributes to cell wall hydrolysis as division is taking place (Yang, Peters et al. 2011, Meisner, Montero Llopis et al. 2013).

FtsE is required for viability and its' localization to the division site is also required for the assembly of Z rings (Corbin, Wang et al. 2007). The overproduction of FtsEX in *E. coli* leads to cell filamentation and death (de Leeuw, Graham et al. 1999). My results show that the  $\Delta cbrA$  mutant expresses *ftsE* and *minC* at higher levels than WT (Figure 6). The CbrA-dependent increase in expression of both *minC* and *ftsE* may be mediated by the same transcription factor. Both *minC* and *ftsE* have predicted CtrA binding sites in their transcription start sites. Therefore, I hypothesize that CtrA directly mediates the overexpression of *minC* and *ftsE* in  $\Delta cbrA$ .

Attempts at cloning the *ftsZ* promoting region fused to GUS in the pVO155 plasmid into *S. meliloti* were successful. However, cells containing the *ftsZ*::GUS fusion grew minimally on LB/MC agar plates, and not at all in liquid LB/MC. Adding a second *ftsZ* promoter likely created lethal alterations to the cellular division process, perhaps because it led to a change in FtsZ concentration which altered the cell's ability to divide properly, leading to non-viable cells.

CcrM is a DNA methylase whose primary role is to methylate newly synthesized hemimethylated DNA. Consistent with previous results showing no change to the rate of DNA replication initiation in  $\Delta cbrA$  cells, there was no significant difference found between transcription of *ccrM* in WT versus the  $\Delta cbrA$  mutant (Wright, Stephens et al. 1997).

### **Misregulation of regulatory genes in $\Delta cbrA$ free-living *S. meliloti***

The genes I used to gain a more thorough understanding of the differences in the DivK regulatory pathway and its' components when *cbrA* is not present were *cpdR1*, *rcdA*, *pleC*, and *cbrA*. CpdR1 and RcdA are both involved in CtrA degradation, CbrA is a histidine kinase (HK) that acts as a kinase on DivK, and PleC is an HK that acts as a phosphatase of DivK. The results presented here provide insight into the feedback regulation of genes involved in the regulatory network as a whole.

Previous work has shown that the absence of *cbrA* results in increased CtrA protein levels (Sadowski, Wilson et al. 2013) and an increase in phosphorylation of CtrA

in *S. meliloti* (Pini, Frage et al. 2013). This suggests that when *cbrA* is absent there is a decrease in CtrA degradation. In *C. crescentus*, CtrA degradation is facilitated by the binding of RcdA to CtrA and localization to the ClpXP protease, however the exact function of RcdA is unknown (Jenal 2004, Curtis and Brun 2010). The targeting of CtrA to ClpXP also requires the presence PopA in *C. crescentus* but not in *S. meliloti*, which does not have a PopA homolog. Despite known homologs of CpdR1 and RcdA in *S. meliloti*, the exact function of these proteins remains untested. Results presented here show that the  $\Delta cbrA$  mutant, which has higher levels of CtrA, expresses *rcdA* at significantly lower levels compared to WT, suggesting that CtrA may repress transcription of the *rcdA* promoter. In *C. crescentus* CtrA directly regulates RcdA, and in *S. meliloti* a CtrA binding site is predicted in the *rcdA* promoter region (McGrath, Iniesta et al. 2006, Schluter, Reinkensmeier et al. 2013). In contrast with *rcdA*, *cpdR1* transcription is not altered in the absence of *cbrA*. Based on previous results, CtrA degradation is likely decreased when *cbrA* is absent due to the loss of DivK phosphorylation and subsequent increase in CpdR1 phosphorylation. In addition to alteration of the signaling pathway, absence of *cbrA* is predicted to further decrease CtrA degradation rates through transcriptional downregulation of *rcdA* but not *cpdR1*. Thus, we have uncovered two redundant mechanisms for regulating CtrA activity in a CbrA-dependent manner.

The other components of the regulatory network examined were PleC and CbrA. In *C. crescentus*, PleC regulates DivK as a phosphatase leading to decreased phospho-DivK and eventually increased phospho-CtrA (**Figure 4**). My results show that in the

absence of *cbrA*, *pleC* transcript levels are significantly decreased. Predictive data has suggested that *pleC* has a CtrA binding site upstream of the *pleC* TSS. I hypothesize that increased levels of CtrA-P in the absence of *cbrA* leads to downregulation of *pleC* as part of a negative feedback loop. Therefore, when CtrA-P levels reach a certain threshold, CtrA downregulates *pleC* to maintain high levels of CtrA activity.

The novel histidine kinase, CbrA, on the other hand, does not seem to have any transcriptional misregulation when the *cbrA* gene is taken out of the genome. Since CbrA is thought to act upstream of DivK, analogous to DivJ and PleC, and acts through DivK to regulation the cell cycle, it is surprising to see no change. Additionally, *cbrA* showed significantly lower transcription levels when compared to all of the other target genes with the exception of *cpdRI*.

### **Expression of *S. meliloti* cell cycle related genes in *M. sativa***

In addition to an interest in understanding how the cell cycle of *S. meliloti* is regulated in free-living cells, there is great interested in elucidating how the cell cycle is regulated during symbiosis. One of our main goals is to understand how the *S. meliloti* cell cycle is regulated as it transitions into endoreduplication and then G0 phase, and the purpose of this differentiation. To gain insight into the symbiotic cell cycle program, we need to better understand how the cell cycle is regulated during bacteroid development and within fully developed bacteroids. The  $\beta$ -glucuronidase (GUS) fusion strains used to

gain insight into cell cycle regulation of free-living *S. meliloti* cells were also used to study gene expression in root nodules. GUS staining of *M. sativa* root nodules infected with GUS fusion strains proved to be a useful method to track the location and general timing of gene expression during symbiosis. Nodules were assayed at three and five weeks due to a lack of mature nodules prior to three weeks and an inability to maintain ideal growth conditions for *M. sativa* plants after five weeks.

*S. meliloti* cells must first travel through the developed infection thread (IT), and undergo endocytosis into the cytoplasm of the host cell before they can differentiate into nitrogen-fixing bacteroids (**Figure 2A**). Mature nodules have all stages of nodule development present, including invasion, colonization, and bacteroid differentiation. Once differentiation and bacteroid development is complete, the *S. meliloti* cells will exit their cell cycle into G0 phase and gain the ability to fix nitrogen. Thus, mature nodules are ideal for tracking genes expressed at all stages of symbiosis because at this point in development a significant subset of *S. meliloti* will have differentiated into nitrogen-fixing bacteroids.

As previously discussed, FtsE works with FtsX, forming an ABC transporter to recruit amydases for cell wall hydrolysis (Yang, Peters et al. 2011). The requirement for cell wall elongation during cellular division is preceded by the need for the bonds between the existing peptidoglycan strands to be hydrolyzed. FtsE and the FtsEX complex is directly implicated in cell division through cell wall elongation by enabling the hydrolysis of pre-existing bonds, allowing for new cell wall to be synthesized. Reports using *Bacillus subtilis* showed that FtsE contributes to cell wall elongation,



which is consistent with the notion that FtsE defects contribute to generating cells with a filamentous phenotype (Meisner, Montero Llopis et al. 2013). Therefore, *ftsE* should be expressed in cells that are actively growing and dividing and may contribute to bacteroid formation since they have a branching and filamentous morphology indicative of cell growth and elongation.

FtsE is expressed everywhere in the nodules after three and five weeks of growth. This result supports the notion that FtsE is an important protein during filamentation and is therefore also involved in cell wall elongation in bacteroid formation. This is consistent with previous investigations that show filamentatous morphology when FtsE is overexpressed (de Leeuw, Graham et al. 1999).

The septum inhibiting protein MinC, which acts to prevent FtsZ localization everywhere in the cell except its center where septum formation takes place, was not always seen expressed in root nodules. When it was expressed, it was almost always expressed in the IT. FtsZ may be inhibited or downregulated in cells that are differentiating into bacteroids given their endoreduplication and filamentation phenotypes. Therefore, the requirement for MinC is likely diminished as well. Since cells aren't dividing during bacteroid development, FtsZ isn't needed to form the Z-ring and MinC will not be needed to prevent FtsZ from forming the Z-ring at an incorrect location. The data presented here suggests that MinC does not play an active role in bacteroid physiology or bacteroid formation, and I hypothesize that FtsZ does not play an active role in bacteroids either. Thus, FtsE, presumably as the FtsEX complex, is recruited to

work independent of FtsZ in bacteroids as there is a need for increased cell wall growth but not Z-ring formation.

DNA methylation has been found to be involved in various critical functions such as transcriptional regulation, initiation of DNA replication, and genomic imprinting (Campbell and Kleckner 1990, Braaten, Blyn et al. 1991, Razin and Cedar 1994). The most common role for DNA methylation in prokaryotes is DNA restriction-modification, which allows the cell to differentiate between self and foreign DNA (Wilson 1988, Bickle and Kruger 1993). The role of CcrM in *C. crescentus* and *S. meliloti* is a little different however, as it is not involved in DNA restriction-modification but is used to methylate the adenine residue in the sequence GANTC (Zweiger, Marczyński et al. 1994) and is essential to these organisms. CcrM is controlled through transcriptional regulation and proteolysis to restrict its presence to late predivisional cells when two hemimethylated chromosomes are converted into two fully methylated chromosomes. CcrM is activated by the global transcription factor CtrA, and when its' regulation is altered cells display abnormal morphology, a disruption in cell division, and loss of control over DNA replication initiation (Zweiger, Marczyński et al. 1994, Wright, Stephens et al. 1996).

The essential DNA methyl-transferase CcrM, was found exclusively in the IT after three weeks and in only about half of the nodules assayed, with no expression seen in the other half. After five weeks, *ccrM* expression was decreased even more, and was still seen exclusively in the IT. This reinforces the idea that CcrM is important for the free-living cell cycle program but not the bacteroid cell cycle program, as cells in the IT should be dividing like WT. A key component of bacteroid formation is repeated rounds

of DNA replication before exiting the cell cycle into G0 phase (**Figure 2C**). Due to its role in methylating hemimethylated chromosomes and its presence in late pre-divisional cells, it appears that CcrM activity and DNA methylation is not required for the endoreduplication seen during bacteroid development.

### **Expression of *S. meliloti* regulatory genes in *M. sativa***

We already know that the DivK pathway plays a major role in the cell cycle of free-living *S. meliloti*. Therefore, studying the regulatory components of this pathway could provide critical insight into understanding cell cycle regulation during symbiosis. Gene expression of the primary phosphatase to DivK, PleC, and the novel histidine kinase that is required for symbiosis, CbrA, was analyzed in nodules. Additionally, the response regulators directly involved in CtrA degradation, CpdR1 and RcdA, were examined for their transcriptional expression patterns.

PleC ultimately leads to increased levels of activated, phospho-CtrA. After three weeks of growth, *pleC* expression was found throughout the nodule (**Figure 4B**). Nodules examined after five weeks of growth showed a shift such that expression of *pleC* was observed primarily in the IT, along the periphery, and in the senescent zone of the nodules (**Figure 4D**). Cells in the IT are thought to divide like WT, so the presence of PleC within invading cells is not surprising. Based on the more restricted pattern of *pleC*

expression in mature nodules, I hypothesize that PleC is not utilized during or upon completion of bacteroid formation, but may play a later role in bacteroid cell senescence.

Expression of *cbrA*, encoding the novel HK required for symbiosis, was not detected in nodules. I do not conclude from this that *cbrA* is not expressed during any point within nodules, though. The free-living MUG assay showed that *cbrA* is expressed at levels so low that the fusion strain is difficult to distinguish from the negative controls. Previous research has shown that CbrA is required for a successful symbiosis (Sadowski, Wilson et al. 2013). Therefore, I conclude that even though *cbrA* expression was not observed within nodules, it is likely expressed but at levels too low to be detected by GUS staining.

Two other components of the DivK pathway that I used to further elucidate gene expression within nodules were CpdR1 and RcdA. In *C. crescentus*, CpdR1 enables degradation of the transcription factor CtrA by the ClpXP protease. Also in *C. crescentus*, RcdA is required for this localization of CtrA to ClpXP while PopA is required for the signaling mechanism leading to CtrA degradation (Duerig, Abel et al. 2009, Curtis and Brun 2010). Expression of *cpdR1* was seen in just one nodule collected after five weeks of growth and in no nodules harvested after three weeks of growth. Similar to CbrA however, in free living cells *cpdR1* is expressed at significantly lower levels compared to the other genes examined in this study. Therefore, *cpdR1* may also be expressed in nodules at levels too low to be detected by the GUS staining method.

In contrast, *rcdA* expression was detected either ubiquitously throughout the nodule or in the peripheral pattern found when analyzing *pleC*. Comparing *ctrA* and *rcdA*

expression in Figure 5, it appears that *rcdA* expression may determine subsequent *ctrA* expression. Nodules harvested after three weeks of growth show that most *rcdA* expression is detected ubiquitously throughout the nodules, similar to *ctrA*, however there are also nodules that express *rcdA* in the peripheral pattern as well. In nodules assayed after five weeks of growth, *rcdA* expression is found primarily in the peripheral pattern. At five weeks, *ctrA* shows a similar shift with most nodules showing a peripheral pattern of gene expression.

The peripheral pattern of bacterial gene expression within nodules has not been described previously. One explanation for this particular observation may be that the protocol used for detecting nodule gene expression is insensitive to low levels of gene expression. A previous investigation into *S. melilot* gene expression within root nodules of *Medicago* showed *cpdR1* expression throughout the entire nodule (Kobayashi, De Nisco et al. 2009). This is in contrast to the findings presented here, which show a lack of *cpdR1* expression in nodules. In free-living *S. meliloti* transcriptional analysis of *cpdR1*, expression levels were distinctly lower than all of the other genes examined with the exception of *cbrA*. Kobayashi et al. (2009) incubated nodules in substrate buffer for 24 hours whereas in this study nodules were incubated for 1.5 hours in order to identify the nodule pattern representing high gene expression. Therefore, a longer incubation time might reveal *cpdR1* and *cbrA* expression in nodules as well as reveal that the peripheral expression pattern within nodules is due to the limited reaction time of our assay. A future goal will be to optimize the protocol to determine if the peripheral expression pattern disappears with increased incubation times.

### ***S. meliloti* synchronization using allelic replacement**

Attempts at using allelic replacement to replace *S. meliloti dnaE* with the temperature-sensitive *C. crescentus* strain YB1804 *dnaE* were unsuccessful. After mating pK18*mobsacB::YB1804dnaE* (pK18CSH10) into *S. meliloti* strain Rm1021, two Nm<sup>r</sup> colonies were chosen for subsequent sucrose-mediated homologous recombination to force the plasmid out of the chromosome. Amplification using both the CcDnaE OLD Fwd/CcdnaE OLD Rev and Sm5 Fwd/Sm 3 Flank Rev primers showed that YB1804*dnaE* was in these isolates. However, after screening 1000 colonies grown on 5% sucrose and 1000 colonies grown on 10% sucrose, no colonies were found in which the *S. meliloti dnaE* had been excised from the genome and replaced with YB1804*dnaE*. Thus, it appears that *C. crescentus dnaE* may not be able to functionally substitute for *S. meliloti dnaE*.

### **Future direction**

The primary future direction for this project will be to show a direct relationship between CtrA and the genes misregulated in the absence of CbrA. I used  $\Delta cbrA$ , which has an increased amount of CtrA, as a way to test for cell cycle regulation of target genes.

However, it remains possible that the misregulation observed could be CtrA-independent. Cell cycle genes were chosen based on previous research that had either shown CtrA-dependent regulation of these genes or predicted CtrA-dependent regulation based on the presence of CtrA-binding sites (Tsokos and Laub 2012, Schluter, Reinkensmeier et al. 2013). Showing CtrA-dependent regulation of cell cycle target genes can be accomplished through overexpression or depletion of CtrA levels. Since CtrA is required for viability, it cannot be deleted in the absence of a complementing plasmid. Placing *ctrA* under the control of an inducible promoter on a complementing plasmid would allow for precise control over levels of CtrA in *S. meliloti*. In this way, it can be determined whether CtrA regulates transcriptional expression of genes, such as FtsE or PleC. If these target genes show the same misregulation as they did in  $\Delta cbrA$  when CtrA is overexpressed, it will provide convincing evidence that CbrA contributes to regulation of these genes by acting through CtrA or influencing CtrA activity.

One way to test whether CtrA is transcriptionally regulating these cell cycle genes directly would be through a Chromatin immunoprecipitation (ChIP) or DNase footprinting assay. These methods would show the direct interaction of CtrA with the specific DNA sequences in the promoter regions of target genes. Showing the direct binding of target genes by CtrA will provide a more complete understanding of how the DivK pathway functions to regulate the cell cycle in *S. meliloti*.

Another interesting experiment that could be utilized to further understand the cell cycle in *S. meliloti* is to synchronize a population of cells in G1 phase through a new nutrient-deprivation protocol and then analyze cell cycle gene expression using MUG

assays (De Nisco, Abo et al. 2014). After synchronization, cells would be collected at certain time points corresponding to a different part of the cell cycle, such as G1, S, or G2 phase, and subjected to the MUG assay to determine when each gene is being transcribed as cells progression through the cell cycle. This would indicate which of the genes are critical for each part of the cell cycle. For example, in *C. crescentus*, CtrA is highly abundant during G1 phase, transcriptionally downregulated and degraded during S phase, and then transcriptionally upregulated during G2 phase and this expression pattern is correlated with CtrA's role in repressing DNA replication initiation and activating cell division gene expression (Curtis and Brun 2010).

The next step in further elucidating how these target genes function in symbiosis will be to test for an effect of Nodule-specific Cysteine Rich (NCR) peptides on gene expression. Using isolated NCR peptides, it is possible to induce bacteroid differentiation in free-living *S. meliloti* cells (Penterman, Abo et al. 2014). Once cells are differentiated into bacteroids, MUG assays could be done to identify which of the target genes are expressed in bacteroids. Successfully characterizing target gene expression in bacteroids will be very useful in trying to further understand the mechanism by which *S. meliloti* cells perform endoreduplication and then exit from their cell cycle into G0 phase.

The attempt to use allelic replacement to exchange the *S. meliloti dnaE* with the temperature sensitive *dnaE* seemed very promising. The *S. meliloti* DnaE and *C. crescentus* DnaE share close homology sharing a high level of identity at the amino acid level, and other essential genes such as *ctrA* have been exchanged between the two organisms (Barnett, Hung et al. 2001, Lo, van Der Schalie et al. 2004). However, it may



not be possible to replace the *S. meliloti* DnaE with *C. crescentus* DnaE due to its non-functionality in *S. meliloti*. If successful, this method would have provided a convenient and easy protocol to synchronize *S. meliloti* cells for all labs interested in further understanding its' cell cycle. Despite the lack of success using this method, there has been progress in using alternative methods to synchronize *S. meliloti* (De Nisco, Abo et al. 2014).

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