Sinorhizobium meliloti CtrA stability is regulated in a CbrA-dependent manner and influenced by CpdR1.

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Running title: CbrA-dependent regulation of CtrA stability

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ABSTRACT

CbrA is a DivJ/PleC-like histidine kinase of DivK that is required for cell cycle progression and symbiosis in the Alphaproteobacterium Sinorhizobium meliloti. Loss of cbrA results in increased levels of CtrA as well as its phosphorylation. While many of the known Caulobacter crescentus regulators of CtrA phosphorylation and proteolysis are phylogenetically conserved within S. meliloti, the latter lacks the regulator PopA that is required for CtrA degradation in C. crescentus. In order to investigate whether CtrA proteolysis occurs in S. meliloti, CtrA stability was assessed. During exponential growth, CtrA is unstable and therefore likely to be degraded in a cell cycle-regulated manner. Loss of cbrA significantly increases CtrA stability but this phenotype is restored to that of the wild type by constitutive ectopic expression of a CpdR1 variant that cannot be phosphorylated (CpdR1D53A). Addition of CpdR1D53A fully suppresses cbrA mutant cell cycle defects, consistent with regulation of CtrA stability playing a key role in mediating proper cell cycle progression in S. meliloti. Importantly, the cbrA mutant symbiosis defect is also suppressed in the presence of CpdR1D53A. Thus, regulation of CtrA stability by CbrA and CpdR1 is associated with free-living cell cycle outcomes and symbiosis.

IMPORTANCE

The cell cycle is a fundamental process required for bacterial growth, reproduction, and developmental differentiation. Our objective is to understand how a two-component signal transduction network directs cell cycle events during free-living growth and host colonization. The Sinorhizobium meliloti nitrogen-fixing symbiosis with plants is associated with novel cell cycle events. This study identifies a link between the regulated stability of an essential response regulator, free-living cell cycle progression, and symbiosis.
INTRODUCTION

*Sinorhizobium meliloti* is an Alphaproteobacteria that grows free-living in the soil or as a beneficial nitrogen-fixing symbiont in association with legumes within genera of *Medicago*, *Melilotus*, and *Trigonella*. As a free-living organism, *S. meliloti* undergoes an asymmetric cell division with once-and-only-once DNA replication per cell cycle (1-3). However, inside its host this bacterium undergoes differentiation into a bacteroid that includes a novel cell cycle program of repeated DNA replication in the absence of cell division (endoreduplication) (3). The underlying molecular mechanisms that dictate cell cycle progression and differentiation in *S. meliloti* remain to be explored in detail and therefore represent a novel aspect of symbiont physiology. Known and putative *S. meliloti* cell cycle regulators are conserved among other Alphaproteobacteria that also specialize in chronic colonization of eukaryotic hosts, in particular rhizobial species within the genera of *Agrobacterium*, *Bartonella*, and *Brucella* (2, 4). Thus, a deeper understanding of *S. meliloti* cell cycle regulation will provide insight into processes that are broadly important to both cell cycle progression and host-microbe interaction (5-12).

*Caulobacter crescentus* provides an intensively studied model for understanding the molecular mechanisms that underlie cell cycle control amongst Alphaproteobacteria. A complex regulatory network, including a central two-component pathway (Fig 1), plays a critical role in coordinating *C. crescentus* cell cycle progression and asymmetric daughter cell fate (13-16).

CtrA is an essential response regulator that contributes to these processes by regulating DNA replication initiation and methylation, as well as cell division and motility, by binding DNA in a phosphorylation-dependent manner. Since CtrA inhibits DNA replication initiation in G1 cells, its activity must be repressed in order to allow S phase to proceed, and later restored to promote G2 events such as cell division. CtrA activity is then differentially inherited by the two daughter
cells, being present in the small swarmer but not the large stalked cell, and in this way establishes their replicative asymmetry (17). The coordination of these diverse processes requires that CtrA activity be post-translationally regulated in a temporal manner through protein-protein interaction with a transcription inhibitor, phosphorylation, degradation, and cellular localization (14, 18-22).

The DivK response regulator functions as an essential switch to indirectly regulate CtrA activity at the level of phosphorylation and degradation (23, 24) (Fig 1). DivK is inactive when dephosphorylated by the phosphatase PleC, and is active when phosphorylated by the kinase DivJ. Phosphorylated DivK inhibits the CckA hybrid histidine kinase (25); therefore, DivK dephosphorylation allows for CckA autophosphorylation and the subsequent transfer of this phosphate to the histidine phosphotransferase ChpT (26, 27). ChpT then transfers this phosphate to two substrates: CtrA and the response regulator CpdR (26, 27). Once CtrA is phosphorylated it is competent to bind DNA and thereby regulate cell cycle events (28-31). The second target of ChpT, CpdR, is required for ClpXP-mediated degradation of CtrA (32, 33); however, CpdR is inactivated by phosphorylation (34). In this manner, the CckA pathway simultaneously phosphorylates CtrA and protects it from proteolysis to stimulate its activity, or dephosphorylates CtrA and promotes its degradation to repress its activity. Interestingly, phosphorylation and degradation are redundant mechanisms for regulating CtrA activity, although at least one is needed for cell viability (18).

Proteolysis of CtrA plays an important role in mediating cell cycle progression and its recognition as a substrate of the ClpXP protease requires its terminal alanine-alanine dipeptide (35). CpdR-regulated proteolysis of CtrA also requires RcdA (36, 37) and the c-di-GMP receptor PopA (38, 39) (Fig 1). RcdA and PopA form a complex that is activated by a CpdR-dependent
increase in c-di-GMP levels and directly interacts with CtrA to recruit a complex containing CpdR and ClpXP to the old cell pole (36, 38-41), although whether localization per se is critical to regulation of CtrA degradation remains unclear (37).

The two-component signaling pathway required for cell division and the establishment of asymmetric daughter cell fate in *S. meliloti* is just beginning to be characterized in molecular detail. However, *S. meliloti* has homologs of many *C. crescentus* regulators of CtrA activity (2, 4) (Fig 1). For example, the DivJ/PleC homolog CbrA is necessary for proper cell cycle progression such that a null mutant displays aberrant morphologies indicative of cell division defects (9, 42). CbrA promotes DivK phosphorylation and this is likely the reason for increased CtrA levels and phosphorylation in *cbrA* mutants (9, 10). DivJ has a similar function, and interestingly *divJ* and *cbrA* null mutations are synthetically lethal (10). The gene encoding the phosphatase PleC is also essential in *S. meliloti* (43), suggesting that strict regulation of DivK phosphorylation is critical to growth and reproduction. Similarly, the presumed downstream target of DivK regulation, CtrA, is essential as in *C. crescentus* (44).

Although CtrA regulation at the level of phosphorylation has been shown in *S. meliloti* (10), it is unclear from bioinformatics analyses whether CtrA might also be regulated at the level of proteolysis. *S. meliloti* CtrA does have a C-terminal ETA motif (44) consistent with the known sequence requirements for ClpXP substrate recognition (45). *S. meliloti* also has CpdR and RcdA orthologs (4). RcdA function has not been examined yet, but CpdR1 is required for polar localization of ClpX and proper cell cycle progression (8), consistent with a role in CtrA cell cycle regulation. However, CpdR1 regulation of CtrA has not been tested directly and, more importantly, there is no PopA ortholog in *S. meliloti* (41) (Fig 2), leaving regulation of CtrA at the level of proteolysis an open question.
We therefore examined CtrA stability in *S. meliloti* and find that it is unstable during exponential growth. Previous studies observed increased levels of CtrA in *cbrA* mutants (9, 10). Here we show that the increased level of CtrA in a Δ*cbrA* mutant is due, in part, to a significant increase in its stability. We further show that constitutive ectopic expression of an unphosphorylatable version of CpdR1 (CpdR1D53A) restores wild type CtrA instability to the Δ*cbrA* mutant. Thus, CtrA stability is regulated in a CbrA-dependent manner and this stability is further influenced by CpdR1. Importantly, CpdR1D53A also suppresses Δ*cbrA* cell cycle and symbiosis defects, providing a strong link between the regulation of CtrA stability, cell cycle outcomes, and host colonization.

**MATERIALS AND METHODS**

**Microbiological techniques.** Bacterial strains and plasmids are listed in Table 1. *Sinorhizobium meliloti* was grown at 30°C in LB/MC (LB supplemented with 2.5 mM calcium chloride and 2.5 mM magnesium sulfate) and *Escherichia coli* was grown at 37°C in LB. Strains were constructed through tri-parental mating as previously described (46). Exponential phase cultures of *S. meliloti* were obtained by inoculating liquid medium with a single colony, then diluting the overnight culture to an OD<sub>600</sub> of 0.1, and allowing the cells to grow to an OD<sub>600</sub> of 0.6-0.8. LB/MC was supplemented with 0.02% calcofluor (Fluorescent brighter 28, Sigma) and buffered with 10 mM HEPES (pH 7.4) to measure succinoglycan production. The following antibiotics were used at the specified concentration: streptomycin (500 µg/mL), neomycin (200 µg/mL), tetracycline (10 µg/mL), and chloramphenicol (20 µg/mL).

**Bioinformatic identification of PopA and PleD homologs through reciprocal blastp analysis.** *C. crescentus* (NA1000) PopA (YP_002517291.1) was used as a query against the
NCBI non-redundant database to perform a primary blast search for homologs using protein-
protein BLAST (blastp). From the first 5,000 hits, candidate homologs with an e-value less than
0.001 and with at least 21% identity to *C. crescentus* PopA were selected; candidates with a
partial species name were eliminated. These candidates were used as a query in a second blastp
search. Hits from this second query were required to have an e-value less than 0.001, and to have
the PopA sequence (YP_002517291.1) as their first hit of the query. If the query results had
PopA as first hit in this secondary blastp, the candidate was identified as a putative PopA
ortholog, or PopA like (PAL). 40 sequences were obtained from the second blastp search. The
PleD (YP_002517919.1) sequence was added to observe its divergence from PopA and PAL
sequences, and the diguanylate cyclase from *Pseudomonas aeruginosa* (WP_034084052.1) was
used as an outgroup to root the phylogenetic tree. Sequences were aligned using MUSCLE. This
multiple sequence alignment was used to infer evolutionary history using the Maximum
Likelihood Phylogeny (InL) with 1,000 bootstraps based on the JTT matrix model (47). The tree
with the highest log likelihood (-13718.7871) is shown. The percentage of trees in which the
associated taxa clustered together is shown next to the branches. Initial trees for the heuristic
search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix
of pairwise distances estimated using a JTT model and then selecting the topology with superior
log likelihood value. All positions containing gaps and missing data were eliminated. There were
a total of 221 positions in the final dataset. Evolutionary analyses were conducted using the
publically available software MEGA version 5.10 (48).

*S. meliloti* CtrA purification and Western blot analysis. CtrA<sup>HIS</sup> was purified and used as a
positive control for Western blot analysis of cellular CtrA as described previously (9).
Exponential phase cultures were centrifuged at 4°C for 10 min at 5000 x g. Cell pellets were
resuspended in 2x Laemmli loading buffer and boiled for 5 min. The volume of lysate analyzed was normalized to OD$_{600}$, subjected to 4-20% SDS-PAGE (Bio-RAD) 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 C. crescentus anti-CtrA polyclonal antibodies (1:5000 dilution in Licor blocking buffer plus 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 plus 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 Image Study software. Subsequently, the membrane was stripped and reprobed with C. crescentus anti-ClpP polyclonal antibodies (1:25,000 dilution in Licor blocking buffer plus 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 plus 0.2% Tween and 0.1% SDS). Cross-reacting proteins were visualized as described for CtrA. Graphical results represent the average and standard deviation of a minimum of three biological replicates.

**CtrA in vivo stability assay.** Exponential phase cultures were divided and treated with either 20 μg chloramphenicol dissolved in 100% ethanol or 100% ethanol, and then grown for 4 hours. Aliquots were taken at 0, 30, 120, and 240 min. Samples were centrifuged at 4°C for 10 min at 5000 x g. Cell pellets were resuspended in 2x Laemmli loading buffer, boiled for 5 min, and used for CtrA Western blot analysis as described above.

**Motility assay.** Overnight cultures were diluted to an OD$_{600}$ of 0.1 and grown on LB/MC with 0.22% agar. Measurement of the diameter of cell growth was taken after 6 days. Results are the average of 5 biological replicates for each strain.
**Microscopy and fluorescence flow cytometry.** Cell morphology was assessed with exponential cultures and each strain was assayed using three biological replicates. Cell adhesion to a slide was performed as previously described (9). Cells were scored as wild type when they showed the typical *S. meliloti* rod shape with an average size of 2-4 µm in length and 1 µm in width. Any cells that did not follow these criteria were considered non-wild type. All strains assessed for DNA content were grown in biological triplicates and prepared as previously described (9, 49). Cells were acquired on a modified Becton-Dickinson LSRII flow cytometer at The Massachusetts General Hospital Flow Cytometry Research Laboratory. Ten thousand cells were acquired for each replicate. The resulting data was analyzed as previously described using the FlowJo software package (9).

**Symbiosis assay.** Symbiosis was assayed with 8 individual *Medicago sativa* plants per *S. meliloti* strain as previously described (9, 50). Nodule coloration and plant height (the length from the epicotyl stem to the apical node) was assessed 5 weeks post-inoculation.

**RESULTS**

*S. meliloti* and closely-related Alphaproteobacteria lack a PopA ortholog. CtrA is cell cycle regulated at the level of transcription, phosphorylation, and degradation in *C. crescentus* (16). In particular, CtrA degradation is one of the key factors contributing to cell cycle progression and the generation of asymmetric daughter cell fate (14, 15, 20). Consistent with the hypothesis that *S. meliloti* CtrA would also be regulated at the level of proteolysis, *divJ* and *cbrA* mutants with decreased DivK phosphorylation accumulate increased levels of CtrA and display cell cycle defects (9, 10). Moreover, the *cpdR1* null mutant is unable to localize ClpX to the cell pole and also displays severe cell cycle defects, which can be complemented by *C. crescentus* CpdR (8).
Orthologs of *C. crescentus* CpdR and RcdA have been identified in *S. meliloti* (4, 8), suggesting regulated ClpXP-dependent degradation of CtrA could be conserved. In contrast, recent analyses indicated the absence of a PopA ortholog in *S. meliloti* (41), and we were therefore interested in further corroborating this observation using a different bioinformatics approach.

A hallmark of PopA is that it can bind c-di-GMP but lacks catalytic activity due to the absence of a consensus GGDEF domain (38). A protein-protein BLAST (blastp) search of the *S. meliloti* genome was performed using *C. crescentus* PopA, however the most closely-related protein identified was a PleD ortholog containing consensus GGDEF and EAL domains. An extensive search for PopA orthologs was then performed using reciprocal blastp and this revealed the limited presence of PopA orthologs primarily among Caulobacterales and Rhodobacterales (Fig 2; PAL, PopA-like). Although this analysis identified several PopA orthologs in addition to what has been described previously (41), particularly in the Rhodobacterales as well as the distantly-related Cyanobacteria *Scytonema millei* and *Mastigocoleus testarum*, this analysis was similarly unable to identify a PopA ortholog in *S. meliloti*. While CpdR and RcdA are present in Caulobacterales, they do not appear to be highly conserved among CtrA-containing Rhodobacterales (4), indicating that either PopA alone is sufficient for CtrA degradation or PopA has an alternative function in these bacteria.

PopA orthologs appear to be absent from nearly all rhizobacterial families, including Bradyrhizobiaceae, Brucellaceae, and Rhizobiaceae, which have conserved the CtrA proteolysis regulators CpdR and RcdA (4). The one exception is the Bradyrhizobial marine bacterium *Rhodopseudomonas palustris*, although its PopA ortholog is the most divergent within this protein family and retains similarity to PleD (Fig 2). PopA also appears to be absent from the Rickettsiales, however these bacteria also lack CpdR and RcdA (4), suggesting that regulated...
proteolysis of CtrA may not be conserved within this order of Alphaproteobacteria. Based on this analysis, if CtrA is regulated at the level of degradation in *S. meliloti*, then proteolysis occurs through a novel PopA-independent mechanism that may be common to a variety of host-associated rhizobiales.

*S. meliloti* CtrA is unstable during exponential growth. *S. meliloti* divJ and *cbrA* mutants with decreased DivK phosphorylation accumulate increased levels of CtrA and display cell cycle defects (9, 10). However, due to the absence of a PopA ortholog in *S. meliloti*, it was unclear whether the high levels of CtrA in these mutants could be the result of decreased proteolysis. To test for degradation, CtrA stability was measured in a protein shut-off assay by adding the translation inhibitor chloramphenicol to exponentially growing wild type cells and assessing CtrA levels by Western blot over the course of 4 hrs. CtrA levels begin to decrease at 30 min after the addition of chloramphenicol and the amount of CtrA further decreases at each successive time point (Fig 3A). ClpP levels were simultaneously assayed but there was no change in protein level over the course of the experiment (Fig 3A), as observed in *C. crescentus* (30). In order to quantitatively determine the relative amount of CtrA present over time, CtrA levels were normalized to ClpP as an internal loading control with the amount of CtrA at *t*=0 arbitrarily set to 1. The concentration of CtrA is approximately 60% of its starting levels at *t*=30 min and at just under 30% of its starting levels at *t*=240 min (Fig 3C). Thus, *S. meliloti* CtrA is unstable during exponential growth despite the absence of a PopA ortholog.

*S. meliloti* CtrA stability is regulated in a CbrA-dependent manner. CtrA levels are increased in a *cbrA* mutant that has decreased levels of phosphorylated DivK (9, 10). This observation is consistent with a model in which CbrA, through its effect on DivK, promotes the degradation of CtrA (Fig 1). This was tested by measuring CtrA stability in a Δ*cbrA* null mutant.
as described for wild type. Western blot analysis shows that CtrA levels do not significantly decrease after treatment with chloramphenicol (Fig 3B). In fact, CtrA levels in the ΔcbrA mutant are maintained at pretreatment levels for at least 240 min (Fig 3BD) in contrast to what is observed with the wild type (Fig 3AC). These results demonstrate that CtrA is normally unstable during exponential growth but is significantly stabilized in the ΔcbrA mutant. Based on C. crescentus, CbrA-dependent regulation of CtrA stability is most likely indirect and requires a CpdR response regulator ortholog whose activity is controlled by phosphorylation (Fig 1).

**CpdR1<sup>D53A</sup> restores wild type CtrA stability to the ΔcbrA mutant.** In C. crescentus, CpdR plays an essential role in the directed proteolysis of CtrA by ClpXP (26, 33, 34, 40), such that in its unphosphorylated state CpdR promotes CtrA degradation (15). As CpdR can function in the place of S. meliloti CpdR1 (8), it is possible that a partially conserved mechanism for CtrA degradation exists in S. meliloti. We therefore predicted that the unphosphorylatable variant CpdR1<sup>D53A</sup> would function as a dominant-negative factor to promote proteolysis of CtrA. To test this, CpdR1 and CpdR1<sup>D53A</sup> were expressed from a constitutive promoter on a low copy plasmid. We were unable to detect a significant change in CtrA levels in wild type cells containing pcpdR1 compared to pcpdR1<sup>D53A</sup> (Fig 4A). Although somewhat surprising, this is consistent with the subtle cell cycle defects associated with pcpdR1<sup>D53A</sup> in the presence of native cpdR1 on the chromosome (8).

Based on C. crescentus, we predict that CpdR1 phosphorylation is regulated by CbrA indirectly through DivK and the CckA pathway (Fig 1). This model further predicts that a ΔcbrA mutant would have aberrantly high levels of phosphorylated CpdR1 and therefore decreased proteolysis of CtrA. In this case, unphosphorylatable CpdR1<sup>D53A</sup> should function as a dominant extragenic suppressor of CtrA levels in the ΔcbrA mutant. Consistent with this hypothesis, lower
levels of CtrA are present in ΔcbrA pcpdR1\textsuperscript{D53A} compared to ΔcbrA pcpdR1 and reflect a
restoration to wild type levels (Fig 4AB).

To test whether expression of CpdR1\textsuperscript{D53A} in ΔcbrA restores CtrA instability, a protein
shut-off assay was performed using chloramphenicol and CtrA levels were measured by Western
blot at 0 and 240 min post-treatment. At 240 min, there is a significant decrease in CtrA levels in
wild type cells with either pcpdR1 or pcpdR1\textsuperscript{D53A} (Fig 4C). There is also a significant decrease in
CtrA levels in ΔcbrA pcpdR1\textsuperscript{D53A} but not ΔcbrA pcpdR1 cells (Fig 4C). These results
demonstrate that a modest input of constitutively active CpdR1\textsuperscript{D53A} is able to suppress the ΔcbrA
mutation with regard to CtrA levels by restoring a wild type-level of instability, and supports a
model that regulation of CtrA degradation by CbrA occurs indirectly through downstream effects
on CpdR1 activity (Fig 1).

\textbf{CpdR1\textsuperscript{D53A} restores wild type cell cycle progression to the ΔcbrA mutant.} CbrA contributes
to cell cycle regulation such that the ΔcbrA mutant displays filamentous growth indicative of a
cell division defect and is associated with cells containing an altered genome complement (9).
CpdR1\textsuperscript{D53A} is able to restore CtrA instability to wild type levels in the ΔcbrA mutant and may
therefore also restore wild type cell cycle progression. As a preliminary test, we examined the
ΔcbrA mutant calcofluor-bright phenotype that reflects overproduction of the exopolysaccharide
EPS I (also referred to as succinoglycan) (9, 51). Wild type cells with either pcpdR1 or
pcpdR1\textsuperscript{D53A} produce normal levels of EPS I and are dim on calcofluor medium (Fig 5A, strains 1
and 2). ΔcbrA pcpdR1 retains its mutant calcofluor-bright phenotype while ΔcbrA pcpdR1\textsuperscript{D53A}
cells display a wild type dim phenotype (Fig 5A, strains 3 and 4). The dim phenotype of ΔcbrA
pcpdR1\textsuperscript{D53A} is indistinguishable from that of the ΔcbrA pLAFR2070 complemented strain and
therefore reflects complete suppression of EPS I overproduction (Fig 5A, strains 4 and 8). The 
ΔcbrA motility defect (Fig 5B) is also fully suppressed by pcpdR1<sup>D53A</sup> but not pcpdR1 (Fig 5B).

As described previously (9), the ΔcbrA mutant displays an aberrant filamentous 
morphology (Fig 6A, column 2) at an increased frequency compared to wild type (Fig 6B), and this phenotype is not altered by the addition of pcpdR1 (Fig 6AB). There is a modest but observable cell morphology defect associated with constitutive ectopic expression of CpdR1<sup>D53A</sup>, but not CpdR1, in an otherwise wild type background (Fig 6A, column 2) such that 5% of cells are highly filamentous (Fig 6B) (8). CpdR1<sup>D53A</sup> suppresses the ΔcbrA cellular filamentation defect (Fig 6A) such that when pcpdR1<sup>D53A</sup> is combined with ΔcbrA the rate of filamentation resembles that of pcpdR1<sup>D53A</sup> in wild type cells (Fig 6B). Similarly, combining pcpdR1<sup>D53A</sup>, but not pcpdR1, with ΔcbrA results in reversion to the bimodal 1N-2N DNA distribution of cells observed in unsynchronized wild type populations (Fig 6CD). We therefore find that constitutive ectopic expression of CpdR1<sup>D53A</sup>, which restores wild type levels of CtrA to the ΔcbrA mutant, is also able to fully suppress known ΔcbrA cell cycle defects during free-living growth.

CpdR1<sup>D53A</sup> restores wild type symbiosis to the ΔcbrA mutant. The cbrA mutant is unable to properly establish a symbiosis with Medicago sativa (51). The underlying reason for this defect has been unclear (52), however we now predict that it may be due to high levels of CtrA and associated cell cycle defects (9). Since the ΔcbrA pcpdR1<sup>D53A</sup> mutant has wild type levels of CtrA, we investigated whether it would be able to establish an effective symbiosis with M. sativa in order to test this prediction. Plants were inoculated with strains containing either pcpdR1 or pcpdR1<sup>D53A</sup> and were grown for five weeks. The percentage of pink nodules and total plant height were used as a set of metrics to assess the effectiveness of symbiosis. The extremely low percentage of pink nodules and decreased plant height observed with ΔcbrA pcpdR1 shows that
this strain fails to establish a functional symbiosis (Fig 7AB), similar to ΔcbrA alone (9). However, ΔcbrA pcpdR1D53A shows a complete rescue of symbiosis to the level observed with wild type (Fig 7AB). These results show that constitutively active CpdR1D53A is able to overcome the ΔcbrA symbiosis defect and are consistent with the model that this defect is due to misregulation of CtrA.

DISCUSSION

A molecular understanding of the components that govern *S. meliloti* cell cycle progression during free-living growth is necessary to develop a model for how this program may be modified during host colonization. Several two-component histidine kinases function as part of the DivK pathway required for both free-living cell cycle progression and symbiosis. CbrA and DivJ function as kinases to phosphorylate DivK while PleC functions as a DivK phosphatase (9, 10) (Fig 1). While *pleC* is essential in *S. meliloti* (43), the individual loss of either *divJ* or *cbrA* is tolerated and leads to filamentation and ploidy defects during free-living growth as well as the loss of symbiosis (9, 10, 51). These phenotypic defects are correlated with increased levels and phosphorylation of the essential response regulator CtrA (9, 10, 44), suggesting that the DivK pathway serves to regulate the cell cycle by controlling CtrA activity (Fig 1). Since DivK-mediated regulation of CtrA at the level of phosphorylation appears to be conserved in *S. meliloti* (10), we examined whether CtrA regulation at the level of degradation is also conserved. CtrA degradation in *C. crescentus* is carried out by the ClpXP protease and requires CpdR, RcdA, and c-di-GMP-bound PopA for enhanced recognition of CtrA as a proteolytic substrate (40). Although *S. meliloti* has putative CpdR and RcdA orthologs (4, 8),
there is no recognizable PopA ortholog and it was therefore unclear whether CtrA would be regulated by proteolysis in *S. meliloti* (41) (Fig 2).

We measured CtrA stability during exponential growth and found that CtrA is in fact unstable (Fig 3A) despite the absence of PopA. Given that PopA serves as a c-di-GMP sensor (38), it is therefore unclear whether *S. meliloti* CtrA levels are responsive to fluctuations in this particular second messenger through an alternative c-di-GMP receptor or if this aspect of CtrA regulation is specific to *C. crescentus* and its more closely related Alphaproteobacteria. We also measured CtrA stability in a ΔcbrA mutant and observed a significant stabilization of the protein (Fig 3B), showing that CtrA stability is regulated in a CbrA-dependent manner.

CpdR1 is hypothesized to be a downstream target of CbrA regulation that is required to direct ClpXP-mediated degradation of CtrA in its unphosphorylated state (Fig 1) (8). We tested this by examining whether addition of unphosphorylatable CpdR1<sup>D53A</sup> would impact CtrA levels. Consistent with this prediction, introduction of CpdR1<sup>D53A</sup> to the ΔcbrA mutant restores CtrA to wild type levels (Fig 4AB) and leads to an increase in CtrA instability (Fig 4CD). Thus, CpdR1<sup>D53A</sup> behaves as a dominant factor that can overcome the loss of CbrA regulation. We were unable to directly test the epistatic relationship between *cbrA* and *cpdR1* due to the severe growth defect of the ΔcpdR1 mutant (8), however we favor a model in which CpdR1 functions downstream of CbrA as a target of DivK-mediated regulation of the CckA pathway (Fig 1). Moreover, since CpdR1 influences ClpX localization (8), it is likely that unphosphorylated CpdR1 directly promotes ClpXP-mediated proteolysis of CtrA, although this remains to be tested directly.

Since CpdR1<sup>D53A</sup> restores wild type CtrA levels to the ΔcbrA mutant, we examined whether it would also have an impact on the cell cycle. We find that CpdR1<sup>D53A</sup> is able to
suppress ΔcbrA mutant free-living phenotypes (Fig 5AB). Importantly, the ΔcbrA mutant cell
361 cycle defects of filamentous cell morphology (Fig 6AB) and aberrant DNA content are fully
362 suppressed by the addition of CpdR1D53A (Fig 6CD). These observations provide a strong link
363 between CbrA-dependent regulation of CtrA stability and free-living cell cycle outcomes.
364 However, C. crescentus CpdR promotes the regulated degradation of multiple ClpXP targets,
365 including the σ54-dependent response regulator TacA (53), the c-di-GMP phosphodiesterase
366 PdeA (39, 54), and CtrA (33). It is therefore possible that CpdR1-dependent degradation of a
367 target other than CtrA is responsible for CpdR1D53A suppression of ΔcbrA mutant phenotypes.
368 Nevertheless, the simplest model is that a single event is responsible for CpdR1D53A suppression
369 of all ΔcbrA phenotypes, including CtrA stability, which implies that regulated CtrA degradation
370 is directly responsible for cell cycle outcomes (Fig 1).

In C. crescentus, CtrA regulation is central to cell cycle progression and asymmetric cell
division and our results support a model in which this role is conserved in S. meliloti. Although it
is possible that CtrA is not the only factor required for CbrA-dependent cell cycle regulation, it
does remain highly likely that CtrA plays a role in cell cycle progression. CtrA is essential (44),
so its presence is needed for cell viability and the identification of putative CtrA binding sites
points towards a role in promoting cell division (4, 55).

We also find that addition of CpdR1D53A restores symbiosis to the ΔcbrA mutant (Fig
7AB), which suggests the regulation of CtrA stability may be critical to symbiosis between S.
380 meliloti and M. sativa. While typical growth and cell division of S. meliloti has been suggested as
381 the mechanism for host invasion (56), a novel cell cycle of bacterial endoreduplication has been
382 observed during the intracellular colonization process of bacteroid formation (3). Given that
383 CbrA is a regulator of CtrA and the cell cycle (9), and is absolutely required for symbiosis (51),

17
it appears likely that regulation of the bacterial cell cycle plays an essential role in establishing
the symbiosis. More specifically, it may be that endoreduplication requires CtrA activity be
repressed so that DNA replication can proceed in the absence of cell division, or that bacterial
exit from the cell cycle into a permanent non-reproductive G0 state involves CtrA regulation.

Several prior observations support this hypothesis that repression of CtrA is critical to
symbiosis. We were unable to examine the CtrA phenotype of a ΔcpdR1 mutant due its severe
growth defect. However, based on our observation that CpdR1^{D53A} decreases the stability of
CtrA, it is likely that loss of cpdR1 results in increased levels of CtrA and this may be why the
ΔcpdR1 mutant is able to infect host nodules but unable to properly differentiate into a bacteroid
(8). More recently, it was shown that a nodule-specific cysteine-rich (NCR) peptide that is
produced by the host within nodules and is able to induce free-living cells to differentiate into
bacteroids ex planta is also able to alter the expression level of putative CtrA gene targets (57).
This is of broad significance given that CpdR1 and CtrA are conserved among rhizobial bacteria
that engage in host-microbe interactions (4), particularly since regulation of the bacterial cell
cycle is associated with the colonization process (3, 5).

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REFERENCES


Figure 1. Two-component pathway model of CtrA cell cycle regulation. The *S. meliloti* genome encodes many of the two-component regulators of CtrA that have been identified in *C. crescentus* (black font). However, there are several points of divergence between these two distantly related Alphaproteobacteria. *S. meliloti* contains an additional DivJ/PleC-like histidine kinase, CbrA, that promotes DivK phosphorylation (blue font). Additionally, *S. meliloti* lacks the c-di-GMP receptor PopA that is required to regulate CtrA proteolysis in *C. crescentus* (green font).

Figure 2. *S. meliloti* lacks a PopA ortholog. *C. crescentus* PopA was used to perform a reciprocal blastp search for orthologs. Sequences with a minimum of 21% identity were used to create a Maximum Likelihood Phylogeny tree. If more than one ortholog was found in the same organism, the sequences were randomly labeled 1 and 2. *C. crescentus* PleD was included in the final sequence alignment and phylogenetic analysis for comparison. PopA is present in Caulobacterales and Rhodobacterales, but absent from many alphaproteobacteria that engage in host-microbe interaction, including Bradyrhizobiaceae, Brucellaceae, and most Rhizobiaceae families within the Rhizobiales order, as well as the Rickettsiales. Several species have a PopA ortholog that is more homologous to those present in a different order and two PopA orthologs were identified outside the alphaproteobacterial division: *Woodsholea maritima* is a Caulobacterales, **Scytonema millei** and ***Mastigocoleus testarum* are from the division Cyanobacterium, *****Polymorphum gylvum* is an unclassified alphaproteobacterium with a PopA ortholog most closely related to those of the Rhodobacterales.

Figure 3. CtrA stability is regulated during exponential growth in a CbrA-dependent manner. A, B) Western blots of whole cell lysates were probed with α-CtrA and α-ClpP. Lane 1: purified *S. meliloti* CtrAHIS; lanes 2-9: protein levels were assayed by Western blot at different times (0, 30, 120, and 240 minutes) post-treatment of cells with (+) and without (-) chloramphenicol (CHL). C, D) CtrA signal in WT and ΔcbrA cells was first normalized to the ClpP loading control signal, and then independently normalized to 1 using their 0 min value. Graphical results are the average of at least three biological replicates and error bars represent the standard deviation. Solid line: no chloramphenicol control. Broken line: chloramphenicol treatment. * Significant, p <0.0001

Figure 4. CtrA instability is restored to the ΔcbrA mutant by constitutive ectopic expression of unphosphorylatable CpdR1D53A. CtrA levels during exponential growth were assayed by Western blot A) in batch culture or C) at different time points (0 and 240 mins) post-treatment of cells with (+) and without (-) chloramphenicol (CHL). The first lane (far left) of each Western blot is purified *S. meliloti* CtrAHIS as a positive control for α-CtrA. Each Western was probed with α-CtrA and α-ClpP. B) For each strain indicated, CtrA signal from three biological replicates was normalized to its internal ClpP loading control, and then normalized to 1 using WT pcpdR1 for comparison. *Significant, p <0.0001 compared to the three other strains. D) For each strain indicated, CtrA signal from three biological replicates was normalized to its internal loading control, ClpP, and then independently normalized to 1 using its 0 min value. *Significant, p <0.0001 compared to its non-chloramphenicol treatment control.
Figure 5. Free-living phenotypes of the ∆cbrA mutant are rescued by constitutive ectopic expression of unphosphorylatable CpdR1D53A. A) Succinoglycan (EPS I) production was assessed on LB/MC medium supplemented with calcofluor. Strain genotypes are as follows: 1- WT pcpdR1, 2- WT pcpdR1D53A, 3- ∆cbrA pcpdR1, 4- ∆cbrA pcpdR1D53A, 5- WT pLAFR1, 6- WT pLAFR2070, 7- ∆cbrA pLAFR1, 8- ∆cbrA pLAFR2070. B) Motility was assessed using 0.2% LB/MC agar. *Significant, p <0.0001.

Figure 6. Cell cycle phenotypes of the ∆cbrA mutant are rescued by constitutive ectopic expression of unphosphorylatable CpdR1D53A. A) Cellular morphology was observed and classified as either rod-shaped (1-3 μM long and 1 μM wide; column 1) or aberrant (round, or branched and filamentous; column 2). B) The frequency of aberrant cell morphology in an exponentially growing population of cells was quantified. C) Flow cytometry was performed to measure cellular genome content per cell. D) The frequency of aberrant DNA content (either <1N or >2N) in an exponentially growing population of cells was quantified as the average percentage and its standard deviation.

Figure 7. The symbiosis defect of ∆cbrA is rescued by constitutive ectopic expression of unphosphorylatable CpdR1D53A. Symbiosis was assayed with Medicago sativa plants five weeks post-inoculation. A) The average percentage of pink nodules per total nodules per plant and its standard deviation. B) The average plant height and its standard deviation. *Significant, p <0.0001 to WT and WT pcpdR1D53A; *significant, p <0.0007 to ∆cbrA pcpdR1D53A.
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### B

#### Cellular Morphology

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### C

![Graph showing DNA content distribution for WT and ΔcbrA strains](image)

### D

#### Cellular Genome Content

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Values are percentages (± standard deviation) of biological triplicates.