# Global analysis of cell cycle gene expression of the legume symbiont *Sinorhizobium meliloti*

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**In α-proteobacteria, strict regulation of cell cycle progression is necessary for the specific cellular differentiation required for adaptation to diverse environmental niches. The symbiotic lifestyle of *Sinorhizobium meliloti* requires a drastic cellular differentiation that includes genome amplification. To achieve polyploidy, the *S. meliloti* cell cycle program must be altered to uncouple DNA replication from cell division. In the α-proteobacterium *Caulobacter crescentus*, cell cycle-regulated transcription plays an important role in the control of cell cycle progression but this has not been demonstrated in other α-proteobacteria. Here we describe a robust method for synchronizing cell growth that enabled global analysis of *S. meliloti* cell cycle-regulated gene expression. This analysis identified 462 genes with cell cycle-regulated transcripts, including several key cell cycle regulators, and genes involved in motility, attachment, and cell division. Only 28% of the 462 *S. meliloti* cell cycle-regulated genes were also transcriptionally cell cycle-regulated in *C. crescentus*. Furthermore, CtrA- and DnaA-binding motif analysis revealed little overlap between the cell cycle-dependent regulons of CtrA and DnaA in *S. meliloti* and *C. crescentus*. The predicted *S. meliloti* cell cycle regulon of CtrA, but not that of DnaA, was strongly conserved in more closely related α-proteobacteria with similar ecological niches as *S. meliloti*, suggesting that the CtrA cell cycle regulatory network may control functions of central importance to the specific lifestyles of α-proteobacteria.**

cell cycle regulation | symbiosis | alpha-proteobacteria

The α-proteobacteria class includes bacteria adapted to a wide range of lifestyles and environments (1). To thrive in their specific ecological niches, α-proteobacteria have developed specialized cellular differentiation programs. For example, *Sinorhizobium meliloti* undergoes a drastic cellular differentiation during its ecologically and agriculturally important nitrogen-fixing symbiosis with *Medicago, Melilotus*, and *Trigonella* legume hosts (2–4). *S. meliloti* initially elicits nodules on the roots of compatible legumes and then invades these nodules through host-derived infection threads. The bacteria proliferate within the infection thread as it extends and, after reaching the interior of the developing nodule, bacteria at the tip of the infection thread are endocytosed individually into compartments termed "symbiosomes." Within this compartment, *S. meliloti* undergoes a striking cellular differentiation to become a nitrogen-fixing bacteroid. This differentiation involves an alteration of the bacterial cell cycle, as not only are cell size and membrane permeability altered in bacteroids but multiple equivalents of the tripartite *S. meliloti* genome accumulate (5, 6). A large family of defensin-like nodule-specific cysteine-rich (NCR) peptides have been recently discovered to play key roles in orchestrating this differentiation process, however their molecular mechanism is largely unknown (7, 8). Work described by Penterman et al. in ref. 9 suggests that these NCR peptides may act in part by altering the transcriptional profiles of key cell cycle regulators and remodeling the transcriptome to favor symbiosis.

Several lines of evidence suggest that modulation of the *S. meliloti* cell cycle is critically important for the cellular differentiation during symbiosis. For example, it has been shown that altering the expression of genes central to *S. meliloti* cell cycle processes (i.e., *ftsZ*, *dnaA*, *minE*, and *ccrM*) produces bacteroid-like polyploid cells (10–13) and that mutation of conserved cell cycle regulators (*cbrA*, *cpdR1*, and *divJ*) blocks bacteroid formation and symbiosis (14–16). Furthermore, in the α-proteobacterium *Caulobacter crescentus,* the cellular differentiation program governing morphological and replicative asymmetry in progeny cells is genetically integrated with the cell cycle (17). This is achieved partially through coordinate expression of genes involved in cell cycle processes and cellular differentiation in a cell cycle phase-dependent manner (18). The transcriptional regulatory proteins at the top of this genetic network include the response regulator, CtrA, which modulates morphological and replicative asymmetry, and the DNA replication initiation protein DnaA (19–21).

Because the regulatory factors that govern the *C. crescentus* cell cycle are highly conserved in α-proteobacteria, the *C. crescentus* paradigm of transcriptional control of cell cycle progression has been postulated to also be conserved in most α-proteobacteria (22, 23). However, this hypothesis does not readily explain how a strictly conserved cell cycle regulatory circuit could accommodate the extremely variable lifestyles and cellular differentiation processes found in this diverse group of bacteria. To date, it has been difficult to test whether the *C. crescentus* paradigm of cell cycle regulation is conserved in *S. meliloti* on a global scale because no method existed to obtain synchronized cultures. Although single-gene studies have indicated that many cell cycle regulators including CtrA, DnaA, CcrM, DivJ, GcrA, and PleC

## Significance

**The bacterium *Sinorhizobium meliloti* establishes an agriculturally and ecologically important nitrogen-fixing symbiosis with leguminous plants. During symbiosis, the bacterial cells undergo drastic cellular differentiation and alter their cell cycle regulation such that they become highly polyploid. Cell cycle research has been limited in *S. meliloti* because there has been no method to generate synchronous cell populations. Here we describe a robust method to synchronize *S. meliloti* and present a global analysis of *S. meliloti* cell cycle gene expression. The results of this study suggest that the *S. meliloti* cell cycle transcriptional regulatory network, especially the regulon of the master regulator CtrA, has adapted specifically to fit its lifestyle both within the soil and its legume host.**

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Data deposition: The microarray data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE54208).

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are essential and/or functionally conserved in *S. meliloti*, our understanding of the role of transcriptional regulation in the *S. meliloti* cell cycle and the link between the cell cycle and cellular differentiation during symbiosis has been limited (11, 12, 24, 25).

Here we describe an efficient method for the synchronization of *S. meliloti* cell populations via nutrient downshift and present a microarray based gene expression analysis of the *S. meliloti* cell cycle. This analysis identified 462 genes exhibiting strong periods of up-regulation and down-regulation during the *S. meliloti* cell cycle. These genes include conserved cell cycle regulators as well as large number of genes involved in motility, attachment, and cell division. Importantly, these data firmly support the extension of the general model of *C. crescentus* cell cycle-regulated transcription to *S. meliloti* and possibly other α-proteobacteria. We also identified 126 cell cycle-regulated transcripts common to both *C. crescentus* and *S. meliloti,* which may represent a conserved core set of cell cycle-regulated genes in α-proteobacteria. However, the large divergence in cell cycle-regulated transcripts, as well as in the major differences between the predicted regulons of CtrA and DnaA between *C. crescentus* and *S. meliloti*, indicates that this network has specifically evolved in each species to facilitate their unique cellular differentiation programs and lifestyles.

## Results

**Synchronization of *S. meliloti* Cell Populations via Nutrient Downshift.** Previous attempts to use the *C. crescentus* method of differential centrifugation to isolate G1-arrested daughter cells (26) from *S. meliloti* cultures proved unsuccessful. However, an effective method for synchronizing populations of *Escherichia coli* involves growing cells in conditions that promote the highly conserved stringent response, which induces a G1 arrest (27). The stringent response of *S. meliloti* is stimulated by both carbon and nitrogen starvation and is dependent on ppGpp synthesis by the enzyme $Rel_{Sm}$ (28, 29). To test if stringent conditions would produce G1-arrested *S. meliloti* cells, early log phase Rm1021 cells were transferred to medium lacking preferred carbon and nitrogen sources (28). DNA content per cell was monitored via flow cytometry and after 4.5 h, ∼95% of the cells had one copy of their genome (1C) (Fig. 1 *A* and *B*). Arrested cells were pelleted and resuspended in rich medium, and after 40–50 min *S. meliloti* cells initiated DNA replication in unison and proceeded to replicate their genomes (Fig. 1 *A* and *B*). The synchronized cells reached mid-S phase by 100–120 min and completed replication by 140 min. At 160 min, cells underwent division and the 1C peak reemerged. Synchronous growth was thus maintained through one full cell cycle (Fig. 1*B*). This cell cycle progression is illustrated in Fig. 1*A*.

**Microarray Analysis of Synchronized *S. meliloti* Cultures Identifies 462 Cell Cycle-Regulated Transcripts.** To test whether cell cycle-regulated gene expression was conserved in *S. meliloti,* gene expression was monitored in synchronized cultures of *S. meliloti* as they progressed through the cell cycle (18). RNA was isolated from cultures arrested in G1 ($t = 0$) and at 20-min intervals starting at the end of G1 ($t = 40$) until cell division ($t = 160$). We directly compared the expression of 6,046 *S. meliloti* genes in distinct phases of the cell cycle with the expression of these genes in early-log phase asynchronous culture via two-color microarray analysis. An SD cutoff was applied to the expression data to identify *S. meliloti* genes whose transcription varies significantly as a function of the cell cycle. Average replicate log ratio values of each gene for each time point were used to calculate the SD for all *S. meliloti* genes. These SD values were compared with SD values generated from random permuted gene expression profiles and genes with statistically significant SDs were chosen (30). To minimize the effect of the stringent response on variance, only values from the 40–160 time points were used to calculate the SD of expression for each gene.

Our analysis identified 462 genes whose expression varies as a function of the cell cycle (Dataset S1). The majority of cell cycle-regulated genes (320) are located on the chromosome, whereas 107 and 36 are located on the symbiotic megaplasmids pSymB and pSymA, respectively. Cell cycle-regulated transcription thus controls 9.5% of chromosomal genes, 6.8% of pSymB genes, and 2.7% pSymA genes. These data suggest that the chromosome is enriched for genes with cell cycle-regulated expression, an inference that was verified by a hypergeometric test ($P < 0.001$). The especially low percentage of genes demonstrating cell cycle-regulated transcription on pSymA compared with the chromosome and pSymB could be a result of pSymA being a more recently acquired, less domesticated replicon than pSymB (6, 31).

A fuzzy *c*-means clustering algorithm was used to group cell cycle-regulated genes with similar expression patterns to identify groups of genes involved in similar cell cycle functions (32, 33). This analysis yielded a total of six gene clusters that are displayed in Fig. 1*C*. Many genes with similar functions clustered together, especially the flagellar and chemotaxis machinery found in clusters 4 and 5, respectively. Importantly, each cluster represents a specific period of transcriptional activation and repression during the cell cycle—similar to the pattern of cell cycle gene expression observed in *C. crescentus,* (18). These data clearly demonstrate that temporally regulated gene expression during the cell cycle is conserved between *S. meliloti* and *C. crescentus,* which supports the extension of this paradigm to other α-proteobacteria.

**Many *S. meliloti* Genes Exhibit Peak Expression Corresponding with the Timing of Their Cellular Function.** Fig. 2*A* illustrates the expression profiles of genes involved in DNA replication, recombination, repair, and chromosome segregation; genes denoted by an asterisk did not have a strong enough variance in their expression to be included in our list of cell cycle-regulated transcripts. Most of the genes encoding components of the DNA replication machinery did not exhibit strong cell cycle-regulated expression, which could mean that the activity of these factors is regulated posttranscriptionally or that the cell is sensitive to small differences in the expression of a particular gene. For example, transcription of *dnaA*, which did not make our cutoff, shows a slight up-regulation at 60 min corresponding with the start of S phase observed via flow cytometry (Figs. 1*B* and 2*A*). The calculated SD of the expression of the chromosome segregation genes *parA* and *parB*, which show early up-regulation in the heat map, was also not large enough to pass the stringent cutoff required by our analysis. However, our analysis did reveal cell cycle-regulated expression of the *repABC* genes, which govern the replication and segregation of pSymA and pSymB and thus play important roles in coordinating the replication of *S. meliloti*'s tripartite genome (34). These genes fall into cluster 3 with peak up-regulation occurring in early S phase between $t = 80$ and $t = 100$ (Fig. 1*B*). Specifically, the expression of *repC1* and *repC2* (which are required for the initiation of replication of pSymB and pSymA, respectively) occurred after the observed peak expression of *dnaA*, suggesting that initiation of the megaplasmid origins may follow that of the chromosomal origin instead of occurring simultaneously (illustrated in Fig. 1*A*). This phenomenon has precedence in bacteria with multipartite genomes, as it has been demonstrated in *Vibrio cholerae* that the replication initiation of the smaller chromosome occurs after that of the larger chromosome to ensure that replication of both chromosomes terminates at the same time (35).

Another set of genes whose cell cycle-regulated transcription was not strongly conserved between *S. meliloti* and *C. crescentus* were those involved in nucleotide biosynthesis and DNA repair (18). Only the gene encoding *lexA,* a regulator of the SOS

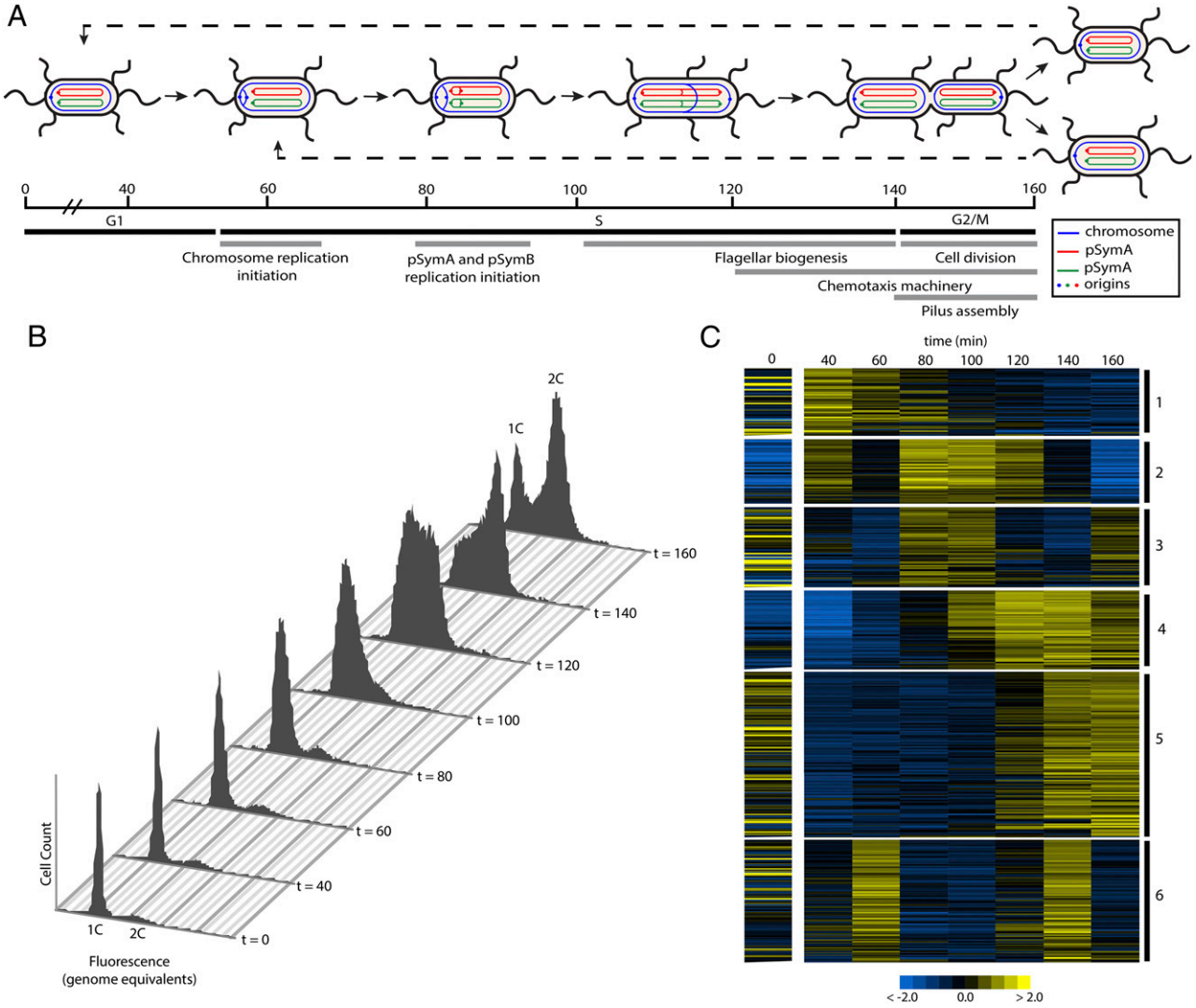


**Fig. 1.** Synchronization of *S. meliloti* and microarray analysis of cell cycle-regulated gene expression. (*A*) Illustration of *S. meliloti* cell cycle progression. The timing of cell cycle events is based on expression patterns of specific genes as described in results. *S. meliloti* is peritrichously flagellated and divides asymmetrically (23, 56). (*B*) FACS profiles from cell synchronization. At $t = 0$, 95% of cells are arrested in G1. At $t = 60$, replication has initiated and S phase starts. Cells have completed S phase at $t = 140$ and divide at $t = 160$. (*C*) Heatmap of the clustering of 462 cell cycle-regulated transcripts. Clusters are indicated on the right. Each row represents a single gene. The color scale bar corresponding to log-fold expression is included at the bottom of the heatmap.

response (36), demonstrated strong cell cycle-regulated transcription in both *C. crescentus* and *S. meliloti* (Fig. 2*A*) (18). In *S. meliloti, lexA* expression peaks late in the cell cycle suggesting a function for LexA at the end of S phase. A possible function of LexA during the *S. meliloti* cell cycle would be to inhibit the SOS response as the replicating chromosomes and megaplasmids are resolved.

Components of the *S. meliloti* cell division machinery fell into two separate clusters suggesting the existence of early and late cell division genes (37). The homologs of *E. coli* division genes *ftsA*, *ftsZ*, and *minC* were in cluster 4, whereas *ftsQ*, *ftsI*, *ftsK*, *minD*, and *minE* were in cluster 5 (Fig. 1*C*). Consistent with their predicted function in *S. meliloti*, these genes were most highly expressed coincident with the timing of septum formation (Figs. 1*A* and 2*B*). The expression pattern of the *minCDE* operon, which is required to restrict the septum to midcell in *S. meliloti*, was surprising (37). It has been demonstrated that *minCDE* genes are coexpressed from a promoter directly upstream of *minC* (13), but our data indicate that *minC* expression greatly precedes that of *minDE* during the cell cycle. This uncoupling of expression may be due to a terminator sequence or the 43-bp partial rhizobium-specific intergenic mosaic element (RIME) in the 101-bp region between *minC* and *minD* (13). Repetitive elements similar to the rhizobia-specific RIME element have been shown to alter gene expression in polycistronic operons (38).

The set of genes demonstrating the most robust cell cycle-regulated transcription were motility genes. Flagellar biosynthesis genes dominated cluster 4, whereas chemotaxis genes were present in cluster 5 (Figs. 1*C* and 2*C*). In *S. meliloti,* swimming motility is restricted to the exponential phase of growth by a three-class hierarchy of flagellar and chemotaxis genes (39). Class I regulators VisNR and Rem are at the top of the hierarchy controlling the expression of class II genes (*flg*, *flh*, *fli*, and *mot*) and class III genes (*che* and *fla*) (39). Our analysis revealed that the motility genes are highly repressed in the beginning of the cell cycle and that expression of class I and II genes is activated between 100–120 min and expression of class III genes is activated at 140 min (Fig. 2*C*). This pattern of gene expression is consistent with the known regulatory hierarchy, but raises the

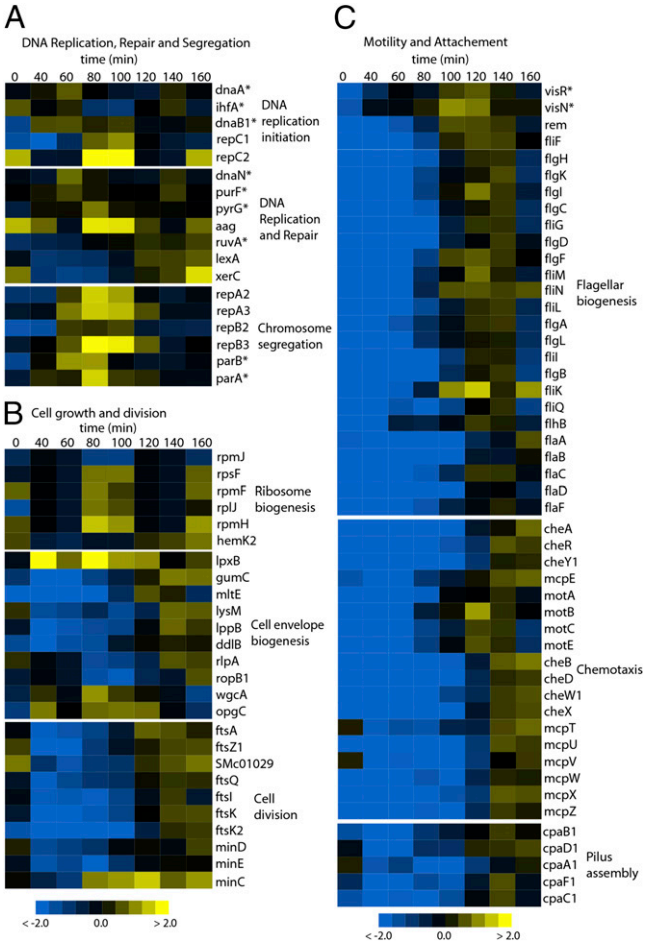


**Fig. 2.** Cell cycle gene expression of genes involved in various cellular processes. (*A*) Expression of genes involved in DNA processes (replication, repair, and segregation) in *S. meliloti*. Genes not included in our list of cell cycle-regulated transcripts are denoted by an asterisk (*). The color scale bar corresponding to log-fold expression is included at the bottom of the heatmaps. Values represent raw log fold change values. (*B*) Cell cycle expression patterns for genes involved in cell growth (ribosome and cell envelope) and division. (*C*) Cell cycle gene expression patterns of genes involved in motility and attachment including flagellar biosynthesis genes (and regulators), chemotaxis machinery, and genes required for pili biogenesis.

intriguing question of how the expression of these genes during the cell cycle is regulated.

**Transcription of Important Cell Cycle Regulatory Genes Occurs Late in the Cell Cycle.** Homologs of the many well-characterized genes involved in the *C. crescentus* cell cycle regulatory network have cell cycle-regulated transcripts in *S. meliloti* (Fig. 3). These conserved regulatory genes fell into three clusters: clusters 4 (*pleC* and *podJ1*), cluster 5 (*ctrA*, *sciP*, *divK*, *divJ*, *ccrM*, and *chpT*), and cluster 6 (*cpdR1*) (Fig. 1*B*). As indicated by the clustering and heatmap in Fig. 3, many of these genes are repressed until mid-S phase, which is consistent with their defined roles in establishing morphological and replicative asymmetry in the *C. crescentus* predivisional cell (17). The *S. meliloti* homologs of genes required for the cell type-specific phosphorylation of CtrA in *C. crescentus*, *pleC*, *divJ*, and *divK*, demonstrated robust cell cycle-regulated transcription (40). Transcription of *pleC* was activated at 120 min, whereas transcription of *divJ* and *divK* was activated at 140 min (Fig. 3). This pattern of transcription combined with recently published biochemical and genetic analyses, help to solidify the role of DivK, DivJ, and PleC in regulating CtrA activity in the *S. meliloti* predivisional cell (25, 40).

In addition, the gene encoding SciP is first up-regulated during G1 arrest and then again up-regulated in predivisional cells, which is consistent with its function in *C. crescentus* (Fig. 3). In *C. crescentus,* SciP accumulates specifically in swarmer cells to inhibit the activity of CtrA as a transcriptional activator, but not its ability to bind to the origin and repress the initiation of DNA replication (41). Surprisingly, the expression of *ctrA* during the *S. meliloti* cell cycle was less pronounced and occurred later than its counterpart in the *C. crescentus* cell cycle (Fig. 3 and Fig. S1). It is also interesting that the expression of the sensor histidine kinase CbrA is high in G1 cells, but is not significantly cell cycle regulated. Loss of CbrA activity in *S. meliloti* has been shown to reduce the polar localization of the response regulator DivK and to be detrimental to symbiosis with *Medicago sativa* (14, 16). Thus, a function for CbrA may be to mediate DivK activity in G1 cells and/or during the starvation response, which may be important during symbiosis.

**A Small Core of Cell Cycle-Regulated Transcripts Is Conserved Between the Mostly Divergent *S. meliloti* and *C. crescentus* Cell Cycle Transcriptional Regulons.** To understand the conservation of transcriptional regulation in cell cycle progression in α-proteobacteria, bidirectional BLAST and clusters of orthologous groups (COG) analysis was used to compare the *S. meliloti* set of 462 genes with cell cycle-regulated gene expression with the set of 553 genes previously identified in *C. crescentus* (18). Because different methods were used to synchronize the two bacteria, genes with cell cycle-regulated transcripts conserved between the two species not only represent a core set of α-proteobacterial cell cycle-regulated genes, but also represent genes demonstrating cell cycle-regulated transcription independent of synchronization method. Our analysis revealed 126 genes with cell cycle-regulated transcripts that are conserved between *S. meliloti* and *C. crescentus* (Fig. 4 and Dataset S2), which included cell division genes (*ftsZ*, *ftsI*, *ftsA*, and *ftsQ*), cell cycle regulators (*ctrA*, *pleC*, *divK*, *divJ*, *cpdR*, and *chpT*), and many flagellar and chemotaxis genes (Fig. 4). Genes encoding phosphate and phosphonate transporters (*pho*/*phn*), chaperones (*dnaJ* and *groELS*), and cell envelope proteins (*lppB*, *sleB*, and *pss*) were also transcriptionally regulated as a function of the cell cycle in

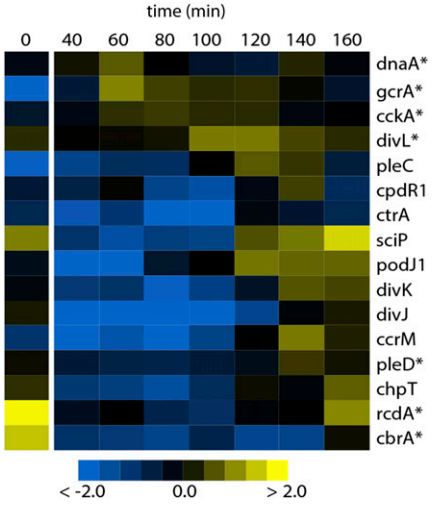


**Fig. 3.** Expression profiles of conserved cell cycle regulators. Values depicted represent raw log fold change values. The color scale bar corresponds to log-fold expression change in comparison with unsynchronized culture. Genes not included in our list of cell cycle-regulated transcripts are denoted by an asterisk (*).

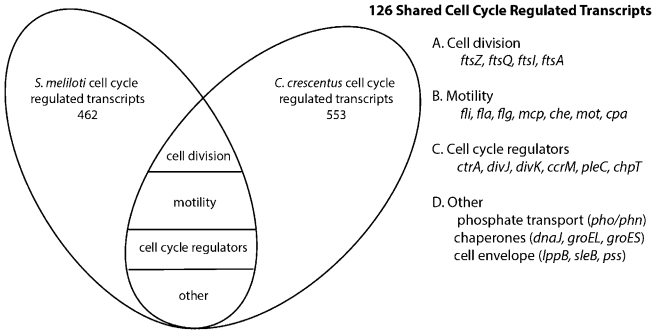


**Fig. 4.** Comparison of genes demonstrating cell cycle-regulated transcription in *S. meliloti* and *C. crescentus*. Diagram of genes conserved between the *S. meliloti* and *C. crescentus* cell cycle-regulated datasets (18). One hundred twenty-six cell cycle-regulated genes were conserved between the two species and fell into the functional groups as illustrated in the Venn diagram.

both species. The processes controlled by these conserved genes are fundamental to the proliferation and architecture of α-proteobacteria and thus likely represent a core set of genes with cell cycle-regulated transcription conserved in many α-proteobacteria, despite their differing specific ecological niches.

Intriguingly, the above analysis revealed that many of the 336 cell cycle-regulated genes specific to *S. meliloti* are crucial for its distinctive lifestyle. For example, megaplasmid replication and segregation genes (*repABC*) and a putative cyclic β-1,2 glucan ABC transporter (*ndvA*) do not have homologs in *C. crescentus* and are required for symbiosis (42). Also, several cell envelope genes (*lpxB*, *olsB*, *opgC*, *ddl*, and *rlpA*) and the genes coding for the *Min* system (*minCDE*) had cell cycle-regulated transcripts in *S. meliloti*, but either did not have homologs in *C. crescentus* or did not demonstrate cell cycle-regulated transcription in *C. crescentus,* demonstrating that the cell cycle regulon is highly specialized in each species.

**Binding Site Analysis Reveals Conserved CtrA- and DnaA-Binding Sites Among Genes with Cell Cycle-Regulated Transcripts.** In *C. crescentus,* CtrA directly controls the transcription of 95 genes in 55 operons primarily involved in late cell cycle events and in establishing asymmetry, whereas DnaA controls the transcription of 40 genes primarily involved in early cell cycle events, including replication and early cell polarity (20, 21). To determine which of the genes with cell cycle-regulated expression in *S. meliloti* may be directly controlled by CtrA and DnaA, we searched for CtrA- and DnaA-binding motifs in the upstream regulatory regions of the 462 *S. meliloti* cell cycle-regulated genes using previously described position weight matrices describing the 16 nucleotide CtrA and 9



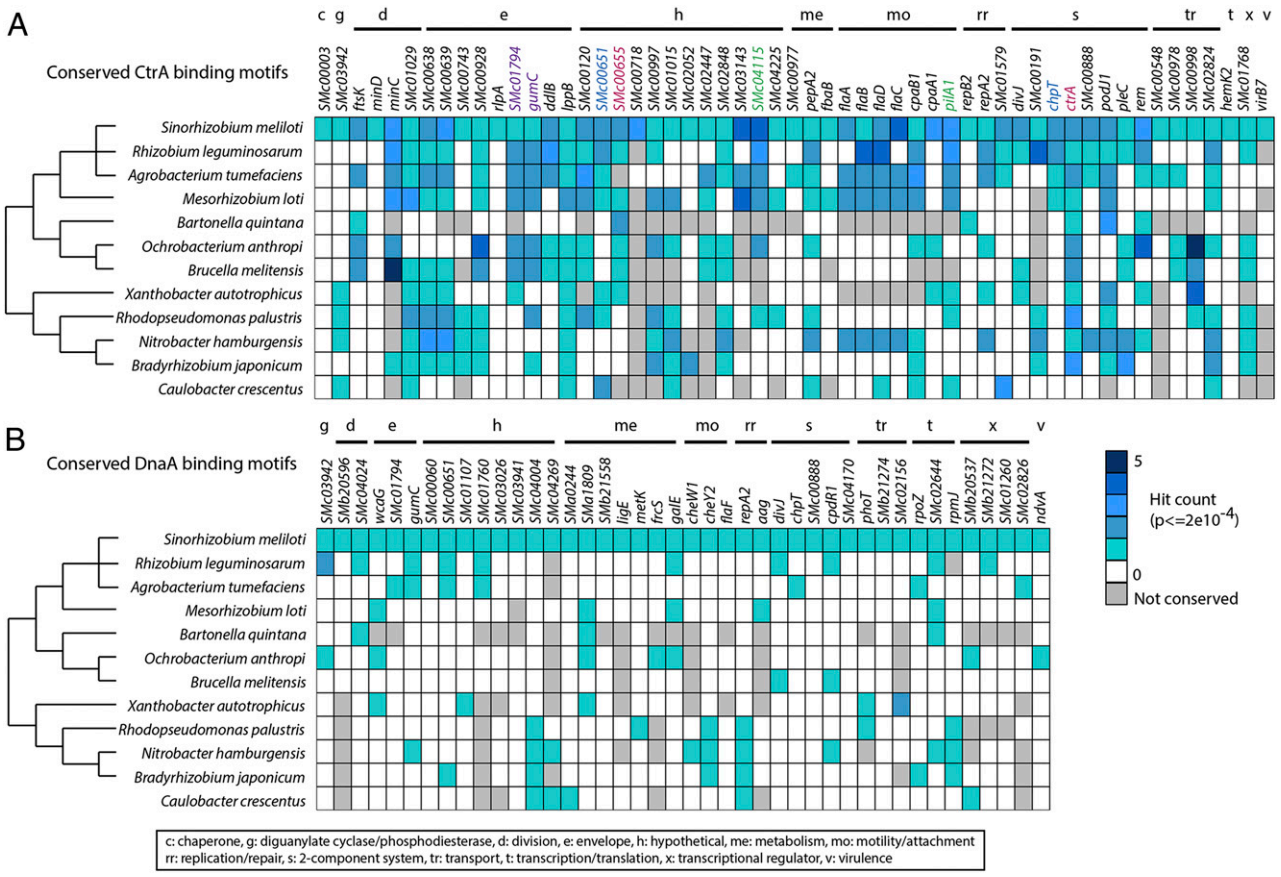


**Fig. 5.** Conservation of putative CtrA- and DnaA-binding sites in cell cycle-regulated genes. The upstream regulatory regions of genes with cell cycle-regulated transcripts were scanned for CtrA-and DnaA-binding motifs. "Hit count" signifies the number of significantly conserved full-length binding motifs that occur within the examined DNA segment. Homologs of genes containing hits from 11 related α-proteobacteria were also scanned for conserved CtrA and DnaA motifs. The tree to the left of the species' names represents their evolutionary relationship as described in ref. 22. (*A*) *S. meliloti* cell cycle-regulated genes with the highest-scoring CtrA-binding motifs are displayed and the number of hits is denoted by different shades of blue as described in the legend. The presence of CtrA-binding sites in the homologous genes of related species is denoted in the same manner. Color-coded genes (i.e., *pilA* and *SMc04115*) share promoter regions. (*B*) *S. meliloti* cell cycle-regulated genes with the highest-scoring DnaA-binding motifs. The number of binding motifs and conservation of these motifs in other α-proteobacteria are denoted in the same manner as in *A*. A key explaining the putative function of the various genes displayed is provided at the bottom of the figure.

nucleotide DnaA-binding motifs in α-proteobacteria (22). This analysis revealed 64 genes with predicted CtrA-binding sites and 47 with predicted DnaA-binding sites (Fig. 5 *A* and *B* and Dataset S3 *A* and *B*). Several CtrA-binding motifs previously identified in *S. meliloti* were found, including motifs in the promoter regions of *minC*, *chpT*, *pleC*, *ftsK*, *flaC*, *flaA*, *flaD*, *SMc00651*, and *podJ1* (22, 43). Surprisingly, there was very little overlap between previously defined *C. crescentus* CtrA and DnaA direct regulons and the corresponding predicted CtrA and DnaA cell cycle direct regulons of *S. meliloti*. Only eight genes were shared in the CtrA regulons and only four genes were shared in the DnaA regulons of the two species (20, 21).

Because the predicted *S. meliloti* CtrA and DnaA cell cycle regulons were so strikingly diverged from the corresponding *C. crescentus* regulons, we decided to test the conservation of these regulons in a sampling of α-proteobacterial species more closely related to *S. meliloti*. Homologs of the *S. meliloti* cell cycle-regulated genes with strong CtrA- and DnaA-binding motifs were found in 11 such α-proteobacterial species and then scanned for conserved CtrA- and DnaA-binding motifs. The results for a selection of genes with the highest-scoring motifs are displayed in Fig. 5 (Dataset S3 *A* and *B*). Although the CtrA-binding motifs in *S. meliloti* genes with cell cycle-regulated transcripts identified by this analysis were not well conserved in *C. crescentus,* they were much more strongly conserved in α-proteobacterial species more closely related to *S. meliloti* (Fig. 5*A*) (20). The conservation of the CtrA regulon in these α-proteobacterial species strongly implies that the regulation of these genes by CtrA during the cell cycle is critical in these species. For example, in all organisms in which the septum formation regulator *minC* is conserved, the CtrA-binding motifs in its promoter region are also conserved (Fig. 5*A*). Therefore, not only is the transcription of *minC* cell cycle regulated in *S. meliloti*, but the role of CtrA in controlling the timing of *minC* expression is highly conserved and likely crucial in α-proteobacteria with the Min system as was previously suggested in ref. 23. This analysis also identified many genes of unknown function as predicted members of the CtrA regulon, such as SMc04115, which is an uncharacterized gene with homology to the N terminus of pili genes. SMc04115 shares a promoter region with pilin gene *pilA,* which also harbors CtrA-binding motifs. In addition, SMc04115 has a very strong co-occurrence in the genome with *pilA1* and the pilus assembly genes of the *cpa* operon in α-proteobacteria suggesting a conserved function in pilus assembly.

A primary role of CtrA in *C. crescentus* is to establish morphological asymmetry in the predivisional cell, which CtrA accomplishes by directly regulating the expression genes involved in flagellar biogenesis and chemotaxis. Interestingly, the binding sites found in the regulatory regions of the motility genes of *C. crescentus* are not conserved in *S. meliloti.* Instead, there are conserved CtrA-binding motifs preceding the gene encoding the motility regulator Rem. This suggests that cell cycle regulation of flagellar and chemotaxis genes in *S. meliloti* may be achieved indirectly through regulation of Rem by CtrA, instead of through direct regulation of the flagellar and chemotaxis genes by CtrA. Rem has already been described as an important node for the regulation of *S. meliloti* flagellar gene expression in response to several environmental cues (44). Furthermore, the CtrA-binding motifs upstream of *rem* are conserved in many α-proteobacteria surveyed in our analysis, including the two organisms most closely related to *S. meliloti, Rhizobium leguminosarum* and *Agrobacterium tumefaciens* (Fig. 5*A*). Thus, in certain α-proteobacteria, including *S. meliloti,* CtrA likely regulates the expression of flagella and chemotaxis genes indirectly during the cell cycle through the intermediate regulator Rem.

Another interesting deviation from the *C. crescentus* cell cycle regulatory network is the absence of CtrA-binding motifs upstream of the gene encoding the essential cell cycle-regulated methylase, CcrM (12, 22). In *C. crescentus,* CcrM methylates newly replicated DNA at the adenine of GANTC site and the activation of *ccrM* transcription by CtrA at the end of S phase results in the remethylation of the chromosome (45). Our data indicate that the expression of *ccrM* in *S. meliloti* is indeed cell cycle regulated (Fig. 3), but conserved CtrA-binding motifs are absent from the upstream regulatory region of *ccrM* (Fig. 5*A*). Previous analysis identified GANTC sites upstream of *ccrM*, suggesting a method of autoregulation, but it is still unclear how transcription of *ccrM* is up-regulated after completion of S phase in *S. meliloti* (12).

The DnaA-binding motifs identified in *S. meliloti* genes with cell cycle-regulated transcripts were much less conserved in the α-proteobacterial species we surveyed (Fig. 5*B*). This suggests that the function of DnaA as a transcriptional regulator in α-proteobacteria might be much less constrained by evolution than that of CtrA. For example, the CtrA-binding motif upstream of the polarity factor *podJ1* is conserved among α-proteobacterial species, but the DnaA-binding motif upstream of *C. crescentus podJ* is not (Fig. 5 *A* and *B*) (21). A previously verified DnaA-binding motif upstream of the *repABC* operon of pSymA was identified by this analysis, as well as a conserved CtrA-binding motif 90 bp downstream of the DnaA site (11). The presence of these binding motifs suggests a possible interplay between CtrA and DnaA in the regulation of expression of this *repABC* operon on pSymA during the cell cycle. It is surprising that neither DnaA nor CtrA motifs were found in the regulatory regions of *repC1* or *repAB3* on pSymB because the expression of these genes is also cell cycle regulated. How the expression of these genes is coordinated with the *S. meliloti* cell cycle remains unclear.

## Discussion

This study describes the first published method for the robust synchronization of *S. meliloti* growth as well as detailed microarray analysis of global gene expression during the *S. meliloti* cell cycle, the only published analysis for an α-proteobacterium with multiple replicons. The microarray analysis revealed that the expression of 462 genes is modulated as a function of the cell cycle. Comparing this set of *S. meliloti* genes with the list of 553 previously identified *C. crescentus* genes with cell cycle-regulated transcripts (18), a core set of 126 genes exhibiting conserved cell cycle-dependent transcription in both bacteria was discovered. Despite this conservation, 72% of the *S. meliloti* cell cycle-regulated genes identified in our analysis either did not have counterparts or were not cell cycle-regulated in *C. crescentus* and there was very little overlap between the predicted CtrA and DnaA cell cycle-dependent regulons between the two species. These observations suggest that, despite the conservation of core elements of the cell cycle regulatory machinery, the cell cycle transcriptional regulons of α-proteobacteria are as diverse as their lifestyles and the environmental niches they occupy (22, 23).

**Cell Cycle-Regulated Transcription in α-Proteobacteria.** Temporal regulation of transcription during the cell cycle was at one time thought to be a purely eukaryotic phenomenon (46). However, groundbreaking work by Laub et al. (18) demonstrated that temporally regulated transcription is also used by the α-proteobacterium *C. crescentus* to control cell cycle progression. Due to the conservation of the genes encoding the cell cycle regulatory circuit in a large percentage of α-proteobacteria, it was postulated that transcriptional control of cell cycle progression was conserved throughout the α-proteobacterial group (22, 23). However, the degree to which this paradigm is globally conserved had remained untested. Therefore, our discovery that cell cycle-regulated transcription is conserved in *S. meliloti,* which lacks the morphological asymmetry of *C. crescentus* and also thrives in an ecological niche completely different than *C. crescentus,* is highly significant. The conservation of cell cycle-regulated transcription in *S. meliloti* also strongly suggests that the function of temporally regulated gene expression in α-proteobacteria is not simply to produce dimorphism.

Genes with cell cycle-regulated transcripts conserved between *C. crescentus* and *S. meliloti* include many of the components of the complex core regulatory circuit that governs cell cycle progression in *C. crescentus.* The transcription of these factors (which include *ctrA, pleC, divK, divJ, cpdR, chpT*, and *sciP*) was mostly upregulated coincident with the timing of their prescribed function in *C. crescentus.* This observation, along with previous molecular studies, strongly suggests that the basic functions of these cell cycle proteins are well conserved between *S. meliloti* and *C. crescentus* (16, 24, 25, 40). Interestingly, many of the *S. meliloti* genes with cell cycle-regulated transcripts that are either not conserved or not cell cycle regulated in *C. crescentus,* are required for the symbiotic relationship between *S. meliloti* and its legume host—including the *repABC* genes controlling megaplasmid replication and maintenance, as well as genes involved in cyclic glucan production.

**CtrA as a Modulator of Asymmetry and Cellular Differentiation in α-Proteobacteria.** Because CtrA is such an important regulator of morphological and replicative asymmetry in *C. crescentus,* it is remarkable that CtrA-binding sites found in *C. crescentus* are only conserved in eight genes with cell cycle-regulated transcripts in *S. meliloti.* A striking illustration of this divergence is the difference in cell cycle regulatory schemes for the flagella and chemotaxis genes in the two organisms. In *C. crescentus,* CtrA directly regulates genes in all four tiers of the flagellar regulatory hierarchy including *flg, fli, flm* and flagellin genes (20). In *S. meliloti,* however, CtrA-binding motifs are only present in the promoters of the motility regulator *rem* and of the flagellin genes (*flaABCD*). It has been shown that *S. meliloti* is able to up- or down-regulate production of its flagella and chemotaxis machinery in response to different environmental cues through the intermediate regulator Rem (39, 44, 47). Thus, *S. meliloti* CtrA likely exerts a major influence on the transcription of these genes during the cell cycle by directly regulating *rem* transcription. This regulatory strategy could allow for environmental cues to be integrated with or even override the cell cycle-coordinated production of flagella and chemotaxis machinery, which might be crucial not only for *S. meliloti* to adapt to life in the soil, but also life within the plant during symbiosis. It is well known that flagellar proteins can activate innate immunity in eukaryotic hosts, so this indirect regulatory adaption may complement the rhizobial strategy of reducing flagella recognition by using Nod factors to suppress the plant innate immune system (48).

Interestingly, our analysis also revealed a high degree of conservation of the predicted *S. meliloti*-direct CtrA regulon in α-proteobacteria whose lifestyles are more similar to *S. meliloti* than to *C. crescentus.* One example is the Min system for regulation of septation, which is present in *S. meliloti* and a number of other α-proteobacteria, but not *C. crescentus.* CtrA-binding motifs are present upstream of the *minCDE* operon in every α-proteobacterium surveyed in this analysis that contains the operon. The conservation of these motifs lends support to the theory first presented in Hallez et al. (23) that CtrA mediates cell division through direct regulation of *minC* transcription in α-proteobacteria containing the Min system and through direct regulation of *ftsZ* in *C. crescentus* and other α-proteobacteria that lack the Min system. It is intriguing that a single regulatory factor can retain its role in a process so highly conserved as cell division but simultaneously demonstrates the flexibility to modulate diverse cellular differentiation events central to the lifestyles of α-proteobacteria, which can range from cyst cell formation in *Rhodospirillum centenum* to intracellular pathogenesis in *Brucella* (49, 50).

Further research will be required to gain a more complete understanding of the wiring of the cell cycle regulatory circuit in *S. meliloti* and how this regulatory circuit is modified by NCR peptides and perhaps other plant factors during symbiosis to achieve the specific cellular differentiation required during bacteroid development. In this issue of PNAS, Penterman et al. (9) found that a specific NCR peptide (NCR247) can alter *S. meliloti* cell cycle progression to cause a cell division block by at least in part manipulating the CtrA regulatory circuit. It will be important to continue to probe the role of the *S. meliloti* cell cycle regulation during symbiosis, including carrying out in vivo experiments to verify the predicted CtrA and DnaA regulons, as well as further elucidating the molecular mechanisms by which NCR247 and other NCR peptides modulate the *S. meliloti* cell cycle and prepare the organism for its important symbiotic role.

## Methods

**Growth Conditions and Cell Synchronization.** *S. meliloti* strain Sm1021 was cultured at 30 °C in LBMC media [LB with 2.5 mM magnesium sulfate ($MgSO_4$) and 2.5 mM $CaCl_2$] or Mops-GS media lacking mannitol (50 mM Mops, 1 mM $MgSO_4$, 0.25 mM $CaCl_2$, 19 mM glutamic acid, and 0.004 mM biotin, pH 7.4) on a platform shaker. For synchronization, 1 L of LBMC was inoculated with 100 μL of saturated Sm1021 liquid culture and grown to $OD_{600}$ = 0.10–0.15. Cells were then collected by centrifugation at 6,500 rpm (Sorvall Slc-4000) for 5 min, washed twice with 0.85% saline, and resuspended in modified Mops-GS. Cells arrested in G1 after a 270-min incubation in Mops-GS. Cells arrested in G1 were then collected by centrifugation and cultured in LBMC.

**Flow Cytometry.** To quantify DNA content per cell in *S. meliloti* cultures, 200 μL culture was fixed in 933 μL of 100% ethanol. Cells were collected by centrifugation and incubated for 2 h in 1 mL 50 mM sodium citrate with 3.3 μg/mL RNase A at 50° C to eliminate cellular RNA. To label DNA, 1 μL 8.3-μM SYTOX Green dye was added to each sample. Samples were analyzed with a BD FACScan flow cytometer (BD Biosciences) and data were analyzed with FlowJo 9.6.3 software (Tree star).

**RNA Isolation and Gene Expression Profiling.** RNA was obtained from 10 mL time points taken from four separate synchronized cultures and from a control ($OD_{600}$ = 0.15) unsynchronized cultures by first treating with Qiagen RNAprotect bacterial reagent to stabilize the RNA and then using the Qiagen RNeasy plus kit for RNA isolation. A total of 600 ng of RNA was used for cDNA synthesis (Ambion MessageAmp II-Bacterial kit) and cDNA labeling with Cy3 and Cy5 fluorophores was carried out using the Ambion Amino Allyl MessageAmp II cRNA Amplification kit. Labeled cDNA was hybridized to a custom Agilent gene expression array (Smexpr1:00 AMADID: 036667) of optimized 60-mer probes to 6,046 *S. meliloti* ORFs (51). The array data were processed using the *limma* package in R. Median red/green hybridization signals were background corrected and the red/green hybridization signal log-ratios, $M = \log_2(R/G)$, were normalized for each array using locally weighted scatterplot smoothing. Normalization between arrays was also performed to increase consistency. The fourth biological replicate of time point 140 was removed from this analysis due to poor correlation with the other replicates.

**Selection and Clustering of Genes with Cell Cycle-Regulated Transcription.** The replicate log ratio values for time points 40–160 were averaged and used to calculate the SD for each gene. The SDs were compared with the distribution of SDs generated from 10,000 randomly permuted gene expression profiles and genes with empirical *P* values <0.05 were denoted as cell cycle regulated and considered for clustering. Fuzzy *c*-means clustering was applied using R software package e1071 (http://cran.r-project.org/web/packages/e1071/index.html). The gene expression profiles were mean centered normalized and the fuzzy parameter was estimated as described previously (32). The optimal cluster number (six) was determined using methods described in ref. 52 and a bagged clustering wrapper, bclust (http://cran.r-project.org/web/packages/bclust/index.html), was used to reduce the variability in the clustering results.

***S. meliloti* and *C. crescentus* Cell Cycle Gene Comparisons.** The amino acid sequences for 6,201 Sm1021 genes were acquired and blasted against 3,737 CB15 protein sequences. The BLAST results were used to determine which of the 553 *C. crescentus* cell cycle-regulated genes identified in ref. 18 were similar to the 462 identified in *S. meliloti*. The BLAST analysis was complemented with COGs (53). The results were filtered using E-values $<1 \times 10^{-20}$, as well as manually filtered, to match genes and remove replicates.

**CtrA- and DnaA-Binding Motif Discovery.** CtrA and DnaA motifs (22) were used in find individual motif occurrences (FIMO) (54) with the 400 bp upstream and 100 bp downstream of the *S. meliloti* cell cycle gene promoters. A *P* value cutoff of $2 \times 10^{-4}$ was used to establish the

*S. meliloti* genes containing the motifs. The protein sequences of these genes were reciprocal blasted against the proteins of 11 closely related species to identify homologous genes and those with protein BLAST E-values <0.05 were considered for FIMO analysis.

**Quantitative PCR Analysis.** Primers were designed to amplify a 100-bp region of the *ctrA* gene (SMc00654) and control gene SMc00128, which demonstrated stable expression across the cell cycle (55). To quantify cell cycle gene expression, RNA was isolated from four representative time points in synchronized *S. meliloti* cultures ($t = 40$, 80, 120, and 160 min). RNA was isolated by the same method as it was for microarray analysis. cDNA was generated using the BioRad iScript cDNA Synthesis kit, and qPCR analysis was carried out on a Roche LightCycler 480 using Roche LightCycler 480 SYBR Green I Master Mix. The $\log_{10}$ expression ratios were calculated for each time point with respect to the expression of the SMc00128, such that log expression ratio = log[ctrA] − log[SMc00128].

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