Microbiology (2013), **159**, 1552–1563 **DOI** 10.1099/mic.0.067504-0

The *Sinorhizobium meliloti* sensor histidine kinase CbrA contributes to free-living cell cycle regulation

Craig S. Sadowski,[1] Daniel Wilson,[1]† Karla B. Schallies,[1] Graham Walker[2] and Katherine E. Gibson[1]

Correspondence
Katherine E. Gibson
katherine.gibson@umb.edu

[1]Department of Biology, 100 Morrissey Boulevard, University of Massachusetts Boston, Boston, MA 02125, USA

[2]Department of Biology, 31 Ames Street, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

Sinorhizobium meliloti is alternately capable of colonizing the soil as a free-living bacterium or establishing a chronic intracellular infection with its legume host for the purpose of nitrogen fixation. We previously identified the *S. meliloti* two-component sensor histidine kinase CbrA as playing an important role in regulating exopolysaccharide production, flagellar motility and symbiosis. Phylogenetic analysis of CbrA has highlighted its evolutionary relatedness to the *Caulobacter crescentus* sensor histidine kinases PleC and DivJ, which are involved in CtrA-dependent cell cycle regulation through the shared response regulator DivK. We therefore became interested in testing whether CbrA plays a role in regulating *S. meliloti* cell cycle processes. We find the loss of *cbrA* results in filamentous cell growth accompanied by cells that contain an aberrant genome complement, indicating CbrA plays a role in regulating cell division and possibly DNA segregation. *S. meliloti* DivK localizes to the old cell pole during distinct phases of the cell cycle in a phosphorylation-dependent manner. Loss of *cbrA* results in a significantly decreased rate of DivK polar localization when compared with the wild-type, suggesting CbrA helps regulate cell cycle processes by modulating DivK phosphorylation status as a kinase. Consistent with a presumptive decrease in DivK phosphorylation and activity, we also find the steady-state level of CtrA increased in *cbrA* mutants. Our data therefore demonstrate that CbrA contributes to free-living cell cycle regulation, which in light of its requirement for symbiosis, points to the potential importance of cell cycle regulation for establishing an effective host interaction.

Received 5 March 2013
Accepted 24 May 2013

INTRODUCTION

The cell cycle is a fundamental process required for growth, reproduction and developmental differentiation in all organisms. Not only is it important to understand how bacterial cells reproducibly carry out an orderly progression of complex cell cycle events, but also how the canonical cell cycle of an invasive bacterium can be customized to promote host colonization. *Sinorhizobium meliloti* is alternately capable of colonizing the soil rhizosphere as a free-living bacterium and invading the roots of leguminous plants as a symbiont to establish a chronic intracellular infection that results in nitrogen fixation (Gibson *et al.*, 2008). Among *Alphaproteobacteria*, *S. meliloti* has emerged as a model organism in which to identify and characterize diverse requirements for host infection (Domenech *et al.*, 2009; Roop *et al.*, 2002; Ugalde, 1999). This model bacterium also represents a critical link between cell cycle regulation and host colonization as it commences a novel cell cycle programme once it has invaded the tissues of its eukaryotic host (Mergaert *et al.*, 2006).

As observed in the alphaproteobacterium *Caulobacter crescentus* (Curtis & Brun, 2010; Tsokos & Laub, 2012), free-living *S. meliloti* tightly coordinates DNA replication with cell division to effect a once-and-only-once replication of its genome per cell division (Mergaert *et al.*, 2006). During host colonization, there are several novel adaptations to this cell cycle that impact both DNA replication and cell division. After invading its host, *S. meliloti* is taken up into the host cell cytoplasm where it differentiates into specialized cells, called bacteroids, which are then capable of performing nitrogen fixation. The bacteroid differentiation programme includes repeated rounds of DNA replication in the absence of cell division, termed

†Present address: Department of Biology, Regents Hall of Natural Science and Mathematics, 1520 St. Olaf Avenue, Northfield, MN 55057, USA.

Abbreviations: DIC, differential interference contrast; DOC, deoxycholate; GFP, green fluorescence protein; HK, histidine kinase.

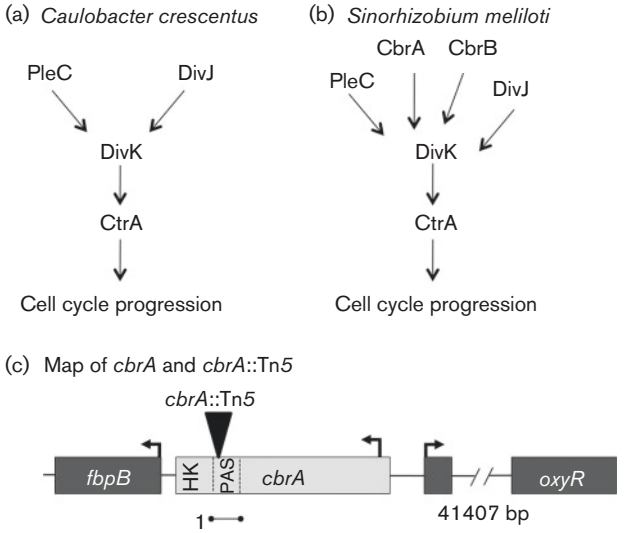
Two supplementary tables are available with the online version of this paper.

endoreduplication, and is associated with enlarged and filamentous cells (Mergaert *et al.*, 2006). Once endoreduplication is completed, *S. meliloti* bacteroids permanently exit the cell cycle and become terminally differentiated cells capable of nitrogen fixation but no longer capable of reproduction (Mergaert *et al.*, 2006). These modifications to the bacterial cell cycle are elicited by host production of nodule-specific cysteine-rich peptides that are taken up into the bacterial cytoplasm (Van de Velde *et al.*, 2010; Haag *et al.*, 2011), although their molecular mechanism of action has yet to be determined. Thus, the cell cycle of *S. meliloti* allows this bacterium to effect an orderly progression of cell cycle events in a manner that is sensitive to host colonization and is capable of producing differentiated cell types.

While the molecular mechanisms that govern cell cycle progression in *S. meliloti* have just begun to be explored (Barnett *et al.*, 2001; Fields *et al.*, 2012; Kobayashi *et al.*, 2009; Lam *et al.*, 2003), its cell cycle shares several features with *C. crescentus*, which serves as an intensively studied model organism for understanding the molecular events that underlie cell cycle coordination. *C. crescentus* undergoes an asymmetrical cell division that produces two daughter cells with distinct fates: a small swarmer cell is born into G_1 phase and is motile while a large stalked cell is born into S phase and is sessile. A complex two-component signal transduction pathway contributes to the regulation of *C. crescentus* cell cycle events and the generation of this asymmetry upon cell division (Curtis & Brun, 2010; Jenal, 2009; McAdams & Shapiro, 2009; Skerker & Laub, 2004; Tsokos & Laub, 2012).

Within this pathway, the sensor histidine kinases (HKs) DivJ and PleC modulate the phosphorylation status of a shared response regulator, DivK, with DivJ functioning as a kinase and PleC functioning as a phosphatase (Fig. 1a) (Hecht *et al.*, 1995; Lam *et al.*, 2003). DivK localizes to the old cell pole when phosphorylated by DivJ (Jacobs *et al.*, 2001; Lam *et al.*, 2003; Matroule *et al.*, 2004), and this drives the transition from G_1 to S phase. Phosphorylated DivK regulates the activity of the response regulator CtrA by repressing both its phosphorylation and its stability (Biondi *et al.*, 2006; Domian *et al.*, 1997; Hung & Shapiro, 2002; Jacobs *et al.*, 1999), which DivK does indirectly through a series of phosphorelay events (Biondi *et al.*, 2006; Chen *et al.*, 2009; Jacobs *et al.*, 2003; Tsokos *et al.*, 2011). CtrA is an essential DNA-binding protein (Quon *et al.*, 1996; Wu *et al.*, 1998) that functions directly to repress DNA replication initiation by binding the origin and blocking DnaA access in S phase cells (Bastedo & Marczynski, 2009; Jonas *et al.*, 2011; Quon *et al.*, 1998), and to promote cell division through the activation of genes such as *ftsZ* in predivisional S/G_2 cells (Laub *et al.*, 2002; Sackett *et al.*, 1998; Wortinger *et al.*, 2000).

As *C. crescentus* cells divide, they acquire an asymmetrical distribution of active DivK due to the localization of DivJ and PleC at opposite cell poles (Jacobs *et al.*, 2001; Matroule *et al.*, 2004; Wheeler & Shapiro, 1999). The DivJ kinase and phosphorylated DivK are localized to the old pole in S phase stalked cells, while the PleC phosphatase is



(e) PCR confirmation of Δ*cbrA*::*cat*

PCR reaction	5′ primer	3′ primer
1	W743FBm	T880RXh
2	cbrA up	cat 2
3	cat 1	cbrA dn
4	Smc00777	cat 2

Fig. 1. Model of CbrA cell cycle function and construction of the Δ*cbrA*::*cat* null mutant. (a) *C. crescentus* HKs DivJ and PleC regulate DivK phosphorylation status. Once activated by phosphorylation, DivK represses phosphorylation and stability of CtrA indirectly through a series of phosphorelay events. Loss of CtrA is required for G_1 cells to enter S phase and subsequent reactivation of CtrA in predivisional cells is required for cell division. (b) A model of the predicted *S. meliloti* DivK-dependent signal transduction pathway that includes CbrA. (c) The chromosomal region surrounding *cbrA* with relevant genes highlighted; the unlabelled gene upstream of *cbrA* is annotated as Smc00777. The *cbrA* gene encodes a large N terminus of unknown function, a PAS domain and a C-terminal HK domain. The *cbrA*::Tn*5* insertion site is indicated for reference. Bar 1 represents an expected PCR product from amplification of the PAS domain and confirms the presence of *cbrA* (PCR 1 in e). (d) The chromosomal region surrounding Δ*cbrA*::*cat* with the same genes highlighted. Each bar represents an expected PCR product that confirms the presence of Δ*cbrA*::*cat* in the correct region of the chromosome (PCRs 2–4 in e). (e) The primer sets used to carry out confirmatory PCRs.

localized to the old cell pole in G_1 phase swarmer cells so that unphosphorylated DivK is distributed throughout the cytoplasm. Upon cell division, this asymmetrical distribution of phosphorylated DivK creates an asymmetry in CtrA activity and contributes to the production of daughter cells with distinct fates (Chen *et al.*, 2011; Jonas *et al.*, 2011). DivK-dependent regulation of CtrA thereby helps regulate DNA replication initiation and cell division during cell cycle progression, and is a key regulatory component of daughter cell asymmetry.

The free-living *S. meliloti* cell cycle is similarly associated with an asymmetrical generation of two distinct daughter cells, which can be observed both morphologically and molecularly (Hallez *et al.*, 2004; Lam *et al.*, 2003). *S. meliloti* produces a small, presumably G_1 phase, and a large, presumably S phase, daughter cell during each cell cycle (Hallez *et al.*, 2004). As observed in *C. crescentus*, *S. meliloti* DivK is localized in a phosphorylation-dependent manner to the old cell pole in the large daughter cell, while in the small daughter cell DivK is distributed evenly throughout the cytoplasm (Lam *et al.*, 2003). The factors that regulate DivK phosphorylation have not been previously identified. However, the asymmetrical distribution of phosphorylated DivK, possibly through its impact on CtrA activity, may help generate distinct cell fates. Interestingly, the specific pattern of *S. meliloti* DivK localization during predivisional S/G_2 cell cycle progression differs from that of *C. crescentus* (Lam *et al.*, 2003), indicating an evolutionary divergence in the control of this key regulator of cell cycle events and asymmetrical cell fate.

There is phylogenetic conservation of several key regulatory components of the DivK signal transduction pathway amongst a variety of *Alphaproteobacteria* (Brilli *et al.*, 2010; Hallez *et al.*, 2004). For example, DivJ and PleC orthologues are present in species of *Sinorhizobium*, *Brucella*, *Mesorhizobium* and *Agrobacterium*. Interestingly, there are also several novel HKs predicted to regulate DivK activity in these host-associated bacteria, which might reflect a need to modify the free-living cell cycle for adaptation to the host niche. In particular, *S. meliloti* has four DivJ/PleC-like HKs that are predicted to regulate DivK (Fig. 1b): CbrA (SMc00776), CbrB (SMc04212), DivJ (SMc0059) and PleC (SMc2369) (Hallez *et al.*, 2004).

We previously identified CbrA as playing an important role in the regulation of exopolysaccharide production, flagellar motility and symbiosis (Gibson *et al.*, 2006, 2007). The *Agrobacterium tumefaciens* orthologue, PdhS1, may not be required for pathogenesis, but has been linked to regulation of motility and exopolysaccharide production with increased succinoglycan synthesis in mutants of both species (Gibson *et al.*, 2006; Kim *et al.*, 2013). CbrA is a 120 kDa protein with an N′-terminal domain of unknown function, at least one internal PAS domain, and a C′-terminal two-component sensor HK domain (Fig. 1c). In *Brucella abortus*, the N′-terminal domain is required for periodic old-pole localization of the CbrA orthologue, PdhS (Hallez *et al.*, 2007), and this may also hold true for CbrA. While *pdhS* is an essential gene, a temperature-sensitive loss-of-function allele results in a severe growth arrest at the restrictive temperature and a fivefold decrease in the frequency of DivK polar localization (Van der Henst *et al.*, 2012). The growth arrest due to *pdhS* loss of function is not associated with altered cell morphology (Van der Henst *et al.*, 2012), but overexpression of PdhS does produce a filamentous cell growth phenotype (Hallez *et al.*, 2007; Van der Henst *et al.*, 2012). PdhS overexpression also increases the frequency of DivK polar localization and decreases CtrA steady-state levels (Hallez *et al.*, 2007). Together, these observations suggest that PdhS functions as a kinase to phosphorylate DivK, which thereby has an impact on CtrA activity and cell cycle progression. Loss of function for the *A. tumefaciens cbrA* orthologue, *pdhS1*, also results in a filamentation phenotype (Kim *et al.* 2013), although its specific role in cell cycle regulation is not yet understood.

As with many bacteria, free-living *S. meliloti* exhibits a general morphological response to cell cycle perturbation that is observed as filamentous cell growth. In *S. meliloti*, as well as the closely related species *A. tumefaciens* and *B. abortus*, disruption of cell cycle processes presents itself morphologically as a branching filamentation phenotype (Bellefontaine *et al.*, 2002; Cheng *et al.*, 2007; Fields *et al.*, 2012; Hallez *et al.*, 2007; Kahng & Shapiro, 2001; Kobayashi *et al.*, 2009; Latch & Margolin, 1997; Robertson *et al.*, 2000; Van der Henst *et al.*, 2012; Wright *et al.*, 1997), in contrast to the classic linear filamentous growth of bacteria such as *Escherichia coli* and *C. crescentus*. This is due to localized budding growth of membrane materials at the new cell pole of daughter cells in rhizobial *Alphaproteobacteria* (Brown *et al.*, 2012). *S. meliloti* becomes filamentous when the process of DNA replication is inhibited by exposure to a DNA-damaging agent (mitomycin C) (Latch & Margolin, 1997), or when DNA replication initiation events are aberrantly increased by overexpression of either DnaA (Sibley *et al.*, 2006) or CcrM (Wright *et al.*, 1997). Cells also grow as filaments when cell division is blocked. Treatment with an inhibitor of septum formation (cephalexin) (Latch & Margolin, 1997), overexpression of the tubulin homologue FtsZ1 or FtsZ2 (Latch & Margolin, 1997), and over-expression of certain components of the MinCDE system (Cheng *et al.*, 2007) each induce filamentation. Thus, it is clear that *S. meliloti* grows as branching filaments in response to a variety of cell cycle perturbations that specifically affect either DNA replication, whether repressed or overstimulated, or cell division.

In this study, we address the question of whether CbrA plays a role in cell cycle regulation during free-living growth of *S. meliloti* as predicted by its homology to *B. abortus* PdhS, as well as *C. crescentus* PleC and DivJ. Consistent with this prediction, we observe a significant increase in branching filamentous cell growth upon loss of *cbrA*. Thus, cell division is perturbed in *cbrA* null mutants. Branching filamentation of the *cbrA* null mutant is

associated with a significantly increased subpopulation of cells that accumulate either a <1N or >2N complement of the genome, indicating that there may be an additional defect in DNA segregation. We find CbrA is required for efficient localization of DivK to the cell pole, which is consistent with the hypothesis that CbrA functions as a cognate HK of DivK, probably through kinase activity. Finally, we observe an increase in CtrA accumulation in *cbrA* null mutants, suggesting that loss of CtrA regulation in the *cbrA* mutants may be ultimately responsible for the cell cycle defects we observe. Combined, these observations suggest strongly that CbrA is an important contributor to cell cycle regulation in *S. meliloti* during free-living growth.

METHODS

Microbiological techniques. All bacterial strains and phage used in this work are described in Table S1 (available in *Microbiology* Online). *E. coli* was grown in Luria-Bertani (LB) at 37 °C, and *S. meliloti* was grown in LB supplemented with $MgSO_4$ and $CaCl_2$ at a final concentration of 2.5 mM each (LB/MC) at 30 °C unless described otherwise. Exponential phase cultures of *S. meliloti* were obtained by diluting an overnight culture to an OD_{600} of 0.1 and growing the cells to an OD_{600} of between 0.6 and 0.8. To assay relative succinoglycan production, *S. meliloti* was grown on LB/MC supplemented with 0.02 % calcofluor and 10 mM HEPES (pH 7.4). *S. meliloti* sensitivity to the detergent deoxycholate (DOC) was assessed at a concentration of 3 mM. When appropriate, growth medium was supplemented with the following antibiotics: chloramphenicol (20 μg ml^{-1}), gentamicin (50 μg ml^{-1}), kanamycin (*E. coli*: 50 μg ml^{-1}), neomycin (*S. meliloti*: 200 μg ml^{-1}), oxytetracyclin (0.75 μg ml^{-1}), rifampicin (50 μg ml^{-1}), tetracyclin (*S. meliloti*: 10 μg ml^{-1}; *E. coli*: 20 μg ml^{-1}) and streptomycin (500 μg ml^{-1}).

Genetic techniques and DNA manipulations. Strains were constructed through either triparental mating or ΦM12 transduction using standard methods (Finan *et al.*, 1984; Leigh *et al.*, 1985). The Δ*cbrA*::*cat* allele was PCR amplified from pLAFR2070Δ*cbrA* using cbrA up and cbrA dn primers (Table S1). The resulting PCR product was directly ligated into *Sma*I-digested pK18*mobsacB* (Schäfer *et al.*, 1994) to generate pK18DW001, and this suicide plasmid was conjugated into Rm1021. Following successful integration of pK18DW001 into the Rm1021 chromosome, the pK18*mobsacB* backbone was removed through *sacB*-mediated sucrose selection on LB/MC agar supplemented with 5 % sucrose (Bina & Mekalanos, 2001). Colonies were purified on LB/MC agar supplemented with 5 % sucrose, and then tested for the loss of pK18*mobsacB*-associated neomycin resistance. Neomycin-sensitive colonies were screened for chloramphenicol resistance, and the presence of the Δ*cbrA*::*cat* allele was confirmed by PCR using primers cbrAup, cbrAdn, cat1 and cat2 (Table S1, Fig. 1d, e). The absence of *cbrA*$^+$ was confirmed by lack of PCR amplification of its PAS domain using primers G743FBm and T880Rxh (Table S1, Fig. 1d, e). Strains that had undergone allelic replacement were chosen based on the presence of the Δ*cbrA*::*cat* allele and the absence of the *cbrA*$^+$ PAS domain. The correct location of Δ*cbrA*::*cat* on the chromosome was confirmed by PCR using the external primer Smc00777 in combination with cat2 (Table S1, Fig. 1d, e), and by co-transduction tests with the *cbrA*-linked marker *oxyR*::mTn*5* (Fig. 1c, d). pLAFR1 (empty vector) and pLAFR2070 (pLAFR1 *cbrA* complementation vector) were conjugated into the relevant strains through triparental matings for complementation assays.

Fluorescence flow cytometry. Fluorescence flow cytometric assays were used to assess the DNA content of exponentially growing cells as described by Kahng & Shapiro (2001). Triplicate cultures of all strains were diluted to an OD_{600} of 0.10 and fixed in a 1 : 5 dilution of fixative (12.5 % paraformaldehyde, 150 mM $NaPO_4$) for 15 min at room temperature. Cells were washed twice with LB to remove excess fixative, resuspended in 1.0 ml cold 70 % EtOH and incubated overnight in the dark at 4 °C. After approximately 12–18 h the EtOH was removed, cells were washed once in TMS buffer (10 mM Tris/HCl, pH 7.2, 1.5 mM $MgCl_2$, 150 mM NaCl), and then resuspended in 1.0 ml TMS supplemented with 0.5 μM SYTOX Green (Molecular Probes). Cells were incubated with SYTOX Green for at least 12 h at 4 °C in the dark. Stained cells were washed once with TMS and then resuspended in 1.0 ml TMS. Fifty thousand cells from each strain were acquired on a Becton Dickinson FACSCanto II flow cytometer and analysed using FlowJo 7.x software (Tree Star). Four categories of cells were grouped based on DNA content: (i) cells with less than a single copy of the genome (<1N), (ii) cells with a single genome copy (1N), (iii) cells that have two copies of the genome (2N) and (iv) cells that have more than two copies of the genome (>2N). The average per cent of cells located within each subpopulation was calculated from triplicate cultures using identical gates for each strain with FlowJo software.

Differential interference contrast (DIC) and fluorescence microscopy. Microscopy was performed with exponentially growing cells to assess cell morphology and protein localization, and each strain was assayed on at least three separate occasions. Cells were adhered to glass slides pretreated with 0.1 % poly-L-lysine (Sigma-Aldrich). Adherent cells were stained with DAPI nucleic acid dye (1 μg ml^{-1}; Molecular Probes) for 5 min at room temperature in the dark. Excess DAPI was removed from the slide with sterile water and cell membranes were stained by covering adherent cells with chilled FM1-43 membrane dye (5 μg ml^{-1}; Molecular Probes) for 1 min on ice as per the manufacturer's instructions. Excess dye was removed with sterile water and slides were immediately observed on a Zeiss Axioskop 2 mot plos microscope (Carl Zeiss Microscopy). The frequency of DivK green fluorescent protein (GFP) polar focus formation was quantified in exponentially growing cells, which express the fusion protein from a low copy plasmid (Lam *et al.*, 2003), cultured in M9 minimal medium supplemented with 0.4 % succinate and 0.1 % Casamino acids. Fluorescence and DIC images were captured with a Hamamatsu Orca-ER camera (Hamamatsu Photonics) using Openlab image software (PerkinElmer) and manipulated using the NIH publicly available software ImageJ. Cells were scored as having wild-type morphology if they exhibited a typical rod shape between 2 μm (G_1/S phase cells) and 4 μm (predivisional G_2 phase cells) in length and approximately 1 μm in width. Rod-shaped cells were scored as having aberrant morphology if they exhibited filamentation, which we defined as either linear growth resulting in cells of greater than 5 μm in length or branching growth resulting in cells with three or more poles. Cells were sometimes associated with a somewhat swollen, or rounded, morphology as well.

Symbiosis assay. Symbiosis was assayed with individual *Medicago sativa* plants on buffered NOD medium (BNM) agar (Ehrhardt *et al.*, 1992). Bacteria were grown to exponential phase, washed, and resuspended in half-strength BNM (½ × BNM) at a final OD_{600} of 0.10, and a 100 μl aliquot was inoculated directly onto plant roots; negative control plants were inoculated with 100 μl of ½ × BNM. At 28 days post-inoculation, plants were measured for height and nodule formation. For each plant, height was measured as the length of the epicotyl stem to the apical node, and nodule formation was observed as total nodules and the percentage of pink nodules per plant.

CtrA purification and Western blot analysis. Gateway cloning for *S. meliloti ctrA* purification was performed using pENTER::*ctrA* (Schroeder *et al.*, 2005) and pDEST17 according to the manufacturer's instructions (Invitrogen), and the presence of *ctrA* in pDEST17::*ctrA* was confirmed by PCR and sequencing.

pDEST17::*ctrA* was transformed into BL21 cells according to the manufacturer's instructions (Invitrogen), and ten transformants were combined and grown in LB supplemented with ampicillin. CtrA-HIS production was induced with 0.2 % L-arabinose for 4 h and protein purification was performed as described by Laub *et al.* (2007). Protein concentration was determined using Coomassie Plus Protein Assay and protein purity was confirmed by SDS-PAGE followed by Coomassie staining. For *S. meliloti* CtrA Western blot analysis, exponential cultures were centrifuged at 4 °C for 10 min at 5000 × g. Cell pellets were resuspended in 2 × Laemmli loading buffer and boiled for 5 min. The volume of sample loaded was normalized to OD_{600}, subjected to 8 % SDS-PAGE with MES running buffer (50 mM Tris Base, 50 mM MES, 1 mM EDTA, 0.1 % SDS) at constant 60 V for 3 h, and then transferred onto nitrocellulose membrane with Tris-glycine transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol) at constant 50 V for 1 h. The membrane was probed with *C. crescentus* anti-CtrA polyclonal antibodies (1 : 10 000 dilution in TBST; gift from P. Chien) for 16 h at 4 °C, and subsequently probed with goat anti-rabbit IgG conjugated to horseradish peroxidase (1 : 50 000 dilution in TBST; Jackson ImmunoResearch Laboratories). Cross-reacting proteins were visualized with an ECL Western blotting detection system (Thermo Scientific), and images were acquired using a Kodak 4000R Image Station camera.

RESULTS

Construction and characterization of a *cbrA* null mutant

The sensor HK *cbrA* was initially identified as playing a critical role in nitrogen-fixing legume symbiosis through a transposon-mediated screen for mutants with a calcofluor-bright phenotype indicative of succinoglycan overproduction (Gibson *et al.*, 2006). The Tn*5* is located within the *cbrA* ORF and separates the N-terminal domain of unknown function from the C-terminal HK domain (Fig. 1c), leaving open the possibility that either portion of the protein may retain some residual function. We subsequently demonstrated that the *cbrA*::Tn*5* mutation is a recessive allele for all known phenotypes (Gibson *et al.*, 2006). However, an early N-terminal transposon insertion within the sensor HK *exoS* produces a recessive gain-of-function allele due to low-level expression of an unregulated kinase domain (Cheng & Walker, 1998). We therefore wanted to determine whether *cbrA*::Tn*5* is a loss-of-function allele and addressed this by constructing a strain that is deleted for the *cbrA* gene. Allelic exchange was performed to replace the entire *cbrA* ORF with the chloramphenicol resistance gene *cat*, generating the Δ*cbrA*::*cat* null allele (Fig. 1d). The presence and correct location of the Δ*cbrA*::*cat* mutation was confirmed through PCR analysis of the *fbpB*-SMc0077 region of the genome that contains *cbrA* (Fig. 1c–e), and by co-transduction with the linked *oxyR*::mTn*5* mutation (Fig. 1c, d, data not shown).

We characterized the Δ*cbrA*::*cat* null mutant for several known *cbrA*::Tn*5* phenotypes (Gibson *et al.*, 2006, 2007). While the Δ*cbrA*::*cat* mutant has a mild growth defect compared with the wild-type on rich medium (Fig. 2a), it displays a dramatic hypersensitivity to the detergent DOC as seen previously with the *cbrA*::Tn*5* mutant (Fig. 2c). The Δ*cbrA*::*cat* mutant also has a calcofluor-bright phenotype that is indistinguishable from the *cbrA*::Tn*5* mutant (Fig. 2b), which results from overproduction of the exopolysaccharide succinoglycan (Gibson *et al.*, 2006). Finally, the Δ*cbrA*::*cat* mutant has a defect in establishing an effective symbiosis with the host *Medicago sativa*. The Δ*cbrA*::*cat* mutant elicits nodule formation at the same frequency as the wild-type, but shows a fivefold decrease in the formation of elongated pink nodules and supports minimal plant growth on nitrogen-depleted medium (Table S2). Importantly, each of these Δ*cbrA*::*cat* phenotypes is fully complemented in the presence of a low copy plasmid containing *cbrA* expressed from its native promoter (Fig. 2a–c, Table S2). Based on these observations, we conclude that the *cbrA*::Tn*5* mutation is a recessive loss-of-function allele that phenocopies the Δ*cbrA*::*cat* null allele, and therefore retains minimal residual activity within either the N-terminal or the C-terminal domain fragments.

Loss of *cbrA* results in branching filamentous cell growth and swollen cell morphology

Bioinformatic analysis suggests CbrA may play a role in regulating cell cycle processes through modulation of DivK phosphorylation status (Fig. 1b) (Brilli *et al.*, 2010; Hallez *et al.*, 2004). To begin testing whether CbrA plays a role in free-living cell cycle regulation, we performed DIC and fluorescence microscopy to compare the cellular morphology of wild-type cells with those of both *cbrA* mutants during exponential phase growth. We observed a significant increase in the proportion of *cbrA*::Tn*5* cells growing as branching filaments (35.1 %) (Fig. 3b, Table 1) in comparison with the wild-type (<1 %) (Fig. 3a, Table 1). The branching *cbrA*::Tn*5* filamentous cells appear to have DNA dispersed throughout the cytoplasm (Fig. 3b). The Δ*cbrA*::*cat* mutant displays cell morphology defects similar to the *cbrA*::Tn*5* mutant (Fig. 3c, Table 1). The filamentation phenotype of Δ*cbrA*::*cat* is complemented by the presence of *cbrA* expressed from its own promoter on a low copy plasmid (Fig. 3d, e, Table 1). These morphological

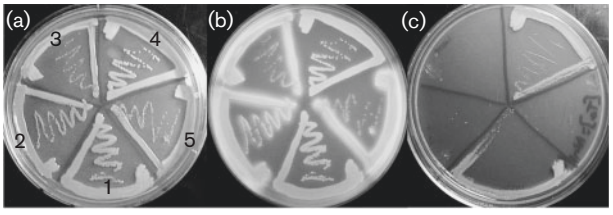


Fig. 2. The *cbrA* mutants have a succinoglycan overproduction phenotype and are sensitive to DOC. (a) Growth assay on LB/MC rich medium. (b) Succinoglycan production assay on LB/MC rich medium supplemented with calcofluor. (c) Growth assay on LB/MC rich medium supplemented with DOC. Strains are arranged in the following order: (1) wild-type, (2) *cbrA*::Tn*5*, (3) Δ*cbrA*::*cat*, (4) Δ*cbrA*::*cat* pLAFR1 *cbrA* and (5) Δ*cbrA*::*cat* pLAFR1.

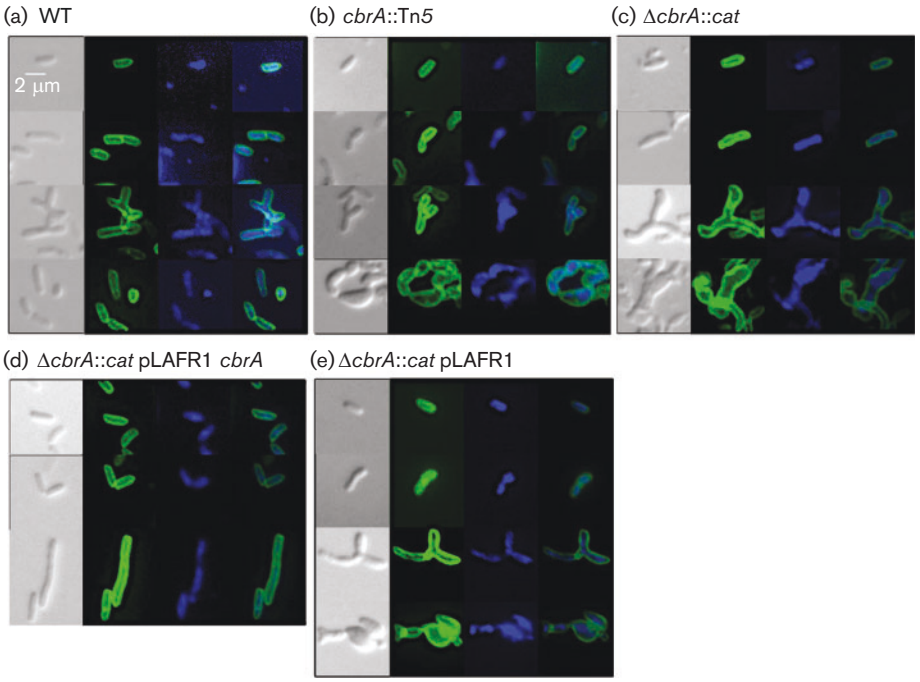


Fig. 3. The *cbrA* mutants display aberrant and filamentous morphology. The first column of each panel is a DIC image of representative cells, the second column shows the same cells stained with FM1-43 to localize the cytoplasmic membrane and septa (green), the third column shows the same cells stained with DAPI to localize DNA (blue), and the fourth column is a merged image of columns 2 and 3. The top two rows demonstrate that all strains can achieve wild-type morphology and cell division. The bottom rows show characteristic filamentous and otherwise aberrant morphologies displayed by each strain: (a) wild-type; (b) *cbrA*::Tn*5*; (c) Δ*cbrA*::*cat*; (d) Δ*cbrA*::*cat* complemented by pLAFR1 *cbrA* with the exception of a low frequency of cells growing as elongated linear filaments (bottom panel, 2 % of total); (e) Δ*cbrA*::*cat* not complemented by pLAFR1 empty vector.

observations suggest strongly that *cbrA* mutants are unable either to initiate or to complete cell division.

Loss of *cbrA* generates cells with an altered genome complement

We quantified the relative DNA content of asynchronous and exponentially growing populations of wild-type and *cbrA* mutant cells using fluorescence flow cytometry as a means of following the process of DNA replication. As expected, we observed two predominant subpopulations with wild-type cells: those cells in G_1 phase with a 1N genome complement and those in late S/G_2 phase with 2N genome complement (Fig. 4a, Table 2). If loss of *cbrA* function significantly delays DNA replication initiation, we would expect to see an increased proportion of cells containing 1N genomes and a corresponding decrease in the proportion containing 2N genomes. In contrast, aberrant overinitiation of DNA replication would be observed as an increase in the proportion of cells with greater than 2N copies of the genome. However, we find *cbrA* mutants retain a subpopulation of cells corresponding to genome complements of 1N and 2N, although their overall percentage within the population is decreased (Fig. 4d, g, Table 2). In contrast, the *cbrA* mutants are twice as likely as the wild-type to contain greater than 2N copies of the genome (Fig. 4a, d, g, Table 2). We also observe a three- to fourfold increase in the number of mutant cells with a genome complement of less than 1N compared with the wild-type (Fig. 4a, d, g, Table 2). Both of these *cbrA* mutant phenotypes are complemented by the presence of cbrA expressed from its own promoter on a low copy plasmid (Fig. 4c, f, i, Table 2), but not by the empty vector alone (Fig. 4b, e, h, Table 2). These observations combined are inconsistent with the hypothesis that *cbrA* mutants undergo filamentation primarily as a result of DNA replication initiation rates being significantly altered. Instead, the mutants may have a mild defect in either DNA segregation or septum localization, resulting in 34–40 % of cells containing either less than or more than a full genome complement and representing a twofold increase over wild-type cells.

Loss of *cbrA* results in a decreased frequency of DivK polar localization and an increased accumulation of CtrA

Periodic polar localization of DivK during cell cycle progression has been shown to depend on phosphorylation in both *C. crescentus* and *S. meliloti*. In *C. crescentus*, the

Table 1. Distribution of aberrant and filamentous cell morphology as a percentage of the total cell population

NA, Not applicable.

Genotype	Total cell count	% WT	% Non-WT	% Filament	% Swollen	% Swollen filament
cbrA^{+}	909	98.3	1.65	0.88	NA	NA
cbrA::Tn*5*	282	58.1	41.8	35.1	15.6	4.96
Δ*cbrA*::*cat*	214	55.1	44.9	35.5	13.6	2.8
Δ*cbrA*::*cat* pLAFR1	156	64.1	36.0	28.8	8.97	3.21
Δ*cbrA*::*cat* pLAFR1 cbrA	1028	97.9	2.14	2.04	0.19	0.1

HKs DivJ and PleC modulate cell cycle localization of DivK through an effect on its phosphorylation status. We therefore sought to determine whether the DivJ/PleC homologue CbrA is an integral component of the signal transduction network required for DivK localization in *S. meliloti*. With a low copy plasmid constitutively expressing DivK-GFP (Lam *et al.*, 2003), we assessed the frequency of DivK polar focus formation in wild-type and *cbrA*::Tn*5*

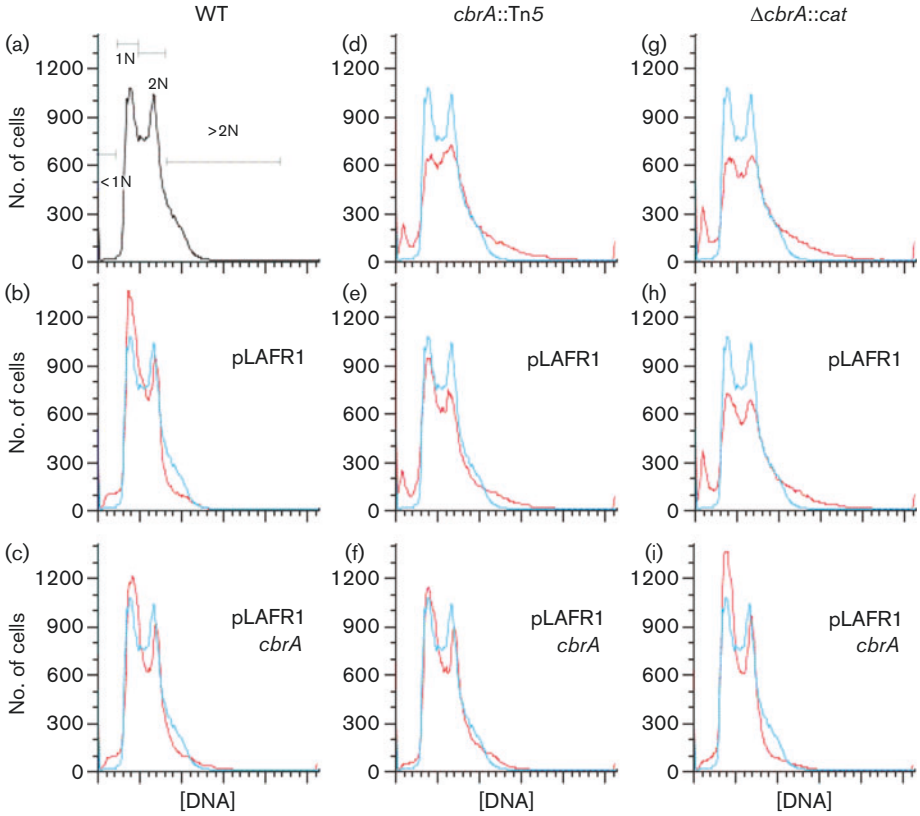


Fig. 4. Loss of *cbrA* results in an increased proportion of cells containing aberrant DNA content. Fluorescence flow cytometry of asynchronous exponential cultures labelled with SYTOX Green was performed to measure DNA content ([DNA]) per cell. Representative distributions were chosen from each set of triplicates and a wild-type distribution (blue) was overlaid on the distribution from a representative replicate of each additional strain (red) for direct comparison of DNA content. (a) The wild-type displays two predominant subpopulations: one peak (1N bar) represents cells in G_1 phase with a 1N complement of the genome, and a second peak (2N bar) represents predivisional cells in late S/G_2 phase with a 2N complement of the genome. Subpopulations of cells containing either less than a full genome (<1N bar) or greater than a full genome (>2N bar) were also analysed. (b, c) Wild-type that contains either the control plasmid pLAFR1 or the complementation plasmid pLAFR1 *cbrA* is largely unaffected. (d, g) The *cbrA*::Tn*5* and Δ*cbrA*::*cat* mutants contain a significant proportion of cells with either a <1N or a >2N genome complement in comparison with the wild-type. (e, f, h, i) The *cbrA*::Tn*5* and Δ*cbrA*::*cat* mutant phenotype is complemented by pLAFR1 *cbrA* (f, i), but not by the empty pLAFR1 vector (e, h).

Table 2. Distribution of DNA content per cell (mean ± SD) as a percentage of the total cell population

Genotype	% <1N	% 1N	% 2N	% >2N	% 1N or 2N	% <1N or >2N
No plasmid						
WT	2.3 ± 0.1	32 ± 2.0	47 ± 1.0	14 ± 0.6	81 ± 0.6	17 ± 0.5
cbrA::Tn*5*	7.9 ± 0.2	23 ± 0.6	40 ± 0.8	26 ± 0.2	63 ± 0.3	34 ± 0.4
Δ*cbrA*::*cat*	11 ± 0.4	21 ± 1.0	37 ± 0.3	28 ± 1.3	58 ± 1.0	39 ± 1.0
pLAFR1						
WT	4.9 ± 0.2	41 ± 3.5	43 ± 1.6	8.4 ± 2.4	84 ± 2.4	13 ± 2.2
cbrA::Tn*5*	9.4 ± 0.4	31 ± 1.1	38 ± 0.3	19 ± 0.7	69 ± 1.1	28 ± 1.0
Δ*cbrA*::*cat*	12 ± 0.6	27 ± 2.0	37 ± 0.3	22 ± 1.9	64 ± 1.9	33 ± 1.6
pLAFR1 *cbrA*						
WT	5.3 ± 0.4	36 ± 0.6	43 ± 1.1	13 ± 1.2	78 ± 1.6	18 ± 1.6
cbrA::Tn*5*	4.0 ± 0.3	34 ± 1.0	43 ± 0.4	15 ± 1.1	77 ± 0.8	20 ± 0.9
Δ*cbrA*::*cat*	4.3 ± 0.2	40 ± 1.1	43 ± 0.9	9.7 ± 0.6	83 ± 0.6	14 ± 0.6

cells using fluorescence microscopy. Approximately 30 % of wild-type cells contain DivK-GFP localized at the cell pole, as expected based on previous findings (Fig. 5a, b). In contrast, only 5 % of *cbrA*::Tn*5* cells contain a DivK-GFP polar focus (Fig. 5c, d). Using polyclonal antibodies against GFP, we confirmed by Western blot analysis that DivK-GFP levels are equivalent in wild-type and *cbrA*::Tn*5* cells (data not shown). These observations are consistent with CbrA playing a role in regulating DivK phosphorylation status, and further suggest that CbrA may function directly as a DivK kinase. We therefore suggest that loss of *cbrA* function leads to decreased levels of phosphorylated DivK, and thus decreased DivK activity.

As a primary function of DivK in *C. crescentus* is to negatively regulate CtrA levels and activity, we hypothesize that decreased DivK activity due to loss of *cbrA* would lead to an increase in CtrA accumulation. We performed Western blot analysis to assay steady-state levels of CtrA in exponentially growing cultures of the wild-type and *cbrA* mutants. Consistent with our hypothesis, we observed increased levels of CtrA in both the *cbrA*::Tn*5* and the Δ*cbrA*::*cat* mutant (Fig. 6, lanes 3 and 4) compared with the wild-type (Fig. 6, lane 2). Importantly, increased accumulation of CtrA in the Δ*cbrA*::*cat* mutant is complemented by pLAFR1 *cbrA* but not by the pLAFR1 empty vector (Fig. 6, lanes 6 and 7). CtrA levels remain unaffected in the wild-type containing either pLAFR1 *cbrA* or the pLAFR1 empty vector (Fig. 6, lanes 8 and 9), suggesting there is no significant increase in the overall level of CbrA activity upon addition of pLAFR1 *cbrA*. Together, our results suggest that loss of *cbrA* function leads to decreased levels of phosphorylated DivK, and a resulting increase in CtrA accumulation and activity.

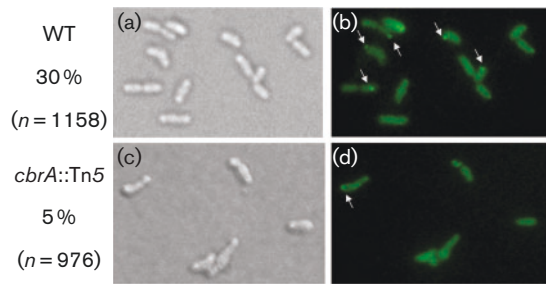


Fig. 5. The loss of *cbrA* results in a decreased rate of DivK-GFP polar focus formation. Asynchronous exponential cells of wild-type (a, b) and *cbrA*::Tn*5* (c, d) strains were assayed for DivK localization by following a DivK-GFP fusion constitutively expressed from a low copy plasmid. (a, c) DIC image of cells. (b, d) DivK-GFP localization with each arrow indicating a polar focus.

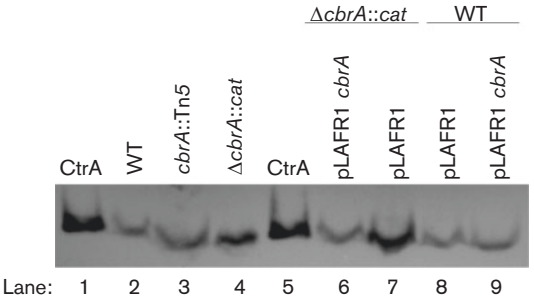


Fig. 6. The loss of *cbrA* results in increased levels of CtrA. Asynchronous exponential cultures were assayed for CtrA levels by Western blot. Lanes 1 and 5: *S. meliloti* CtrA was cloned into a HIS-tag overexpression vector. The resulting CtrA-HIS fusion was expressed in BL21, purified by affinity chromatography on nickel resin and used as a positive control for identification of *S. meliloti* CtrA. Lanes 2–4: the level of CtrA is greater in the *cbrA* mutants than in the wild-type. Lanes 6 and 7: wild-type levels of CtrA are restored in the Δ*cbrA*::*cat* mutant with pLAFR1 *cbrA* complementation but not by pLAFR1 empty vector. Lanes 8 and 9: wild-type with either pLAFR1 empty vector or pLAFR1 *cbrA* complementation displays the same level of CtrA accumulation.

DISCUSSION

Bioinformatic analyses suggest CbrA is a homologue of the DivJ/PleC cell cycle regulators of DivK activity in *C. crescentus* (Fig. 1a, b; Brilli *et al.*, 2010; Hallez *et al.*, 2004). In fact, the *B. abortus* CbrA orthologue, PdhS, is required for proper cell cycle progression presumably through its effect on DivK activity (Hallez *et al.*, 2007; Van der Henst *et al.*, 2012), which thereby influences CtrA levels (Hallez *et al.*, 2007). Given that *S. meliloti* enters a novel set of cell cycle pathways during bacteroid differentiation in the host (Mergaert *et al.*, 2006), and that CbrA is required to establish an effective symbiosis (Gibson *et al.*, 2006), we were interested to determine whether CbrA contributes to cell cycle regulation. Consistent with this prediction, we find *cbrA* mutants display a 40-fold increase in filamentous cell growth compared with the wild-type (Table 1), which is indicative of a serious perturbation in cell division that inhibits cytokinesis. In addition, we observe a twofold increase in the proportion of mutant cells that accumulate an aberrant genome complement in comparison with the wild-type (Table 2). This observation suggests the process of either DNA segregation or septum localization is also affected in *cbrA* mutants. In support of the latter possibility, it was recently shown that *A. tumefaciens* Δ*pdhS1* mutants are observed with a marked increase in Z-ring mislocalization (Kim *et al.*, 2013). Thus, our data and work in related organisms strongly implicate CbrA and its orthologues as important regulatory factors in several cell cycle processes during free-living growth.

The DivJ/PleC HKs of *C. crescentus* regulate aspects of the cell cycle as well as asymmetrical cell fate through opposing effects on the activity of the shared response regulator DivK. DivJ functions as a DivK kinase (Hecht *et al.*, 1995; Jacobs *et al.*, 2001), and the absence of DivJ results in decreased phosphorylated DivK levels with a corresponding decrease in DivK polar foci (Jacobs *et al.*, 2001; Lam *et al.*, 2003). In contrast, PleC functions as a DivK phosphatase such that the absence of PleC leads to increased phosphorylated DivK levels and a corresponding presence of DivK polar foci in both daughter cells (Jacobs *et al.*, 2001; Lam *et al.*, 2003). In *S. meliloti*, DivK also forms polar foci and in a phosphorylation-dependent manner (Lam *et al.*, 2003). We find the frequency of DivK polar focus formation is decreased sixfold in the *cbrA* mutant, such that only 5 % of mutant cells form a focus (Fig. 5d) in comparison with 30 % of wild-type cells (Fig. 5b). These observations are consistent with a model in which CbrA functions as a cognate HK of DivK, and more specifically that it functions as a DivK kinase to promote DivK activity and localization.

The loss of either DivJ or DivK function results in cellular filamentation and an increased genome complement in *C. crescentus* (Wheeler & Shapiro, 1999), similar to what we observe with *cbrA* mutants. Loss of PleC function, by contrast, primarily results in asymmetrical cell fate defects with the absence of a stalk, pili and functional flagella, resulting in reduced swarmer cell motility. Although *cbrA* mutants do have decreased motility (Gibson *et al.*, 2007), their phenotype of filamentous cell growth, ploidy defects and decreased DivK-GFP focus formation more closely resemble the *C. crescentus divJ* mutant rather than a *pleC* mutant.

In *C. crescentus*, DivK contributes to cell cycle progression and asymmetrical cell division through its effect on activity of the response regulator CtrA, which has been shown to directly regulate several cell cycle processes (Tsokos & Laub, 2012; Tsokos *et al.*, 2011). When phosphorylated, DivK inhibits a CckA-dependent phosphorelay, which, when active, serves to phosphorylate CtrA and increase its stability by repressing ClpXP-mediated degradation (Biondi *et al.*, 2006; Chen *et al.*, 2009; Jacobs *et al.*, 2003; Tsokos & Laub, 2012). Although the nature of the *S. meliloti* pathway regulating CtrA has not been functionally probed in detail yet, most of the *C. crescentus* regulators have orthologues in *S. meliloti*, including the CckA phosphorelay and the ClpXP proteolytic machinery (Brilli *et al.*, 2010; Hallez *et al.*, 2004). We therefore sought to test whether CtrA levels would be affected in the *cbrA* mutants as they display decreased DivK localization, and presumably activity. Consistent with a loss of DivK activity, we find *cbrA* mutants accumulate increased levels of CtrA (Fig. 6), suggesting the *S. meliloti* CckA pathway downstream of DivK retains a similar role in CtrA regulation as observed in *C. crescentus*.

S. meliloti null mutants for the *cpdR1* gene, which in *C. crescentus* functions downstream of CckA to repress CtrA at the level of stability, grow as filamentous cells containing greater than a 2N genome complement (Kobayashi *et al.*, 2009). In *C. crescentus*, PodJ is required for polar localization of PleC, and thus *podJ* mutants display defects in pilus synthesis and flagellar motility (Curtis *et al.*, 2012; Levi & Jenal, 2006; Viollier *et al.*, 2002). Interestingly, *pleC* is essential in *S. meliloti*, although there are two non-essential PodJ homologues: PodJ1 and PodJ2 (Fields *et al.*, 2012). PodJ1 localizes to the cell pole, and in its absence DivK is inefficiently recruited to the opposite cell pole (Fields *et al.*, 2012). In addition to loss of DivK localization, the *podJ1* null mutant shares several additional phenotypes with *cbrA* mutants: overproduction of succinoglycan, filamentous cell morphology, reduction in motility, and sensitivity to detergents such as DOC (Fields *et al.*, 2012). Thus, *cbrA* mutants share a similar set of cell cycle defects with both *cpdR1* and *podJ1* mutants, which are predicted to have derepression of CtrA activity, although this has yet to be shown. However, we do observe a significant increase in CtrA accumulation within *cbrA* mutants (Fig. 6), indicating that the DivK-dependent pathway regulating CtrA is likely to be intact in *S. meliloti* as predicted by bioinformatic analysis (Brilli *et al.*, 2010).

Based on our present data, we suggest the primary role of CbrA is to regulate cell division through its effect on DivK activity and CtrA regulation. In predivisional *C. crescentus*

cells, loss of DivK repression allows CtrA to coordinate the expression of genes that play an essential role in formation of the FtsZ ring and cell division, including *ftsZ*, *ftsQA* and *ftsW* (Kelly *et al.*, 1998; Laub *et al.*, 2002; Wortinger *et al.*, 2000). CtrA loss-of-function was characterized using a conditional temperature-sensitive *ctrA* allele (Jacobs *et al.*, 1999, 2003), and this mutant loses the ability to undergo cytokinesis, developing a filamentous morphology that is accompanied by cells containing an increased genome complement. While CtrA-depleted cells are specifically inhibited for cell division, they initiate new rounds of DNA replication with the same periodicity as wild-type cells and therefore become polyploid (Jonas *et al.*, 2011). A consensus CtrA binding site is conserved across several species of *Alphaproteobacteria*, including *S. meliloti* (Brilli *et al.*, 2010; Hallez *et al.*, 2004). Based on the location of these sites, CtrA is predicted to regulate genes involved in the process of cell division throughout most *Alphaproteobacteria*, which has been borne out in *B. abortus* (Bellefontaine *et al.*, 2002). However, the exact subset of cell division genes within the CtrA regulon varies between different species, and perhaps due to differences in the lifestyle of these bacteria. It will therefore be of interest to develop a deeper understanding of how *S. meliloti* regulates CtrA activity and the nature of the CtrA regulon, both for a deeper understanding of the mechanisms underlying cell cycle regulation and for an evolutionary perspective on how the fundamental process of reproduction is adapted to promote differing lifestyles.

ACKNOWLEDGEMENTS

We are grateful to Dr Frederic Preffer and the clinical flow cytometry lab at Massachusetts General Hospital for allowing us the use of their fluorescence flow cytometer. We also extend our thanks to Dr Linda Huang (University of Massachusetts Boston, Department of Biology) and her laboratory for use of their fluorescence microscope and microscopy expertise; and Dr Lyle Simmons (University of Michigan, Department of Molecular, Cellular and Developmental Biology) for his microscopy expertise and helpful discussions. We also thank Dr Peter Chien (University of Massachusetts Amherst, Department of Biochemistry and Molecular Biology) for the generous gift of *C. crescentus* anti-CtrA polyclonal antibodies. This work was supported by funding to K. E. G. from the University of Massachusetts Boston, the NIH (1 R15 GM099052-01) and the NSF (IOS 1119866); to D. W. from the NSF through its Research Experiences for Undergraduates (REU) grant to the University of Massachusetts Boston (DBI 1062748); and to G. W. from the NIH (GM31030). G. W. is an American Cancer Society Professor.

REFERENCES

Barnett, M. J., Hung, D. Y., Reisenauer, A., Shapiro, L. & Long, S. R. (2001). A homolog of the CtrA cell cycle regulator is present and essential in *Sinorhizobium meliloti*. *J Bacteriol* **183**, 3204–3210.

Bastedo, D. P. & Marczynski, G. T. (2009). CtrA response regulator binding to the *Caulobacter* chromosome replication origin is required during nutrient and antibiotic stress as well as during cell cycle progression. *Mol Microbiol* **72**, 139–154.

Bellefontaine, A. F., Pierreux, C. E., Mertens, P., Vandenhaute, J., Letesson, J. J. & De Bolle, X. (2002). Plasticity of a transcriptional regulation network among alpha-proteobacteria is supported by the identification of CtrA targets in *Brucella abortus*. *Mol Microbiol* **43**, 945–960.

Bina, J. E. & Mekalanos, J. J. (2001). *Vibrio cholerae tolC* is required for bile resistance and colonization. *Infect Immun* **69**, 4681–4685.

Biondi, E. G., Reisinger, S. J., Skerker, J. M., Arif, M., Perchuk, B. S., Ryan, K. R. & Laub, M. T. (2006). Regulation of the bacterial cell cycle by an integrated genetic circuit. *Nature* **444**, 899–904.

Brilli, M., Fondi, M., Fani, R., Mengoni, A., Ferri, L., Bazzicalupo, M. & Biondi, E. G. (2010). The diversity and evolution of cell cycle regulation in alpha-proteobacteria: a comparative genomic analysis. *BMC Syst Biol* **4**, 52.

Brown, P. J., de Pedro, M. A., Kysela, D. T., Van der Henst, C., Kim, J., De Bolle, X., Fuqua, C. & Brun, Y. V. (2012). Polar growth in the Alphaproteobacterial order Rhizobiales. *Proc Natl Acad Sci U S A* **109**, 1697–1701.

Chen, Y. E., Tsokos, C. G., Biondi, E. G., Perchuk, B. S. & Laub, M. T. (2009). Dynamics of two phosphorelays controlling cell cycle progression in *Caulobacter crescentus*. *J Bacteriol* **191**, 7417–7429.

Chen, Y. E., Tropini, C., Jonas, K., Tsokos, C. G., Huang, K. C. & Laub, M. T. (2011). Spatial gradient of protein phosphorylation underlies replicative asymmetry in a bacterium. *Proc Natl Acad Sci U S A* **108**, 1052–1057.

Cheng, H. P. & Walker, G. C. (1998). Succinoglycan production by *Rhizobium meliloti* is regulated through the ExoS-ChvI two-component regulatory system. *J Bacteriol* **180**, 20–26.

Cheng, J., Sibley, C. D., Zaheer, R. & Finan, T. M. (2007). A *Sinorhizobium meliloti minE* mutant has an altered morphology and exhibits defects in legume symbiosis. *Microbiology* **153**, 375–387.

Curtis, P. D. & Brun, Y. V. (2010). Getting in the loop: regulation of development in *Caulobacter crescentus*. *Microbiol Mol Biol Rev* **74**, 13–41.

Curtis, P. D., Quardokus, E. M., Lawler, M. L., Guo, X., Klein, D., Chen, J. C., Arnold, R. J. & Brun, Y. V. (2012). The scaffolding and signalling functions of a localization factor impact polar development. *Mol Microbiol* **84**, 712–735.

Domenech, P., Kobayashi, H., LeVier, K., Walker, G. C. & Barry, C. E., III (2009). BacA, an ABC transporter involved in maintenance of chronic murine infections with *Mycobacterium tuberculosis*. *J Bacteriol* **191**, 477–485.

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

Ehrhardt, D. W., Atkinson, E. M. & Long, S. R. (1992). Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. *Science* **256**, 998–1000.

Fields, A. T., Navarrete, C. S., Zare, A. Z., Huang, Z., Mostafavi, M., Lewis, J. C., Rezaeihaghighi, Y., Brezler, B. J., Ray, S. & other authors (2012). The conserved polarity factor podJ1 impacts multiple cell envelope-associated functions in *Sinorhizobium meliloti*. *Mol Microbiol* **84**, 892–920.

Finan, T. M., Hartweig, E., LeMieux, K., Bergman, K., Walker, G. C. & Signer, E. R. (1984). General transduction in *Rhizobium meliloti*. *J Bacteriol* **159**, 120–124.

Gibson, K. E., Campbell, G. R., Lloret, J. & Walker, G. C. (2006). CbrA is a stationary-phase regulator of cell surface physiology and legume symbiosis in *Sinorhizobium meliloti*. *J Bacteriol* **188**, 4508–4521.

Gibson, K. E., Barnett, M. J., Toman, C. J., Long, S. R. & Walker, G. C. (2007). The symbiosis regulator CbrA modulates a complex

regulatory network affecting the flagellar apparatus and cell envelope proteins. *J Bacteriol* **189**, 3591–3602.

Gibson, K. E., Kobayashi, H. & Walker, G. C. (2008). Molecular determinants of a symbiotic chronic infection. *Annu Rev Genet* **42**, 413–441.

Haag, A. F., Baloban, M., Sani, M., Kerscher, B., Pierre, O., Farkas, A., Longhi, R., Boncompagni, E., Hérouart, D. & other authors (2011). Protection of *Sinorhizobium* against host cysteine-rich antimicrobial peptides is critical for symbiosis. *PLoS Biol* **9**, e1001169.

Hallez, R., Bellefontaine, A. F., Letesson, J. J. & De Bolle, X. (2004). Morphological and functional asymmetry in α-proteobacteria. *Trends Microbiol* **12**, 361–365.

Hallez, R., Mignolet, J., Van Mullem, V., Wery, M., Vandenhaute, J., Letesson, J. J., Jacobs-Wagner, C. & De Bolle, X. (2007). The asymmetric distribution of the essential histidine kinase PdhS indicates a differentiation event in *Brucella abortus*. *EMBO J* **26**, 1444–1455.

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

Hung, D. Y. & Shapiro, L. (2002). A signal transduction protein cues proteolytic events critical to *Caulobacter* cell cycle progression. *Proc Natl Acad Sci U S A* **99**, 13160–13165.

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

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

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

Jenal, U. (2009). The role of proteolysis in the *Caulobacter crescentus* cell cycle and development. *Res Microbiol* **160**, 687–695.

Jonas, K., Chen, Y. E. & Laub, M. T. (2011). Modularity of the bacterial cell cycle enables independent spatial and temporal control of DNA replication. *Curr Biol* **21**, 1092–1101.

Kahng, L. S. & Shapiro, L. (2001). The CcrM DNA methyltransferase of *Agrobacterium tumefaciens* is essential, and its activity is cell cycle regulated. *J Bacteriol* **183**, 3065–3075.

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

Kim, J., Heindl, J. E. & Fuqua, C. (2013). Coordination of division and development influences complex multicellular behavior in *Agrobacterium tumefaciens*. *PLoS ONE* **8**, e56682.

Kobayashi, H., De Nisco, N. J., Chien, P., Simmons, L. A. & Walker, G. C. (2009). *Sinorhizobium meliloti* CpdR1 is critical for co-ordinating cell cycle progression and the symbiotic chronic infection. *Mol Microbiol* **73**, 586–600.

Lam, H., Matroule, J. Y. & Jacobs-Wagner, C. (2003). The asymmetric spatial distribution of bacterial signal transduction proteins coordinates cell cycle events. *Dev Cell* **5**, 149–159.

Latch, J. N. & Margolin, W. (1997). Generation of buds, swellings, and branches instead of filaments after blocking the cell cycle of *Rhizobium meliloti*. *J Bacteriol* **179**, 2373–2381.

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

Laub, M. T., Biondi, E. G. & Skerker, J. M. (2007). Phosphotransfer profiling: systematic mapping of two-component signal transduction pathways and phosphorelays. *Methods Enzymol* **423**, 531–548.

Leigh, J. A., Signer, E. R. & Walker, G. C. (1985). Exopolysaccharide-deficient mutants of *Rhizobium meliloti* that form ineffective nodules. *Proc Natl Acad Sci U S A* **82**, 6231–6235.

Levi, A. & Jenal, U. (2006). Holdfast formation in motile swarmer cells optimizes surface attachment during *Caulobacter crescentus* development. *J Bacteriol* **188**, 5315–5318.

Matroule, J. Y., Lam, H., Burnette, D. T. & Jacobs-Wagner, C. (2004). Cytokinesis monitoring during development; rapid pole-to-pole shuttling of a signaling protein by localized kinase and phosphatase in *Caulobacter*. *Cell* **118**, 579–590.

McAdams, H. H. & Shapiro, L. (2009). System-level design of bacterial cell cycle control. *FEBS Lett* **583**, 3984–3991.

Mergaert, P., Uchiumi, T., Alunni, B., Evanno, G., Cheron, A., Catrice, O., Mausset, A. E., Barloy-Hubler, F., Galibert, F. & other authors (2006). Eukaryotic control on bacterial cell cycle and differentiation in the *Rhizobium*–legume symbiosis. *Proc Natl Acad Sci U S A* **103**, 5230–5235.

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

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

Robertson, G. T., Reisenauer, A., Wright, R., Jensen, R. B., Jensen, A., Shapiro, L. & Roop, R. M., II (2000). The *Brucella abortus* CcrM DNA methyltransferase is essential for viability, and its overexpression attenuates intracellular replication in murine macrophages. *J Bacteriol* **182**, 3482–3489.

Roop, R. M., II, Robertson, G. T., Ferguson, G. P., Milford, L. E., Winkler, M. E. & Walker, G. C. (2002). Seeking a niche: putative contributions of the *hfq* and *bacA* gene products to the successful adaptation of the brucellae to their intracellular home. *Vet Microbiol* **90**, 349–363.

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

Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G. & Pühler, A. (1994). Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**, 69–73.

Schroeder, B. K., House, B. L., Mortimer, M. W., Yurgel, S. N., Maloney, S. C., Ward, K. L. & Kahn, M. L. (2005). Development of a functional genomics platform for *Sinorhizobium meliloti*: construction of an ORFeome. *Appl Environ Microbiol* **71**, 5858–5864.

Sibley, C. D., MacLellan, S. R. & Finan, T. (2006). The *Sinorhizobium meliloti* chromosomal origin of replication. *Microbiology* **152**, 443–455.

Skerker, J. M. & Laub, M. T. (2004). Cell-cycle progression and the generation of asymmetry in *Caulobacter crescentus*. *Nat Rev Microbiol* **2**, 325–337.

Tsokos, C. G. & Laub, M. T. (2012). Polarity and cell fate asymmetry in *Caulobacter crescentus*. *Curr Opin Microbiol* **15**, 744–750.

Tsokos, C. G., Perchuk, B. S. & Laub, M. T. (2011). A dynamic complex of signaling proteins uses polar localization to regulate cell-fate asymmetry in *Caulobacter crescentus*. *Dev Cell* **20**, 329–341.

Ugalde, R. A. (1999). Intracellular lifestyle of *Brucella* spp. Common genes with other animal pathogens, plant pathogens, and endosymbionts. *Microbes Infect* **1**, 1211–1219.

Van de Velde, W., Zehirov, G., Szatmari, A., Debreczeny, M., Ishihara, H., Kevei, Z., Farkas, A., Mikulass, K., Nagy, A. & other authors (2010). Plant peptides govern terminal differentiation of bacteria in symbiosis. *Science* **327**, 1122–1126.

Van der Henst, C., Beaufay, F., Mignolet, J., Didembourg, C., Colinet, J., Hallet, B., Letesson, J. J. & De Bolle, X. (2012). The histidine kinase PdhS controls cell cycle progression of the pathogenic alphaproteobacterium *Brucella abortus*. *J Bacteriol* **194**, 5305–5314.

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

Wheeler, R. T. & Shapiro, L. (1999). Differential localization of two histidine kinases controlling bacterial cell differentiation. *Mol Cell* **4**, 683–694.

Wortinger, M., Sackett, M. J. & Brun, Y. V. (2000). CtrA mediates a DNA replication checkpoint that prevents cell division in *Caulobacter crescentus*. *EMBO J* **19**, 4503–4512.

Wright, R., Stephens, C. & Shapiro, L. (1997). The CcrM DNA methyltransferase is widespread in the alpha subdivision of proteobacteria, and its essential functions are conserved in *Rhizobium meliloti* and *Caulobacter crescentus*. *J Bacteriol* **179**, 5869–5877.

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

Edited by: I. Oresnik