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# The *Caulobacter crescentus ctrA* P1 promoter is essential for the coordination of cell cycle events that prevent the overinitiation of DNA replication

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The master regulator CtrA oscillates during the *Caulobacter* cell cycle due to temporally regulated proteolysis and transcription. It is proteolysed during the G1–S transition and reaccumulates in predivisional cells as a result of transcription from two sequentially activated promoters, P1 and P2. CtrA reinforces its own synthesis by directly mediating the activation of P2 concurrently with repression of P1. To explore the role of P1 in cell cycle control, we engineered a mutation into the native *ctrA* locus that prevents transcription from P1 but not P2. As expected, the *ctrA* P1 mutant exhibits striking growth, morphological and DNA replication defects. Unexpectedly, we found CtrA and its antagonist SciP, but not DnaA, GcrA or CcrM accumulation to be dramatically reduced in the *ctrA* P1 mutant. SciP levels closely paralleled CtrA accumulation, suggesting that CtrA acts as a rheostat to modulate SciP abundance. Furthermore, the reappearance of CtrA and CcrM in predivisional cells was delayed in the P1 mutant by 0.125 cell cycle unit in synchronized cultures. High levels of *ccrM* transcription despite low levels of CtrA and increased transcription of *ctrA* P2 in the *ctrA* P1 mutant are two examples of robustness in the cell cycle. Thus, *Caulobacter* can adjust regulatory pathways to partially compensate for reduced and delayed CtrA accumulation in the *ctrA* P1 mutant.

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## INTRODUCTION

Oscillating master regulators drive both the prokaryotic and the eukaryotic cell cycles. These transcription factors are themselves regulated at multiple levels to ensure that they perform critical functions only during the correct time of the cell cycle. In the cell cycle of the aquatic Gram-negative bacterium *Caulobacter crescentus*, three proteins (DnaA, GcrA and CtrA) control the expression of over 200 (Holtzendorff *et al.*, 2004; Hottes *et al.*, 2005; Laub *et al.*, 2002) of *Caulobacter*'s 553 cell cycle-regulated genes (Laub *et al.*, 2000). The CcrM adenine DNA methyltranferase has been suggested as a fourth cell cycle master regulator for its effect on transcription via methylation of promoters, creating a complete core circuit (Collier *et al.*, 2007). The temporal oscillation of the accumulation of the four core master regulators during the cell cycle is depicted in Fig. 1.

The *Caulobacter* cell cycle is programmed to yield cells of two shapes and fates: a motile swarmer cell and a stationary stalked cell. Swarmer cells swim in active search of nutrients and are incapable of division until they differentiate into stalked cells. During this process, they eject their flagellum, retract their pili, and grow a stalk at the same pole formerly occupied by the flagellum and pili. At the tip of the stalk is a holdfast, one of the strongest known biological adhesives (Tsang *et al.*, 2006). Thus, stalked cells attach to a surface and will bud off new swarmer cells in the presence of nutrients. New-born swarmer cells are developmentally regulated to either immediately re-enter the cell cycle (forming a new stalked cell) or prolong the swarmer cell stage, and use chemotaxis to search for a more nutrient-rich environment (Ausmees & Jacobs-Wagner, 2003).

CtrA is abundant in swarmer cells, where it inhibits the initiation of DNA replication by directly binding to CtrA target sites within the origin of replication (Quon *et al.*, 1998). Once a swarmer cell is exposed to sufficient nutrients, it differentiates into a stalked cell. As part of this transition, CtrA is degraded by the dynamically localizing protease

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Abbreviations: QPCR, real-time quantitative PCR; 5′ RACE, 5′ rapid amplification of cDNA ends.

 

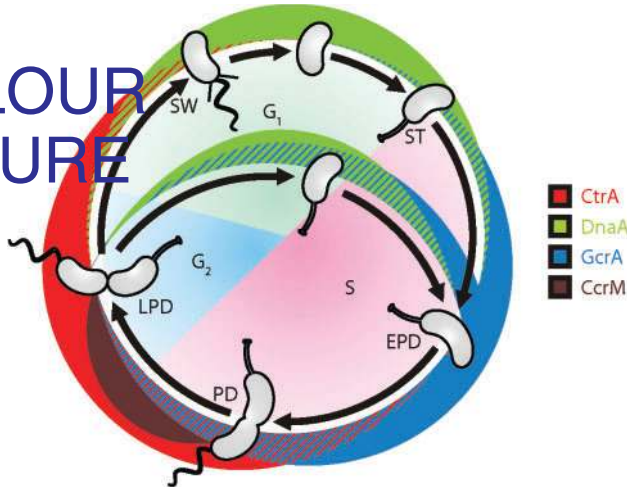


**Fig. 1.** The four core oscillating master regulators of the *Caulobacter* cell cycle. The stages of the *Caulobacter* cell cycle are analogous to those of the eukaryotic cell cycle with $G_0$, $G_1$ and S phases. DnaA notably initiates DNA replication, GcrA activates the transcription of genes involved in DNA replication elongation and chromosome segregation, CtrA represses the initiation of DNA replication and controls genetic modules involved in cell polarity and cell division, and CcrM, a DNA $N^6$-adenine methylase, regulates genes in an epigenetic manner based on their location in the chromosome. SW, swarmer cell; ST, stalked cell; PD, predivisional cell; EPD, early predivisional cell; LPD, late predivisional cell.

ClpXP (Iniesta *et al.*, 2006; Jenal & Fuchs, 1998; McGrath *et al.*, 2006), while DnaA continues to accumulate (Collier *et al.*, 2006, 2007). Without CtrA to block DnaA binding to the origin of replication, activated DnaA (Collier & Shapiro, 2009; Jonas *et al.*, 2011) initiates DNA replication and activates the transcription of genes at the swarmer-to-stalked cell transition (Hottes *et al.*, 2005). One of these genes encodes GcrA, which activates or represses different genetic modules in stalked cells (Collier *et al.*, 2006; Hottes *et al.*, 2005). GcrA in turn inhibits the production of DnaA (Holtzendorff *et al.*, 2004). GcrA notably activates genes involved in DNA replication elongation and chromosome segregation and contributes to the expression of CtrA by activating the first promoter of *ctrA*, P1, which is regulated by $N^6$-adenosine methylation (Reisenauer & Shapiro, 2002). When *ctrA* P1 becomes hemimethylated during DNA replication (Domian *et al.*, 1999; Stephens *et al.*, 1996), GcrA can activate its transcription (Holtzendorff *et al.*, 2004). The newly synthesized strand is unmethylated because the CcrM methyltransferase is only active at the end of the cell cycle. After CtrA accumulates, it represses its own transcription from P1 and activates transcription from P2 (Domian *et al.*, 1999). P1 is activated in early predivisional cells, whereas P2 is activated in late predivisional cells. As CtrA accumulates to high levels in late predivisional cells, it activates transcription of the gene encoding the CcrM DNA methyltransferase, which methylates adenines in GANTC target sequences in the newly replicated DNA strand, thus making the chromosome fully methylated (Stephens *et al.*, 1996). This genetic circuit resets the cell cycle by promoting the expression of *dnaA*, which requires a fully methylated promoter for transcriptional activation (Collier *et al.*, 2007).

A recent review (McAdams & Shapiro, 2011) describes a fifth (Christen *et al.*, 2011; Tan *et al.*, 2010) regulator, SciP, a CtrA antagonist that inhibits the transcription of at least 58 CtrA-activated genes (Gora *et al.*, 2010; Tan *et al.*, 2010) in swarmer cells, limiting their expression to the predivisional stage of the cell cycle. SciP integrates with the oscillating core cell cycle regulators via CtrA and CcrM (Gora *et al.*, 2010; Tan *et al.*, 2010). SciP is a small protein that is co-conserved with CtrA and physically interacts with CtrA. CtrA activates the transcription of *sciP*, whose gene product acts as a rheostat to prevent the overaccumulation of CtrA. As SciP accumulates, it represses *ctrA* transcription, as well as the transcription of a subset of CtrA-activated genes, including *ccrM* and those involved in flagellum and pili biogenesis (Gora *et al.*, 2010; Tan *et al.*, 2010), thus limiting their transcription to predivisional cells. Although it is clear that SciP represses *ctrA* transcription, the specific effect of SciP on the transcription of the *ctrA* P1 and P2 promoters has yet to be reported.

When *ctrA* was moved from its normal chromosomal location (near the origin) to a position near the terminus (to prolong the time that P1 spends in the fully methylated state), 42 % of swarmer cells were elongated and there was a 20 min delay in the reappearance of CtrA during the latter half of the cell cycle (Reisenauer & Shapiro, 2002). The physiological consequences arising from dysregulation of P1 were confounded by the discovery of a third promoter, $P2^{FM\ (fully\ methylated)}$, which is only active when *ctrA* is near the terminus (Reisenauer & Shapiro, 2002). Thus, the contribution of *ctrA* P1 to cell cycle progression was unclear.

Based on current models of the *Caulobacter* cell cycle (Laub *et al.*, 2007; Shen *et al.*, 2008), we hypothesized that the *ctrA* epigenetic P1 promoter would be critical to jump-start the synthesis of CtrA in early predivisional cells, which is important for retaining the temporal and spatial control of cell cycle events regulated by CtrA (Domian *et al.*, 1997; Quon *et al.*, 1996, 1998). To explore the role of P1 in cell cycle progression, we inactivated the P1 promoter at the native *ctrA* locus by inserting the same 5 bp spacer between the −35 and −10 sites of the P1 promoter that had previously been shown to prevent transcription from P1, but not P2, on a plasmid-based transcriptional reporter (Domian *et al.*, 1999). Here, we characterize the effect of this mutation at the native *ctrA* locus. We show that the loss of *ctrA* P1 activity results in striking growth defects and a mis-coordination of cell cycle events that arise from the altered accumulation of CtrA during the cell cycle.

**Table 1.** Bacterial strains

| Strain | Genotype and phenotype | Source or reference |
|---|---|---|
| ***C. crescentus* strains** | | |
| NA1000 (CB15N) | Wild-type | Gift from Lucy Shapiro |
| CMS33 | pBGS18T in CC3039/CC3040 intergenic region ($kan^R$) | West *et al.* (2002) |
| JC130 | P*dnaA-lacZ*/pRKlacZ290 (transcriptional reporter) ($tet^R$) | Collier *et al.* (2007) |
| LS178 | Δ*bla6 rsaA2* P*ccrM-lacZ*/pGZ4 integrant (transcriptional reporter) ($kan^R$) | Zweiger *et al.* (1994) |
| LS2222 | *ctrA*::pXPC15 (=P*xylX*::*ctrA*) ($gent^R$) | Quon *et al.* (1996) |
| LS4185 | *rcdA*::Ω*hyg* | McGrath *et al.* (2006) |
| MHT68 | P*xylX*::*sciP*/pMT464 (high copy vector) | Tan *et al.* (2010) |
| SM904 | P*xylX*::*ctrA ctrA*P1 ($gent^R$) | Sucrose selection of *ctrA* P1/pNPTS138 |
| SM921 | *ctrA* P1 | This study, see Methods |
| SM948 | P*xylX*::*ctrA ctrA* P1 pBGS18T in CC3039/CC3040 intergenic region ($gent^R$ $kan^R$) | PhiCr30(CMS33)→SM904 |
| SM1093 | P*ccrM-lacZ*/pGZ4 integrant (transcriptional reporter) ($kan^R$) | PhiCr30(LS178)→NA1000 |
| SM1095 | *ctrA* P1 P*ccrM-lacZ*/pGZ4 integrant (transcriptional reporter) ($kan^R$) | PhiCr30(LS178)→SM921 |
| SM1099 | P*dnaA-lacZ*/pRKlacZ290 (transcriptional reporter) ($tet^R$) | This study, conjugation of P*dnaA-lacZ*/pRKlacZ290 into NA1000 |
| SM1101 | *ctrA* P1 P*dnaA-lacZ*/pRKlacZ290 (transcriptional reporter) ($tet^R$) | This study, conjugation of P*dnaA-lacZ*/pRKlacZ290 into SM921 |
| SM1228 | P*ctrA* P2-*lacZ*/pRKlacZ290 (transcriptional reporter) ($tet^R$) | This study, conjugation of P*ctrA* P2-*lacZ*/pRKlacZ290 into NA1000 |
| SM1230 | P*gcrA* (−78+92)-*lacZ*/pRKlacZ290 (transcriptional reporter) ($tet^R$) | This study, conjugation of P*gcrA-lacZ*/pRKlacZ290 into NA1000 |
| SM1235 | *ctrA* P1 P*ctrA* P2-*lacZ*/pRKlacZ290 (transcriptional reporter) ($tet^R$) | This study, conjugation of P*ctrA* P2-*lacZ*/pRKlacZ290 into SM921 |
| SM1237 | *ctrA* P1 P*gcrA*(−78..+92)-*lacZ*/pRKlacZ290 ($tet^R$) | This study, conjugation of P*gcrA-lacZ*/pRKlacZ290 into SM921 |
| SM1268 | pMT464 (high copy vector) ($kan^R$) | Gift from Martin Thanbichler* |
| SM1324 | P*ctrA290* (=*ctrA* P1+P2-*lacZ*/pRKlacZ290 ($tet^R$) | This study, conjugation of P*ctrA-lacZ*/pRKlacZ290 into NA1000 |
| SM1345 | pMT464 P*ctrA* P2-*lacZ*/pRKlacZ290 (transcriptional reporter) ($kan^R$ $tet^R$) | This study, conjugation of P*ctrA* P2-*lacZ*/pRKlacZ290 into SM1268 |
| SM1347 | P*xyl*::*sciP*/pMT464 P*ctrA* P2-*lacZ*/pRKlacZ290 (transcriptional reporter) ($kan^R$ $tet^R$) | This study, conjugation of P*ctrA* P2-*lacZ*/pRKlacZ290 into MHT68 |
| SM1402 | P*ctrA* $P1^{wt}$ $P2^{wt}$-*lacZ*/pJC326C (translational reporter) ($tet^R$) | This study, electroporation of P*ctrA* $P1^{wt}$ $P2^{wt}$-*lacZ*/pJC326C into NA1000 |
| SM1403 | P*ctrA* $P1^{mut}$ $P2^{wt}$-*lacZ*/pJC326C (translational reporter) ($tet^R$) | This study, electroporation of P*ctrA* $P1^{mut}$ $P2^{wt}$-*lacZ*/pJC326C into NA1000 |
| SM1404 | P*ctrA* $P1^{wt}$ $P2^{mut}$-*lacZ*/pJC326C (translational reporter) ($tet^R$) | This study, electroporation of P*ctrA* $P1^{wt}$ $P2^{mut}$-*lacZ*/pJC326C into NA1000 |
| SM1405 | P*ctrA* P1-*lacZ*/pRKlacZ290 (transcriptional reporter) ($tet^R$) | This study, conjugation of P*ctrA* P1-*lacZ*/pRKlacZ290 into NA1000 |
| SM1407 | *ctrA* P1 P*ctrA* $P1^{mut}$ $P2^{wt}$-*lacZ*/pJC326C (translational reporter) ($tet^R$) | This study, electroporation of P*ctrA* $P1^{mut}$ $P2^{wt}$-*lacZ*/pJC326C into SM921 |
| ***E. coli* strains** | | |
| TOP10 | $F^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*ΔM15 Δ*lacX74 nupG recA1 araD139* Δ(*ara-leu*)7697 *galE15 galK16 rpsL*($Str^R$) *endA1* $\lambda^-$ | Invitrogen |
| DH10B | $F^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara leu*) 7697 *galU galK rpsL nupG* $\lambda^-$ | Invitrogen |
| S17-1 | $F^-$, lambda(−), *thi*, *pro*, *recA*, restriction (−), modification (+), RP4 derivative integrated into the chromosome with *Tet*::Mu, *Km*::Tn*7* | Simon *et al.* (1983) |
| LS1914 | DH10B pNPTS138 ($kan^R$) | C. Mohr, R. Roberts and L. Shapiro |
| LS2882 | S17-1 P*ctrA* $P1^{wt}$ $P2^{mut}$(referred to as *ctrA* P1-*lacZ*/pRKlacZ290) ($tet^R$) | Domian *et al.* (1999) |
| LS3146 | S17-1 P*ctrA* $P1^{wt}$ $P2^{mut}$ (referred to as *ctrA* P1) *lacZ*/pRKlacZ290 ($tet^R$) | Domian *et al.* (1999) |
| LS3147 | S17-1 P*ctrA* $P1^{mut}$ $P2^{wt}$ (referred to as *ctrA* P2) *lacZ*/pRKlacZ290 ($tet^R$) | Domian *et al.* (1999) |
| LS4322 | DH10B pJC326C (translational reporter vector) ($tet^R$) | Chen *et al.* (2006) |

**Table 1.** cont.

| Strain | Genotype and phenotype | Source or reference |
|---|---|---|
| LS4225 | S17-1 P*gcrA* (−78..+92)-*lacZ*/pRKlacZ290 (tet$^R$) | Collier *et al.* (2006) |
| SM1087 | S17-1 P*dnaA*-*lacZ*/pRKlacz290 (tet$^R$) | This study, electroporation of P*dnaA*-*lacZ*/pRKlacZ290 into S17-1 |
| SM1382 | *ctrA* P1 mutation/pNPTS138 (kan$^R$) | This study |
| SM1399 | TOP10 P*ctrA* P1$^{wt}$ P2$^{wt}$-*lacZ*/pJC326C (translational reporter) (tet$^R$) | This study |
| SM1400 | TOP10 P*ctrA* P1$^{mut}$ P2$^{wt}$-*lacZ*/pJC326C (translational reporter) (tet$^R$) | This study |
| SM1401 | TOP10 P*ctrA* P1$^{wt}$ P2$^{mut}$-*lacZ*/pJC326C (translational reporter) (tet$^R$) | This study |

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## METHODS

**Bacterial strains, plasmids, media, and synchronization.** Strains and plasmids are described in Table 1. *Caulobacter* cultures were grown in either peptone yeast extract (PYE) or minimal (M2G) medium (Ely, 1991), supplemented with 0.3 % (w/v) xylose (called PYEX or M2GX), tetracycline (1 μg ml$^{-1}$) or kanamycin (5 μg ml$^{-1}$) in a 28 °C incubator on a shaking platform rotating at 125 r.p.m. Ludox AS-40 colloidal silica (Sigma-Aldrich) density gradients were used to isolate swarmer cells from exponentially growing cultures grown in M2G broth (Evinger & Agabian, 1977). Swarmer cells were then used to inoculate M2G broth cultures.

**Mutational inactivation of the *ctrA* P1 promoter using PCR cloning.** The following primers were used to create a loss-of-function mutation (5 bp insertion, underlined) in the *ctrA* P1 promoter by sequential PCR. The upstream (US) and downstream (DS) primer sets were used to respectively amplify the US and DS sequences. Then a third PCR using the US and DS PCR products as template and the CtrAΔp1USFwd and CtrAΔp1DSRev primers produced a PCR product which was digested and ligated into the *sacB*-containing suicide vector pNPTS138. The primer sequences were as follows: CtrAΔp1USFwd, 5′-AAAAAAGCTTAACACGGCTCGCGCCTGGAGATGGGGCCGTT-3′; CtrAΔp1USRev, 5′-CGTCGGAGGAATGGTTAATCTGATGAAGCTTGCGAATCGGGTGCAAGCCGCGT-3′; CtrAΔp1DSFwd, 5′-ACGCGGCTTGCACCCGATTCGCAAGCTTCATCAGATTAACCATTCCTCCGACG-3′; CtrAΔp1DSRev, 5′-AAAACTTAAGTTGATGACCGACTGGGCGTGACCCTTCGAA-3′. The resulting PCR fragment was cloned into pNPTS138, electroporated into NA1000, and resolved using sucrose selection (Lesley & Shapiro, 2008). DNA sequencing of PCR products using the following primers identified colonies carrying the P1 mutation: CtrA-FL-Fwd, 5′-TGGCGTCATCGGCAGTCAACTT-3′; CtrA-FL-Rev, 5′-CTTAACGCTGTAACGCCGTTCG-3′.

**Construction of CtrA–LacZ protein fusion vectors.** The following primers were used to create wild-type *ctrA*, *ctrA* P1 only and *ctrA* P2 only translational reporters in pJC326C (Chen *et al.*, 2006). The forward primer amplifies the same nucleotides that are found in the 5′ end of the *ctrA* transcriptional reporters created earlier (Domian *et al.*, 1999). To amplify the wild-type and *ctrA* P1 mutant promoters, the following primers were used: *ctrA*-TNL-US-Fwd, 5′-AAAAAGATCTAGGCCTCGATTTTCTCGATTTCTT-3′; *ctrA*-TNL-DS-Rev, 5′-AAAACTGCAGATCCTCGATCAACAGTACGCGCAT-3′. The following primers were used to create a loss-of-function mutation (Domian *et al.*, 1999) (specific mutated nucleotides underlined) in the *ctrA* P2 promoter by sequential PCR. The upstream (US) and downstream (DS) primer sets were used to respectively amplify the US and DS sequences (relative to the mutation). Then a third PCR using the US and DS PCR products as template and the *ctrA*-TNL-US-Fwd and *ctrA*-TNL-DS-Rev primers (described above) produced a PCR product, which was digested and ligated into pJC326C. The primer pairs were as follows: US PCR, *ctrA*-TNL-US-Fwd (see above) and *ctrA*-TNL-US-P2mut-Rev (5′- TCCGCCGGTGAAACCCTTCGGCCACCCGGCCGGAGAGTTAATTTAAGACT-3′); DS PCR, *ctrA*-TNL-DS-P2mut-Fwd (5′-AGTCTTAAATTAACTCTCCGGCCGGGTGGCCGAAGGGTTTCACCGGCGGA-3′) and *ctrA*-TNL-DS-Rev (see above).

**Growth curves.** Single colonies from a streak plate were inoculated into 3 ml PYE or PYEX broth and were incubated overnight in a 28 °C incubator with 125 r.p.m. of rotational movement. $OD_{660}$ was measured (path length=1.5 cm) with an Amersham Novaspec III spectrophotometer. Cultures were diluted to $OD_{660}$ 0.300 and allowed to double before starting the growth curve at $OD_{660}$ 0.100. $OD_{660}$ was measured and dilutions were plated on PYE agar to determine c.f.u. ml$^{-1}$ at regular time intervals. For growth curves in M2G, colonies were first inoculated into 3 ml PYE or PYEX broth and then subcultured into M2G broth. The P*xylX*::*ctrA ctrA* P1 (SM904) depletion strain was grown in PYEX, then M2GX, as described above, and washed three times in M2G broth before being split into M2GX and M2G media. Cultures were incubated as described above.

**Microscopy.** Strains were grown as described above for growth curves. Cells were fixed for microscopy using a solution of 30 mM sodium phosphate buffer (pH 7.5) and 2.5 % formaldehyde, and viewed on 1 % agarose slides. Cell morphology was observed with a Zeiss AxioVision microscope with a Hamamatsu ORCA-ER digital camera using differential interference contrast settings. Fixed cells were incubated in the dark for 10 min with 2 μg DAPI ml$^{-1}$ to detect DNA and were aliquoted onto a 1 % agarose pad.

**Fluorescence activated cell sorting (FACS).** Samples for flow-cytometry were isolated from exponentially growing cells treated with rifampicin for 3 h as described by Winzeler & Shapiro (1995), except that DyeCycle Orange (Invitrogen) was used to stain the DNA as in a recent publication (Lesley & Shapiro, 2008). Data were collected using a FACStar Plus machine (Becton Dickinson) and analysed using FlowJo software (Tree Star). FACS results were obtained from triplicate or quadruplicate independent cultures.

**5′ Rapid amplification of cDNA ends (RACE) mapping.** RNA was isolated (Argueta *et al.*, 2006) from exponentially growing NA1000 and SM921 in M2G broth. 5′ RACE was performed as described by Argueta *et al.* (2006). The following *ctrA*-specific primers were used: 5′ RACE CtrA Rev, 5′-CCGCAGGGTGCGCAGAACATCGAT-3′; 5′ RACE CtrA Nested Rev, 5′-GCAGGATAAGATCGTAGTCGTAGAT-3′. The 5′ RACE CtrA nested Rev primer was used to sequence the 5′ RACE PCR products.

**Measurement of transcription.** The β-galactosidase activities (Miller units) of promoter–*lacZ* fusions were measured in exponentially

growing cultures in M2G supplemented with the appropriate antibiotic (Miller, 1972). $OD_{660}$ was measured and LacZ activity was recorded at 420 nm (path length=1 cm) using a Beckman Coulter DU 640 UV–visible spectrophotometer. *dnaA*, *gcrA* or *ctrA* transcription was measured from *lacZ* reporters on plasmid pRKlacZ290, whereas *ccrM* transcription was measured from a chromosomal *lacZ* reporter at the native locus (Table 1). Relative promoter activity was calculated by considering the mean Miller units for the wild-type for each transcriptional fusion as 100 % activity. Experiments were performed in triplicate or quadruplicate. Paired or unpaired Student's *t* tests were used to determine whether changes in transcription were significant. *P* values $<0.05$ were considered to be significant.

**Immunoblotting.** Immunoblot samples were taken from exponentially growing cells and were normalized based on optical density. To shut off P*xylX* expression of *ctrA* in SM904 (P*xylX*::*ctrA ctrA* P1), exponentially growing cells in M2GX were washed three times in M2G and then grown for 31 h before immunoblot samples were taken from exponentially growing cells. DnaA protein was resolved on 8 % SDS-PAGE, CcrM was resolved with 12 % SDS-PAGE, and GcrA/CtrA/SciP were resolved using 15 % SDS-PAGE (Sambrook *et al.*, 1989). Proteins were transferred to PVDF membranes (Millipore). Polyclonal antibodies (gifts from Lucy Shapiro, Stanford School of Medicine) reacting with DnaA, GcrA, CtrA, CcrM and SciP were detected with donkey anti-rabbit conjugated to horseradish peroxidase (Jackson ImmunoResearch). Serum for anti-DnaA was diluted 1 : 20 000; anti-GcrA was diluted 1 : 4000; anti-CtrA was diluted 1 : 20 000; anti-CcrM was diluted 1 : 5000; and anti-SciP was diluted 1 : 4000. A chemiluminescent reagent (PerkinElmer) and HyBlot CL autoradiography films (Denville Scientific) were used to view the blots. Films were scanned and image contrast and brightness were optimized with Photoshop (Adobe), and relative band intensities were determined using ImageJ (NIH, Bethesda, MD) (Barboriak *et al.*, 2005).

**Translation index.** Translational index experiments were performed as previously described (Anderson & Gober, 2000). Briefly, β-galactosidase activity from exponentially growing *Caulobacter* strains in M2G-tetracycline broth was measured as described above. The total Miller units from protein fusions were divided by the total Miller units produced from the promoter fusions to calculate the translation index. The protein fusions connect the first eight codons of CtrA to LacZ (and contain the native *ctrA* Shine–Dalgarno sequence), whereas the promoter fusions do not include the *ctrA* Shine–Dalgarno or any CtrA codons.

**CtrA half-life.** CtrA stability was measured using pulse–chase experiments. Exponentially growing cells were labelled for 10 min with 10 μCi $ml^{-1}$ (370 kBq) [$^{35}$S]methionine (PerkinElmer) and then chased with 1 mM unlabelled methionine and 0.2 mg Casamino acids $ml^{-1}$. One millilitre of culture was centrifuged for 2 min in a microfuge at 8000 r.p.m. every 10 min and frozen on dry ice. Labelled CtrA protein was immunoprecipitated as described by McGrath *et al.* (2006) and resolved on a 15 % SDS-PAGE gel. HyBlot CL autoradiography films (Denville Scientific) were used. Films were scanned and band intensities were measured using Image J (NIH, Bethesda, MD) (Barboriak *et al.*, 2005).

**Protein stability analysis by immunoblotting.** Protein stability was estimated using exponentially growing cells treated with 2 μg chloramphenicol $ml^{-1}$. Whole cell samples were taken every 30 min for 210 min and analysed by immunoblotting. Immunoblots were scanned and analysed with ImageJ (NIH, Bethesda, MD) to detect changes in protein accumulation over time.

**Real-time quantitative PCR (QPCR) analysis.** RNA was isolated from 1.5 ml of exponentially growing *Caulobacter* cultures using TRIzol (Life Technologies), following the manufacturer's recommended protocols. We used Superscript II Reverse Transcriptase (Life Technologies) to generate cDNAs. QPCR was performed as previously described (Cuajungco *et al.*, 2012) with minor modifications to suit our experiments. The amplification reactions were done in a Bio-Rad CFX96 machine using SensiMix SYBR Green Master Mix (Bioline) with the following thermocycling conditions: 5 min 95 °C followed by 40 cycles (30 s at 95 °C, 45 s at 55 °C, 30 s at 72 °C). The primer sets for *ctrA* and CC2677 (normalization control) genes were obtained from a recent publication (Tan *et al.*, 2010): *ctrA* Fwd, 5′-CCGACGACTACATGACCAAG-3′; *ctrA* Rev, 5′-AGCATCTGGTACTCCTTGCC-3′; CC2677 Fwd, 5′-TCTACTATCGCGGCTATCCC-3′; CC2677 Rev, 5′-GTACATGCGCGAGGTGTC-3′. The QPCR efficiency (*E*) and correlation coefficient ($R^2$) were: $E=94\pm2$ %, $R^2=0.99$ for *ctrA* ($n=2$); and $E=99\pm7$ %, $R^2=0.99$ for CC2677 ($n=2$).

## RESULTS

### The *ctrA* P1 mutant has severe growth and morphological defects

Five basepairs were inserted between the −35 and −10 sites of the *ctrA* P1 promoter to prevent transcription from P1, but not P2 (Domian *et al.*, 1999). This construct (in plasmid pNPTS138) was integrated into both wild-type and P*xylX*::*ctrA C. crescentus*. Screening of recombinants from *sacB* selections revealed that 5 % (2/40) of wild-type colonies contained the mutation in P1 compared with 50 % (10/20) of the P*xylX*::*ctrA* strain grown in the presence of xylose. The low percentage of mutants obtained in the wild-type was far from that expected for non-lethal mutations (50 %) because P1 mutants have a significantly slower doubling time (described below) than wild-type. We also linked a kanamycin marker to the P1 mutation (creating strain SM948) and were able to cotransduce the P1 mutation into wild-type NA1000.

The *ctrA* P1 mutant and wild-type strains were grown in both rich (PYE) and minimal (M2G) media to observe their growth characteristics. As shown in Fig. 2(a), growth of the *ctrA* P1 mutant in PYE was severely impaired in both biomass accumulation (measured by optical density) and doubling time (calculated from c.f.u. $ml^{-1}$) compared with wild-type. In PYE, the wild-type strain had a doubling time of $97\pm19$ min and the *ctrA* P1 mutant had a doubling time of $135\pm7$ min (1.4× longer). In contrast (Fig. 2b), in M2G, although no significant difference in biomass accumulation was observed, the doubling time of the wild-type was $113\pm10$ min and that of *ctrA* P1 was $141\pm15$ min (1.2× longer).

Microscopic observation revealed that the *ctrA* P1 mutant was elongated in PYE broth (Fig. 3a), with a mean length of $3.81\pm1.88$ μm ($n=176$; SD is high due to the presence of filamentous cells) and width of $0.76\pm0.09$ μm ($n=126$) compared with the mean length of $2.67\pm0.53$ μm ($n=100$) and width of $0.78\pm0.08$ μm ($n=99$) for wild-type. In contrast, in M2G, the *ctrA* P1 mutant cell length was $4.39\pm2.89$ μm ($n=1567$; SD is high due to the presence of

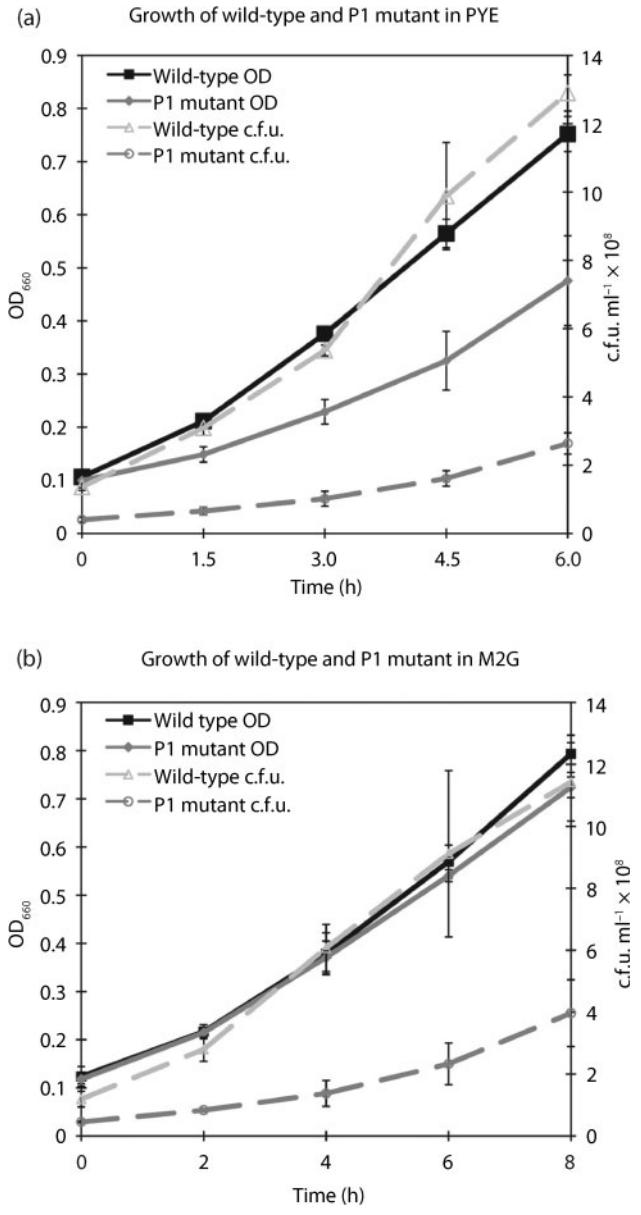


**Fig. 2.** Growth curves of the wild-type and *ctrA* P1 mutant in rich and minimal media. Exponentially growing NA1000 (wild-type) and SM921 (*ctrA* P1) were grown in PYE or M2G broth. Optical density (OD) was used to measure biomass and dilutions were plated onto PYE agar to determine c.f.u. $ml^{-1}$. NA1000 OD is shown with filled squares/solid lines; SM921 OD is shown with filled mid-grey diamonds/solid lines; NA1000 c.f.u. $ml^{-1}$ is shown with open light-grey triangles/dashed lines; SM921 c.f.u. $ml^{-1}$ is shown with open mid-grey circles/dashed lines. Means of triplicate experiments are shown with *y*-error bars. (a) The *ctrA* P1 mutant has significant biomass and c.f.u. growth defects in rich media broth. (b) The *ctrA* P1 mutant has significant c.f.u., but not biomass, growth defects in minimal media broth.

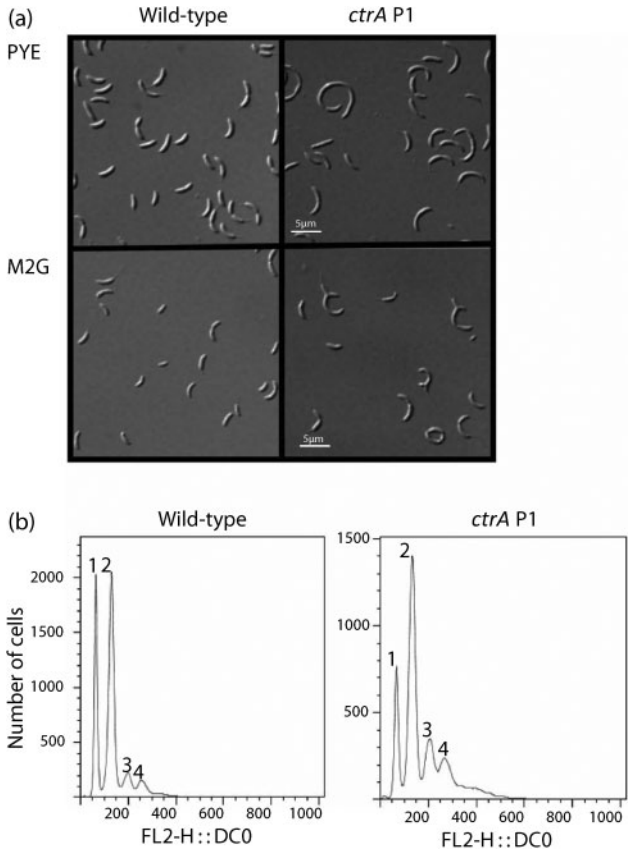


**Fig. 3.** (a) The *ctrA* P1 mutant is elongated and a subpopulation contains ectopic poles in both PYE and M2G broth; bars, 5 μm. (b) The *ctrA* P1 mutant cannot effectively silence the initiation of DNA replication. The number of cells containing one, two, three or four chromosomes in rifampicin-treated unsynchronized cultures is shown for the wild-type (NA1000) and *ctrA* P1 (SM921) strains. FL2-H : : DCO indicates peak emission values for DyeCycle Orange. Graphs are representative of FACS analysis from triplicate or quadruplicate cultures.

filamentous cells) compared with $2.25 \pm 0.62$ μm ($n$=1949) in wild-type in M2G broth. Cell width remained unchanged at $0.66 \pm 0.11$ μm ($n$=200) for *ctrA* P1 and $0.67 \pm 0.06$ μm ($n$=200) for wild-type. Additionally 1.4 % ($n$=2167) of *ctrA* P1 cells have ectopic poles compared with 0 % ($n$=2083) in wild-type. Ectopic pole formation presumably arises from defects in polar morphogenesis (Lam *et al.*, 2006) via CtrA-regulated proteins.

Additionally, the growth and morphological defects described here were recapitulated in the P*xylX*::*ctrA ctrA* P1 strain grown in the absence of xylose (which leads to the repression of P*xylX*) (Meisenzahl *et al.*, 1997). In sum, these results show that transcription from the P1 promoter of *ctrA* is required for efficient progression through the cell division cycle in both rich and minimal media. To avoid selection for suppressor mutations, all subsequent experiments were performed in M2G media, where no significant difference was observed in biomass accumulation between the P1 mutant and wild-type (Fig. 2b).

### The *ctrA* P1 mutant accumulates multiple chromosomes

Since the *ctrA* P1 mutant cells had growth defects and morphological abnormalities, we hypothesized that the mutant could have defective repression of DNA replication. To test this hypothesis, FACS analysis of rifampicin-treated mutant and wild-type cells in M2G was performed. The results confirmed our hypothesis, showing that a higher fraction of *ctrA* P1 mutant cells accumulated three to four chromosomes than the wild-type (Fig. 3b). We predicted that the overinitiation of DNA replication could result from reduced levels of CtrA in the P1 mutant.

### 5′ RACE confirms that only the P2 promoter is functional in the *ctrA* P1 mutant

To verify that the altered spacing between the −35 and −10 sites of the native *ctrA* P1 promoter prevents transcription from P1 but not P2 on the chromosome, we performed 5′ RACE. As shown in Fig. 4(a), wild-type 5′ RACE PCR products (confirmed with DNA sequencing) from exponentially growing cultures in M2G broth revealed bands representing P1 and P2 transcripts. In contrast to the wild-type, gels of *ctrA* P1 mutant 5′ RACE PCR products revealed only one strong band (representing P2 transcripts). Furthermore, 5′ RACE produced no evidence for transcription from the $P2^{FM}$ promoter, which was found to be transcribed only when the *ctrA* promoter was moved away from its native locus to a new location near the chromosomal terminus (Reisenauer & Shapiro, 2002). Thus, 5′ RACE confirmed that the 5 bp insertion prevents transcription from *ctrA* P1 but not P2. Interestingly, although the 5′ RACE PCRs were normalized for the quantity of mRNA and PCR reagents, we observed an increase (119 % of wild-type) in P2 PCR product abundance in the P1 mutant compared with wild-type, suggesting increased levels of P2 mRNA (as primers were added in excess, the amount of product may have been related to the quantity of cDNA template).

### Net *ctrA* transcription decreases by 50 % in the P1 mutant despite increased *ctrA* P2 transcription

*lacZ* promoter reporters were used to measure net *ctrA* transcription in the P1 mutant and wild-type. All transcriptional reporter data were tested for significance with paired Student's *t* tests (two-tailed). Fig. 4(b) shows that total *ctrA* transcription was reduced by 50 % ($P=0.001$) in the P1 mutant. These results were confirmed by two independent QPCR experiments, indicating that net *ctrA* transcription was reduced to 52 % of wild-type levels. *lacZ* promoter reporters were subsequently used to test whether there were any transcriptional changes for the core cell cycle master regulators in M2G. As shown in Fig. 4(b), a significant increase in *ctrA* P2 transcription (121 % of wild-type; $P=0.018$) confirmed the results suggested by 5′ RACE, and a significant decrease in *ccrM* transcription (78 % of wild-type; $P=0.020$) was observed in the P1 mutant. Reduced *ccrM* transcription is expected to correlate with reduced CtrA accumulation because CtrA activates the transcription of *ccrM*. Although *gcrA* transcription increased to 115 % of wild-type in the P1 mutant, this difference was not found to be significant ($P=0.147$). Additionally, no significant changes in *dnaA* transcription were observed (108 % of wild-type; $P=0.137$). Significantly, the increase in *ctrA* P2 transcription only partially compensated for the loss of transcription from *ctrA* P1, since the P1 mutant had lower net *ctrA* transcription and growth defects.

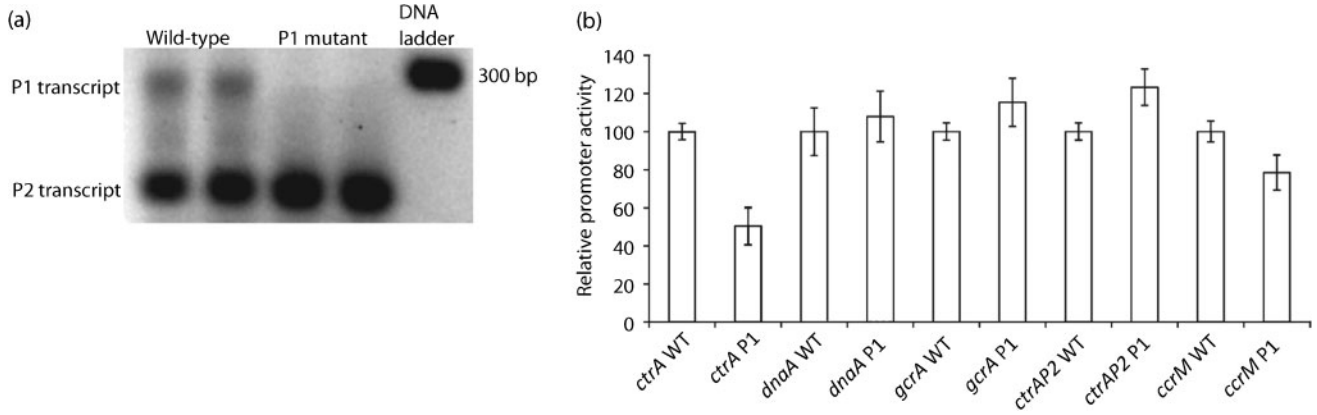


**Fig. 4.** (a) 5′ RACE reveals that the *ctrA* P1 mutant produces P2 but not P1 transcripts. The *ctrA* P1 PCR product is 290 nt, whereas the *ctrA* P2 PCR product is 233 nt. (b) Transcription of cell cycle master regulators in the wild-type and *ctrA* P1 mutant. Results of *lacZ* transcriptional reporters for *ctrA*, *dnaA*, *gcrA*, *ctrA* P2 and *ccrM* in the wild-type (WT) and *ctrA* P1 mutant are shown. For each promoter, transcription levels in the *ctrA* P1 mutant relative to wild-type (normalized to 100 %) are shown. Means of triplicate or quadruplicate experiments are shown with *y*-error bars.

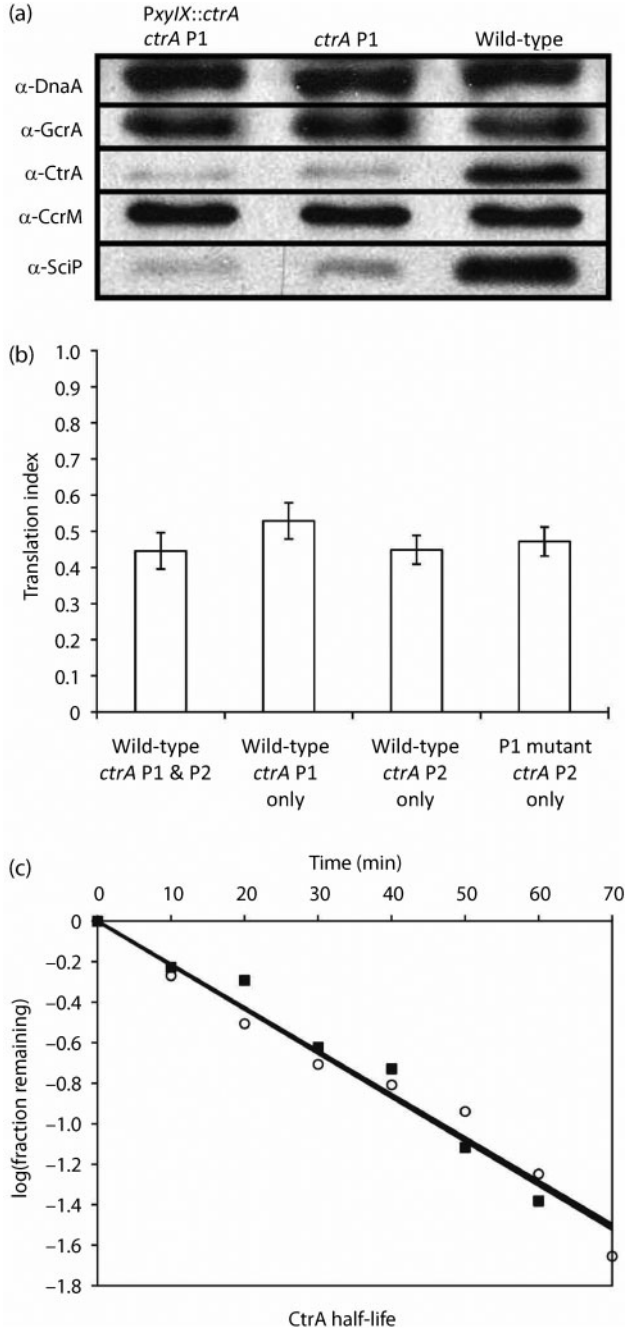


**Fig. 5.** (a) Accumulation of the cell cycle master regulators reveals a similar and significant reduction in CtrA and SciP but not DnaA, GcrA or CcrM accumulation. Immunoblots of cell lysates from exponentially growing wild-type (NA1000), *ctrA* P1 (SM921) and P*xylX*::*ctrA ctrA* P1 (SM904) mutants grown in M2G were normalized based on biomass (optical density) and probed with antibodies against DnaA, GcrA, CtrA, CcrM or SciP. Representative immunoblots (from one culture for each strain) of triplicate independent cultures are shown. (b) *ctrA* P1 mutants have no defect in *ctrA* translation initiation. The translation index reflects the average Miller units produced by translational reporters divided by the Miller units produced by transcriptional reporters in unsynchronized, exponentially growing cells. *y*-Error bars, SDs from quadruplicate trials. (c) CtrA half-life is similar to wild-type in the *ctrA* P1 mutant. CtrA has a half-life of 47 min in the wild-type (■; $R^2$=0.97) and 46 min in the *ctrA* P1 mutant (○; $R^2$=0.97). Means of results from triplicate experiments are shown.

## The CtrA and SciP master regulators have reduced accumulation in the *ctrA* P1 mutant, but the CcrM, DnaA and GcrA master regulators accumulate to wild-type levels in minimal media

To test if the transcriptional changes noted in Fig. 4(b) affected protein accumulation, whole-cell protein lysates were normalized based on cell density and probed by immunoblotting for the DnaA, GcrA, CtrA, CcrM and SciP master regulators (Fig. 5a). The blots shown are from one representative culture from each strain. Relative accumulation was determined by averaging the results from six independent cultures for *ctrA* P1 (three of SM921 and three of SM904) and three independent cultures of the wild-type (NA1000). As expected, CtrA levels were significantly reduced, reaching only 22±7 % of wild-type levels in both the *ctrA* P1 and P*xylX*::*ctrA ctrA* P1 mutants in M2G (the *xylX* promoter is not transcribed in M2G; Meisenzahl *et al.*, 1997). Interestingly, SciP levels decreased to 19±9 % of wild-type in the *ctrA* P1 mutants, which is very similar to the reduction in CtrA accumulation. The accumulation of DnaA, GcrA and CcrM was similar in the wild-type and *ctrA* P1 mutants, despite the drastic reduction in CtrA and SciP in the P1 mutants.

## *ctrA* translational initiation and CtrA half-life are unaffected in the *ctrA* P1 mutant

In an attempt to address the discrepancy between net *ctrA* transcription (50 % of wild-type) and CtrA abundance (22 % of wild-type) in the P1 mutant, we determined the translation index (Anderson & Gober, 2000) for *ctrA*. As shown in Fig. 5(b), no significant changes were noted between *ctrA* translation initiation in wild-type and *ctrA* P1 mutant cells. Both strains had a translation index of ~0.46. Finding no change in translation initiation, we measured CtrA half-life. As shown in Fig. 5(c), CtrA stability was unaltered in the *ctrA* P1 mutant, with a half-life of ~46.5 min in both strains. Thus, neither translation initiation nor protein stability explains the discrepancy between *ctrA* transcription and CtrA accumulation in the P1 mutant, suggesting that differences exist at another level of regulation.

## DnaA, GcrA and CcrM stability appear to be unaltered in the *ctrA* P1 mutant

CcrM protein levels were similar in wild-type and *ctrA* P1 mutant cultures (Fig. 5a), despite a reduction in *ccrM*

transcription (78 % of wild-type in Fig. 4b). We thus hypothesized that CcrM could be more stable in the P1 mutant compared with wild-type. To test this hypothesis and to see if there were any changes in the stability of DnaA or GcrA, we treated exponentially growing *Caulobacter* cells grown in M2G with 2 μg chloramphenicol $ml^{-1}$, which prevents *de novo* protein synthesis, and followed the accumulation of the four core *Caulobacter* master regulators over time by immunoblot analyses. We compared CtrA stability in a *rcdA*::Ω mutant (LS4185; CtrA lacks cell cycle-regulated degradation in this mutant) and wild-type, and found that CtrA was significantly stabilized in the RcdA mutant using this approach (results not shown). However, no significant differences were found in triplicate experiments comparing the stability of DnaA, GcrA, CtrA and CcrM by immunoblot analyses (results not shown).

## Reduced SciP accumulation in the *ctrA* P1 mutant likely contributes to increased *ctrA* P2 transcription

Based on recent studies that show that SciP inhibits the transcription of *ctrA* (Gora *et al.*, 2010; Tan *et al.*, 2010) and the 81 % decrease in SciP accumulation in our *ctrA* P1 mutants, we hypothesized that the increase in *ctrA* P2 transcription could be due to decreased levels of SciP. To test this, we measured *ctrA* P2 transcription for 6 h in a strain that overexpressed SciP and found that transcription was significantly reduced to 65±7 % ($P$=0.023) of the levels found in an isogenic strain carrying an empty vector (Fig. 6a). The significant reduction in SciP accumulation in the P1 mutant likely contributes to increased *ctrA* P2 transcription. We were unable to create a SciP depletion strain under the control of either the xylose or vanillate promoter at the *sciP* locus and therefore were unable to measure *ctrA* P2 transcription when SciP was depleted.

## CtrA functions as a rheostat to control SciP levels

Since CtrA and SciP accumulation decreased by similar levels in the P1 mutant, we suspected that CtrA may act as a rheostat for SciP, just as SciP has been shown to be a rheostat for controlling CtrA accumulation (Tan *et al.*, 2010). To test this hypothesis, we partially depleted CtrA in our P*xylX*::*ctrA ctrA* P1 mutant (SM904) and monitored CtrA and SciP accumulation over time. We found that the relative abundance of CtrA and SciP decreased in synchrony (Fig. 6b). Thus, the levels of CtrA and SciP are tightly and coordinately regulated.

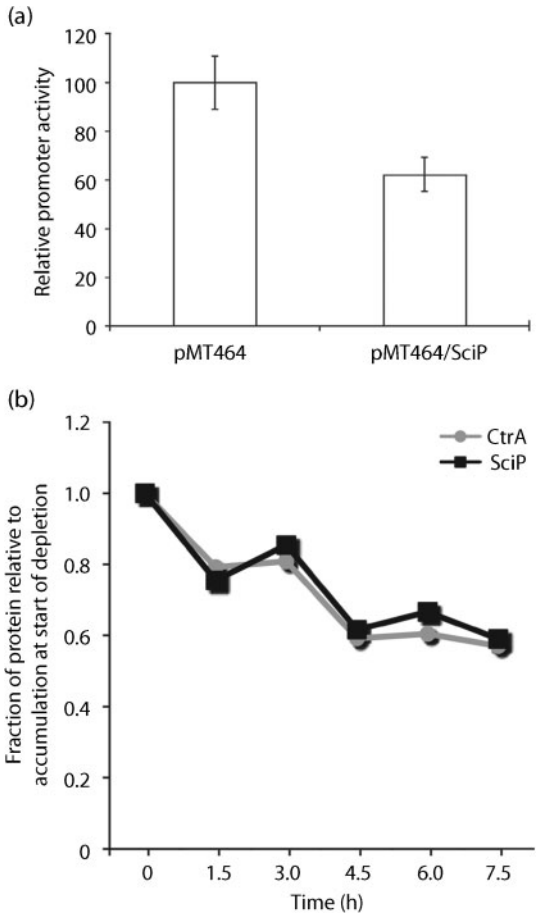


**Fig. 6.** (a) SciP represses transcription of *ctrA* P2, suggesting that reduced accumulation of SciP in the *ctrA* P1 mutant may lead to increased *ctrA* P2 transcription. Xylose (0.3 %, w/v) was added to exponentially growing cultures of SM1345 (NA1000 pMT464 P*ctrA*P2-*lacZ*/pRKlacZ290) and SM1347 (NA1000 P*xylX*::*sciP*/pMT464 P*ctrA*P2-*lacZ*/pRKlacZ290) in PYE broth supplemented with kanamycin and tetracycline. *ctrA* P2 transcription was measured with a β-galactosidase assay at 6 h after P*xylX*::*sciP* overexpression with xylose. Means from quadruplicate independent cultures are shown with *y*-error bars. (b) SciP and CtrA accumulation decrease in synchrony as CtrA protein levels are reduced. SM904 (P*xylX*::*ctrA ctrA* P1) was grown in M2G xylose, washed three times, and split into M2G and M2G xylose. Immunoblot samples normalized for optical density were taken from M2G broth every 1.5 h for 7.5 h. Relative levels of CtrA and SciP were quantified using ImageJ (NIH, Bethesda, MD). Relative CtrA abundance is represented by grey circles/lines and relative SciP abundance is indicated with black squares/lines. Data points represent the average of duplicate trials.

## *ctrA* transcription peaks 0.125 cell unit later during the cell cycle in the *ctrA* P1 mutant

The experiments described above focused on the quantity of CtrA produced. To detect temporal changes in *ctrA* transcription during the cell cycle, *ctrA* mRNA levels were assessed relative to CC2677 mRNA, which is not cell cycle-regulated (Tan *et al.*, 2010), using QPCR. Since the wild-type and P1 mutant have different doubling times that reflect their distinct rates for cell cycle progression, the cell cycles were normalized. Samples were taken every 20 min from wild-type but every 23 min from the *ctrA* P1 mutant in M2G broth (determined by synchronizing

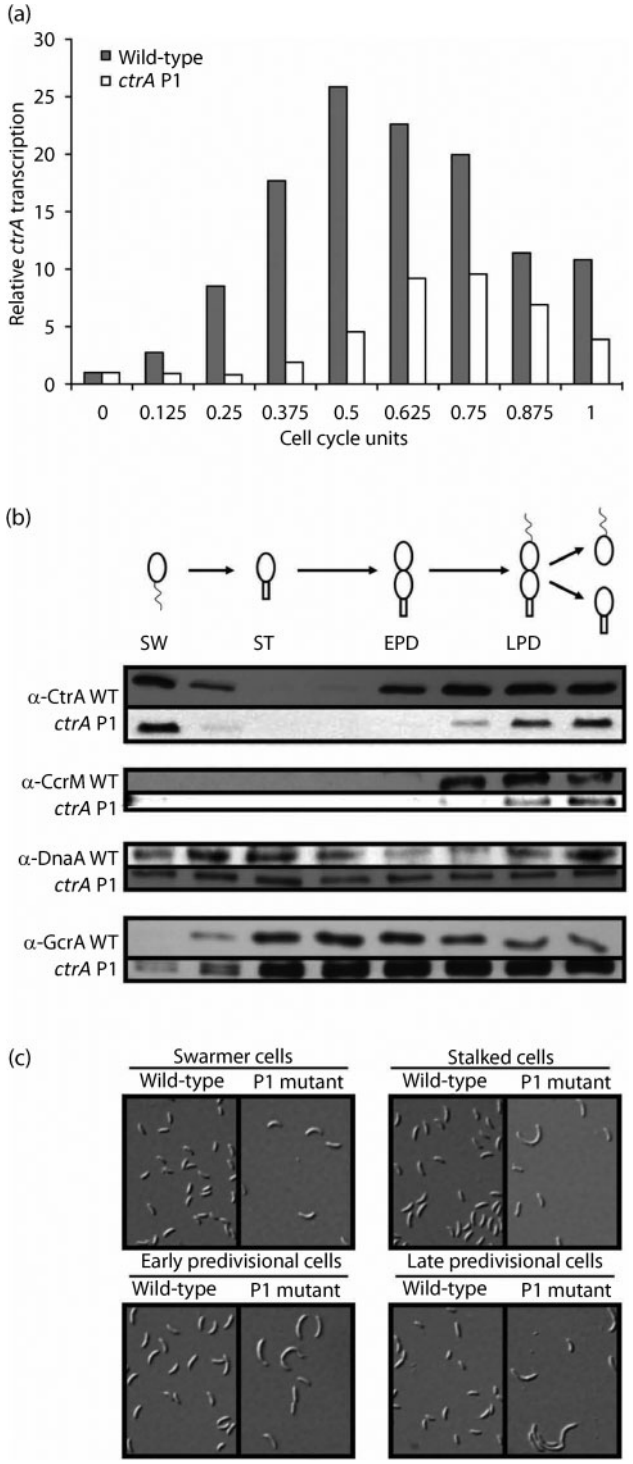


**Fig. 7.** (a) Transcription of *ctrA* during the cell cycle: *ctrA* transcription peaks 0.125 cell unit later during the cell cycle in the *ctrA* P1 mutant. Wild-type (NA1000) and *ctrA* P1 (SM921) cultures were synchronized. RNA samples for QPCR were taken from the wild-type every 20 min and from the *ctrA* P1 mutant every 23 min. *ctrA* mRNA levels were normalized to the amount of *ctrA* mRNA present at 0 min (swarmer cells), representing one unit. Averages from independent duplicate experiments are shown. (b) CtrA and CcrM have a 0.125 cell cycle unit delay in their accumulation in predivisional cells. Immunoblot samples were probed with antisera for CtrA, CcrM, DnaA and GcrA as indicated. The blots shown are from one representative culture of each strain. WT, wild-type. (c) *ctrA* P1 mutant cells are ~1.3× elongated at all stages of the cell cycle. Synchronized cells were fixed at 0 (SW), 40 (ST), 80 (EPD) and 120 min (LPD) for wild-type (NA1000), and at 0 (SW), 46 (ST), 92 (EPD) and 138 min (LPD) for *ctrA* P1 (SM921). Differential interference contrast images are from one synchrony of wild-type and one of *ctrA* P1. They are representative of three independent experiments for each strain. Abbreviations used above: SW, swarmer, ST, stalked, EPD, early predivisional, LPD, late predivisional cells.

cultures and observing the optical density increase over time). This allows for the comparison of cell cycle units. As shown in Fig. 7(a), *ctrA* mRNA peaked at 0.500 cell cycle unit in wild-type (corresponding to 80 min). In contrast, *ctrA* mRNA peaked at 0.625 unit in the P1 mutant (corresponding to 115 min).

### There is a 0.125 unit delay in the reappearance of both CtrA and CcrM in synchronized *ctrA* P1 cultures

To test whether the temporal accumulation of master regulators was altered, as expected for CtrA by the delay in *ctrA* transcription, *ctrA* P1 and wild-type cultures were synchronized in M2G broth. The blots shown are from one representative culture of each strain. Immunoblots for CtrA, CcrM, DnaA and GcrA are shown in Fig. 7(b) for synchronized cultures of wild-type and *ctrA* P1. All master regulators oscillate, but there is a 0.125 cell cycle unit delay in the reappearance of both CtrA and CcrM at the end of the cell cycle. A similar delay in the reappearance of CtrA was also observed when *ctrA* was moved from its normal location, near the origin, to a new location near the terminus that remains fully methylated during the cell cycle (Reisenauer & Shapiro, 2002). However, the only morphological change noted for the *ctrA* terminus mutant was elongated swarmer cells. In contrast, the defects in our P1 mutant at the native locus were more pronounced. Cells at all stages of the cell cycle, not just swarmer cells (as found when *ctrA* was moved near the terminus), were elongated ~1.3-fold (Fig. 7c). This suggests that CtrA accumulation and/or oscillation contribute to the coordination of molecular events that modulate cell size.

## DISCUSSION

This study illustrates the robustness of the *Caulobacter* cell cycle. Although CtrA levels decrease by 78 % and SciP levels decrease by 81 % in the *ctrA* P1 mutant, accumulation of CcrM, DnaA and GcrA is similar to wild-type. Decreased CtrA, but not DnaA, protein levels result in a population of P1 mutant cells accumulating three to

four chromosomes per cell. When CtrA is depleted in a P*xylX*::*ctrA ctrA* P1 mutant, SciP levels decrease in a synchronized manner with CtrA, suggesting that CtrA acts as a rheostat to control the accumulation of SciP. Two examples of robustness include high levels of *ccrM* transcription (78 % of wild-type) despite low CtrA accumulation (22 % of wild-type) and increased *ctrA* P2 transcription (121 % of wild-type) in the P1 mutant. Although Reisenauer *et al.* (1999) showed that CtrA has lower affinity for P*ccrM* than P*fliQ*, our results suggest that CtrA may have a higher affinity for the *ccrM* promoter than for many other CtrA targets. The fact that low CtrA levels do not translate to low CcrM levels allows for *Caulobacter* to continue the cell cycle with high levels of most master regulators. If CcrM levels were reduced as much as CtrA, the other cell cycle regulators would likely follow suit, possibly resulting in more severe growth defects. Reisenauer *et al.* (1999) proposed that the lower affinity of CtrA for P*ccrM* than P*fliQ* contributes to the 0.125 cell cycle unit delay in the transcription of *ccrM* after the reappearance of CtrA in early predivisional cells. In the *ctrA* P1 mutant, a similar delay in CcrM accumulation is observed, suggesting that delayed *ccrM* transcription results from temporally altered CtrA accumulation. In the second example of robustness, we showed that CtrA acts as a rheostat to control the levels of SciP. Presumably, increased transcription from *ctrA* P2 arises from reduced repression by SciP. Interestingly, SciP represses transcription from P*ccrM* (Gora *et al.*, 2010). Thus, reduced SciP-mediated repression of P*ccrM* in the P1 mutant may contribute to the robustness of *ccrM* transcription.

There are five CtrA binding sites within the *Caulobacter* chromosomal origin of replication (*Cori*) (Bastedo & Marczynski, 2009; Spencer *et al.*, 2009). It has been reported that CtrA binding to three of these sites represses DNA replication on plasmids but not on the chromosome (Bastedo & Marczynski, 2009). This suggests that there are additional, chromosome-specific factors that help CtrA to repress DNA replication because there is still a block in the initiation of replication at *Cori* even when CtrA cannot bind to *Cori*. Our FACS results (showing that decreased CtrA accumulation results in the overinitiation of chromosomal DNA replication) suggest that CtrA directly or indirectly controls these other factors that help CtrA to restrict DNA replication to once, and only once, per cell cycle.

The reduced levels of CtrA are not solely explained by a reduction in *ctrA* transcription. In an attempt to resolve the discrepancy between *ctrA* transcription (50 % of wild-type) and CtrA accumulation (22 % of wild-type), we tested for a defect in translation initiation and for increased proteolysis in the P1 mutant. However, no changes between translation initiation and proteolysis were observed. Therefore, we hypothesize that translation elongation may be affected; however, our translational constructs only contained the first eight codons of CtrA. It is possible that the missing *ctrA* codons in our constructs could provide a basis for regulation of translation/mRNA stability by factors such as small RNAs. If substantiated, this would provide yet another level of regulation for CtrA synthesis.

In sum, this study (a) identified two examples of regulation that contribute to the robustness of the cell cycle; (b) suggests that the proposed non-CtrA DNA replication repressors (Bastedo & Marczynski, 2009) may be CtrA regulated; and (c) hints at possible regulation of *ctrA* translation elongation or mRNA stability. The robustness of the cell cycle is quite impressive, and our data suggest an uncharacterized genetic circuitry that allows survival under stressful conditions. *Caulobacter* is clearly well prepared for the challenge, as its ability to adapt lies in the robustness and flexibility of its cell cycle genetic circuitry.

## ACKNOWLEDGEMENTS

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