

# DNA methylation affects the cell cycle transcription of the CtrA global regulator in *Caulobacter*

Ann Reisenauer<sup>1</sup> and Lucy Shapiro

Developmental Biology, Stanford University, Stanford, CA 94305-5329, USA

<sup>1</sup>Corresponding author  
e-mail: reisen@cmgm.stanford.edu

**The *Caulobacter* chromosome changes progressively from the fully methylated to the hemimethylated state during DNA replication. These changes in DNA methylation could signal differential binding of regulatory proteins to activate or repress transcription. The gene encoding CtrA, a key cell cycle regulatory protein, is transcribed from two promoters. The P1 promoter fires early in S phase and contains a GAnTC sequence that is recognized by the CcrM DNA methyltransferase. Using analysis of CcrM mutant strains, transcriptional reporters integrated at different sites on the chromosome, and a *ctrA* P1 mutant, we demonstrate that transcription of the P1 promoter is repressed by DNA methylation. Moreover moving the native *ctrA* gene to a position near the chromosomal terminus, which delays the conversion of the *ctrA* promoter from the fully to the hemimethylated state until late in the cell cycle, inhibited *ctrA* P1 transcription, and altered the time of accumulation of the CtrA protein and the size distribution of swarmer cells. Together, these results show that CcrM-catalyzed methylation adds another layer of control to the regulation of *ctrA* expression.**

**Keywords:** *Caulobacter crescentus*/CcrM/CtrA response regulator/DNA methylation

## Introduction

The interaction of regulatory proteins and methylated DNA is important to cell physiology in both eukaryotes and prokaryotes. In eukaryotes, a major consequence of chromosome methylation is transcriptional silencing (Bird and Wolffe, 1999). In prokaryotes, DNA methyltransferases (MTases) are best known for their role in restriction–modification systems (Bickle and Kruger, 1993). However, these enzymes also have regulatory roles in the bacterial cell. Two examples of regulatory MTases are the *Escherichia coli* Dam and the *Caulobacter crescentus* CcrM proteins. Neither Dam nor CcrM have known cognate restriction enzymes, but rather these proteins act to coordinate cell cycle events. Dam methylation governs several cellular functions, including the initiation of DNA replication (Barras and Marinus, 1989; Boye and Lobner-Olesen, 1990) and the transcription of certain genes, such as the *pap* pili operon in uropathogenic *E. coli* (Nou *et al.*, 1993; Braaten *et al.*, 1994) and plasmid-encoded fimbriae (Pef) in *Salmonella typhimurium* (Nicholson and Low, 2000). In addition,

Dam methylation regulates Tn10 transposition by altering the activity of the transposase promoter (Roberts *et al.*, 1985). Dam is also required for virulence in *S. typhimurium*, where it either directly or indirectly controls the expression of a number of genes that are induced during infection (Garcia-Del Portillo *et al.*, 1999; Heithoff *et al.*, 1999).

The CcrM MTase, which methylates the adenine in GAnTC target sequences, is widespread among  $\alpha$ -proteobacteria and has been shown to be essential for viability in *C. crescentus*, *Sinorhizobium meliloti*, *Brucella abortus* and *Agrobacterium tumefaciens* (Stephens *et al.*, 1996; Wright *et al.*, 1997; Robertson *et al.*, 2000; Kahng and Shapiro, 2001). CcrM activity is cell cycle regulated in both *C. crescentus* and *A. tumefaciens* (Stephens *et al.*, 1996; Kahng and Shapiro, 2001). In *Caulobacter*, this enzyme is present and is active only at the end of S phase when it brings the newly replicated DNA from the hemimethylated to the fully methylated state (Stephens *et al.*, 1996). CcrM is restricted to this period of the cell cycle by three regulatory mechanisms: activation of *ccrM* transcription by the CtrA response regulator (Quon *et al.*, 1996; Reisenauer *et al.*, 1999), inhibition of *ccrM* transcription by methylation of the GAnTC sites immediately downstream of the transcription start site (Stephens *et al.*, 1995), and rapid proteolysis of the CcrM protein (Wright *et al.*, 1996). In mutants that express CcrM throughout the cell cycle, the control of DNA replication initiation is relaxed and the cells have abnormal morphology (Zweiger *et al.*, 1994), suggesting that differential CcrM methylation helps to regulate these processes. Although CcrM is required for viability, the essential functions of this MTase are unknown.

In *Caulobacter*, cell differentiation is coordinated with progression through the cell cycle (see Figure 3B). The motile swarmer cell present in G<sub>1</sub> phase ejects its flagella and differentiates into a non-motile stalked cell. During the swarmer to stalked cell (G<sub>1</sub>–S) transition, chromosome replication is initiated on a fully methylated chromosome. As the stalked cell progresses through S phase, a new flagellum is assembled at the pole opposite the stalk. Consequently, two distinct cell types are produced at each cell division, a replication-repressed swarmer cell and a stalked cell, which immediately begins another round of DNA synthesis (Hung *et al.*, 2000). The chromosome is fully methylated at the start of replication, but progressively becomes hemimethylated as replication proceeds bidirectionally from the origin to the terminus (Dingwall and Shapiro, 1989). Re-methylation of the newly synthesized DNA is restricted to the end of S phase when the CcrM MTase is synthesized (Stephens *et al.*, 1996; Marczyński, 1999). This successive change in the methylation state of the chromosome during S phase reflects the progression of DNA replication.

There are nearly 4500 GAnTC sites in the *Caulobacter* genome, whereas ~12 000 sites are expected statistically (Nierman *et al.*, 2001). In addition, 22% of these sites are found in the 10% of the genome located between open reading frames. The concentration of the limited number of GAnTC sites in intergenic DNA suggests that changes in the methylation state of these sites may alter the interactions of regulatory proteins with their target DNA. To explore the possibility that DNA methylation plays a role in controlling transcription in *Caulobacter*, we examined temporally regulated genes that have GAnTC sites in their promoter regions. These genes include *ctrA*, encoding a global transcriptional regulator (Quon *et al.*, 1996), *ftsZ*, encoding a tubulin-like protein required for cell division (Quardokus *et al.*, 1996), and *groESL*, encoding a molecular chaperone (Avedissian and Gomes, 1996). Of these candidate genes, the transcription of *ctrA* and *ftsZ* changed in response to changes in the methylation state of the chromosome.

The CtrA response regulator directly controls the transcription of at least 55 operons (Laub *et al.*, 2002), including those required for DNA methylation (*ccrM*), cell division (*ftsZ*), and flagella and pili biogenesis (Quon *et al.*, 1996; Kelly *et al.*, 1998; Laub *et al.*, 2000; Skerker and Shapiro, 2000). CtrA also prevents the initiation of DNA replication in swarmer cells by binding to the *Caulobacter* origin of replication (*Cori*; Quon *et al.*, 1998). CtrA activity during the cell cycle is highly regulated. The transcription of *ctrA* is controlled by feedback regulation (Figure 1A). At the beginning of S phase, *ctrA* is transcribed from the *ctrA* P1 promoter, which contains a GAnTC site near the -35 region. As CtrA protein accumulates during S phase, it activates transcription from the *ctrA* P2 promoter and represses the P1 promoter (Domian *et al.*, 1999). The activity of this global transcriptional regulator in turn is governed by temporally controlled phosphorylation and targeted proteolysis

(Domian *et al.*, 1997). Here we show that the methylation state of the P1 promoter adds another layer of control to the regulation of CtrA expression.

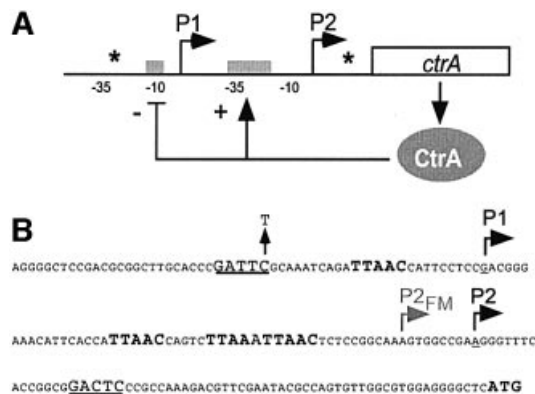
## Results

### *ctrA* promoter activity is altered in *CcrM* mutant strains

The gene encoding the CtrA response regulator is transcribed from two promoters, P1 and P2, which are expressed at different times during the *Caulobacter* cell cycle (Domian *et al.*, 1999). CtrA binds to consensus motifs in each promoter (Figure 1A, light gray boxes), repressing the transcription of the early P1 promoter and activating P2 transcription. In addition, there are two GAnTC sites (shown by the asterisks in Figure 1A) that are found at -29 relative to the P1 promoter and at +16 relative to the P2 promoter. Their location in the *ctrA* promoter suggests that changes in the methylation of these sites could influence *ctrA* transcription.

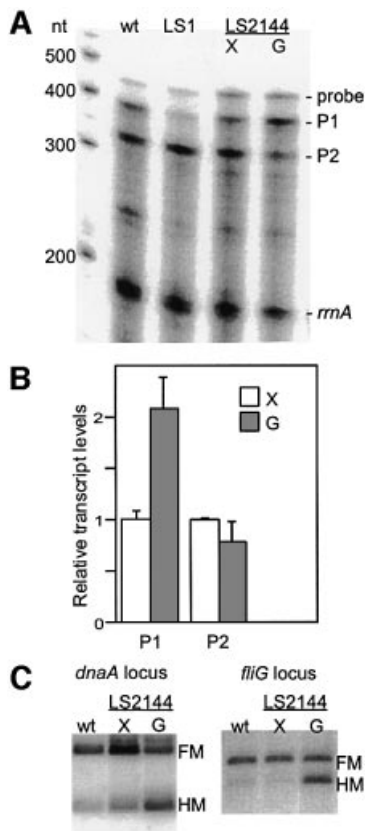
To test the effect of maintaining the chromosome in the fully methylated state throughout the cell cycle on *ctrA* transcription, we expressed *ccrM* constitutively using strain LS1. In the LS1 strain, two copies of *ccrM* are present on the chromosome: one is expressed from its native promoter, and the other is expressed from a constitutive  $P_{lac}$  promoter. As a result, *ccrM* is transcribed continually and chromosomal DNA is maintained in the fully methylated state throughout the cell cycle (Zweiger *et al.*, 1994). RNase protection assays showed that the *ctrA* P1 transcript was significantly reduced but the P2 transcript was unaffected under these conditions (Figure 2A). In this experiment, RNA isolated from the wild-type and LS1 strains was probed with  $^{32}P$ -labeled antisense RNA probes for *ctrA* and *rrnA*. The 16S ribosomal RNA (*rrnA*) probe is an internal control used to normalize for differences in the amount of RNA applied to each lane.

To analyze *ctrA* P1 and P2 promoter activity when CcrM is depleted, we used strain LS2144 in which the chromosomal *ccrM* locus is inactivated and *ccrM* under the control of the conditional *xylX* promoter is present on a low copy number plasmid (Stephens *et al.*, 1996). Transcription of  $P_{xylX}$  is induced by xylose (Meisenzahl *et al.*, 1997). When LS2144 cultures are shifted from growth in peptone-yeast extract (PYE) + 0.1% xylose (PYEX) to PYE + 0.1% glucose (PYEG), cell viability drops after 4 h and cell growth ceases after 6–8 h (Stephens *et al.*, 1996). In addition, CcrM protein levels fall. To confirm that growth of this strain in PYEG reduces CcrM activity and methylation of the chromosome, we examined the methylation state of two sites on the chromosome, the *dnaA* and *fliG* loci, using an overlapping restriction site assay (Campbell and Kleckner, 1990; Zweiger and Shapiro, 1994). As shown in Figure 2C, hemimethylated DNA (HM) at these sites increased 4- to 6-fold when cultures were shifted to PYEG for 3 h, demonstrating that methylation of the chromosome was impaired when CcrM was depleted. We used RNase protection assays to compare *ctrA* P1 and P2 mRNA levels in the wild-type strain and strain LS2144 with xylose-dependent expression of CcrM. As shown in



**Fig. 1.** Feedback control of *ctrA* transcription. (A) Diagram of the *ctrA* promoter region. The P1 and P2 transcription start sites are indicated by bent arrows, GAnTC sites are marked by asterisks, and CtrA binding sites are shown as gray boxes. As CtrA protein (gray oval) accumulates during S phase, it activates transcription from P2 and inhibits P1 transcription. (B) Nucleotide sequence of the *ctrA* promoter. The P1 and P2 transcription start sites (bent black arrows) and the P2<sub>FM</sub> alternate start site described in this study (bent gray arrow) are marked. CcrM methylation sites are underlined and the C(-25)T mutation in P1 is indicated. CtrA recognition motifs and the translation start site are in bold.

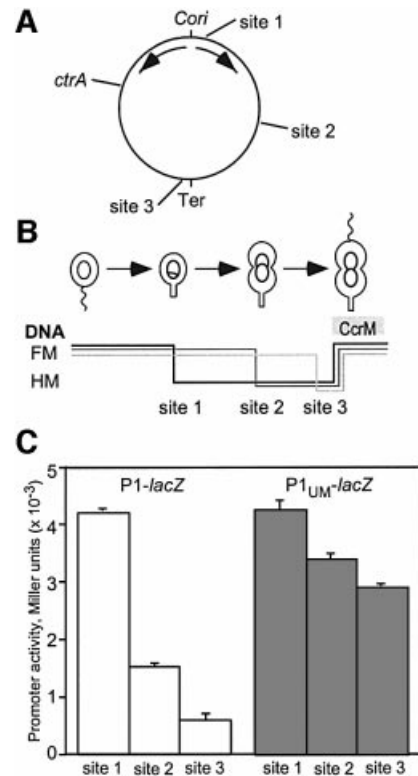
Figure 2A, transcription from the *ctrA* P1 promoter increased when CcrM was depleted (LS2144 cultures grown in PYEG). The ratio of P1 to total (P1 + P2) *ctrA* transcripts in this experiment was 0.38 (wild-type cultures), 0.14 (LS1 cultures), 0.34 (LS2144 cultures grown in PYEX) and 0.61 (LS2144 cultures grown in PYEG). The bar graph in Figure 2B summarizes the results of three separate RNase protection experiments using the CcrM depletion strain LS2144. As CcrM was depleted, *ctrA* P1 transcript levels doubled, but there was no substantial change in *ctrA* P2 mRNA levels. Thus both depleting CcrM and expressing the enzyme throughout the cell cycle altered *ctrA* P1 transcription, suggesting that CcrM methylation either directly or indirectly affects the transcription of this promoter. Because P2 transcription did not change in these experiments, we focused on the effect of the DNA methylation state on P1 transcription in subsequent experiments.



**Fig. 2.** *ctrA* transcript levels in CcrM mutant strains. (A) Representative phosphorimage of *ctrA* and *rrmA* transcripts assayed by RNase protection in wild-type (wt), LS1 and LS2144 cultures grown in PYE + 0.1% xylose (X) or PYE + 0.1% glucose for 3 h (G). The excess, undigested *ctrA* probe and transcripts from the P1 and P2 promoters are marked. The 16S ribosomal RNA (*rrmA*) was probed as an internal control.  $^{32}$ P-labeled ssDNA markers are shown on the left. (B) Quantitation of *ctrA* P1 and P2 transcripts in LS2144 cultures grown in PYE + xylose (X) or glucose (G) using a PhosphorImager. Values were normalized using the *rrmA* internal control and expressed relative to the PYEX value. Data are the mean  $\pm$  SD of three experiments. (C) Southern blots showing the methylation state of the *dnaA* and *fliG* chromosomal loci in wild-type cultures (wt), and in LS2144 cultures grown in PYE + xylose (X) or glucose (G). FM and HM mark fully methylated and hemimethylated DNA, respectively.

### Transcription of a *ctrA* P1-*lacZ* fusion integrated at different chromosomal locations

The previous experiments using *ccrM* mutant strains showed that changing the timing or level of CcrM expression altered *ctrA* P1 transcription. To test the possibility that *ctrA* P1 transcription is directly regulated by the methylation state of the GAnTC site in the *ctrA* P1 promoter, we constructed a P1 transcription probe containing an  $\Omega$ -*ctrA* P1-*lacZ* transcriptional fusion (pAR263). This reporter was integrated at three different sites on the chromosome: near the origin (site 1, generating NA1000 *hrcA* $\Omega$ ::pAR263), approximately halfway between the origin and the terminus (site 2, generating NA1000 *recA*::Tn5 $\Omega$ ::pAR263), and near the terminus (site 3, generating NA1000 *trpE*::Tn5 $\Omega$ ::pAR263). The position of these sites relative to *Cori* is shown in Figure 3A. In each of these strains, chromosomal *ccrM* is transcribed from its native promoter so the timing and

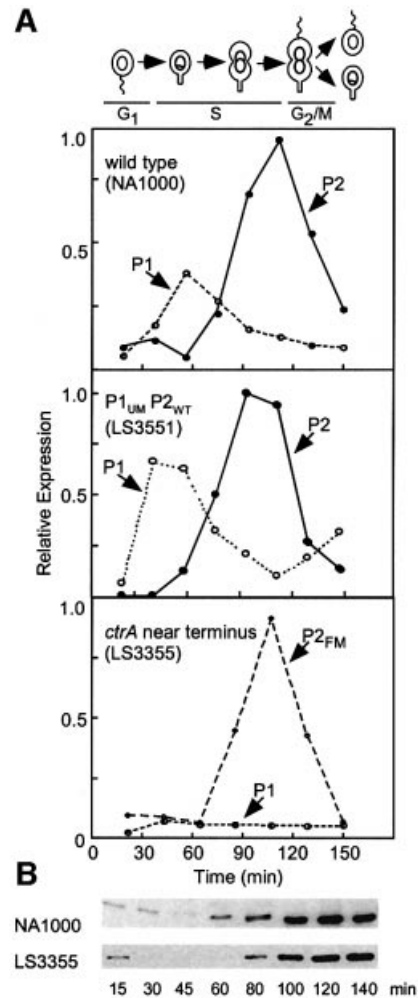


**Fig. 3.** Activity of the *ctrA* P1-*lacZ* and P1<sub>UM</sub>-*lacZ* transcriptional reporters integrated at different sites on the chromosome. (A) Diagram of the *C. crescentus* chromosome showing the locations of the origin of replication (*Cori*), the terminus region (Ter), the *ctrA* gene and the *hrcA* (site 1), *recA* (site 2), and *trpE* (site 3) integration sites. (B) Schematic of the methylation state of GAnTC motifs at the three integration sites during the cell cycle. All sites are fully methylated (FM) in the swarmer cell. After the initiation of DNA replication, the time when each GAnTC site becomes hemimethylated (HM) depends on its distance from *Cori*. Near the end of S phase, CcrM (shown as a gray bar) methylates the newly synthesized DNA strand, restoring the chromosome to the fully methylated state. The *Caulobacter* cell cycle is shown schematically. The  $\theta$  and ring structures inside the cells represent replicating and non-replicating DNA, respectively. (C) Activity of the wild-type *ctrA* P1 (P1-*lacZ*) and unmethylated P1 (P1<sub>UM</sub>-*lacZ*) transcription probes integrated into sites 1, 2 and 3. Promoter activity is the mean  $\pm$  SD of three experiments.

amount of CcrM expression is normal. Previous studies have demonstrated that DNA methylation at the sites used in this study varies during the cell cycle. GAnTC sites near *Cori* become hemimethylated soon after the initiation of DNA replication and remain hemimethylated until the end of S phase when the CcrM MTase is present and active (Stephens *et al.*, 1996; Marczynski, 1999). In contrast, the GAnTC sites engineered into a transposon-based methylation probe integrated near the terminus (site 3) are hemimethylated only for a short period at the end of S phase. When this methylation probe was integrated midway between the origin and terminus (site 2), the GAnTC sites are hemimethylated for an intermediate period of time (Marczynski, 1999). The changes in the methylation state of these sites during the cell cycle are shown in Figure 3B.

Activity of the P1-*lacZ* transcription probe varied dramatically when integrated at these three sites on the chromosome (Figure 3C, left panel).  $\beta$ -galactosidase activity was maximal when the probe was integrated near *Cori* (site 1), reduced by 60% when the reporter was integrated 1.1 Mb from *Cori* (site 2), and reduced by 85% when it was integrated near the terminus (site 3). Thus, *ctrA* P1 activity correlates well with the position of the P1 transcription probe on the chromosome, and reflects the period of time that GAnTC sites at these locations remain in the fully methylated state during the cell cycle (Marczynski, 1999). These results support the previous experiments indicating that hemimethylation of the *ctrA* P1 promoter is required for its full expression and further suggest that the effect is direct.

To assess the role of CcrM in the direct regulation of *ctrA* P1 transcription, we generated a C(-25)T mutation in the P1 promoter that eliminated the GATTC methylation site (see Figure 1B). We then constructed a *lacZ* transcriptional fusion plasmid containing only the mutant P1 promoter (pP1<sub>UM</sub>-*lacZ*), introduced the plasmid into wild-type cells and measured promoter activity. Mutating the methylation site had little effect on P1 activity; promoter activity was  $3250 \pm 100$  and  $3980 \pm 160$  Miller units in wild-type cultures bearing the *pctrA*-P1 and pP1<sub>UM</sub>-*lacZ* transcriptional fusion plasmids, respectively. This is not surprising since the timing of P1 transcription was similar for both the wild-type and unmethylated promoters (Figure 4A). We also constructed a transcription probe containing the mutant *ctrA* P1<sub>UM</sub> promoter fused to *lacZ* (pAR579), integrated this reporter at the same three sites on the chromosome, and measured  $\beta$ -galactosidase activity. As shown in Figure 3C, the activity of the P1<sub>UM</sub>-*lacZ* transcription probe was maximal at site 1 and reduced by 20 and 32% at sites 2 and 3, respectively. This modest drop in promoter activity reflects the changes in the copy number of the reporter during DNA replication. During most of S phase, there are two copies of the reporter integrated near the origin (site 1), but only one copy of the reporter integrated near the terminus (site 3). The activity of both the wild-type P1-*lacZ* and the unmethylated P1<sub>UM</sub>-*lacZ* reporters was similar at site 1, reflecting the similar timing of the transcription of these promoters (Figure 4A). However, activity at sites 2 and 3 increased 2- and 5-fold, respectively, when P1 contained a point mutation that blocks CcrM methylation. These data indicate that changes in the



**Fig. 4.** The timing of *ctrA* transcription when P1 cannot be methylated and when the *ctrA* gene is relocated near the chromosomal terminus. (A) The *Caulobacter* cell cycle is shown schematically. In synchronized wild-type cultures, *ctrA* P1 (open circles) and P2 (closed circles) transcription was monitored using plasmids *pctrA*-P1 and *pctrA*-P2 (Domian *et al.*, 1999). Samples were pulse-labeled with [<sup>35</sup>S]methionine at the indicated times, and  $\beta$ -galactosidase synthesis was assessed by immunoprecipitation. To determine the timing of P1 and P2 transcription in synchronous cultures with *ctrA* transcribed from an unmethylated P1 promoter (LS3551) or with *ctrA* located near the terminus (LS3355), total cellular RNA was isolated at 20 min intervals and *ctrA* transcript abundance was analyzed by primer extension. The primer extension products were resolved on a sequencing gel and quantitated with a PhosphorImager. (B) Immunoblot of CtrA in cells from synchronized wild-type and LS3355 cultures. An equivalent cell mass (based on OD<sub>600</sub>) was applied to each lane. Cell proteins were separated on SDS-12% polyacrylamide gels, and probed with an antibody to the *C.crescentus* CtrA protein.

methylation of P1 are responsible for the inhibition of promoter activity in the P1-*lacZ* strains at sites 2 and 3 and imply that full methylation of the P1 promoter represses *ctrA* transcription. When promoter activity is corrected for approximate gene dosage, we observed that P1-*lacZ* activity at site 3 was still significantly reduced.

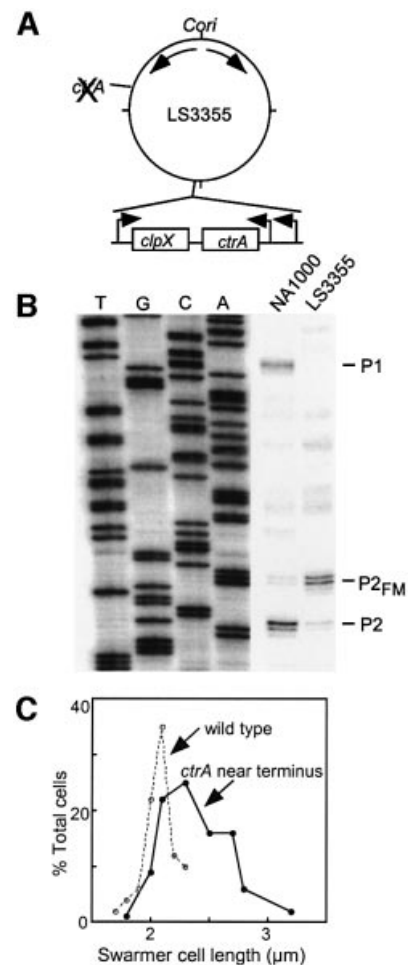
#### Temporally controlled transcription from the *ctrA* P1<sub>UM</sub> promoter

In wild-type cells, *ctrA* transcription is temporally regulated during the cell cycle. P1 transcription is maximal early in S phase, while P2 transcription peaks in mid to late

S phase (Figure 4A; Domian *et al.*, 1999). To test the effect of expressing the intact *ctrA* gene from the unmethylated P1 promoter (P1<sub>UM</sub>) *in vivo*, we constructed a strain (LS3551) in which *ctrA* was inactivated on the chromosome, but that contained a plasmid-borne *ctrA* gene transcribed from the C(-25)T mutant P1 promoter and the wild-type P2 promoter. Primer extension and S1 analysis showed that both P1<sub>UM</sub> and P2 transcripts initiated from their native start sites (data not shown). To determine whether cells bearing an unmethylated P1 promoter show the same temporal pattern of transcription as those bearing the wild-type P1 promoter, we synchronized LS3551 cultures and monitored transcription by primer extension. As shown in Figure 4A, transcription from both the wild-type and the unmethylated P1 promoters was maximal early in the cell cycle. In addition, P2 was transcribed late in S phase in both wild-type and LS3551 cultures. Therefore, both the activity and the timing of *ctrA* P1 transcription remained unchanged when methylation of the P1 promoter was eliminated. However, as shown above, P1 transcription is repressed when the promoter is in the fully methylated state. Since P1 transcription is inhibited by feedback regulation from CtrA (Figure 1; Domian *et al.*, 1999), full methylation must affect the initiation of P1 transcription.

#### Changing the position of *ctrA* on the chromosome altered the temporal pattern of its transcription

To determine whether the transcription of *ctrA* P1 is delayed when the chromosome is in the fully methylated state, we changed the chromosomal location of the native *ctrA* gene. We constructed a strain (LS3355) in which *ctrA* was inactivated at its wild-type position and instead placed close to the terminus of the chromosome, a site that remains fully methylated throughout most, if not all, of the cell cycle (Marczynski, 1999). The native *ctrA* gene and its promoters were integrated within the 400 bp region between the *clpX* and *lon* genes by homologous recombination (see Figure 5A). The *clpX* and *lon* genes are located 1.9 Mb from *Cori*, placing these genes near the terminus. Genomic PCR using primers located within the *ctrA* gene and in the sequence flanking either *ctrA* or *clpX* was used to confirm that *ctrA* was absent from its wild-type location and present adjacent to *clpX* (data not shown). To determine whether the chromosomal position of the *ctrA* gene influences its transcription *in vivo*, we isolated RNA from wild-type and LS3355 cultures, and assessed *ctrA* transcript levels by S1 nuclease assays. As shown in Figure 5B, the *ctrA* P1 and P2 transcripts were present in wild-type cells and initiated at the sites previously described (Domian *et al.*, 1999). However, when *ctrA* was located near the chromosome terminus, transcription from the P1 and P2 promoters dropped and the bulk of *ctrA* transcription originated at P2<sub>FM</sub>, a new transcription start site located eight nucleotides upstream of the P2 start site (Figure 5B). These results were confirmed by primer extension analysis (data not shown). The location of the P2<sub>FM</sub> start site is shown in Figure 1B. Thus when P<sub>*ctrA*</sub> is moved to a site that remains fully methylated throughout most of the cell cycle, P1 is inactivated. This is consistent with our observation that P1 transcription is repressed when the chromosome is in the fully methylated state.



**Fig. 5.** Moving *ctrA* near the terminus changes the mRNA start sites and affects swarmer cell size. (A) Diagram of strain LS3355. The chromosomal copy of *ctrA* was inactivated and the *ctrA* gene and its promoters were integrated between the *clpX* terminator and the *lon* gene. (B) S1 nuclease mapping of *ctrA* transcription start sites in wild-type (NA1000) and LS3355 cultures. The first four lanes show a sequencing ladder generated using a primer with the same 5' end as the S1 probe. The P1 and P2 transcription start sites were detected in wild-type cells, while P2<sub>FM</sub> was the predominant start site in LS3355 cultures. (C) The size distribution of swarmer cells in wild-type and LS3355 cultures. Swarmer cells were isolated from synchronized cultures at 20 min into the cell cycle, fixed in buffered neutral formalin, and examined by DIC microscopy. To estimate cell size, the length of at least 50 cells in each culture was measured.

Furthermore, in the LS3355 strain, the native *ctrA* gene is transcribed from an alternate start site.

To determine whether moving *ctrA* to a site near the chromosomal terminus affects the temporal regulation of *ctrA* transcription, we synchronized cultures of LS3355 and monitored *ctrA* mRNA levels by primer extension analysis. When the native *ctrA* gene was located close to the terminus, P1 transcription was negligible throughout the cell cycle, while P2<sub>FM</sub> was transcribed in mid to late S phase (Figure 4A). Immunoblot analysis showed that CtrA was present in swarmer cells, rapidly degraded at the G<sub>1</sub>-S transition, and reappeared in pre-divisional cells in both wild-type and LS3355 cultures (Figure 4B). However, the reappearance of CtrA protein was delayed in the LS3355 cultures, reflecting the delay in *ctrA*

transcription. Thus the period during which CtrA protein is absent during the cell cycle was prolonged when the only copy of the *ctrA* gene is moved to a position on the chromosome that remains fully methylated for the majority of the cell cycle. Although variability in the cell cycle could affect the timing of CtrA expression, it is unlikely to cause both the earlier disappearance and later reappearance of CtrA observed in the LS3355 strain.

#### **Changing the chromosomal location of *ctrA* affected the distribution of swarmer cell size**

Swarmer cells were isolated from wild-type and LS3355 cultures and examined by light microscopy. In LS3355 cultures, the swarmer cells were elongated and exhibited a broad distribution of cell lengths; 42% of the swarmer cells were longer than their wild-type counterparts (Figure 5C). This change in cell size was also observed in stalked and pre-divisional cells. In the LS3418 control strain, with the vector alone integrated between the *clpX* and *lon* genes, swarmer cell length was not affected ( $2.0 \pm 0.2 \mu\text{m}$ ). In addition, swarmer cell length was normal when a plasmid containing the native *ctrA* gene and promoter region (pSAL290) was introduced into strain LS3355. These data indicate that the cell elongation is due to faulty expression of *ctrA* P1 and not to changes in the expression of the *clpX* or *lon* genes. Thus prolonging the period of the cell cycle in which the cells remain free of CtrA results in an abnormal cell size distribution, suggesting that changes in the DNA methylation state of the *ctrA* promoter and the subsequent changes in the timing of CtrA expression influence cell physiology. However, it is possible that other effects of its chromosomal position may alter CtrA expression and contribute to the observed phenotype.

## **Discussion**

The CtrA response regulator is a critical component of cell cycle control in *Caulobacter*. This DNA-binding protein directly regulates the transcription of 55 operons (Laub *et al.*, 2002) and directly or indirectly controls ~25% of the 550 cell cycle-regulated genes (Laub *et al.*, 2000). CtrA also represses DNA replication initiation in swarmer cells by binding to the origin of replication (Quon *et al.*, 1998). Not surprisingly, this essential protein is under multiple levels of control: cell cycle-regulated *ctrA* transcription, CtrA phosphorylation, and proteolysis of phosphorylated CtrA (CtrA~P) (Domian *et al.*, 1997, 1999). The *ctrA* gene is transcribed from two promoters, P1 and P2, which are active at different times in the cell cycle (Figure 4). Here we present evidence that DNA methylation silences the transcription of the *ctrA* P1 promoter and that this inhibition of P1 transcription affects cell physiology. Consequently, the methylation state of the chromosome adds another layer of regulation to the temporal expression of CtrA.

Because the *Caulobacter* chromosome changes progressively from the fully methylated state at the start of S phase to the hemimethylated state at the end of S phase (Stephens *et al.*, 1996; Marczynski, 1999), the differential methylation state of specific promoters could contribute to the cell cycle timing of transcription in this bacterium. In this report, we demonstrate that the *ctrA* P1 promoter,

which contains a CcrM methylation site near the -35 region, is repressed when the chromosome is fully methylated, and active when it is unmethylated or hemimethylated. These conclusions are based on the following observations. P1 transcription decreased when CcrM was expressed constitutively, resulting in the maintenance of the chromosome in the fully methylated state throughout the cell cycle, and increased when CcrM was depleted, preventing re-methylation of the replicating chromosome. The effects of DNA methylation on the activity of the *ctrA* P1 promoter in these *ccrM* mutant strains could be direct or indirect. Although we cannot measure the actual changes in the methylation of the GAnTC site in the P1 promoter, two experiments that change the chromosomal position of the P1 promoter indicate that the effect is direct. First, the activity of a *ctrA* P1 transcription probe was highest when the probe was integrated near the origin of replication, a site that remains hemimethylated throughout S phase, and lowest when the probe was integrated near the terminus, a site that is fully methylated throughout most of the cell cycle. When the methylation site in P1 was eliminated by a point mutation, the activity of the probe integrated near the terminus increased nearly 5-fold, providing a direct link between the methylation state of the P1 promoter and its activity. Second, moving the native *ctrA* gene and its promoters to a site near the chromosomal terminus inhibited P1 transcription and prolonged the period of time that CtrA was absent from the cell during the G<sub>1</sub>-S transition. Instead, *ctrA* was transcribed from an alternate promoter, P2<sub>FM</sub>, which fired later in the cell cycle, at the same time that P2 is normally expressed.

We reasoned that the passage of the replication fork through the *ctrA* promoter could play a role in initiating *ctrA* P1 transcription by converting the GAnTC site at -29 from the fully methylated to the hemimethylated state. In the G<sub>1</sub>-phase swarmer cell, the chromosome is fully methylated and *ctrA* is not transcribed. P1 is transcribed early in S phase, shortly after the initiation of DNA replication and the subsequent transition of the origin-proximal region of the chromosome to the hemimethylated state. However, when the methylation site in the P1 promoter was eliminated, the early pulse of *ctrA* transcription occurred at the normal time. Therefore, the temporally controlled activation of P1 transcription occurs when the promoter is in the unmethylated or hemimethylated state, but not when it is maintained in the fully methylated state. Hence, the conversion of the P1 promoter to the hemimethylated state alone cannot signal the initiation of P1 transcription. It is possible that an as yet unidentified transcriptional activator preferentially binds to the hemimethylated P1 promoter early in S phase and initiates *ctrA* transcription, but does not bind P1 when it is in the fully methylated state. It is unlikely that methylation affects the binding of CtrA to P1 because the GAnTC site is upstream of the region footprinted by CtrA (Domian *et al.*, 1999).

Here we show that moving *ctrA* to a region of the chromosome that remains in the fully methylated state has two consequences: first, the initial burst of *ctrA* transcription in early S phase was eliminated and the reappearance of CtrA protein was delayed, and second, cells were longer and exhibited a wide distribution of

sizes. Therefore, the accumulation of CtrA at the right time in the cell cycle is important for the temporal regulation of cell growth. There is precedent for changes in the methylation state of a promoter altering the timing of gene transcription during the cell cycle in *Caulobacter*. The *ccrM* gene is transcribed during a narrow window of the cell cycle. Normally, transcription of *ccrM* is initiated by CtrA~P late in S phase and is terminated just before cell division. When the tandem GAnTC sites in the mRNA leader region are mutated so that this region of the DNA is never methylated, *ccrM* transcription continues until CtrA is degraded at the G<sub>1</sub>-S transition (Stephens *et al.*, 1995).

Changes in the methylation state of specific regions of bacterial chromosomes can modify the interaction of regulatory proteins with DNA. In *E.coli*, for example, Dam methylation alters the binding of Lrp and PapI to the *papBA* promoter, which regulates the expression of adhesive pili (Nou *et al.*, 1993). The SeqA protein, which prevents the re-initiation of DNA replication by binding to and sequestering the origin, specifically binds to hemimethylated DNA (Kang *et al.*, 1999). Similarly, the MutH endonuclease binds to hemimethylated DNA during methyl-directed mismatch repair and cleaves the unmethylated strand (Au *et al.*, 1992). The CcrM MTase itself has a distinct preference for hemimethylated DNA as compared with unmethylated DNA as a substrate (Berdis *et al.*, 1998).

As is expected for a global regulator, CtrA activity is under complex regulatory control. Not only is its transcription temporally controlled, but CtrA activity is governed by phosphorylation and targeted proteolysis. Here we report an additional element in the control of CtrA expression: the methylation state of the chromosome. We propose that the pattern of DNA methylation affects the cell cycle indirectly by altering the expression of the CtrA response regulator at a critical time. With the recent publication of the *Caulobacter* genome (Nierman *et al.*, 2001), we are in a position to evaluate the genome-wide distribution of GAnTC sites and to determine the extent to which CcrM-catalyzed DNA methylation plays a role in regulating gene transcription during the cell cycle.

## Materials and methods

### Bacterial strains, plasmids and growth conditions

*Caulobacter crescentus* NA1000 (a synchronizable derivative of the wild-type strain CB15) and derivative strains were grown in PYE complex media or M2 minimal salts-glucose (M2G) minimal media at 30°C (Ely, 1991). Strains and plasmids used are listed in Table I. Antibiotics used include tetracycline (2 µg/ml), kanamycin (20 µg/ml) and spectinomycin (25 µg/ml). Plasmids were mobilized from *E.coli* strain S17-1 into *C.crescentus* by bacterial conjugation (Ely, 1991).

### Promoter activity and cell cycle transcription analysis

The β-galactosidase activity of strains containing the promoter-*lacZ* plasmids or integrated Ω-*ctrA* P1-*lacZ* transcriptional fusions was assayed at 30°C in log-phase cultures (Miller, 1972). Transcription

**Table I.** Strains and plasmids

Strains		
NA1000	Synchronizable derivative of <i>C.crescentus</i> CB15	Evinger and Agabian (1977)
GM1254	NA1000 <i>recA</i> ::Tn5Ω-MP	Marczynski (1999)
GM1258	NA1000 <i>trpE</i> ::Tn5Ω-MP	Marczynski (1999)
LS1	NA1000, constitutive transcription of <i>ccrM</i>	Zweiger <i>et al.</i> (1994)
LS2144	NA1000 Δ <i>ccrM</i> pCS226 (P <sub>cyt</sub> :: <i>ccrM</i> )	Stephens <i>et al.</i> (1996)
LS2293	NA1000 <i>hrcA</i> Ω	Roberts <i>et al.</i> (1996)
LS3317	NA1000 <i>hrcA</i> Ω::pAR263	This study
LS3318	NA1000 <i>recA</i> ::Tn5Ω::pAR263	This study
LS3319	NA1000 <i>trpE</i> ::Tn5Ω::pAR263	This study
LS3336	NA1000 <i>ctrA</i> Δ2 pSAL290	This study
LS3355	NA1000 <i>ctrA</i> Δ2::pAR358	This study
LS3418	NA1000 <i>clpX</i> ::pAR427	This study
LS3551	NA1000 <i>ctrA</i> Δ2 pP1 <sub>UM</sub> - <i>ctrA</i>	This study
LS3561	NA1000 <i>hrcA</i> Ω::pAR579	This study
LS3562	NA1000 <i>recA</i> ::Tn5Ω::pAR579	This study
LS3563	NA1000 <i>trpE</i> ::Tn5Ω::pAR579	This study
Plasmids		
pAR263	Ω- <i>ctrA</i> P1- <i>lacZ</i> in pNPTS138	This study
pAR358	3' region of <i>clpX</i> and all of <i>ctrA</i> in pNPT228	This study
pAR427	3' region of <i>clpX</i> in pNPT228	This study
pAR579	Ω- <i>ctrA</i> P1 <sub>UM</sub> - <i>lacZ</i> in pNPTS138	This study
<i>ctrA</i> -P1	<i>ctrA</i> P1- <i>lacZ</i> in pRKlac290 (3 bp substitution in the -10 region of P2)	Domian <i>et al.</i> (1999)
<i>ctrA</i> -P2	<i>ctrA</i> P2- <i>lacZ</i> in pRKlac290 (5 bp insertion at -20 of P1)	Domian <i>et al.</i> (1999)
pMR10	Low copy number vector, replicates in <i>Caulobacter</i>	R.Roberts
pNPT228	pLitmus28-derived integration vector	M.R.K.Alley
pNPTS138	pLitmus38-derived integration vector	M.R.K.Alley
pP1 <sub>UM</sub> - <i>ctrA</i>	P1 <sub>UM</sub> and wild-type P2 promoters driving <i>ctrA</i> in pMR10	This study
pP1 <sub>UM</sub> - <i>lacZ</i>	<i>ctrA</i> P1 C(-25)T mutant in pRKlac290	This study
pRKlac290	<i>lacZ</i> transcriptional fusion vector	Gober and Shapiro (1992)
pRW72	<i>SacI</i> fragment containing <i>clpX</i> and <i>lon</i> in pBluescript	R.Wright
pSAL10	<i>Sall</i> - <i>Hin</i> II <i>ctrA</i> fragment in pMR10	This study
pSAL290	<i>Sall</i> - <i>Hin</i> II <i>ctrA</i> fragment in pRK290-20R	Quon <i>et al.</i> (1996)
pSALFI	<i>Sall</i> - <i>Hin</i> II <i>ctrA</i> fragment in pBluescript	K.Quon

during the cell cycle was measured in synchronous cultures by monitoring  $\beta$ -galactosidase synthesis in strains bearing *lacZ* transcriptional fusions (Jenal *et al.*, 1994) or by primer extension (Ausubel *et al.*, 1989). Radiolabeled  $\beta$ -galactosidase or RNA was quantitated using a PhosphorImager.

### Methylation state of chromosomal loci

The methylation state of the *dnaA* and *flhG* chromosomal loci was assessed using the overlapping restriction site assay (Stephens *et al.*, 1996). Genomic DNA was isolated from strains NA1000, LS1 and LS2144 grown in PYEX (PYE + 0.1% xylose) or PYEG (PYE + 0.1% glucose) for 3 h using PureGene (Gentra Systems).

### Transcript analysis

RNase protection assays were performed with the Ambion RPAIII kit following the manufacturer's instructions. Briefly, total cellular RNA was isolated from NA1000 and LS1 cultures grown in PYE and from LS2144 cultures grown in PYEX or PYEG using the Qiagen RNeasy Midi Kit. RNA antisense probes were produced by *in vitro* transcription using T7 RNA polymerase, [<sup>32</sup>P]CTP and PCR products as templates as described in the Ambion MAXIScript kit. All probes were gel purified before use. The primers *ctrA*314 (5'-AATGAATTCAGGGGCTCCGA-3') and *ctrA*T7.rev (5'-TAATACGACTCACTATAGGTCCTGACCTTGGTGT-3') were used for making the *ctrA* template, and *rrnA*2.for (5'-CTCTTCGATCCTGGGTCTCC-3') and *rrnA*T7.rev (5'-TAATACGACTCACTATAGGAGAAAGTCGGCCAATC-3') for making the *rrnA* template. The labeled *ctrA* and *rrnA* antisense probes were hybridized with sample RNA (10  $\mu$ g) overnight at 42°C, and digested with RNase A–RNase T1 for 30 min at 37°C. The protected fragments were separated on 5% acrylamide–8 M urea gels and quantitated using a PhosphorImager. The *rrnA* probe was included in each hybridization reaction and used as an internal control. Relative transcript levels were calculated as the Phosphor volume of the *ctrA* P1 or P2 transcript divided by the volume of the *rrnA* band. Data were normalized to the transcript levels of LS2144 cells grown in PYEX and are presented as the mean  $\pm$  SD of three experiments.

S1 nuclease protection and primer extension analysis were performed as described previously (Ausubel *et al.*, 1989) using total cellular RNA isolated from mid-log phase cultures with the RNeasy Mini Kit (Qiagen). The DNA probe for S1 mapping was generated by PCR using primers *ctrA*243 (5'-AGGCCTCGATTTTCTCGATT-3') and *ctrA*523R (5'-CATCCTCGATCAACAGTACG-3'). Primer extension assays were performed as described previously (Domian *et al.*, 1999). Annealing temperatures of 45 and 55°C were used for primer extension and S1 mapping, respectively.

### Construction of the $\Omega$ -*ctrA* P1–*lacZ* and $\Omega$ -*ctrA* P1<sub>UM</sub>–*lacZ* chromosomal integrants

The  $\Omega$ -*ctrA* P1–*lacZ* and  $\Omega$ -*ctrA* P1<sub>UM</sub>–*lacZ* transcriptional fusions were constructed by isolating the ~4 kb *Bam*HI–*Dra*I fragments containing *ctrA* P1 and *lacZ* from plasmids *pctrA*-P1 and *pP1<sub>UM</sub>-lacZ*, respectively. These fragments were ligated into the integration vector pNPTS138, and the  $\Omega$  cassette from pHP45 $\Omega$  (Prentki and Kirsch, 1984) was inserted upstream of the promoter–*lacZ* fragment creating plasmids pAR263 and pAR579. These plasmids were then integrated into the chromosomal  $\Omega$  cassette of strains LS2293 (NA1000 *hrcA*:: $\Omega$ ), GM1254 (NA1000 *recA*:: $\Omega$ -MP) and GM1258 (NA1000 *trpE*:: $\Omega$ -MP) by a single integration event. A control plasmid lacking the *ctrA* P1 promoter but retaining the  $\Omega$ -cassette and *lacZ* was also integrated into these strains. The activity of the control plasmid (~1000 Miller units) was subtracted from P1–*lacZ* and P1<sub>UM</sub>–*lacZ* activity.

### Site-directed mutagenesis of *ctrA* P1

A C(–25)T site-directed mutation in the GAnTC site in P1 was generated by PCR, changing the GATTC methylation site to GATTT. For the first round of PCR, we used the mutagenic primers 5'-TTGC-ACCCGATTTGCAAATC-3' and 5'-GATTTGCAAATCGGGTG-CAA-3', and the flanking primers *ctrA*243 and *ctrA* P2-10m.R2 (5'-GTGAAACCCTTCGGCCACCCGGCCGGAGAG-3'). For the second round of PCR, we used these two PCR products as the template and the same flanking primers. The final PCR product was sequenced and cloned into pRKLac290, resulting in a transcriptional fusion of the mutant promoter to a promoterless *lacZ* gene and creating plasmid *pP1<sub>UM</sub>-lacZ*.

Double PCR was also used to construct *pP1<sub>UM</sub>-ctrA*. We used the mutagenic primers shown above, *ctrA*243 and *ctrA*724R (5'-GGAATTCATGATGGGGCTGTGATCTT-3'). Sequencing confirmed that the PCR product contained the mutant P1 promoter, the

wild-type P2 promoter and the 5' end of the *ctrA* gene. This promoter fragment was then cloned into the *Bg*III site within *ctrA* in pSAL10 creating plasmid *pP1<sub>UM</sub>-ctrA*. To create strain LS3351, *pP1<sub>UM</sub>-ctrA* was first mated into NA1000. Then the *ctrA* deletion allele (*ctrA* $\Delta$ 2::spec) was transduced into NA1000 *pP1<sub>UM</sub>-ctrA* to inactivate the chromosomal copy of *ctrA*. We confirmed that *ctrA* was absent on the chromosome by PCR using genomic DNA from LS3351 as the template, a primer within the *ctrA* gene and a primer located 5' of the *ctrA* promoter fragment in *pP1<sub>UM</sub>-ctrA*.

### Moving *ctrA* to the chromosomal terminus

We constructed strain LS3355 in which the *ctrA* gene was deleted at its wild-type position and inserted into the 400 bp intergenic region between the *clpX* terminator and the *lon* gene. Because *ctrA* is essential, we first constructed strain LS3336 in which the chromosomal copy of *ctrA* was inactivated and the native *ctrA* gene was present on pSAL290. We then constructed plasmid pAR358 by cloning a 1 kb *Stu*I–*Eco*RI fragment from pSALFI containing the *ctrA* gene and its promoter, and a 1.2 kb *Spe*I–*Xho*I fragment from pRW72 containing the 3' region of *clpX* and its terminator into the integration vector pNPT228. We integrated pAR358 into LS3336 by a single integration event, generating strain LS3355. To lose pSAL290, we grew the cells overnight in PYE without selection and isolated tetracycline-sensitive colonies. We confirmed that *ctrA* was adjacent to *clpX* in LS3355 by PCR using genomic DNA from this strain as the template, a primer within the *ctrA* gene and a primer located 5' of the *clpX* fragment in pAR358. We also constructed a control strain (LS3418) in which the 1.2 kb *Spe*I–*Xho*I *clpX* fragment alone was cloned into pNPT228 and integrated into NA1000.

### Synchronization and microscopy

Swarmer cells were isolated from wild-type, LS3355, LS3355 pSAL290 and LS3418 cultures by density gradient centrifugation (Jenal *et al.*, 1994). Samples were taken for phase microscopy at 20 min. The cells were fixed in buffered neutral formalin (3.7% formaldehyde, 145 mM NaCl, 30 mM KH<sub>2</sub>PO<sub>4</sub> and 45 mM Na<sub>2</sub>HPO<sub>4</sub>). Nomarski differential interference contrast (DIC) images were taken using a Nikon E800 microscope with a 100 $\times$  DIC objective. To determine average cell length, at least 50 swarmer cells in wild-type and LS3418 cultures, and 85 swarmer cells in LS3355 cultures, were measured.

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