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Craig Stephens
Santa Clara University, cstephens@scu.edu

Ann Reisenaur

Rachel Wright

Lucy Shapiro

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A cell cycle-regulated bacterial DNA methyltransferase is essential for viability

CRAIG STEPHENS, ANN REISENAUER, RACHEL WRIGHT, AND LUCY SHAPIRO

Department of Developmental Biology, Beckman Center for Molecular and Genetic Medicine, Stanford University, Stanford, CA 94305-5427

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ABSTRACT **The CcrM adenine DNA methyltransferase, which specifically modifies GANTC sequences, is necessary for viability in *Caulobacter crescentus*. To our knowledge, this is the first example of an essential prokaryotic DNA methyltransferase that is not part of a DNA restriction/modification system. Homologs of CcrM are widespread in the α subdivision of the Proteobacteria, suggesting that methylation at GANTC sites may have important functions in other members of this diverse group as well. Temporal control of DNA methylation state has an important role in *Caulobacter* development, and we show that this organism utilizes an unusual mechanism for control of remethylation of newly replicated DNA. CcrM is synthesized *de novo* late in the cell cycle, coincident with full methylation of the chromosome, and is then subjected to proteolysis prior to cell division.**

Chromosomal DNA methylation is widespread in prokaryotes and eukaryotes and can affect critical processes such as DNA replication (1, 2), transcription (3–6), and repair of mutational lesions (7). We are examining the regulation and function of a DNA methyltransferase, CcrM*, found in *Caulobacter crescentus*, a bacterium that undergoes cellular differentiation during each cell cycle (9). *Caulobacter* chromosomal DNA exhibits cell cycle-dependent patterns of methylation. Constitutive expression of the *ccrM* gene, yielding chromosomes that are fully methylated throughout the cell cycle, results in an altered developmental program, indicating that variations in methylation state are of regulatory significance (10). Understanding the role of DNA methylation in growth and development has been an elusive goal in many systems. There is abundant evidence correlating the level of cytosine methylation of eukaryotic DNA with gene expression and/or differentiation states (3–5, 11–13), but only recently has the role of DNA methylation in eukaryotic organisms been addressed genetically. A deficiency in cytosine methylation results in embryonic lethality in mice (14); in contrast, mutations resulting in deficiencies in DNA methylation in *Arabidopsis thaliana* (15, 16) and *Neurospora crassa* (17) are not lethal but cause abnormalities in chromosome segregation behavior. In prokaryotes, the only "regulatory" DNA methyltransferase that has been extensively examined has been the Dam methyltransferase of *Escherichia coli* and related enterics. Dam methylation is important for temporal control of chromosomal replication (1, 2) and for directing mismatch repair (7) but is dispensable for growth (18). We present herein evidence that the *Caulobacter* CcrM methyltransferase, which like Dam is not a component of a DNA restriction/modification (R/M) system, is essential for cellular viability. Furthermore, we have found that CcrM homologs are widely distributed in the α subdivision of Proteobacteria, in which the genus *Caulobacter* resides, suggesting that the critical function of DNA methylation in *Caulobacter* may similarly be conserved in other bacteria.

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The *ccrM* (*c*ell-cycle *r*egulated *m*ethyltransferase) locus encodes a DNA methyltransferase (CcrM) responsible for N^6 methylation of adenine in GANTC sequences (10). In *Caulobacter*, the single chromosome replicates just once during the cell cycle (19, 20). We have shown previously that the remethylation of newly replicated (and thereby hemimethylated) GANTC sites is restricted to the predivisional cell (10), near completion of chromosome replication, and that transcription of the *ccrM* gene occurs during a similar time frame (8, 10). We show here that the CcrM DNA methyltransferase is present only in predivisional cells. Because chromosome replication initiates on fully methylated DNA and the passage of the replication fork progressively generates hemimethylated sites, we demonstrate that the time of appearance of hemimethylation reflects the location of sites relative to the origin of replication.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Growth Conditions. *E. coli* strains were grown at 37°C in Luria–Bertani (LB) broth supplemented with ampicillin (50 μg/ml), tetracycline (10 μg/ml), or kanamycin (50 μg/ml) as necessary. *C. crescentus* strains were grown at 30°C in either peptone/yeast extract (PYE) or M2 minimal salts/glucose medium (M2G) (21).

The sources of strains used for isolation of chromosomal DNA are as follows: *Caulobacter* isolates (other than NA1000) and marine *Hyphomonas* sp. 3, John Smit (University of British Columbia); *Rhizobium* species and *Agrobacterium tumifaciens*, R. Fisher and S. Long (Stanford University); *Asticcacaulis excentricus* and *Asticcacaulis biprosthecum*, S. Green and Y. Brun (Indiana University); *Hyphomicrobium zavarzinii*, L. Tuhela (Ohio State University); *Rhodobacter capsulatus*, C. Bauer (Indiana University); *Legionella pneumophila*, D. Martin (Stanford); *Campylobacter jejuni*, E. Segal (Stanford); *Synecchococcus* PCC7942 and *Fremyella diplosiphon*, C. Niyogi and A. Grossman (Stanford); *Bacillus subtilis*, J. Sarcero and C. Yanofsky (Stanford); *Streptomyces lividans*, E. S. Kim and S. Cohen (Stanford); and *Acinetobacter calcoaceticus*, R. Roberts (Stanford).

Allelic Replacement Experiments. An interrupted copy of *ccrM* was constructed by using plasmid pCS179, which contains the *ccrM* coding region and 0.5 kb upstream in pBluescript (SK+) (Stratagene). pCS179 was digested with *Bst*BI and *Eco*47III, deleting 262 bp of the *ccrM* coding region. A 2-kb *Sma* I fragment containing the spectinomycin-resistant Ω cassette (22) was inserted between these sites. The *sacB* gene was then ligated downstream of *ccrM*, in the opposite orientation, as a 1.5-kb *Sac*I–*Eco*RV fragment (23), to generate pCS190. pCS190 was electroporated into LS101 (NA1000 Δ*bla*), and ampicillin- and spectinomycin-resistant integrants

Abbreviation: R/M, restriction/modification.

*We have discontinued reference to the CcrM DNA methyltransferase as M.CcrII (8), a designation based on the convention for DNA methyltransferase components of restriction/modification systems. There is no cognate restriction enzyme in *Caulobacter* that cleaves GANTC sites (see *Results*).

were selected that contained tandem copies of wild-type and mutant *ccrM* genes separated by plasmid sequence. The structure of the *ccrM* region in several integrants was determined by restriction and Southern blot analyses. Strain LS1823, in which pCS190 integrated by recombination of the 5′ side of Ω, and LS1824, in which pCS190 integrated on the 3′ side of Ω, were kept for further experiments. (A clear bias was seen for recombination on the 5′ side of Ω, which in pCS190 has 900 bp of homologous DNA available for recombination, while the 3′ side has only 700 bp.) Excision of the integrated plasmid was selected for by growth on 3% sucrose, inducing the toxic activity of the *sacB*-encoded levansucrase. The structure of the *ccrM* locus in ampicillin-sensitive and sucrose-resistant isolates was verified by Southern blot analysis.

Conditional Expression of *ccrM*. A promoterless *ccrM* gene was placed downstream of a 0.9-kb fragment containing the *C. crescentus xylA* promoter (A. Meisenzahl, U. Jenal, and L.S., unpublished results) in the low copy number pRKlac290 vector, generating plasmid pCS226. Expression of the *xylA* promoter was induced by growth on PYE containing 0.1% xylose. This promoter is maximally repressed by growth on 0.1% glucose in the absence of xylose. pCS226 was mated into LS1824 with selection for tetracycline resistance. Recombinants that were sucrose- and spectinomycin-resistant and ampicillin-sensitive and had lost the wild-type *ccrM* locus from the chromosome were isolated as described above. Xylose (0.1%) was added to all plates during isolation of this strain to induce expression of *ccrM* from pCS226.

Preparation of Anti-CcrM Serum. The polymerase chain reaction (PCR) was used to generate a 750-bp DNA fragment encoding the N-terminal 250 amino acids of CcrM. Primer ccrT7PCR (GGGACCATGAATTCGGGCCGG) overlaps the *ccrM* start site and contains an *Eco*RI site; primer ccrHis-PCR (GGCGTGCTCGAGGTCTTCGG) is complementary to the coding strand and contains an *Xho* I site. The PCR product was cloned into the *Eco*RI/*Xho* I sites of pET21a (Novagen) to generate pCSAR1. This creates an in-frame fusion of the T7 leader to the third codon of the *ccrM* coding sequence, and a fusion of residue 249 of CcrM to a polyhistidine tag at the C terminus. Expression of the fusion protein in *E. coli* BL21(λDE3) under control of a T7 promoter (24) was induced indirectly by addition of isopropyl B-D-thiogalactoside. Cells were lysed by sonication and centrifuged at 20,000 × *g* for 15 min. The majority of CcrM-His pelleted with the cell debris fraction in inclusion bodies. CcrM-His was purified under denaturing conditions by chromatography using His-Bind resin (Novagen), according to the manufacturers protocol. The 30-kDa polypeptide band was excised from polyacrylamide gels and used to immunize rabbits at Josman Laboratories (Napa, CA) by conventional protocols. The antiserum recognizes specifically a *C. crescentus* protein of 41 kDa, consistent with that of CcrM (predicted to be 39.6 kDa).

Western Blots. Cellular proteins from samples taken during the cell cycle were resolved by SDS/PAGE and transferred to Immobilon P membrane by standard Western blot protocols (25). Membranes were blocked with T-TBS (20 mM Tris·HCl, pH 7.4/150 mM NaCl/0.05% Tween 20) containing 5% (wt/vol) milk powder. Primary antiserum (1:5000 dilution) was added directly to the blocking solution. Membranes were washed with T-TBS, and secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG, Boehringer Mannheim) was added. After further washes with T-TBS, bound antigen was visualized by chemiluminescence.

Assay of Methylation State and CcrM Levels in Synchronized Cultures. Cultures for synchronization were grown in M2G medium, and swarmer cells were isolated by Ludox density gradient centrifugation (26). Swarmers were released into M2G medium at 30°C at an OD_{600} of 0.3–0.4. Progression through the cell cycle was monitored microscopically. Samples for immunoprecipitation were labeled and prepared as described (27). Samples for immunoblot analysis were harvested by centrifugation, and cell pellets were frozen on dry ice. To assay methylation state of chromosomal loci, samples were taken at each time point and cells were collected by centrifugation. DNA was isolated by using the EluQuik kit (Schleicher & Schuell) and a modified protocol. The overlapping restriction site assay to determine methylation state has been described (10, 28). The *dnaA Hin*fI site located upstream of the coding region (29) is overlapped by a *Hin*dII site; Southern blots of *Hin*dII-digested DNA were probed with a randomly labeled (T7 QuickPrime kit, Pharmacia) 530-bp *Xba*I–*Hin*dIII fragment. The *Hin*fI site in the *fliG* coding region (30) is overlapped by a *Sal* I site; Southern blots of *Sal* I/*Pst* I-digested DNA were probed with a 1.3-kb *Sal* I–*Pst* I fragment containing the 3′ end of *fliF* and the 5′ portion of *fliG*. The *Hin*fI site at the *pbpA* locus also is overlapped by a *Sal* I site (P. Kang and L.S., unpublished results); Southern blots of *Sal* I/*Bam*HI-digested DNA were probed with a 1.1-kb *Bam*HI–*Sal* I fragment.

Detection of CcrM Homologs in Other Species. Chromosomal DNA from each species was isolated as above, digested with *Nco* I, electrophoretically separated, and transferred to Hybond N+ membrane (Amersham) (25). The *ccrM* probe was an internal 0.7-kb *Hin*dIII–*Pml* I fragment randomly labeled with [α-^{32}P]-dCTP. The probe (2×10^7 cpm) was used for hybridization at 65°C for 12 h in 525 mM sodium phosphate, pH 7.2/1 mM EDTA/7% SDS. Blots were washed with 2 × SSC/0.1% SDS at 22°C and exposed to film (25).

RESULTS

CcrM Is Essential for Viability. To understand the function of DNA methylation by CcrM, we sought to construct a null mutation in *ccrM* to eliminate methylation at GANTC sites. A system employing the counterselectable *sacB* gene (22, 23, 31) was used in attempts to isolate recombinant strains in which the wild-type *ccrM* locus had been replaced by a nonfunctional *ccrM* allele. It was not possible to delete the wild-type chromosomal *ccrM* locus unless a functional episomal copy of *ccrM*

Table 1. Chromosomal *ccrM* locus can only be inactivated in the presence of a functional copy of *ccrM* in trans

Strain	Plasmid	Chromosomal genotype after pCS190 excision (number of isolates)	
		ccrM$^+$	*ccrM*::Ω
LS1823	None	177	0
LS1824		124	0
LS1823	pMR10	22	0
LS1824		51	0
LS1823	pCS194 (*ccrM*$^+$)	193	21
LS1824		27	25

Strains LS1823 and LS1824 contain plasmid pCS190 integrated by homologous recombination, resulting in both a wild-type copy of *ccrM* (*ccrM*$^+$) and a nearby copy of *ccrM* interrupted by a spectinomycin-resistant Ω cassette (*ccrM*::Ω). LS1823 and LS1824 differ in that pCS190 integrated at the chromosomal *ccrM* locus via recombination on opposite sides of the Ω insertion. Cells were plated on 3% sucrose to induce toxicity of the pCS190-encoded *sacB* gene; recombinants in which the plasmid excised were sucrose-resistant and ampicillin-sensitive. When the excision occurred on the same side of Ω as the original integration, wild-type *ccrM* was retained and the resulting colony was spectinomycin-sensitive; if it excised on the opposite side, *ccrM*::Ω was retained and the colony was spectinomycin-resistant. Sucrose selection was carried out in strains containing no plasmid, pMR10, or pCS194 (pMR10 + *ccrM*).

was present (Table 1). It appeared therefore that cells require DNA methylation by CcrM for growth. Because a strain completely lacking CcrM could not be isolated, one was constructed in which the level of CcrM could be manipulated. A plasmid-borne copy of *ccrM* was put under the control of a xylose-inducible promoter (plasmid pCS226), and a strain (LS2144) containing pCS226 and a nonfunctional chromosomal *ccrM* locus was isolated. The *xylA* promoter is maximally repressed when xylose is removed and glucose is added (A. Meisenzahl, U. Jenal, and L.S., unpublished results). When strain LS2144 was shifted from growth on PYEX (PYE/0.1% xylose) to PYEG (PYE/0.1% glucose) (Fig. 1*A*), detectable DNA methylation activity ceased within 1 h (data not shown), and growth and DNA replication halted after 6–8 h. The number of viable cells in the PYEG culture increased for one generation and then precipitously declined. By 11 h after the shift, there was a 300-fold difference in viable cell counts between the PYEG and PYEX cultures (Fig. 1*B*). An isogenic strain containing pCS226 and an intact chromosomal *ccrM* locus showed no difference in viability under these conditions; thus, cell death in the presence of glucose only occurs in a strain dependent on the *xylA* promoter for expression of *ccrM*. We conclude that the CcrM protein is necessary for cellular viability.

CcrM is an essential bacterial DNA methyltransferase that is not part of a DNA R/M system. The methyltransferase component of an R/M system is essential for protection of the host genome from cleavage by the cognate endonuclease. This point is relevant in that the amino acid sequence of CcrM shows considerable homology to M.*Hin*fI, the methyltransferase component of the *Hin*fI R/M system (10, 32). However, there is compelling evidence arguing against the presence of a GANTC-specific endonuclease in *C. crescentus*. Components of R/M systems tend to be tightly linked genetically, but no gene encoding an endonuclease homolog has been identified near *ccrM* on the chromosome (8, 10). More to the point, intact plasmid DNA containing unmethylated GANTC sites is readily detectable in *C. crescentus in vivo* (10), and genomic DNA isolated from LS2144 11 h after shifting to PYEG, though significantly undermethylated, is not detectably degraded (data not shown).

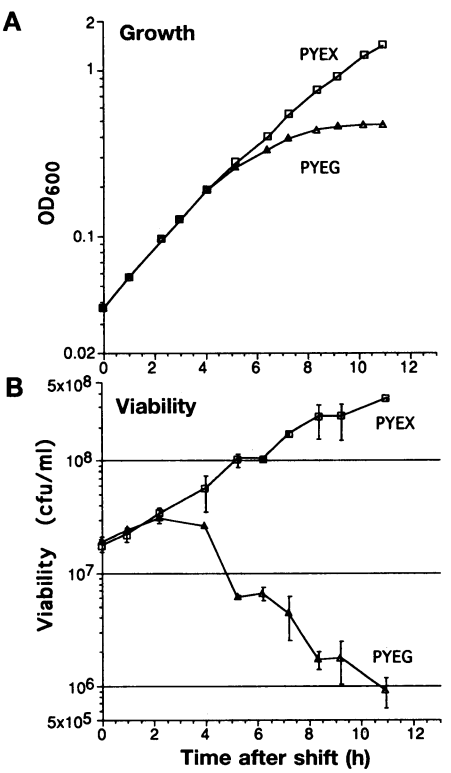

FIG. 1. Elimination of CcrM is lethal. Plasmid pCS226 expresses *ccrM* from the *xylA* promoter, which is induced on PYEX medium (0.1% xylose) and repressed on PYEG (0.1% glucose). The chromosomal *ccrM* locus is nonfunctional in LS2144. Cultures were grown in PYEX, washed twice with PYE, and resuspended in PYEX or PYEG at $t = 0$. Cultures were maintained in logarithmic phase by periodic dilution. (*A*) Growth measured by optical density (600 nm). (*B*) Viable cell counts. cfu, Colony-forming units. Samples were plated in triplicate on PYEX agar containing spectinomycin (50 μg/ml). Bars indicate the SEM from three plates.

CcrM Homologs Are Present in Other Bacteria. Proteins that are critical for growth tend to be conserved during evolution. We therefore searched for homologs of the CcrM DNA methyltransferase in other bacteria known to be phylogenetically linked to *C. crescentus* (33, 34). Chromosomal DNA from 20 species that are members of the α subdivision of the purple Gram-negative bacteria was examined, and all were found to be modified at GANTC sites (Fig. 2*A*). Ten representative species outside the α subdivision were also examined, and none of the species were found to be modified at this

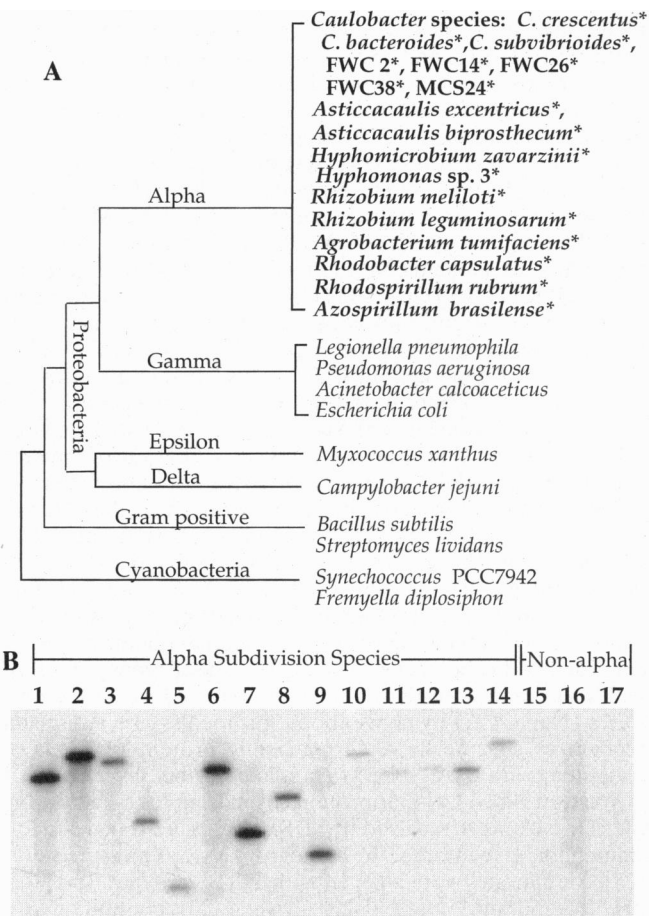

FIG. 2. Phylogenetic distribution of CcrM homologs. (*A*) Species in which chromosomal DNA is resistant to cleavage with *Hin*fI, indicating modification of the GANTC recognition sequence, are listed in boldface type and marked by asterisks. Freshwater (FWC) and marine (MCS) *Caulobacter* species and marine *Hyphomonas* sp. 3 are described in ref. 33. (*B*) Southern blot showing hybridization of the *C. crescentus ccrM* probe to chromosomal DNA from various species digested with *Nco* I. Lanes: 1, *C. crescentus*; 2, FWC26; 3, FWC2; 4, FWC38; 5, *Caulobacter bacteroides*; 6, FWC14; 7, MCS24; 8, Marine *Hyphomonas* sp. 3; 9, *Caulobacter subvibrioides*; 10, *Asticcacaulis biprosthecum*; 11, *Agrobacterium tumifaciens*; 12, *Rhizobium meliloti*; 13, *Rhizobium leguminosarum*; 14, *Rhizobium* sp. ANU 265; 15, *E. coli* TG1; 16, *Pseudomonas aeruginosa*; 17, *Acinetobacter calcoaceticus*.

sequence. To determine whether the species showing modification of GANTC sites indeed encode a homolog of CcrM, genomic DNA was examined by Southern blot analysis using a fragment of the *ccrM* gene as a probe. Hybridization was seen to a single *Nco* I fragment in all of the α subdivision species tested, a subset of which is shown in Fig. 2*B*, but no hybridization was seen to non-α species.

DNA Methylation and CcrM Levels Are Cell Cycle-Regulated. The bacterial cell cycle is readily studied in synchronous populations of *C. crescentus* cells (9). The *Caulobacter* cell cycle is shown diagramatically in Fig. 3. DNA replication (19, 20) and DNA methylation (10) exhibit discrete, overlapping temporal boundaries during the cell cycle. Different GANTC sequences exhibit distinct temporal patterns of hemimethylation during the cell cycle (10). A model to explain these cell cycle patterns needs to account for two temporal factors: namely, the time at which a given gene is replicated and goes from the fully methylated to the hemimethylated state and the timing of remethylation to the fully methylated state. The time at which a given chromosomal DNA site becomes hemimethylated should be dependent on its location on the chromosome relative the origin, as the time required for the replication fork to reach the site should be proportional to its distance from the origin. By using a single synchronous culture, methylation patterns of GANTC sites at three chromosomal loci were assayed as a function of the cell cycle. The chromosomal locations of these loci (*dnaA*, *fliG*, and *pbpA*) are shown in Fig. 3*A*, and the abundance of hemimethylated DNA at each site as a function of the cell cycle is plotted in Fig. 3*B*. The site near *dnaA* became hemimethylated at the transition from swarmer to stalked cells. Hemimethylated *fliG* DNA was first seen 45 min later, and the *pbpA* locus became hemimethylated 15 min after *fliG*. The order of hemimethylation of these sites, and previously examined sites (10), thus reflects their position on the chromosome.

The *ccrM* gene is expressed from a promoter that is activated in the predivisional cell (8, 10), coincident with the period of remethylation of newly replicated chromosomes (Fig. 3*B*). Western blots using a polyclonal antiserum generated against the CcrM protein showed negligible levels of CcrM in swarmer and early stalked cells (Fig. 3*B*). Enzyme levels increased dramatically in the predivisional cell, coinciding with the drop in hemimethylation of the *dnaA* locus. Replication of the *fliG* and *pbpA* genes overlaps the early expression of *ccrM*, such that hemimethylation of these sites initially increases even as *dnaA* is being remethylated. On average, the period of hemimethylation is roughly 40% of the cell cycle for *dnaA*, 20% for *fliG*, and $\leq$10% for *pbpA*. In contrast, very few Dam methylation sites are hemimethylated for significant fractions of the *E. coli* cell cycle, exceptions being the origin of replication and the nearby *dnaA* locus (35), and some sites that are completely unmethylated (36–38).

The levels of CcrM protein reflect closely the level of transcription of *ccrM*, as indicated by *lacZ* fusions to the *ccrM*

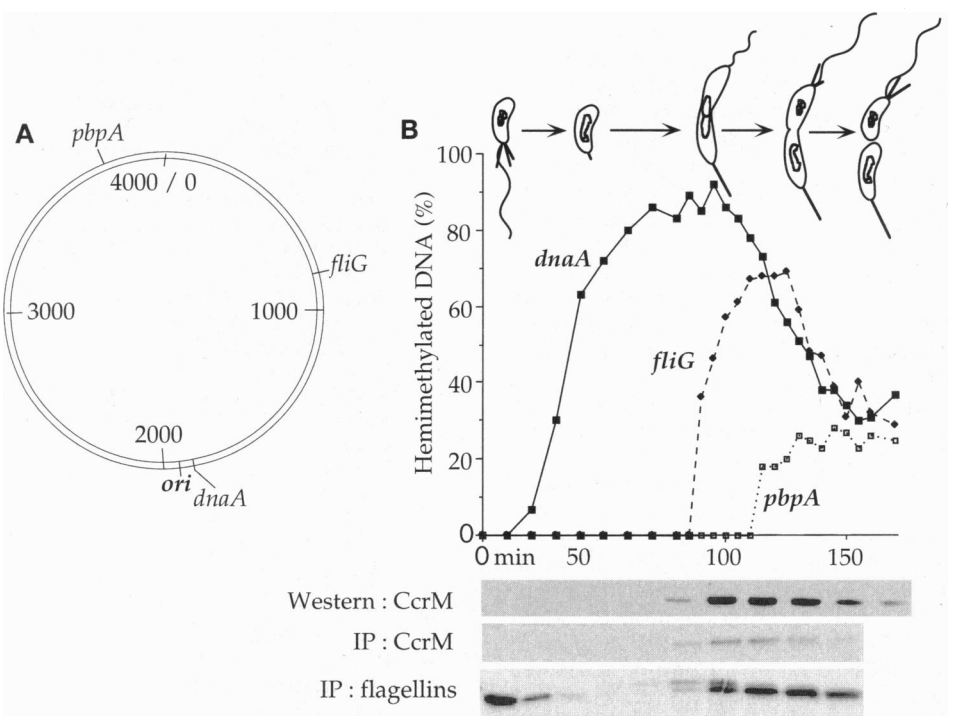

FIG. 3. Chromosomal methylation patterns and cell cycle regulation of CcrM. (*A*) *C. crescentus* NA1000 chromosomal map showing the location of the origin of replication and three methylation sites assayed. (*B*) (*Upper*) Levels of hemimethylated DNA and CcrM over the course of the cell cycle. A schematic of the cell cycle is shown for orientation, depicting landmark events of the cell cycle (flagellum loss, stalk synthesis, chromosome replication, and flagellum biogenesis). Synchronized swarmer cells, in which the chromosomal DNA is fully methylated, were released into M2G medium at 30°C at t = 0. Methylation state of DNA samples was assayed by using overlapping restriction sites (10). Bands on Southern blots representing fully and hemimethylated DNA were quantified with a PhosphoImager (Molecular Dynamics). As the probes used detected only one of the hemimethylated products of replication (10), the signal from the band representing hemimethylated DNA was multiplied by two and plotted as a percentage of total signal (full + hemimethylated). The maximum of 100% hemimethylation is not achieved experimentally at any site because individual cells in the population of "synchronous" swarmer cells vary slightly in cell cycle age, and additional slight variations in the length of the cell cycle causes asynchrony to increase as cells approach division. In the case of *dnaA*, hemimethylation occurs early while the culture is maximally synchronous, and more than 90% of the DNA is detected as hemimethylated. For *fliG* and *pbpA*, the overlap of replication with expression of CcrM results in shorter times of hemimethylation, reducing observable hemimethylated DNA. (*Lower*) Western blot and immunoprecipitation results are shown beneath the plot. Anti-CcrM polyclonal antiserum was used to probe blots of samples taken during the cell cycle. Immunoprecipitation of radiolabeled CcrM and flagellins from samples labeled for 5 min with [^{35}S]methionine at 15-min intervals during the cell cycle is shown. The flagellins serve as a control for cell cycle progression.

promoter (8, 10) and by immunoprecipitation of CcrM from synchronous cells pulse-labeled at successive stages of the cell cycle (Fig. 3*B*). The precipitous drop in CcrM levels as cells approach division suggests that the protein is subject to rapid degradation. In confirmation of this, we have found the *in vivo* half-life of CcrM protein in nonsynchronous cultures grown in M2G minimal medium at 30°C to be less than 10 min (unpublished data). This instability is sufficient to account for its elimination during the terminal phase of the cell cycle.

DISCUSSION

We have found that a *Caulobacter* DNA methyltransferase (CcrM) that, like the *E. coli* Dam methyltransferase, is not part of a R/M system, is essential for viability. Mutations in the *E. coli* Dam methyltransferase, which has been the *de facto* model for understanding functions for DNA methylation in prokaryotes, are not lethal but cause a loss of temporal control of DNA replication (ref. 2 and references therein) and require enhanced constitutive activity of the SOS system to respond to DNA damage (18, 39). We do not yet understand the physiological role(s) of methylation of GANTC sites in *Caulobacter* that result in *ccrM* being an essential gene. We speculate that DNA methylation may be involved in repression of genes that are deleterious when expressed at high levels or inappropriate times, as suggested for the mouse CpG methyltransferase during development (40). Additionally, factors necessary for expression of essential genes, or DNA replication, may require a methylated DNA substrate.

A DNA methyltransferase homologous to CcrM appears to be present in all of the α subdivision species of bacteria sampled. This enzyme was likely present in the progenitor of this group, a diverse assemblage that includes among its members free-living stalked bacteria (such as *Caulobacter*), phototrophs, plant pathogens and symbionts, and animal pathogens. The broad distribution of CcrM homologs suggests that some of the physiological functions of CcrM may be conserved as well. Dam DNA methyltransferase homologs, by comparison, are found in the branch of the γ subdivision of Proteobacteria containing *E. coli* (41). It will be of interest to examine the role, and regulation, of DNA methylation by CcrM homologs in the cell cycle of organisms with such varied lifestyles and to compare these with known functions of Dam methylation in *E. coli*.

In *C. crescentus*, chromosomal replication is tightly regulated, with initiation occurring only once per cell cycle in the stalked cell. Remethylation of hemimethylated GANTC sites after replication fork passage occurs later in the predivisional cell, near completion of chromosome replication but prior to cell division. Rapid degradation of CcrM prior to cell division presumably prevents premature remethylation of the origin region in the progeny stalk cell, which immediately reinitiates replication. We have recently identified a protease whose activity is necessary for CcrM degradation (R.W., C.S., L.S., and M.R.K. Alley, unpublished results). Constitutive expression of the *ccrM* gene throughout the cell cycle effectively overcomes rapid degradation of the CcrM protein and yields chromosomes that remain fully methylated throughout the cell cycle, resulting in developmental abnormalities including relaxation of the control of DNA replication, aberrant cell shape, and frequent failure of dividing cells to separate completely (10). Prolonged maintenance of hemimethylated DNA is thus important for normal progression of the cell cycle. One scenario is that the hemimethylated state of one or more "signposts" on the chromosome, as read by methylation-sensitive DNA binding proteins, could serve a timing function with feedback to a master regulatory circuit for cell cycle progression. Because the timing of entry into the hemimethylated state is dependent on chromosomal location, it could be used to monitor the progress of DNA replication. Hemimethylation of a site at or near the origin could indicate that replication has successfully initiated, whereas a hemimethylated site at or near the terminus, though existing only transiently, could signal that replication is near completion. An important challenge for the future is to identify and characterize such signposts and the signal transduction pathway linking them to the cell cycle.

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