JOURNAL OF BACTERIOLOGY, Sept. 1997, p. 5869–5877 Vol. 179, No. 18
0021-9193/97/$04.00+0

The CcrM DNA Methyltransferase Is Widespread in the Alpha Subdivision of Proteobacteria, and Its Essential Functions Are Conserved in *Rhizobium meliloti* and *Caulobacter crescentus*

RACHEL WRIGHT, CRAIG STEPHENS,† AND LUCY SHAPIRO*

Department of Developmental Biology, Stanford University, Stanford, California

Received 23 May 1997/Accepted 7 July 1997

The *Caulobacter crescentus* DNA methyltransferase CcrM (M.*Ccr*MI) methylates the adenine residue in the sequence GANTC. The CcrM DNA methyltransferase is essential for viability, but it does not appear to be part of a DNA restriction-modification system. CcrM homologs are widespread in the alpha subdivision of gram-negative bacteria. We have amplified and sequenced a 258-bp region of the *ccrM* gene from several of these bacteria, including *Rhizobium meliloti*, *Brucella abortus*, *Agrobacterium tumefaciens*, and *Rhodobacter capsulatus*. Alignment of the deduced amino acid sequences revealed that these proteins constitute a highly conserved DNA methyltransferase family. Isolation of the full-length *ccrM* genes from the aquatic bacterium *C. crescentus*, the soil bacterium *R. meliloti*, and the intracellular pathogen *B. abortus* showed that this sequence conservation extends over the entire protein. In at least two alpha subdivision bacteria, *R. meliloti* and *C. crescentus*, CcrM-mediated methylation has important cellular functions. In both organisms, CcrM is essential for viability. Overexpression of CcrM in either bacterium results in defects in cell division and cell morphology and in the initiation of DNA replication. Finally, the *C. crescentus* and *R. meliloti ccrM* genes are functionally interchangeable, as the complemented strains are viable and the chromosomes are methylated. Thus, in both *R. meliloti* and *C. crescentus*, CcrM methylation is an integral component of the cell cycle. We speculate that CcrM-mediated DNA methylation is likely to have similar roles among alpha subdivision bacteria.

DNA methylation has been identified in both prokaryotes and eukaryotes and has been implicated in many critical cellular processes, including transcriptional regulation (4, 40), initiation of DNA replication (3, 6, 28) and genomic imprinting (33, 34). In mammals and plants, DNA methylation is essential for development. Mice with a null mutation in a cytosine DNA methyltransferase die as embryos (19), and reduced cytosine methylation in *Arabidopsis thaliana* results in major defects in vegetative and reproductive development (12, 35). In prokaryotes, DNA methylation is largely associated with DNA restriction-modification (R/M) systems, where its main function is to allow the cell to differentiate between self and foreign DNA (2, 41). However, bacterial DNA methylation does have other significant biological roles. In *Escherichia coli*, the Dam DNA methyltransferase (see reference 25 for a recent review), which is not part of an R/M system, regulates several cellular processes, including mismatch repair (15, 24), control of initiation of DNA replication (3, 20), and the regulation of gene expression (30, 40). Although Dam methylation is involved in a variety of important physiological functions, it is not essential for viability (23). The *E. coli* Dam methyltransferase is present in several related enteric bacteria, where its function is probably conserved (1).

In the bacterium *Caulobacter crescentus*, the CcrM (M.*Ccr*MI) DNA methyltransferase methylates the adenine residue in the sequence GANTC (44). The role of the CcrM DNA methyltransferase is unusual in that it is essential for viability, yet it does not appear to be part of a DNA R/M system (38, 44). DNA methylation catalyzed by CcrM is tightly controlled during the cell cycle. CcrM is present only in the predivisional cell; consequently, the methylation state of the chromosome varies dramatically during the cell cycle (38). Replication is initiated on a fully methylated chromosome, bidirectional replication yields two hemimethylated chromosomes, and the generation of two fully methylated chromosomes occurs in the late predivisional cell (38). The confinement of the CcrM protein to the late predivisional cell is controlled at two levels: transcription and proteolysis. The *ccrM* gene is transcribed only in the predivisional stage of the cell cycle (38), where it is activated by the CtrA response regulator (32). The CcrM protein is degraded by the constitutive Lon-dependent pathway by the time cell division occurs (43). Strict temporal control of DNA methylation in *C. crescentus* is required for proper progression of the cell cycle and cell division. If CcrM is present throughout the cell cycle, as a result of constitutive *ccrM* transcription or in a *lon* null mutant strain, the chromosome is maintained in a fully methylated state. Under these conditions, the cells have an abnormal morphology, cell division is disrupted, and there is a relaxation in the control of DNA replication initiation (43, 44).

CcrM homologs are distributed widely but restricted to the alpha subdivision of proteobacteria (38). In this study, we show that the sequences of the CcrM DNA methyltransferases are highly homologous and have found that in at least two alpha subdivision members, CcrM-mediated methylation has conserved physiological functions. We have isolated partial *ccrM* coding sequences from four alpha subdivision bacterial species (*Caulobacter bacteroides*, *Caulobacter subvibrioides*, *Rhodobacter capsulatus*, and *Agrobacterium tumefaciens*) and the full-length *ccrM* genes from the nitrogen-fixing soil bacterium *Rhizobium meliloti* (13, 21) and the animal pathogen *Brucella abortus* (37). Alignment of the predicted amino acid sequences shows that these CcrM homologs are highly conserved. The physiological roles of CcrM-mediated DNA methylation in

* Corresponding author. Mailing address: Department of Developmental Biology, Beckman Center, Stanford University, Stanford, CA 94305-5427. Phone: (415) 725-7678. Fax: (415) 725-7739.

† Present address: Department of Biology, Santa Clara University, Santa Clara, CA.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
E. coli		
DH10B	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 endA1 recA1 deoR* Δ(*ara leu*)*7697 araD139 galU galK nupG rpsL*	GIBCO BRL
S17	RP4-2, Tc::Mu Km::Tn7 (used for conjugal transfer of plasmids into *C. crescentus*)	36
TGI	F′ *traD36 lacI*^{q} Δ(*lacZ*)M15 *proA*$^+$*B*$^+$/*supE* Δ(*hsdM-mcrB*)*5* (r_K^- m_K^- McrB$^-$) *thi* Δ(*lac-proAB*)	T. J. Gibson
C. crescentus		
NA1000	CB15 *syn-1000*; synchronizable wild-type derivative	10
LS1824	LS107 *ccrM*/*ccrM*::Ω *sacB* Gm^r	38
LS2818	LS1824 *ccrM*::Ω containing plasmid pRW201	This study
LS2866	LS1824 *ccrM*::Ω containing plasmid pCS174	This study
R. meliloti		
1021	Su47 *str-21*	26
LS2590	*ccrM*/*ccrM*::*nptII*. Integration of pRW134 occurred on the 5′ side of *nptII.*	This study
LS2591	*ccrM*/*ccrM*::*nptII*. Integration of pRW134 occurred on the 3′ side of *nptII.*	This study
LS2660	*R. meliloti* 1021 containing plasmid pRW171	This study
LS2764	*R. meliloti* 1021 containing plasmid pMB393	This study
LS2838	LS2590 *ccrM*::*nptII* containing plasmid pRW205	This study
Plasmids		
pBluescript SKI(−)	Amp^r vector	Stratagene
pCS174	pRKlac290 containing the *C. crescentus ccrM* promoter and gene	38
pDnaA	530-bp *Xba*I-*Sal*I fragment containing the end of the *rpsT* gene and the beginning of the *dnaA* gene in pBluescript SKI(−)	43
pJQ200SK	*R. meliloti* suicide vector, *sacB* Gm^r	31
pMB393	High-copy-number vector, Spec^r which replicates in *R. meliloti* (pBBR1MCS derivative [17])	M. Barnett and S. Long
pMB440	Low-copy-number vector, Spec^r which replicates in *R. meliloti*	M. Barnett and S. Long
pRK600	Cm^r, pRK2013, Nm^r::Tn*9* helper plasmid (used for conjugal transfers of plasmids into *R. meliloti*)	11
pRKlac290	pRK290 derivative, *lacZ* transcriptional fusion vector	14
pRW115	pBluescript SKI(−) + a 3.0-kb *Not*I fragment isolated from a positive phage containing the complete *R. meliloti ccrM* gene	This study
pRW134	pJQ200SK + 4.5-kb *Xho*I-*Xba*I fragment containing *R. meliloti ccrM* disrupted with the *nptII* gene	This study
pRW171	pMB393 + the 1.6-kb *Pst*I-*Hin*dIII fragment from pRW115 containing the complete *R. meliloti ccrM* gene	This study
pRW175	pMB440 + the 1.6-kb *Pst*I-*Hin*dIII fragment from pRW115 containing the complete *R. meliloti ccrM* gene	This study
pRW184	pBluescript SKII(+) containing the fusion between the *C. crescentus ccrM* promoter and the *R. meliloti ccrM* gene	This study
pRW201	pRKlac290 + *Eco*RI-*Hin*dIII fragment from pRW184 containing the fusion between the *C. crescentus ccrM* promoter and the *R. meliloti ccrM* gene	This study
pRW205	pMB393 + 2.0-kb *Sac*II fragment from pCS179 containing the complete *C. crescentus ccrM* gene	This study
pRW342	pBluescript SKI(−) + 4.0-kb *Bam*HI fragment containing the complete *B. abortus ccrM* gene	This study

C. crescentus and *R. meliloti* are similar. CcrM is essential for viability in both organisms. Overexpression of CcrM in *R. meliloti* results in defects in cell division, abnormal cell morphology, and loss of normal control of DNA replication initiation. Thus, two bacteria with distinctively different ecological niches, the freshwater bacterium *C. crescentus* (5) and the soil bacterium *R. meliloti* (13, 21), use CcrM-mediated DNA methylation for essential cell cycle functions. We speculate that CcrM-mediated methylation is likely to have similar physiological roles among the members of the alpha subdivision of proteobacteria.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *C. crescentus* strains were grown and synchronized as previously described (9). *R. meliloti* strains were grown at 30°C in LB medium supplemented with neomycin (50 μg/ml), streptomycin (50-500 μg/ml), or spectinomycin (50 μg/ml) where necessary. *R. meliloti* 1021 (Su47 *str-21*) was kindly provided by Sharon Long (Stanford University). The other alpha subdivision species used in this study were obtained as previously described (38).

Isolation of PCR fragments encoding *ccrM* homologs. Degenerate primers for PCR were designed based on regions of homology between the *C. crescentus* CcrM (44) and the *Haemophilus influenzae* Rf M.*Hin*fI DNA methyltransferase sequences (7). Primer 1 [5′-AT(C/T)TT(C/T)GC(C/G/T)GA(C/T)CC(C/G/T)TA-3′] corresponds to amino acids 29 to 34 in the *C. crescentus* sequence; primer 2 [5′-TC(A/G)TT(G/C)A(A/G)(A/G)ATCCA(A/G)AA-3′] corresponds to amino acids 108 to 113. These primers were used in a PCR with genomic DNA from various species as the template, and a 258-bp product was amplified. Conditions for each PCR varied depending on the template. A different set of primers {primer 1 [5′-AT(C/T)TT(C/T)GC(C/G/T)GA(C/T)CC(C/G/T)CC(C/G/T)TA-3′] corresponds to amino acids 28 to 34 in the *C. crescentus* amino acid sequence; primer 2 [5′-CC(A/G)AA(A/G)AA(A/C/G)GG(A/G)TC(G/C)AG-3′] corresponds to amino acids 108 to 113 in the *C. crescentus* amino acid sequence} were used to amplify a fragment from *R. meliloti* to generate a product of 573 bp. The resulting PCR fragments were sequenced by using a Thermosequenase DNA sequencing kit (Amersham).

Cloning the full-length *R. meliloti* and *B. abortus ccrM* genes. The 573-bp PCR product amplified from the *R. meliloti* genomic DNA was used as a specific probe to screen an *R. meliloti* 1021 λFixII library (kindly provided by Sharon Long). Four positive clones were isolated and characterized. A 3.0-kb *Not*I fragment encompassing the complete *R. meliloti ccrM* gene was subcloned and ligated into the *Not*I site of pBluescriptSKI(−), generating pRW115. The *B. abortus ccrM* gene was cloned in a similar manner. The 258-bp PCR product was used to screen a λZAP library (kindly provided by T. Ficht, Texas A&M University), and the complete *B. abortus ccrM* gene was subcloned as a 4.0-kb *Bam*HI fragment into pBluescriptSKI(−), creating pRW342. Both the *R. meliloti* and *B. abortus* full-length *ccrM* genes were sequenced as described above.

A

```
     29                                                                                                  113
C.cr IFADPPYNL QLGGDLLRPD NSKVDAVDDH WDQFESFAAY DKFTREWLKA ARRVLKDDGA IWVIGSYHNI FRVGVAVQDL GFWILN
C.ba ......... .Q........ .......... .......... .......M.E CQ........ L......... ......L... ......
C.su ......... ......N... G.H....T.A ..K.D.L... .A......AE ...I.....T .......... .K..S.I... ......
R.ca ......... ..R...H... .........A ....G..DH. .T...D..A. .K.I..PN.. .........V ..L.AEL.NQ ......
A.tu ......... ....NVH... Q.L......E ....A..D.. .A...A..L. C.....PN.T .......... ....AML.N. D.....
R.me V......... ....T.H... Q.L......D ....A..E.. .A...A..L. C.....PT.T L......... ....AIL... H..V..
B.ab ......... ....D.H... Q.M.S..... .......Q.. .A...A..L. C.....PN.T .......... ....TQL... ...L..
H.in .......FM .TE.K...TN GDEFSG...E ..K.ND.VE. .S.CEL...E CK.I..ST.S ......FQ.. Y.I.YIM.N. D.....
```

B % similarity among the CcrM homologs

	C.ba	C.su	R.ca	A.tu	R.me	B.ab	H.in
C.cr	94	86	81	76	79	82	61
C.ba		83	77	75	77	81	64
C.su			76	75	78	80	60
R.ca				80	79	80	60
A.tu					93	89	65
R.me						89	65
B.ab							62

FIG. 1. The CcrM homologs are a conserved DNA methyltransferase family. (A) Alignment of the CcrM homologs. The deduced amino acid sequence for each partial *ccrM* gene is shown. C.cr, *C. crescentus*; C.ba, *C. bacteroides*; C.su, *C. subvibrioides*; R.ca, *R. capsulatus*; A.tu, *A. tumefaciens*; R.me, *R. meliloti*; B.ab, *B. abortus*; H. in, *H. influenzae*. A dot indicates that the amino acid present in that sequence is identical to that of *C. crescentus* CcrM. (B) Percent similarities among the CcrM homologs derived by using the GAP program in the Genetics Computer Group sequencing package (8).

Construction of the *R. meliloti ccrM* null mutant. *R. meliloti ccrM* was disrupted by inserting the *nptII* gene (which encodes resistance to neomycin [Neo^r]) into the *Eco*RV site in the *ccrM* gene. A 4.5-kb *Xho*I-*Xba*I fragment with the disrupted gene and flanking sequence was ligated into the suicide vector pJQ200SK, containing the *sacB* gene (31), generating pRW134. pRW134 was mated into wild-type *R. meliloti* 1021, and two integrants, LS2590 and LS2591, which were resistant to neomycin and gentamicin were selected. In strain LS2590, pRW134 integrated by recombination at the chromosomal *ccrM* locus on the 5′ side of *nptII*, while in strain LS2591, pRW134 integrated on the 3′ side of *nptII* (see Fig. 3A). Thus, in both strains, a wild-type copy of *ccrM* is separated from the disrupted copy of *ccrM* by a plasmid sequence containing the counterselectable *sacB* gene and the gene encoding gentamicin resistance (Gm^r). To induce excision of the integrated plasmid, the strains were grown on 5% sucrose (16). Sucrose-resistant isolates were tested for Neo^r and sensitivity to gentamicin (Gm^s), which indicated that only the disrupted copy of *ccrM* remained. For a diagram of this technique, see Fig. 3A.

To determine whether the recombination event yielding the disrupted copy of *ccrM* on the chromosome could occur in the presence of the *R. meliloti ccrM* gene on a plasmid, a 1.6-kb *Pst*I-*Hin*dIII fragment encompassing the *R. meliloti ccrM* gene was cloned into plasmid pMB440, generating pRW175. This plasmid was mated into LS2590 and LS2591. Selection for the excision of the integrated plasmid, leaving behind the disrupted *ccrM* gene at this locus, was performed as described above. Parallel experiments were performed with the vector pMB440 without an insert.

Nomarski microscopy and flow cytometry. The 1.6-kb *Pst*I-*Hin*dIII fragment from pRW115 containing the complete *R. meliloti ccrM* gene was ligated into the vector pMB393, and the resulting plasmid was mated to *R. meliloti* 1021, generating LS2660. Logarithmic-phase cells from LS2660 and LS2764 were prepared for Nomarski microscopy as previously described (43) and were photographed, without staining, with 100 ASA Ecktachrome on a Zeiss Axiophot microscope with differential interference contrast (Nomarski) optics.

For flow cytometry analyses, strains LS2660 and LS2764 (*R. meliloti* containing pMB393) were grown to the mid-logarithmic phase, and neomycin was added to a final concentration of 50 μg/ml. The cultures were incubated for a further 3 h, at which time the cells were fixed and stained as previously described (42). For each flow cytometry experiment, the DNA content was measured in a population of 10,000 cells with a Becton Dickinson FACStar Plus machine at 495 nm. The data were collected and analyzed by using the FACS/DESK software (Stanford University, Stanford, Calif.).

Exchanging the *R. meliloti* and *C. crescentus ccrM* genes. To determine whether the *C. crescentus ccrM* gene could replace *R. meliloti ccrM*, a 2.0-kb *Sac*II fragment containing the *C. crescentus ccrM* promoter and gene was ligated into the high-copy-number vector pMB393 and the low-copy-number vector pMB440, generating pRW205 and pRW207, respectively. These plasmids were mated into LS2590, and selection for excision of the integrated plasmid was performed as described above.

To determine if the *R. meliloti ccrM* could replace *ccrM* in *C. crescentus*, a fusion of the *C. crescentus ccrM* promoter and the *R. meliloti ccrM* coding sequence was constructed. The *C. crescentus ccrM* promoter was amplified by PCR (primer 1 [5′-AGAACTCCATGGTCCCACGTCCTCA-3′] and primer 2 [5′-CCGAATTCGAGGAGATCGCCCAGCTCA-3′]). Primer 1 introduced an *Eco*RI site at one end of the fragment, and primer 2 introduced an *Nco*I site overlapping the *C. crescentus ccrM* start codon. The *R. meliloti ccrM* gene was also amplified by PCR (primer 1 [5′-GGACAGCACGATCGCTTTCG-3′] and primer 2 [5′-CAATGCTACGACGCTGCAGC-3′]). Primer 1 introduced an *Nco*I site overlapping the *R. meliloti ccrM* start codon, and primer 2 introduced a *Hin*dIII site directly after the *R. meliloti* stop codon. Digestion of both PCR products with *Nco*I, *Eco*RI, and *Hin*dIII followed by a triple ligation into pBluescriptSKI(−) resulted in a fusion of the *C. crescentus ccrM* promoter and the *R. meliloti ccrM* gene at the start codon (at the introduced *Nco*I sites), generating pRW184. A fragment containing the promoter and coding sequence was ligated into plasmid pRKlac290, creating pRW201. pRW201 was mated into *C. crescentus* LS1824. LS1824 contains the wild-type *ccrM* gene separated from the disrupted copy of *ccrM* by a plasmid sequence with the ampicillin gene and the counterselectable *sacB* gene (38). Growth on 3% sucrose selected for excision of the integrated plasmid. Sucrose-resistant strains which had only the disrupted copy of *ccrM* remaining on the chromosome were selected for spectinomycin resistance and ampicillin sensitivity.

Determining the methylation state of the *dnaA* chromosomal locus in *C. crescentus*. The DNA methylation state of an overlapping *Hin*fI-*Hin*dII site located just upstream of the *dnaA* gene was assessed in strains LS2818 and LS2866. Genomic DNA was isolated at various time points during the *C. crescentus* cell cycle, and the DNA methylation state of the *dnaA*-overlapping *Hin*fI-*Hin*dII site was determined. Southern blots of *Hin*dII-digested chromosomal DNA were probed with a randomly labeled 420-bp *Hin*dII fragment from plasmid pDnaA. When the *Hin*fI site is fully methylated, the *Hin*dII restriction enzyme is unable to cleave the overlapping *Hin*dII site, and thus the probe hybridizes to a 820-bp product. When this site is hemimethylated, the *Hin*dII restriction enzyme is able to cleave this site and the probe will hybridize to a 420-bp product. The percentage of hemimethylated DNA was quantitated by using a Molecular Dynamics PhosphorImager with ImageQuant software. A detailed explanation of this overlapping restriction site DNA methylation assay has been previously provided (27, 44).

Nucleotide sequence accession numbers. The *R. meliloti ccrM* gene has been given GenBank accession no. AF011894, and the *B. abortus ccrM* gene has been assigned accession no. AF011895.

RESULTS

CcrM homologs from alpha subdivision bacteria form a highly conserved family of DNA methyltransferases. Chromosomal DNA from numerous alpha subdivision species is methylated at the adenine residue in the sequence GANTC. Furthermore, the *C. crescentus ccrM* gene hybridizes with genomic DNA from several alpha subdivision species, suggesting that CcrM DNA methyltransferases are widespread among this group of bacteria (38). To examine the sequence conservation among CcrM homologs, PCR was used to isolate 258 bp of the *ccrM* coding region from six alpha subdivision bacteria: *C. bacteroides*, *C. subvibrioides*, *R. capsulatus*, *A. tumefaciens*, *R. meliloti*, and *B. abortus*. Alignment of these sequences showed that the CcrM homologs are highly conserved, with stretches of invariant amino acid residues (Fig. 1A). A determination of pairwise similarities of the CcrM homologs found a range of 75 to 94% similarity in primary sequence (Fig. 1B). The beta subdivision bacterium *Haemophilus influenzae* contains the

```
B.ab  MSLVRLAHEL PIEAPRTAWL DSIIKGDCVS ALERLPDHSV DVIFADPPYN  50
R.me  ..S.VSLA.I SRA.RPLN.. .........A ..NA...... ..V.......
C.cr                  MKFGP ET..H...IE QMNA..EK.. .L........

B.ab  LQLGGDLHRP DQSMVSAVDD HWDQFESFQA YDAFTRAWLL ACRRVLKPNG  100
R.me  .....T.... ...L.D.... D....A..E. .......... ........T.
C.cr  .......L.. .N.K.D.... ........A. ..K...E..K .A.....DD.

B.ab  TIWVIGSYHN IFRVGTQLQD LGFWLLNDIV WRKTNPMPNF RGRRFQNAHE  150
R.me  .L........ .....AI... .H..V....I ....Q.DAEL Q.........
C.cr  A......... .....VAV.. ....I..... ...S...... K.T..A....

B.ab  TLIWASREQK GKGYTFNYEA MKAANDDVQM RSDWLFPICT GSERLKDENG  200
R.me  .....TANA. A......... .......... .........S ......GDD.
C.cr  ......KS.N A.R.....D. L.M...E... ....TI.L.. .E..I.GAD.

B.ab  DKVHPTQKPE ALLARIMMAS SKPGDVILDP FFGSGTTGAV AKRLGRHFVG  250
R.me  K......... ......L... T.....V... .......... ..........
C.cr  Q.A....... ...Y.VILST T......... ...V.....A ......K.I.

B.ab  IEREQPYIDA ATARINAVEP LGKAELTVMT GKRAEPRVAF TSVMEAGLLR  300
R.me  .....D.... .AE..A.... ....T.S... ..K....... NTLV.S..IK
C.cr  ....AE.LEH .K...AK.V. IAPED.D..G S.......P. GTIV.....S

B.ab  PGTVLCDERR RFAAIVRADG TLTANGEAGS IHRIGARVQG FDACNGWTFW  350
R.me  .....T.AK. .YS....... ..ASG..... ...L..K... L.........
C.cr  ..DT.YCSKG THV.K..P.. SI.VGDLS.. ..K...L..S AP......Y.

B.ab  HFEENGVLKP IDALRKIIRE QMAAAGA  377
R.me  ....GS.... ..E..SV..N DL.KLN
C.cr  ..KTDAG.A. ..V..AQV.A GMN
```

FIG. 2. Comparison of the full-length CcrM homologs from *C. crescentus* (C.cr), *R. meliloti* (R.me), and *B. abortus* (B.ab). A dot indicates that the residue in that sequence is identical to the amino acid in *B. abortus.*

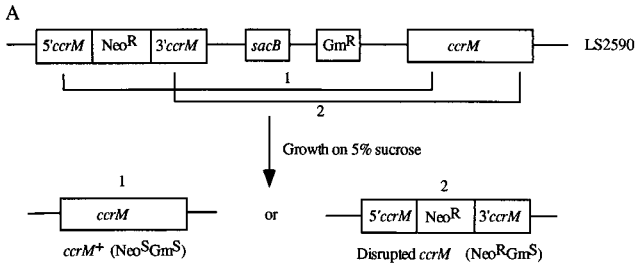
R/M DNA methyltransferase M.*Hin*fI, which has the same recognition sequence as the CcrM DNA methyltransferases (7). Comparison of the M.*Hin*fI sequence and sequences of the CcrM homologs showed 60 to 65% similarity, indicating that the alpha subdivision CcrM proteins are more closely related to each other than to M.*Hin*fI (Fig. 1B).

To determine whether this sequence conservation extends over the entire protein, we cloned and sequenced the full-length *ccrM* genes from two bacteria, the nitrogen-fixing soil bacterium *R. meliloti* and the animal pathogen *B. abortus*. Alignment of the deduced CcrM amino acid sequences with the *C. crescentus* CcrM sequence indeed revealed that there is extensive conservation throughout the entire protein (Fig. 2). The *R. meliloti* and *B. abortus* CcrM homologs are more similar to each other (87% similarity) than to *C. crescentus* CcrM (76 and 78% similarity, respectively). Additionally, both the *R. meliloti* and *B. abortus* homologs have an extended N terminus of 15 amino acids.

***R. meliloti ccrM* is essential for viability.** To determine if the sequence homology among the CcrM DNA methyltransferases extends to similar cellular functions, we compared the physiological roles of CcrM-catalyzed methylation in *R. meliloti* to those previously observed in *C. crescentus. R. meliloti* was selected for further analysis because it is amenable to genetic manipulation and has an interesting life cycle that includes cellular differentiation upon infection of alfalfa plant root hairs (13, 21). Because CcrM is essential for viability in *C. crescentus* (38), we attempted to generate a *ccrM* null allele in *R. meliloti*. The *R. meliloti ccrM* gene was disrupted by insertion of a selectable marker (*nptII*), and the resulting plasmid, pRW134, was integrated in both orientations into the *R. meliloti ccrM* chromosomal locus by homologous recombination, generating strains LS2590 and LS2591. As a result of this recombination event, the wild-type chromosomal copy of *ccrM* was separated from the disrupted *ccrM* by a plasmid sequence containing the counterselectable *sacB* gene (Fig. 3A). Subsequent growth of LS2590 and LS2591 on 5% sucrose selected for excision of the *sacB* gene and a second recombination event between the two *ccrM* copies. Recombinants in which wild-type *ccrM* was replaced by disrupted *ccrM* were selected for by Neo^r and Gm^s. However, as shown in Fig. 3B, it was not possible to obtain strains with a deleted wild-type chromosomal *ccrM* locus. Of 300 sucrose-resistant colonies screened, none contained a disrupted *ccrM*. To determine if a chromosomal disruption of the *ccrM* gene could occur if CcrM activity was provided in *trans*, *ccrM* chromosomal disruptions were selected for in the presence of plasmid pRW175, which contains the wild-type *ccrM* gene. In this experiment, of 250 sucrose-resistant LS2590 and LS2591 isolates screened, 59 and 79%, respectively, retained only the disrupted *ccrM* gene. In the presence of the vector pMB440 alone, no *ccrM* disruption isolates were obtained (Fig. 3B). Therefore, as is the case in *C. crescentus* (38), CcrM is essential for viability in *R. meliloti.*

Overexpression of *ccrM* in *R. meliloti* causes defects in cell division, cell morphology, and the control of DNA replication initiation. In *C. crescentus*, overexpression of CcrM results in the loss of control of initiation of DNA replication and aberrant cell division and cell morphology (43, 44). We examined whether overexpression of CcrM had a similar effect in *R. meliloti*. The *R. meliloti ccrM* gene and promoter were subcloned into the high-copy-number plasmid pMB393, and the

A



B

Strain	Plasmid	Genotype at the *ccrM* chromosomal locus after pRW134 excision (% of isolates)	
		ccrM$^+$ Neo^{S}Gm^S	Disrupted *ccrM* Neo^{R}Gm^S
LS2590	None	300 (100%)	0
LS2591	None	300 (100%)	0
LS2590	pMB440 (vector)	300 (100%)	0
LS2591	pMB440 (vector)	300 (100%)	0
LS2590	pRW175 (*ccrM*$^+$)	105 (42%)	145 (58%)
LS2591	pRW175 (*ccrM*$^+$)	58 (21%)	192 (79%)

FIG. 3. The *R. meliloti ccrM* gene is essential for viability. (A) Strategy for disrupting the *R. meliloti ccrM* gene. Plasmid pRW134 was integrated into the *R. meliloti* 1021 genome at the *ccrM* locus, generating strains LS2590 and LS2591. The map of the *ccrM* locus in LS2590 is shown. Growth on 5% sucrose selects for a second recombination event which can generate two types of isolates. If excision of the plasmid occurs on the 5′ side of *nptII* (model 1), the wild-type *ccrM* is retained and the sucrose-resistant isolates will be Neo^s Gm^s. However, if excision occurs on the opposite side of *nptII* (model 2), then the disrupted *ccrM* gene is retained and the sucrose-resistant isolates will be Neo^r Gm^s. (B). Genotype of the *ccrM* chromosomal locus after growth on sucrose to select for excision of plasmid pRW134. The percentage of isolates that retained wild-type *ccrM* or disrupted *ccrM* for each experiment is shown in parentheses.

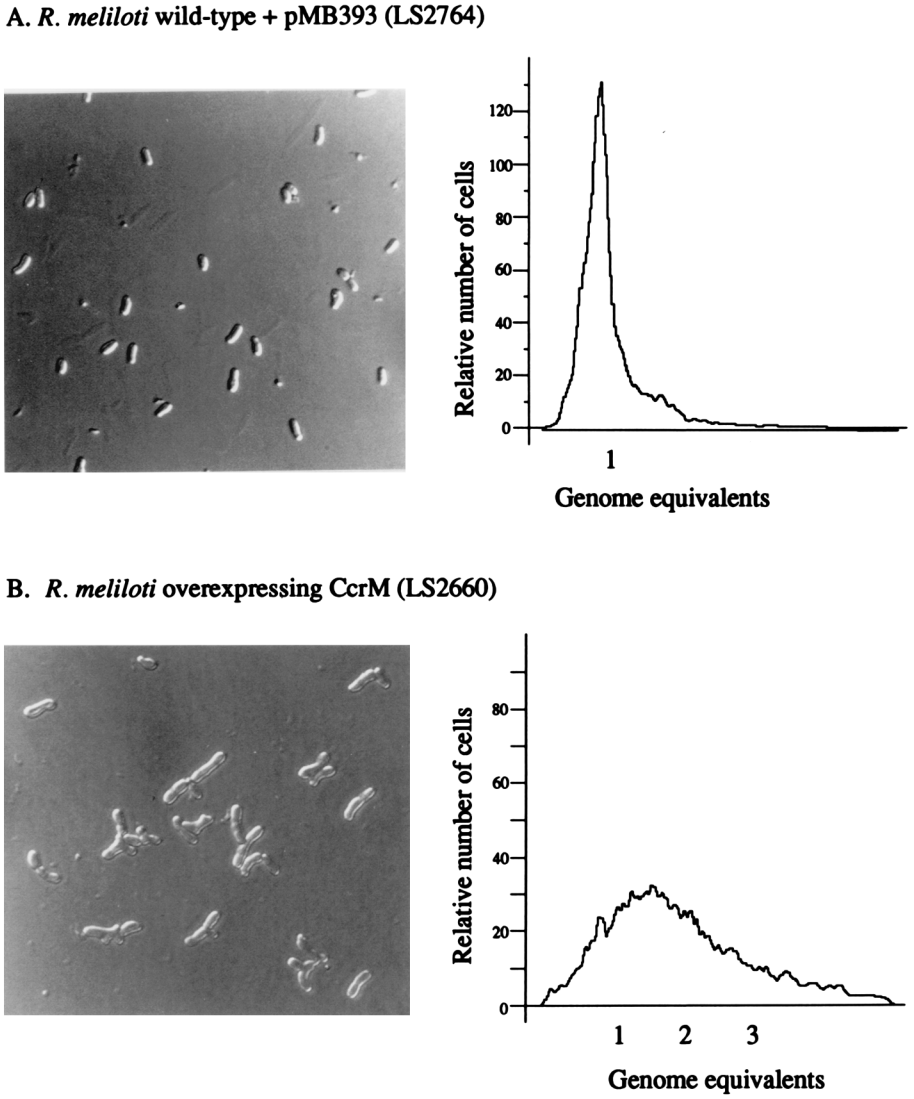


FIG. 4. Overexpression of *R. meliloti* CcrM results in defects in cell division and cell morphology and in the loss of control over the initiation of DNA replication. Shown are Nomarski micrographs and flow cytometry analyses of strains LS2764 (*R. meliloti* and vector alone) (A) and LS2660 (*R. meliloti* overexpressing *ccrM*) (B). For the flow cytometry analyses, the vertical axis shows the relative number of cells measured by fluorescence intensity and the horizontal axis shows the genome equivalents.

resulting construct, pRW171, was mated into wild-type *R. meliloti* to generate strain LS2660. The phenotypes of LS2660 (strain overexpressing *ccrM*) and the control strain LS2764 (*R. meliloti* with vector alone) are shown in Fig. 4. In control strain LS2764, the cells resembled the short rods typical of wild-type cells (Fig. 4A). However, in LS2660, more than 70% of the cells were branched and bloated, and they were two to three times the size of wild-type cells (Fig. 4B). This phenotype has recently been correlated with blocked cell division (18). Therefore, overexpression of *ccrM* in *R. meliloti* results in aberrant cell division and cell morphology.

Flow cytometry was used to assess the effects of overexpression of the *R. meliloti* CcrM homolog on DNA replication by comparing the numbers of initiation events in LS2660 and the wild-type control strain LS2764. Log-phase cultures of LS2660 and LS2764 were treated with neomycin, which allows completion of DNA replication but blocks initiation of new rounds of DNA replication and cell division. In the control strain LS2764, a single peak in DNA content representing the main chromosome and the two large pSym plasmids (pSymA and pSymB) was observed (Fig. 4A). When *R. meliloti* CcrM was overexpressed, a broad peak representing cells with one to three genome equivalents was observed, indicating that additional initiations of DNA replication had occurred (Fig. 4B). Thus, increasing the levels of *R. meliloti* CcrM results in the loss of normal control of DNA replication initiation.

The *R. meliloti* and *C. crescentus ccrM* genes are functionally interchangeable. The conservation of CcrM sequence and function in *R. meliloti* and *C. crescentus* suggested that the two *ccrM* genes might be interchangeable. To determine if the *C. crescentus ccrM* gene could replace *R. meliloti ccrM*, the *C. crescentus ccrM* promoter and gene were ligated into two replicating *R. meliloti* plasmids that are present at high and low copy numbers, generating pRW205 and pRW207, respectively. These plasmids were mated into *R. meliloti* LS2590, and the resulting strains were grown on 5% sucrose. Sucrose-resistant isolates retaining the disrupted copy of *ccrM* on the chromosome were isolated as described in Materials and Methods and

TABLE 2. Summary of the complementation results[a]

Background	Plasmid	Plasmid copy number	Viability	Methylated GANTC sites
C. crescentus Δ*ccrM*	None	N/A	-	N/A
	pCS174 (P C.cr — C.cr *ccrM*)	low	+	+
	pRW201 (P C.cr — R.me *ccrM*)	low	+	+
R. meliloti Δ*ccrM*	None	N/A	-	N/A
	pRW175 (P R.me — R.me *ccrM*)	low	+	+
	pRW207 (P C.cr — C.cr *ccrM*)	low	-	N/A
	pRW205 (P C.cr — C.cr *ccrM*)	high	+	+

[a] N/A, not applicable.

the legend for Fig. 3A. It was possible to recover *R. meliloti* LS2838 bearing the disrupted copy of *ccrM* on the chromosome when *C. crescentus ccrM* was present on the high-copy-number plasmid pRW205. Of 300 individual sucrose-resistant colonies examined, 69% of the isolates retained the disrupted copy of *ccrM* on the chromosome. Approximately 20% of the cells from this complemented strain were branched and bloated compared to wild-type cells (data not shown). Complementation did not occur when *C. crescentus ccrM* was present in the low-copy-number plasmid pRW175. Of 300 sucrose-resistant isolates screened, all retained the wild-type *ccrM* gene on the chromosome. These complementation results suggest that *C. crescentus ccrM* gene is poorly transcribed in *R. meliloti*, and the abnormal cells observed in LS2838 may be the result of reduced expression of *C. crescentus ccrM* in *R. meliloti.*

To show that *C. crescentus* CcrM is functional in *R. meliloti*, we digested the genomic DNA from the complemented strain with restriction endonuclease *Hin*fI (which digests only unmethylated GANTC sequences) and found that the DNA was resistant to *Hin*fI cleavage (Table 2). Therefore, as the genomic DNA in LS2838 must be methylated at GANTC sites, *C. crescentus* CcrM is able to functionally substitute for *R. meliloti* CcrM.

A similar experiment was performed to determine whether the *R. meliloti ccrM* gene could function in *C. crescentus*. In *C. crescentus*, transcription of *ccrM* occurs at a specific time in the cell cycle. Changes in the temporal expression of *ccrM* result in aberrant cell division and cell morphology (43, 44). To ensure proper temporal control of *R. meliloti ccrM* expression in *C. crescentus*, we constructed a fusion between the *C. crescentus ccrM* promoter and the *R. meliloti ccrM* gene on the low-copy-number plasmid pRKlac290, generating pRW201. pRW201 was able to complement the *C. crescentus ccrM* null mutation generated from strain LS1824 after growth on 3% sucrose (38). Of 300 sucrose-resistant colonies, 57% retained the disrupted copy of *ccrM* on the chromosome. The complemented strain, LS2818, was indistinguishable in both morphology and growth rate from the control strain LS2866 (*C. crescentus ccrM* gene on a plasmid in a *C. crescentus ccrM* null background) (data not shown). To verify that *R. meliloti* CcrM is active in *C. crescentus*, genomic DNA from the complemented strain, LS2818, was treated with *Hin*fI and found to be resistant to digestion (Table 2), indicating that *R. meliloti* CcrM catalyzes the methylation of *C. crescentus* DNA. Thus, the *C. crescentus* and *R. meliloti* CcrM homologs are functionally interchangeable. The complementation results are summarized in Table 2.

Turnover of *R. meliloti* CcrM in *C. crescentus*. In *C. crescentus*, the level of CcrM protein is controlled through transcription and protein instability to ensure that CcrM activity occurs only in the predivisional stage of the cell cycle. In a *lon* null strain, or if *ccrM* is transcribed from a constitutively expressed promoter, CcrM is present throughout the cell cycle and the chromosomal DNA remains fully methylated, which leads to developmental abnormalities (43, 44). In this study, we asked if the *R. meliloti* CcrM protein is confined to the predivisional stage of the *C. crescentus* cell cycle when expressed from the *C. crescentus ccrM* promoter in strain LS2818. Because the antiserum generated previously to the *C. crescentus* CcrM protein does not cross-react with *R. meliloti* CcrM, its levels could not be monitored directly by Western blot analyses. To indirectly determine when *R. meliloti* CcrM was present during the cell cycle, the methylation state of the *dnaA* locus was assayed in synchronized populations of strain LS2818 by using a methylation-sensitive restriction site that overlaps the *Hin*fI sequence (38, 44) (Fig. 5A). Parallel experiments performed with a wild-type strain of *C. crescentus* have shown that the *dnaA* locus goes from the fully methylated state to the hemimethylated state soon after the initiation of DNA replication, since *dnaA* lies near the origin of replication. The *dnaA* locus remains hemimethylated until the late predivisional stage of the cell cycle, when it is remethylated by CcrM (38). If the *R. meliloti* CcrM protein in LS2818 were not degraded, it would remain present and active throughout the cell cycle and the *dnaA* site would remain fully methylated. However, if *R. meliloti* CcrM is degraded similarly to native *C. crescentus* CcrM, the methylation state of the *dnaA* locus should vary during the cell cycle. The results shown in Fig. 5B and C demonstrate that the DNA

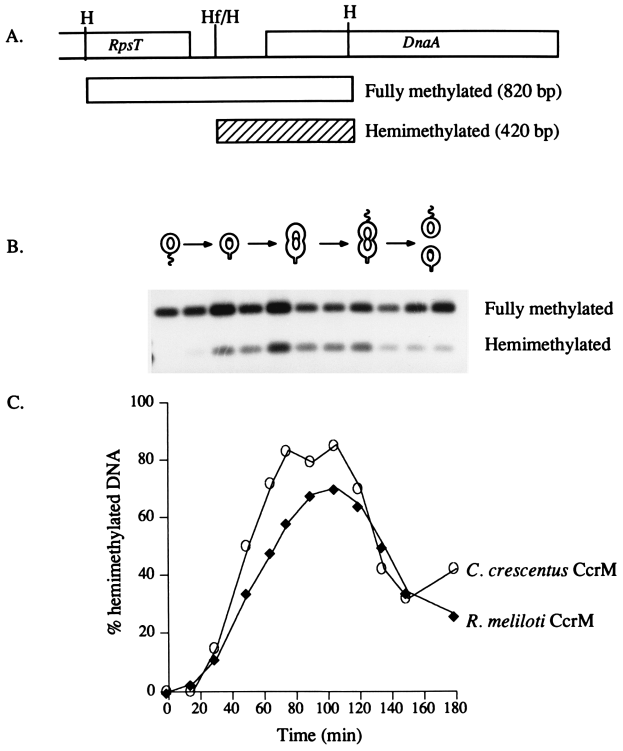


FIG. 5. Cell cycle expression of *R. meliloti* CcrM in *C. crescentus*. Swarmer cells from LS2818 (*R. meliloti ccrM* under the transcriptional control of the *C. crescentus ccrM* promoter in a *C. crescentus ccrM* null background) and LS2866 (*C. crescentus ccrM* promoter and gene in a *C. crescentus ccrM* null background) were isolated by Ludox density centrifugation (9). The cells were resuspended in M2G at 30°C and allowed to proceed through the cell cycle. Cell cycle progression was monitored by microscopic analyses, and cell division occurred at approximately 160 min. (A) Diagram of the *dnaA* chromosomal locus. If the overlapping *Hin*fI/*Hin*dII (Hf/H) site is fully methylated, then the *Hin*dII restriction endonuclease is unable to digest this site. Thus, in a Southern blot of *Hin*dII-digested genomic DNA with the 420-bp *Hin*dII fragment as the probe (hatched box), an 820-bp fragment representing fully methylated DNA is detected. If the Hf/H site is hemimethylated, then the *Hin*dII restriction endonuclease is able to cleave this site, and a 420-bp band representing the hemimethylated DNA is detected by Southern blotting (see Materials and Methods for a detailed description of this technique). (B) The level of hemimethylated DNA at the *dnaA* locus during the cell cycle in LS2818. Southern blots of *Hin*dII-digested genomic DNA from various time points of the LS2818 cell cycle were probed with the 420-bp *Hin*dII fragment from the *dnaA* gene. The top band represents the fully methylated DNA, and the lower band represents hemimethylated DNA. A diagram of the *C. crescentus* cell cycle is shown at the top. (C) Levels of fully methylated and hemimethylated DNA were quantified with a PhosphorImager. Results are plotted as the percentage of hemimethylated DNA relative to the total signal (hemimethylated plus fully methylated). ○, LS2866 (*C. crescentus* containing the *C. crescentus ccrM* gene on pRKlac290); ◆, LS2818 (*C. crescentus* containing the *R. meliloti ccrM* gene on pRKlac290).

methylation state at this locus in LS2818 varies as a function of the cell cycle, as it does in the control strain LS2866 (*C. crescentus ccrM* gene on a plasmid in a *C. crescentus ccrM* null background). Thus, the *R. meliloti* CcrM is either degraded or somehow inactivated as the cell cycle is completed, as its activity is absent in the swarmer cell at the start of the cell cycle. It is possible that *R. meliloti* CcrM is recognized and degraded via the Lon protease pathway as is the case for *C. crescentus* CcrM (43).

DISCUSSION

We have isolated partial- or full-length *ccrM* genes from six alpha subdivision bacteria (*C. bacteroides*, *C. subvibrioides*, *R. capsulatus*, *A. tumefaciens*, *R. meliloti*, and *B. abortus*), and alignment of the deduced amino acid sequences revealed that the CcrM homologs constitute a highly conserved DNA methyltransferase family. Furthermore, there is a correlation between the sequence percent similarities of the CcrM homologs and the bacterial phylogenies determined by 16S rRNA comparisons (29). For instance, in 16S rRNA phylogenies, the *R. meliloti* and *A. tumefaciens* bacteria are more closely related to each other than to *C. crescentus*. This parallels the similarities between the CcrM homologs from these three bacteria: the CcrM homologs from *R. meliloti* and *A. tumefaciens* are 93% similar to one another, whereas they are 79 and 76% similar, respectively, to *C. crescentus.*

The high level of sequence homology among the CcrM DNA methyltransferases suggested that CcrM-mediated DNA methylation has similar roles in alpha subdivision bacteria. We demonstrate here that there are functional similarities in CcrM methylation for at least two of these bacteria, *C. crescentus* and *R. meliloti*. In both organisms, CcrM is essential for viability. Overexpression of CcrM in either *C. crescentus* or *R. meliloti* results in defects in cell division, cell morphology, and the initiation of DNA replication, suggesting that CcrM-catalyzed DNA methylation plays integral roles in the cell cycles of both bacteria. The two *ccrM* genes are interchangeable between these two bacteria, and the resulting CcrM protein is functionally active in the complemented strains. Furthermore, when *R. meliloti ccrM* is under the transcriptional control of the *C. crescentus ccrM* promoter in *C. crescentus*, the chromosome methylation state varies during the cell cycle in a pattern similar to the wild-type pattern. This result suggests that the *R. meliloti* CcrM protein is degraded during the *C. crescentus* cell cycle. We speculate that the sequence conservation among CcrM homologs reflects the requirement not only to encode an active DNA methyltransferase but also to contain the determinants which target CcrM for specific degradation, possibly via the Lon protease as has been shown for *C. crescentus* (43). The functional similarities of CcrM-mediated methylation in *C. crescentus* and *R. meliloti* and the conservation of sequence among the CcrM homologs suggest that CcrM-mediated methylation has similar roles among alpha subdivision bacteria and was probably present in an earlier progenitor of this group of bacteria.

Although the specific physiological role(s) for CcrM-catalyzed methylation has yet to be identified, it is unlikely that CcrM is part of an R/M system in either *C. crescentus* or *R. meliloti*, as there is no cognate restriction endonuclease upstream or downstream of the *ccrM* gene. In addition, we have previously shown for *C. crescentus* that when CcrM-catalyzed DNA methylation is inhibited, the chromosome is not degraded, as would be the case if a cognate restriction enzyme were present (38). In several aspects, the CcrM DNA methyltransferase is functionally analogous to the Dam DNA methyltransferase in *E. coli*. Dam methylation is important in many cellular functions, including regulation of gene transcription (30, 40), DNA replication initiation (3, 20), and mismatch repair (15, 24), although it is not essential for viability. Similarly, CcrM methylation functions in the control of DNA replication initiation and in normal cell division in both *C. crescentus* and *R. meliloti*. It is likely that CcrM methylation is involved in several cellular processes such as in the regulation and coordination of the expression of one or more essential genes.

H. influenzae contains the R/M DNA methyltransferase M.*Hin*fI (7), which has the same DNA recognition sequence and is homologous to the CcrM DNA methyltransferase family. The full-length *R. meliloti*, *C. crescentus*, and *B. abortus*

CcrM homologs are 64 to 66% similar to the M.*Hin*fI DNA methyltransferase. Although M.*Hin*fI is homologous to the CcrM family, it is clear that the CcrM homologs exhibit higher sequence conservation with each other than with M.*Hin*fI (Fig. 1). This is not surprising, as *H. influenzae* is a member of the beta subdivision of proteobacteria and M.*Hin*fI is an R/M DNA methyltransferase (7). Possibly *H. influenzae* acquired M.*Hin*fI by horizontal gene transfer from a member of the alpha subdivision and has since evolved to be part of an R/M system. Dam methyltransferase homologs are present in other bacteria as R/M DNA methyltransferases; examples are the M.*Dpn*II DNA methyltransferase in *Streptococcus pneumoniae* (22) and the M.*Mbo*I DNA methyltransferase from *Moraxella bovis* (39). Thus, homologs for both the Dam and CcrM DNA methyltransferases exist in other bacteria, where they are part of R/M systems. It has been proposed that DNA methyltransferases evolved initially as enzymes with regulatory functions like the CcrM and Dam methyltransferases and that later, restriction endonucleases evolved in the background of a methylated genome (2). It is possible that CcrM and Dam methyltransferases never became part of R/M systems, as they were integral components of their respective cell cycles. Thus two proteobacterial branches possess distinct DNA methylation patterns which are not part of R/M systems but are integral components of the cell cycle: CcrM in the alpha subdivision and Dam in the enteric and related bacteria (1). We speculate that other bacterial lineages have also evolved DNA methylation systems with significant biological functions. Further understanding of bacterial regulatory DNA methylation systems is required before we can fully appreciate the evolution and function of bacterial DNA methylation.

ACKNOWLEDGMENTS

We thank Sharon Long, William Margolin, Valerie Oke, and Melanie Barnett for providing the various strains and plasmids for *R. meliloti* and for helpful discussion and suggestions; we thank T. Ficht, Texas A&M University, for providing the *B. abortus* genomic library. We thank the members of the Shapiro lab, especially Ann Reisenauer, Jeff Skerker, and Chris Mohr, for reading the manuscript.

This work was supported by National Institutes of Health grant GM-52426. R.W. was supported by New Zealand Foundation for Research, Science and Technology grant STA501.

REFERENCES

1. **Barbeyron, T., K. Kean, and P. Forterre.** 1984. DNA adenine methylation of GATC sequences appeared recently in the *Escherichia coli* lineage. J. Bacteriol. **160:**586–590.
2. **Bickle, T. A., and D. H. Kruger.** 1993. Biology of DNA restriction. Microbiol. Rev. **57:**434–450.
3. **Boye, E., and A. Lobner-Olsen.** 1990. The role of dam methyltransferase in the control of DNA replication in *E. coli*. Cell **62:**981–989.
4. **Braaten, B. A., L. B. Blyn, B. S. Skinner, and D. A. Low.** 1991. Evidence for a methylation-blocking factor (*mbf*) locus involved in *pap* pilus expression and phase variation in *Escherichia coli*. J. Bacteriol. **173:**1789–1800.
5. **Brun, Y. V., G. T. Marczynski, and L. Shapiro.** 1994. The expression of asymmetry during *Caulobacter* cell differentiation. Annu. Rev. Biochem. **63:**419–450.
6. **Campbell, J. L., and N. Kleckner.** 1990. *E. coli* oriC and the *dnaA* gene promoter are sequestered from dam methylation following the passage of the chromosomal replication fork. Cell **62:**967–979.
7. **Chandrasegaran, S., K. D. Lunnen, H. O. Smith, and G. G. Wilson.** 1988. Cloning and sequencing the *Hin*fI restriction and modification genes. Gene **70:**387–392.
8. **Devereux, D., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis program for the VAX. Nucleic Acids Res. **12:**387–395.
9. **Ely, B.** 1991. Genetics of *Caulobacter crescentus*. Methods Enzymol. **204:**372–384.
10. **Evinger, M., and N. Agabian.** 1977. Envelope-associated nucleoid from *Caulobacter crescentus* stalked and swarmer cells. J. Bacteriol. **132:**294–301.
11. **Finan, T. M., B. Klunkel, G. F. De Vos, and E. R. Signer.** 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. J. Bacteriol. **167:**66–72.
12. **Finnegan, E. J., W. J. Peacock, and E. S. Dennis.** 1996. Reduced DNA methylation in *Arabidopsis thaliana* results in abnormal plant development. Proc. Natl. Acad. Sci. USA **93:**8449–8454.
13. **Fisher, R. F., and S. R. Long.** 1992. *Rhizobium*-plant signal exchange. Nature **357:**655–660.
14. **Gober, J. W., and L. Shapiro.** 1992. A developmentally regulated *Caulobacter* flagellar promoter is activated by 3′ enhancer and IHF binding elements. Mol. Biol. Cell **3:**913–926.
15. **Herman, G. E., and P. Modrich.** 1981. *Escherichia coli* K-12 clones that overproduce *dam* methylase are hypermutable. J. Bacteriol. **145:**644–646.
16. **Hynes, M. F., J. Quandt, M. P. O'Connell, and A. Puehler.** 1989. Direct selection for curing and deletion of *Rhizobium* plasmids using transposons carrying the *Bacillus subtilis sacB* gene. Gene **78:**111–120.
17. **Kovach, M. E., P. H. Elzer, D. S. Hill, G. T. Robertson, M. A. Farris, R. M. Roop II, and K. M. Peterson.** 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS carrying different antibiotic-resistance cassettes. Gene **166:**175–176.
18. **Latch, J. N., and W. Margolin.** 1997. Generation of buds, swellings, and branches instead of filaments after blocking the cell cycle of *Rhizobium meliloti*. J. Bacteriol. **179:**2373–2381.
19. **Li, E., T. Bestor, and R. Jaenisch.** 1992. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. Cell **69:**915–926.
20. **Lobner-Olesen, A., E. Boye, and M. G. Marinus.** 1992. Expression of the *Escherichia coli dam* gene. Mol. Microbiol. **6:**1841–1851.
21. **Long, S. R.** 1989. *Rhizobium*-legume nodulation: life together in the underground. Cell **56:**203–214.
22. **Mannarelli, B. M., T. S. Balganesh, B. Greenberg, S. S. Springhorn, and S. A. Lacks.** 1985. Nucleotide sequence of the DpnII DNA methylase gene of *Streptococcus pneumoniae* and its relationship to the *dam* gene of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **82:**4468–4472.
23. **Marinus, M. G., and N. R. Morris.** 1973. Isolation of deoxyribonucleic acid methylase mutants of *Escherichia coli* K-12. J. Bacteriol. **114:**1143–1150.
24. **Marinus, M. G., A. Poteete, and J. A. Arraj.** 1984. Correlation of DNA adenine methylase activity with spontaneous mutability in *Escherichia coli* K-12. Gene **28:**123–125.
25. **Marinus, M. G.** 1996. Methylation of DNA, p. 782–791. *In* F. C. Neidhardt, R. Curtiss, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
26. **Meade, H. M., S. R. Long, G. B. Ruvkun, S. E. Brown, and F. M. Ausubel.** 1982. Physical and genetic characterization of symbiotic and auxotrophic mutants of *Rhizobium meliloti* induced by transposon Tn*5* mutagenesis. J. Bacteriol. **149:**114–122.
27. **Nelson, M., C. Christ, and I. Schildkraut.** 1984. Alteration of apparent restriction endonuclease recognition specificity by DNA methylase. Nucleic Acids Res. **12:**5165–5173.
28. **Ogden, G. B., M. J. Pratt, and M. Schaechter.** 1988. The replicative origin of the *E. coli* chromosome binds to cell membranes only when hemimethylated. Cell **54:**127–135.
29. **Olsen, G. J., C. R. Woese, and R. Overbeek.** 1994. The winds of (evolutionary) change: breathing new life into microbiology. J. Bacteriol. **176:**1–6.
30. **Plumbridge, J.** 1987. The role of dam methylation in controlling gene expression. Biochimie **69:**439–443.
31. **Quandt, J., and M. F. Hynes.** 1993. Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. Gene **127:**15–21.
32. **Quon, K. C., G. T. Marczynski, and L. Shapiro.** 1996. An essential bacterial two-component signal transduction protein controls multiple cell cycle events in *Caulobacter*. Cell **84:**83–93.
33. **Razin, A., and H. Cedar.** 1994. DNA methylation and genomic imprinting. Cell **77:**473–476.
34. **Reik, W., and N. D. Allen.** 1994. Imprinting with and without methylation. Curr. Biol. **4:**145–147.
35. **Ronemus, M. J., M. Galbiati, C. Ticknor, J. Chen, and S. L. Dellaporta.** 1996. Demethylation-induced developmental pleiotropy in Arabidopsis. Science **273:**654–657.
36. **Simon, R., U. Prieffer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/Technology **1:**784–790.
37. **Smith, L. D., and T. A. Ficht.** 1990. Pathogenesis of *Brucella*. Crit. Rev. Microbiol. **17:**209–230.
38. **Stephens, C., A. Reisenauer, R. Wright, and L. Shapiro.** 1996. A cell cycle-regulated bacterial DNA methyltransferase is essential for viability. Proc. Natl. Acad. Sci. USA **93:**1210–1214.
39. **Ueno, T., H. Ito, F. Kimizuka, H. Kotani, and K. Nakajima.** 1993. Gene structure and expression of the MboI restriction-modification system. Nucleic Acids Res. **21:**2309–2313.
40. **van der Woude, M. W., B. A. Braaten, and D. A. Low.** 1992. Evidence for

global regulatory control of pilus expression in *Escherichia coli* by Lrp and DNA methylation: model building based on analysis of pap. Mol. Microbiol. **6:**2429–2435.
41. **Wilson, G. G.** 1988. Type II restriction modification systems. Trends Genet. **4:**314–318.
42. **Winzeler, E., and L. Shapiro.** 1995. Use of flow cytometry to identify a *Caulobacter* 4.5S RNA temperature-sensitive mutant defective in the cell cycle. J. Mol. Biol. **251:**346–365.
43. **Wright, R. J., C. Stephens, G. Zweiger, L. Shapiro, and M. R. K. Alley.** 1996. *Caulobacter* Lon protease has a critical role in cell-cycle control of DNA methylation. Genes Dev. **10:**1532–1542.
44. **Zweiger, G., G. Marczynski, and L. Shapiro.** 1994. A *Caulobacter* DNA methyltransfease that functions only in the predivisional cell. J. Mol. Biol. **235:**472–485.