

The *Caulobacter crescentus* DNA-(adenine-N6)-methyltransferase CcrM methylates DNA in a distributive manner

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ABSTRACT

The specificity and processivity of DNA methyltransferases have important implications regarding their biological functions. We have investigated the sequence specificity of CcrM and show here that the enzyme has a high specificity for GANTC sites, with only minor preferences at the central position. It slightly prefers hemimethylated DNA, which represents the physiological substrate. In a previous work, CcrM was reported to be highly processive [Berdis *et al.* (1998) *Proc. Natl Acad. Sci. USA* 95: 2874–2879]. However upon review of this work, we identified a technical error in the setup of a crucial experiment in this publication, which prohibits making any statement about the processivity of CcrM. In this study, we performed a series of *in vitro* experiments to study CcrM processivity. We show that it distributively methylates six target sites on the pUC19 plasmid as well as two target sites located on a 129-mer DNA fragment both in unmethylated and hemimethylated state. Reaction quenching experiments confirmed the lack of processivity. We conclude that the original statement that CcrM is processive is no longer valid.

INTRODUCTION

DNA methylation at position N⁶ of adenine, or at position N⁴ or C⁵ of cytosine bases is a chemical modification of DNA present in a wide variety of prokaryotic and eukaryotic organisms (1,2). The methylation reaction is catalyzed by DNA methyltransferases (MTases) which employ S-adenosine-L-methionine (AdoMet) as methyl group donor. In bacteria, DNA methylation is most often associated with restriction-modification (RM) systems, which protect the bacterial cell against bacteriophages (3). However, there exists a distinct class of

bacterial DNA MTases, known as solitary MTases, which are not part of an RM system. The best known examples of solitary MTases are the *Escherichia coli* DNA adenine MTase (EcoDam) which recognizes GATC sequences and regulates DNA repair, gene expression and DNA replication (1,4), and the *Caulobacter crescentus* cell-cycle regulating MTase (CcrM) which methylates the adenine in GANTC sites and has a central role in the regulation of the bacterial cell division cycle (5–7). Furthermore, CcrM is an essential protein in several α -Proteobacteria, including pathogens, which makes it a potential antibacterial drug target (8–10).

One important property of DNA MTases is their processivity in the methylation of DNA molecules containing more than one target site. Processive enzymes stay bound to one DNA molecule after first turnover and methylate several target sites on that molecule without dissociation. Thereby, they directly convert unmethylated DNA into DNA modified at all target sites. Distributive enzymes, in contrast, always dissociate from the DNA after one methyl group transfer leading to an accumulation of methylation intermediates, i.e. DNA molecules that are modified at some but not all target sites. Since methylation intermediates are not released by processive enzymes, detection of the presence or absence of intermediates is the most direct and reliable experimental approach in processivity analysis. The processivity of DNA methyltransferases has a strong impact on their biological function, because DNA methylation is established in a radically different way by each type of enzyme. The EcoDam enzyme, for example, was shown to be highly processive, thus leading to efficient re-methylation of the GATC sites after DNA replication (11), although particular flanking sequences were shown to reduce processivity (12). T4Dam was shown to be processive as well (13), while most of the methyltransferases associated with RM systems are distributive, which may help prevent the methylation of incoming phage DNA before its cleavage by restriction digestion (1,11).

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In a publication of Berdis *et al.* (14), it was reported that CcrM methylated DNA in a processive manner. The assay applied in that work for detection of CcrM processivity probed the methylation of GANTC sites through protection against HindII (GTYRAC) cleavage at overlapping sites (Figure 2A). However, although the substrate contained two CcrM sites, only one of them was flanked by a HindII site. Therefore, only one GANTC site was being probed for methylation and no conclusion could be drawn toward processivity. It is clear that this error is not just a typographical mistake in the 'Materials and Methods' section of the manuscript, because at the zero time point in Figure 5 of the Berdis *et al.* (14) publication, the long 51-mer HindII cleavage product was observed, which is indicative of the absence of cleavage at the second CcrM site. Since no methylation can be present at this point, this result can only be explained by the absence of the second HindII site. Thus, the issue of processivity of CcrM must be considered open and not resolved. In this work, we have re-investigated the processivity of CcrM and show that it methylates pUC19 plasmid DNA and a linear 129-mer substrate in a distributive manner.

MATERIALS AND METHODS

Protein expression and purification

His₆-tagged *C. crescentus* CcrM cloned into pET28 vector was kindly provided by Dr Xiaodong Cheng (Emory University). The pET28 plasmid encoding His-tagged CcrM was transformed into HMS174 (DE3) cells (Novagen). The cells were grown in LB medium until mid-exponential phase (OD₆₀₀ 0.5–0.6) and expression was induced by addition of 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG), after which the cultures were incubated at room temperature for an additional 3 h. The cells were harvested by centrifugation at 4300g for 15 min, followed by washing with STE buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1 mM EDTA) and an additional centrifugation step. The His-tagged CcrM was purified by affinity chromatography on Ni-NTA Agarose (Qiagen) using sonication/washing buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 0.1 mM DTT, 10% glycerol, 20 mM imidazol) and elution buffer (50 mM HEPES, pH 7.5, 500 mM NaCl, 0.1 mM DTT, 10% glycerol, 220 mM imidazol). The eluate was dialyzed in dialysis buffer I (50 mM HEPES, pH 7.5, 200 mM NaCl, 0.1 mM DTT, 10% glycerol) for 2–3 h, and in dialysis buffer II (50 mM HEPES, pH 7.5, 200 mM NaCl, 0.1 mM DTT, 60% glycerol) for an additional 12 h. The purified protein was at high concentration (~100 μM) and contained only two minor contaminants (Figure 1A).

DNA substrates

The 129-mer DNA substrate containing two CcrM target sites which was used for processivity analysis was produced by PCR using the pBAD24 vector as template. The hemimethylated 129-mer HM substrate was obtained by PCR using an *in vitro* synthesized DNA oligonucleotide as template. The M.TaqI methylation was carried out

at 65°C for 2 h, with 80 μM AdoMet and using NEB4 buffer supplied by New England Biolabs. The methylated DNA was purified using a standard DNA extraction kit (Macherey-Nagel NucleoSpin ExtractII). All substrates described above are shown in Figure 2B–D. The 23-mer substrates used for determining the relative preference for hemimethylated over unmethylated DNA and for the quenching experiment were obtained by heating of an equimolar (20 μM) mixture of complementary oligonucleotides to 95°C for 5 min and allowing the mixture to slowly cool down to room temperature. The quality of the annealing procedure was assessed by polyacrylamide gel electrophoresis.

23-mer: Bt_d(GGCAGCTACGAATCGCAACAGCT)
 23-mer revmet:_d(AGCTGTTGCG^mATTCGTAGC
 TGCC)
 23-mer_rev: d(AGCTGTTGCGATTCGTAGCTGCC)

In addition, 12 double-stranded 23-mer substrates were used, in which the base pairs at positions 1, 3, 4 or 5 were exchanged against all other base pairs. All substrates were biotinylated and hemimethylated with the methylation in the lower strand (except of variants at the fourth position, which did not contain an A in the lower strand). These substrates were used to investigate the sequence specificity of CcrM for the first, third, fourth and fifth position of the GANTC site.

DNA binding experiments

DNA binding was analyzed by using the nitrocellulose filter-binding assay. For the experiment, radioactively labeled hemimethylated 129-mer DNA substrate was prepared by phosphorylation of the hemimethylated DNA with γ-[P³²]-ATP using T4 polynucleotide kinase (NEB) following the recommendations of the supplier. CcrM concentrations were varied between 0 and 10 μM, 10 nM of DNA was used. The binding reactions were incubated in binding buffer (50 mM HEPES pH 7.5, 50 mM NaCl, 1 mM EDTA and 500 μM DTT) supplemented with 200 μM sinefungin (Sigma) for 30 min at ambient temperature. The nitrocellulose filter membrane (Macherey & Nagel, Düren, Germany) was soaked in binding buffer for 30 min. Afterwards the membrane was transferred into the dot blot chamber (BioRad) and the slots were washed with binding buffer. The samples were transferred into the wells of the dot blot apparatus using a multiple pipette, immediately sucked through the nitrocellulose filter membrane, and washed several times with 100 μl of binding buffer. The membranes were dried and the radioactivity of the spots analyzed using a PhosphorImager (Fuji). The results were fitted to the equation describing a bimolecular association equilibrium to determine the binding constant.

CcrM DNA methylation reactions for processivity analysis

All methylation kinetics were performed in a buffer containing 50 mM HEPES pH 7.5, 50 mM NaCl, 1 mM EDTA and 500 μM DTT, in the presence of 5 ng/μl BSA. The AdoMet (Sigma-Aldrich) cofactor was used at a concentration of 200 μM for the processivity

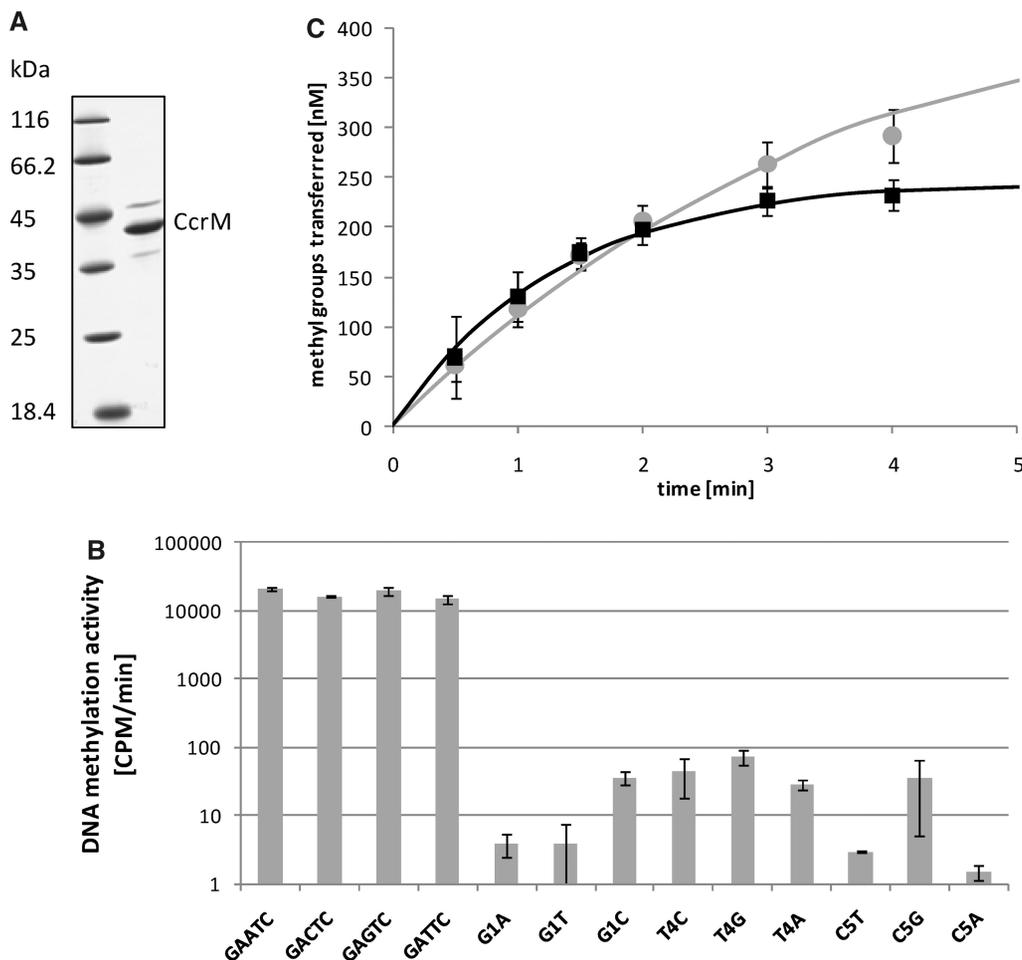


Figure 1. (A) Purification of CcrM. (B) Methylation of various GANTC and near cognate 23-mer substrates by CcrM. The methylation experiments were carried out under single turnover conditions using 1 μ M DNA and 3 μ M CcrM, with 760 nM AdoMet. The experimental data were fitted by linear regression of the initial data points to derive the initial rate of DNA methylation. Experiments were conducted twice, the errors bars display the standard deviation between the individual results. (C) Methylation of hemimethylated (black squares) and unmethylated (gray circles) 23-mer by CcrM. The methylation experiments were carried out as described in B. The experimental data were fitted to an exponential model (black line for hemimethylated DNA, gray line for unmethylated DNA), and the enzymatic turnover rate was extracted. The rate of the methylation reaction carried out using unmethylated DNA as substrate was 0.50 turnovers/min, whereas for hemimethylated DNA the rate was 0.76 turnovers/min. Thus, CcrM has an apparent 1.5-fold preference for hemimethylated, over unmethylated DNA.

experiments. All methylation reactions were carried out at room temperature and started by addition of CcrM. Methylation of pUC19 was carried out using 2 μ M CcrM and 600 nM pUC19. To assess the methylation state of the pUC19 vector, a double digestion was carried out using *Hinf*I and *Nde*I (New England Biolabs), the latter being used for linearization of the vector. The double digestion was carried out overnight at 37°C in buffer NEB4. The methylation reactions with the unmethylated 129-mer were carried out using 1 μ M DNA and 2 μ M CcrM, with the hemimethylated 129-mer HM 1 μ M DNA and 0.5 μ M CcrM were used. Aliquots were taken at various time points and reactions were stopped by shock freezing in liquid nitrogen and purified using PCR purification kit (Macherey-Nagel NucleoSpin Extract II). All restriction endonuclease treatments were carried out for at least 1 h under the appropriate conditions, as recommended by the provider. For the

quenching experiment, a standard methylation was prepared and time points were collected and treated the same way as described above, except that 3 min after starting the reaction 10 μ M double stranded 23-mer competitor substrate was added.

Methylation of oligonucleotide substrates using radioactively labeled AdoMet

In order to study the methylation of the unmethylated and hemimethylated 23-mer substrates by CcrM, the *in vitro* biotin/avidin methylation assay was performed, as previously described (15). The methylation reactions were performed in a 40 μ l total volume, under single turnover conditions using 760 nM 3 H-labeled AdoMet. The enzyme and DNA concentrations are given in the main text. The enzymatic activity was assessed by linear

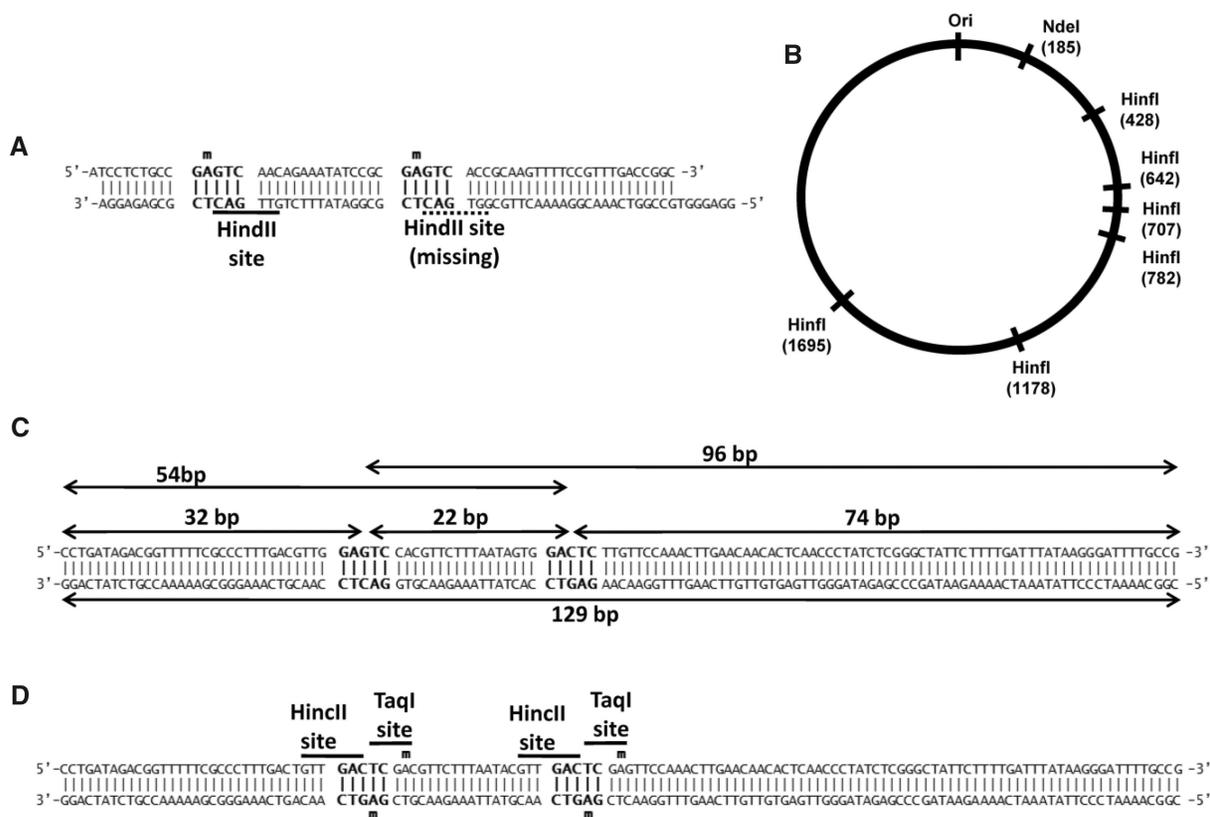


Figure 2. Various substrates used for studying CcrM processivity. (A) Substrate used by Berdis *et al.* (14) to study CcrM processivity, referred to as N⁶ 60/66-mer. Two GANTC target sites are present, hemimethylated on the upper strand. HindII target sites (GTYRAC) coupled to CcrM target sites were used to screen for methylation on the lower strand. However, only one of the two HindII sites is present, making it impossible to probe the methylation state of the second site. (B) The distribution of GANTC sequences (shown as Hinfl target sequences) throughout the pUC19 plasmid. The position of each sequence is indicated relative to the plasmid's replication origin. The vector contains a single NdeI target site, which was used in conjunction with Hinfl for vector linearization, to facilitate viewing of the progression toward fully methylated state. (C) 129-mer substrate containing two CcrM target sites. The expected size of the fragments obtained after Hinfl digestion of completely unmethylated, partially methylated and fully methylated substrates are indicated. (D) 129-mer_{HM} substrate used to probe CcrM activity over hemimethylated GANTC sites. A M.TaqI methylation site (TCGA), as well as a HincII restriction site (GTYRAC) were linked to the GANTC site. M.TaqI-established methylation occurs as shown earlier, creating two GANTC sites hemimethylated on the lower strand. CcrM-catalyzed methylation of the upper strand was probed through protection from HincII digestion, which is blocked by hemimethylation.

regression of the initial data points or by fitting the reaction progress curve to the following exponential equation:

$$CPM = BL + F * (1 - e^{-k \cdot t})$$

where *BL* indicates the background signal, *F* is the intensity factor, and *k* represents the catalytic rate constant, expressed as turnovers per minute. Calibration was done with completely methylated DNA, incubated with CcrM for several hours.

RESULTS

We have expressed His₆-tagged CcrM in *E. coli* and purified it using a Ni-NTA column (Figure 1A). We initially investigated the specificity of CcrM by using biotinylated oligonucleotide substrates which were incubated with CcrM and AdoMet, the methyl group of which was radioactively labeled. After methylation, the DNA was purified using an avidin microplate and the incorporation of radioactivity into the DNA was detected.

We used four 23-mer substrates with altered central position (N3) representing the different versions of the GANTC cognate sites. In addition, nine substrates were used which contained variants of the GANTC target site in which one base pair was altered at the G1, T4 or C5 position (near-cognate sites). Substrates were hemimethylated in the lower strand, such that methylation of the upper strand was detected. The results shown in Figure 1B illustrate that CcrM has a high preference for the GANTC sequence, because the best near-cognate substrate was methylated 200-fold less efficiently than the worst cognate one. Many of the near-cognate substrates were methylated more than 1000-fold less efficiently. At the central position we observed only moderate variations in the methylation rate which were close to the experimental fluctuations. Since hemimethylated DNA is the product of DNA replication of methylated GANTC sites *in vivo*, it is the major physiological substrate of CcrM. We compared the activity of CcrM using hemimethylated and unmethylated DNA substrates indicating that CcrM methylates both substrates, but it

displayed a weak preference of ~ 1.5 -fold for methylation of hemimethylated DNA over the average of the methylation rates of both strands of the unmethylated DNA (Figure 1C).

Methylation of a plasmid substrate

In order to assess whether CcrM methylates DNA in a processive or distributive manner, we have performed methylation kinetics using pUC19 plasmid DNA as substrate which contains six GANTC sequences (Figure 2B). After methylation for a defined period of time, the DNA was purified and digested with the *Hinf*I (GANTC) and *Nde*I restriction endonucleases. *Hinf*I cleavage is blocked by the adenine methylation introduced by the CcrM methyltransferase, which allows probing its methylation activity. The *Nde*I cleavage, in contrast, is insensitive to DNA methylation by CcrM and used to linearize the plasmid DNA. In a distributive methylation reaction, the enzyme dissociates from the DNA after methylating each target site, and it needs to re-associate before carrying out another methylation reaction. Since re-association occurs randomly, incompletely methylated DNA molecules, which are protected against *Hinf*I cleavage at some but not all sites, accumulate in the initial phases of the reaction. In contrast, in a processive reaction, the enzyme methylates all available target sequences without releasing the DNA, leading to complete protection of the plasmid DNA against *Hinf*I cleavage. As can be seen in Figure 3, without methylation (i.e. at time point 0.1 min), the pUC19 DNA was completely cleaved by the *Hinf*I and *Nde*I enzymes. Within the first 4 min of the methylation reaction, the plasmid DNA was becoming increasingly protected against the *Hinf*I cleavage. However, we did not observe a direct conversion of the DNA into the fully protected form, but an accumulation of methylation intermediates, leading to the appearance of DNA bands corresponding to incomplete cleavage of the plasmid DNA. Similar this finding clearly indicates that CcrM methylated the plasmid DNA in a distributive manner.

Methylation of a 129-mer substrate

As described earlier, the pUC19 methylation experiments indicated that CcrM functions in a distributive mode. However, the distance between the GANTC target sites is relatively large in pUC19, making processive DNA methylation challenging for the enzyme, because it has to travel a long distance on the DNA after each methylation event to reach the next target site. In order to provide an experimental test system more supportive for a processive reaction mechanism, we have designed and generated a 129-mer oligonucleotide substrate which contains two GANTC target sequences separated by 22 bp and flanked by 32 bp on one side and 74 bp on the other (Figure 2C). The DNA was incubated with CcrM for up to 2 h and aliquots were taken at various time points. The methylation reaction was stopped by flash-freezing in liquid N₂ and the methylation state of the DNA was assessed by digestion with *Hinf*I, as described above. A processive methylation reaction would lead to a gradual appearance of fully protected 129-mer and corresponding loss of unprotected 74-mer, 32-mer and 22-mer without generation of 54-mer and 96-mer, which are obtained from cleavage of partially methylated DNA molecules. As shown in Figure 4A, a large amount of methylation intermediates was observed, indicating that CcrM has a non-processive reaction mechanism. Interestingly, the results shown in Figure 4A also reveal a considerable excess of one of the two expected intermediate fragments, 96 bp in length, and only trace amounts of the shorter 54 bp fragment. This result indicates that the central one of the two CcrM target sites is methylated more efficiently. The second site is also methylated, but with lower rate, since the fully protected form of the 129-mer substrate also appears, and after 2 h almost complete protection of the DNA is achieved (Figure 4A).

Reaction quenching studies

An alternative approach to study processivity is to quench a reaction by addition of an excess of an external substrate

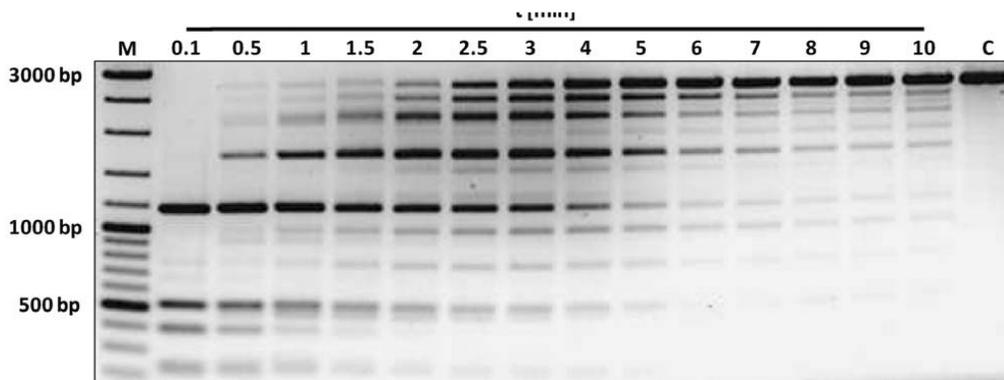


Figure 3. CcrM processivity assayed using pUC19 (Figure 1B) as substrate. A double digestion with *Hinf*I and *Nde*I was performed to assess the methylation state of the plasmid. pUC19 plasmid linearized by *Nde*I digestion was used as a control (lane marked C). A large number of incompletely methylated intermediates are formed throughout the duration of the experiment, supporting the conclusion that CcrM is a distributive, rather than a processive methyltransferase. The marker lane (lane marked M) contains the GeneRuler molecular weight marker, provided by Fermentas. The sizes of the major bands are indicated on the left.

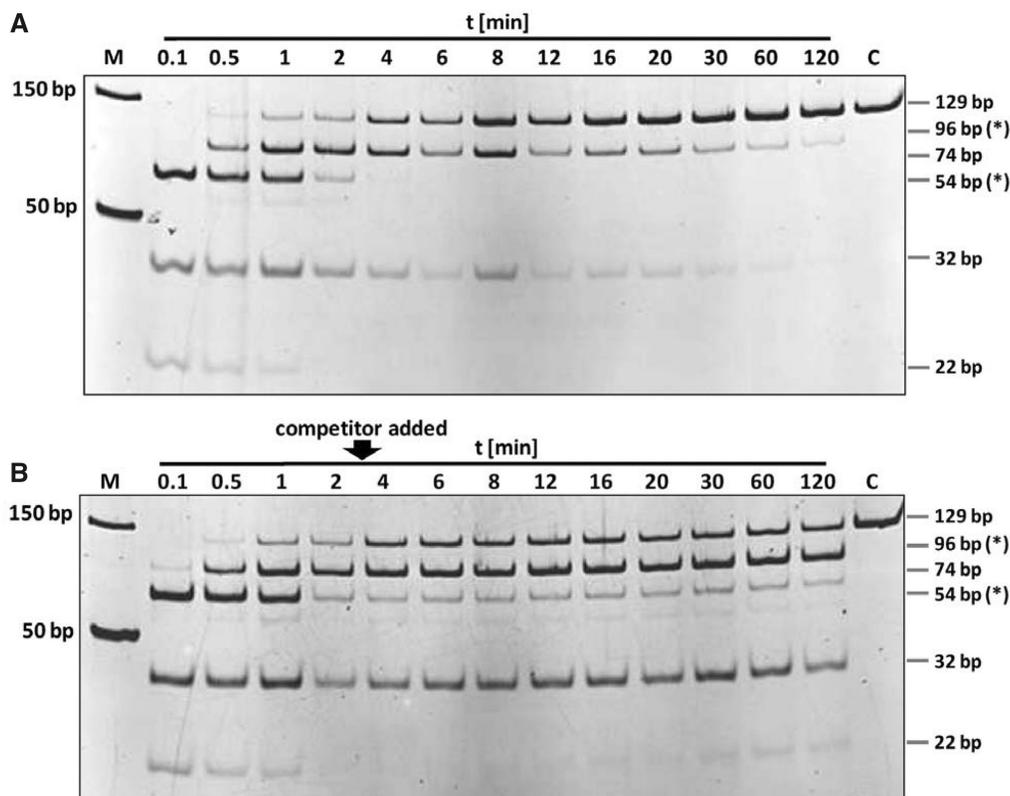


Figure 4. CcrM processivity assays using the 129-mer DNA as substrate (Figure 2C) and conducted in the absence (A) and with the addition (B) of competitor DNA. The expected running distance of partially protected intermediates is indicated on the right by asterisk. Undigested 129-mer DNA was used as control, indicating the expected running distance of fully protected DNA. The marker lane (indicated by M) contained PCR marker provided by New England Biolabs. The sizes of the bands are indicated on the left. (A) Initially all of the DNA is efficiently digested by HinfI (time point 0.1 min). Increasing protection from HinfI digestion is established over time, with nearly complete protection being achieved after 120 min. The 96 bp intermediate is present in large amounts, as well as low levels of the 54 bp intermediate, indicating distributive methylation by CcrM, as well as a preference for one target sequence over the other. (B) Competitor DNA is added after 3 min (indicated by the arrow). The protection state of the 129-mer substrate remains the same after supplementation with competitor, suggesting dissociation of CcrM from incompletely methylated DNA. The weak band appearing in the last lanes at low molecular weights corresponds to the 23-mer competitor which has become methylated and, thereby, protected against HinfI cleavage.

acting as competitor. Thereby, an enzyme working distributively will be trapped and the reaction stopped. In contrast, a processive enzyme should be able to finish the methylation of the substrate it is bound to. In order to implement this assay, the 129-mer methylation reaction was performed as described and after 3 min a 10-fold excess of a competing 23-mer oligonucleotide containing a single, unmethylated GANTC target site was added. Our results show that before the addition of the competitor DNA, the 129-mer substrate was methylated with same kinetics as seen before (compare Figure 4A for methylation without competitor and Figure 4B for methylation with competitor). However, after addition of the competitor, the level of protection of the 129-mer DNA remained constant throughout the duration of the experiment, with no additional methylation taking place. Most importantly, the partially methylated intermediates were not converted into the fully methylated state indicating that the CcrM enzyme cannot move to the second site after methylation of one site of the 129-mer substrate. This behavior is characteristic of a distributive, rather than a processive,

methyltransferase confirming the conclusions from the previous experiments.

Analysis of processivity on hemimethylated DNA

Although CcrM is able to efficiently methylate completely unmodified substrates (Figures 1C, 3 and 4), it could be argued that processive methylation might occur on the preferred substrate. To this end, a CcrM *in vitro* methylation experiment was conducted using a hemimethylated variant of the 129-mer substrate (Figure 2D). Hemimethylation was introduced into the 129-mer HM substrate by using GTTGACTCGA sites which represent overlapping HincII (GTYRAC, position 1–6 of the sequence, inhibited by adenine methylation in either strand), CcrM (GANTC, position 4–9 of the combined sequence) and TaqI sites (TCGA, position 7–11 of the combined sequence). The 129-mer HM DNA was methylated by M.TaqI at the TCGA adenine residues in both strands. In the upper strand, the methylation is outside of the CcrM site and it does not influence GANTC methylation by CcrM. However, M.TaqI

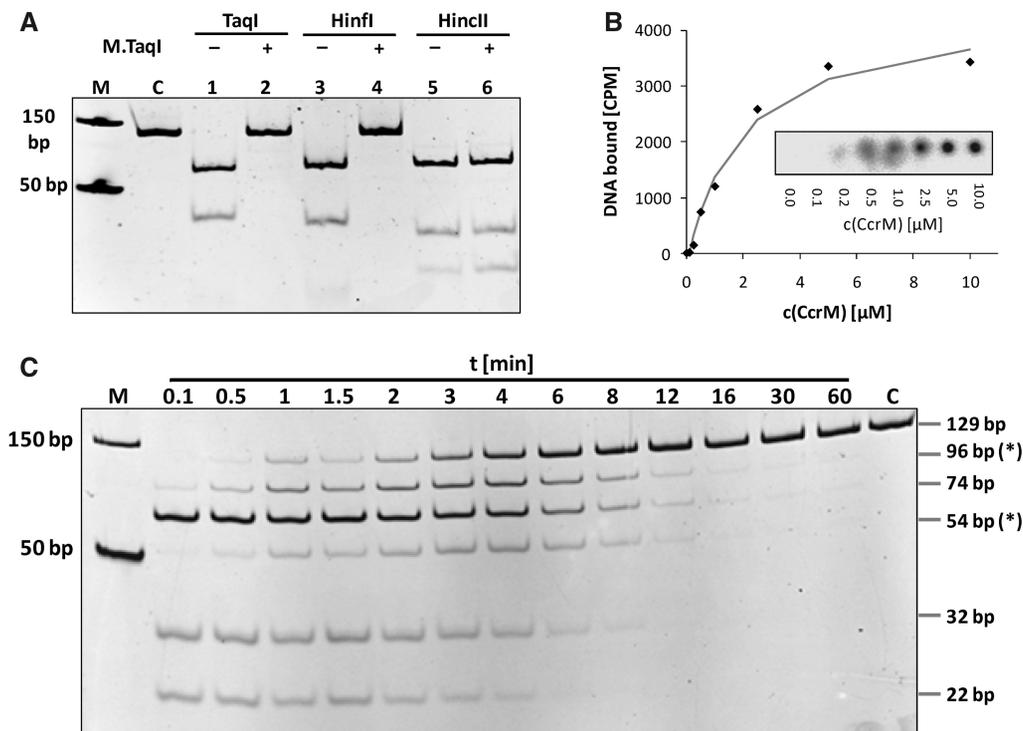


Figure 5. (A) Quality control of M.TaqI methylated 129-mer HM DNA. Digestions were performed of 129-mer HM DNA, before and after methylation with M.TaqI (as indicated), using TaqI, HinfI and HincII. As expected, TaqI could not cleave DNA, after treatment with M.TaqI. HinfI was also unable to cleave methylated DNA, whereas HincII was unhindered by the M.TaqI treatment. Thus, since both HinfI and HincII are blocked by N6-adenine hemimethylation, the CcrM target sites present on the 129-mer HM substrate are both in hemimethylated state after M.TaqI treatment. (B) Binding of CcrM to the hemimethylated 129-mer analyzed by nitrocellulose filter binding. The insert shows one example of primary data, the graph shows the quantitative analysis of one binding experiments. In two independent experiments the binding constant was determined as $5.4 (\pm 0.1) \times 10^5 \text{ M}^{-1}$. (C) CcrM methylation of 129-mer HM DNA. Both the 96-mer and 54-mer methylation intermediates can be seen (indicated by asterisk), thus CcrM functions in a distributive manner on hemimethylated DNA. In both (A) and (B) the control lane (marked C) contained undigested 129-mer HM DNA. The marker lane (indicated by M) contained the same PCR marker (New England Biolabs) used in the previous experiments, shown in Figure 3.

methylation of the lower strand creates a hemimethylated GANTC site. Completeness of the M.TaqI methylation was confirmed by full resistance of the DNA against R.TaqI cleavage after incubation with M.TaqI (Figure 5A). The hemimethylated state of all the CcrM sites was assayed by a control digestion with HinfI, which has the same recognition sequence as CcrM and is inhibited by hemimethylation of its target sequence. As shown in Figure 5A, the 129-mer HM DNA was completely refractory to HinfI cleavage after incubation with M.TaqI, indicating that all GANTC sites were hemimethylated. As expected, M.TaqI methylation did not interfere with HincII cleavage because the hemimethylation resides outside of its recognition sequence (Figure 5A).

In order to characterize the reaction process (cf. 'Discussion' section), we determined the binding constant of CcrM to this substrate by nitrocellulose filter binding after radioactive labeling of the hemimethylated DNA (Figure 5B). We observed relatively weak binding with a binding constant of $K_{\text{Ass}} = 5.4 \times 10^5 \text{ M}^{-1}$.

Next, this substrate containing hemimethylated GANTC sites, which are methylated in the lower DNA strand, was incubated with CcrM, which also leads to the methylation of the upper strand of the GANTC site. Since

the upper strand adenine of the GANTC site overlaps with the HincII site, and HincII is inhibited by adenine methylation in either strand, the conversion of hemimethylated to fully methylated CcrM sites can be followed by protection against HincII cleavage. As before, processive methylation would cause the direct conversion of the 22, 32 and 74 bp fragments into fully protected 129-mer while appearance of 54 and 96 bp fragments which correspond to partially methylated DNA would be expected in case of a distributive reaction mechanism. As shown in Figure 5C, appearance of the 96-mer and 54-mer DNA fragments indeed was observed, which is indicative of distributive DNA methylation. This finding confirms that CcrM is distributive on unmethylated and hemimethylated DNA. Unlike the results shown in Figure 4A, CcrM appears to have no preference for either of the target sites on the 129-mer HM substrate, since the relative abundance of both intermediate fragments is approximately the same throughout the experiment. This is expected, since the inclusion of the HincII and TaqI sites on either side of both GANTC sites has placed them in an identical sequence context, whereas in the original 129-mer DNA, the flanking sequences of the two sites were different (compare Figure 2C and D).

DISCUSSION

Caulobacter crescentus is an established model system to study cell cycle regulation and differentiation in bacteria. In a series of seminal papers in the 1990s, Shapiro and coworkers (5,8,16–20) identified the CcrM DNA methyltransferase and described its biological role in the control of the cell cycle of *Caulobacter*. The enzymatic properties of CcrM were studied by Benkovic and coworkers (14,21), showing that CcrM is active as monomer and dimer and it has some preference for methylation of hemimethylated targets. Here, we show that CcrM has a high specificity for GANTC sites which is similar to results obtained with T4Dam and EcoDam (22,23). At the central position, CcrM prefers GANTC sites 200-fold over the best near cognate substrate and hemimethylated targets are modified about 1.5-fold faster than unmethylated. This slight preference of CcrM for hemimethylated targets makes physiological sense, because replication of the *Caulobacter* chromosome generates hemimethylated GANTC sites, which, therefore, are the main physiological substrate of CcrM. In addition, Berdis *et al.* (14) reported that CcrM methylates DNA in a highly processive manner in a paper that is highly cited. Unfortunately, due to a mistake in the design of the methylation substrates that has been described above (Figure 2A), the conclusion of processive DNA methylation was not justified. Berdis *et al.* (14) based their claim of processive DNA methylation also on a second observation, which is that CcrM methylated a substrate with two CcrM sites 2-fold faster than a single site substrate used at the same concentration. However, this experiment was not conclusive, because the two site substrate offers twice the amount and concentration of target sites, which may explain the higher incorporation of radioactivity.

The question if a DNA MTase acts in a processive or distributive manner has important consequences for its behavior in the biological context of a living cell and strongly influences the pathway of re-establishing DNA methylation after DNA replication. Therefore, we have re-examined the processivity of CcrM and show that CcrM distributively methylated six target sites on the pUC19 plasmid as well as two target sites located on a 129-mer DNA fragment both in unmethylated and hemimethylated state. One technical challenge in detecting potential processivity of CcrM was the relatively weak activity of the enzyme, which may pretend a distributive reaction mechanism because of incomplete reaction progress. However, this argument does not apply to our study, since we observed complete methylation of the substrate in all our experiments (cf. Figures 3–5). Another potential problem, that could obscure a processive reaction mechanism, is that two enzymes may bind on the same substrate and methylate independently of each other or block each others movement on the DNA. To estimate if such problem could have occurred in our experiments, we determined the DNA binding constant of CcrM to the hemimethylated 129-mer and found a K_{Ass} of $5.4 \times 10^5 \text{ M}^{-1}$. In the corresponding methylation kinetics with the hemimethylated 129-mer, we used $1 \mu\text{M}$ DNA

and $0.5 \mu\text{M}$ CcrM, which corresponds to an average occupancy of the DNA of 7.5%. This result indicates that on average only 0.6% of the substrate molecules ($=0.075^2$) had two CcrM molecules bound. Therefore, processive DNA methylation should have been detectable under these conditions. Since, the reaction quenching experiments confirmed the lack of processivity, we conclude that the original statement that CcrM is processive is no longer valid.

Our new finding of a distributive methylation mechanism of CcrM can be interpreted by considering the biological role of the enzyme. Since a processive enzyme methylates several sites after one DNA binding event, it typically has long residence times on the DNA. One paradigm of this type is the EcoDam enzyme (11), which shortly follows the replication fork and, therefore, probably acts on nascent DNA with few other proteins bound. In contrast, distributive enzymes have to bind to and dissociate from the DNA for each methylation event and typically have a short dwell time on the DNA. The distributive mechanism may be better suited for CcrM, because in *C. crescentus* DNA methylation happens after DNA replication when other DNA binding proteins have had enough time to re-bind to the DNA. Under such conditions, a processive DNA methylation may not be ideal, because bound proteins would act as roadblocks and processive MTases would be trapped due their slow dissociation from the DNA. Thereby the entire process of DNA methylation could become impeded. On the other hand, the necessity for multiple DNA binding events of distributive enzymes suggest that there may exist mechanisms for targeting CcrM to the DNA, which so far have not been discovered.

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