Characterization of DNA methyltransferase specificities using single-molecule, real-time DNA sequencing

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ABSTRACT
DNA methylation is the most common form of DNA modification in prokaryotic and eukaryotic genomes. We have applied the method of single-molecule, real-time (SMRT®) DNA sequencing that is capable of direct detection of modified bases at single-nucleotide resolution to characterize the specificity of several bacterial DNA methyltransferases (MTases). In addition to previously described SMRT sequencing of N6-methyladenine and 5-methylcytosine, we show that N4-methylcytosine also has a specific kinetic signature and is therefore identifiable using this approach. We demonstrate for all three prokaryotic methylation types that SMRT sequencing confirms the identity and position of the methylated base in cases where the MTase specificity was previously established by other methods. We then applied the method to determine the sequence context and methylated base identity for three MTases with unknown specificities. In addition, we also find evidence of unanticipated MTase promiscuity with some enzymes apparently also modifying sequences that are related, but not identical, to the cognate site.

INTRODUCTION
Methylation of DNA bases, catalyzed by DNA methyltransferases (MTases), is the most abundant form of post-replicative DNA modification found in the genomes of prokaryotic and higher eukaryotic organisms. Three functional classes of MTases have been identified in bacteria and archaea. Two of these transfer a methyl group from S-adenosyl-L-methionine (SAM) to the exocyclic amino groups of adenine and cytosine bases in duplex DNA, yielding N6-methyladenine (m6A) and N4-methylcytosine (m4C), respectively (1). A third, and mechanistically distinct, class transfers the methyl group of SAM to C5 of cytosine to produce 5-methylcytosine (m5C) (2,3).

Most bacterial and archaeal MTases are associated with sequence-specific restriction-modification (RM) systems that protect the prokaryotic cell from invasion by DNA bacteriophages, and examples have been identified that recognize several hundred distinct DNA sequences (4). However, some well-characterized prokaryotic MTases do not appear to be associated with a cognate restriction endonuclease (REase) and some of these ‘orphan’ MTases perform different cellular functions. For example, the product of the Escherichia coli deoxyadenosine methyltransferase (dam) gene, M.EcoKDam, which modifies adenine residues in the sequence 5'-GATC-3', is involved in both chromosomal replication initiation and in the maintenance of genomic integrity [reviewed earlier (5)].

Most eukaryotic MTases are of the m5C class and are related to their prokaryotic equivalents through a common reaction mechanism that is reflected in the conservation of tertiary structural elements within the enzyme active sites (6). Mammalian m5C-MTase activity is predominantly targeted to CpG dinucleotides and three different enzymes (DNMT1, DNMT3A and 3B) are known (7). Such DNA methylation is a key component of the epigenetic control of gene expression [reviewed earlier (8)]. CpG methylation is also a key player in genomic imprinting and in female X-inactivation (9,10). An additional modified base, 5-hydroxymethylcytosine (5-hmC), was presumed to be a product of DNA damage (11), but has

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recently been found to be a normal component of mammalian DNA (12,13) and appears to be generated by oxidation of m5C in a reaction catalyzed by the ten eleven translocation (Tet)-family of enzymes (13).

A number of diagnostic tools have been developed to detect DNA methylation, to establish what type of modification is associated with a particular MTase and to determine the DNA sequence context in which such methylation occurs. Nucleosides containing m6A, m4C and m5C can be resolved from their unmodified equivalents by chromatographic methods, thereby facilitating their detection in total DNA hydrolysates (14,15). Alternatively, polyclonal antisera that specifically recognize m6A or m4C have been used for immunological detection of such modifications in order to ascribe function to multiple putative MTase genes in Helicobacter pylori (16).

Bioinformatic analysis of a large number of genes that encode well-characterized prokaryotic MTases have identified groups of conserved sequence motifs that are diagnostic for DNA MTases and permit the accurate prediction of m5C-MTases, but the amino m6A- and m4C-MTases cannot be unequivocally distinguished (1,2). In the case of methylation activity that is part of a Type II RM system, the sequence specificity of the MTase is expected to be the same as the cleavage specificity of the associated REase. However, very little experimental evidence has been generated to support this, and biochemical characterization of the target specificity of most MTases remains to be gathered. Furthermore, the exact sites of modification are often uncertain in cases where the recognition sequence contains multiple potential target bases (i.e. A or C) or where separate MTases act on the two strands independently (17).

The experimental approaches currently available to fully characterize prokaryotic and eukaryotic MTases are labor-intensive, requiring radioactive labeling with [3H]S-adenosylmethionine and mapping and sequencing of individual sites (17,18). One method based on Sanger sequencing can also detect methylated bases, but this is not a high-throughput method (19). Methods currently in use to discriminate between m5C and unmodified cytosines at CpG sequences, such as differential sensitivity to cleavage by REases (20) and methylation-specific polymerase chain reaction (PCR) of bisulfite-modified DNA (21,22) do not permit genome-wide analysis. The state-of-the-art method for m5C methylome studies is MethylC-Seq (23,24), but it is indirect and also requires extensive experimentation. In particular, the first requisite is the complete DNA sequence of the DNA to be analyzed. Only then can methylome analysis be undertaken. Some additional new technologies have been described recently that have potential applications in methylome analysis such as methods to map genomic 5-hmC by enzyme-catalyzed glycosylation (25,26) and a family of REases that specifically recognize m5CpG and m5CpWPpG sequences (27). The latter enzymes are of interest because members of this MspII family of REases excise a 32–33 bp fragment that includes the methylated bases in a central position and the products lend themselves to high-throughput sequencing approaches. However, they are partially constrained by the sequence specificity of the REases (27).

A method has been described previously to directly detect methylated DNA bases during single-molecule, real-time (SMRT) DNA sequencing (28). This method takes advantage of kinetic data pertaining to the rate of incorporation of each dNTP in the form of two parameters—the pulse width (PW) and the interpulse duration (IPD). Significant changes in these kinetic parameters were observed during SMRT sequencing when the DNA polymerase encounters m6A, m5C or 5-hmC on the template strand. These distinct kinetic signatures allow for the identification of the type and position of the base modification in the DNA template.

Here, we extend the SMRT sequencing method to combine complete DNA sequence determination and methylated base analysis to characterize MTase specificities in a single operation (28,29). We analyzed a set of 16 DNA substrates that were methylated in vivo by a range of single prokaryotic MTases expressed in an E. coli strain that lacks additional MTase genes. The samples included MTases introducing m6A, m4C or m5C modifications, either from MTases whose substrate specificity was previously known, or from some whose specificity was unknown. The results allowed us to determine the absolute sequence specificity of the MTases, as well as the precise location of the methylated base.

**MATERIALS AND METHODS**

**Materials**

All restriction endonucleases, Phusion-HF DNA polymerase, Antarctic Phosphatase, T4-DNA ligase, SAM, MTase cloning and methods to map genomic 5-hmC by enzyme-catalyzed glycosylation (25,26) and a family of REases that specifically recognize m5CpG and m5CpWPpG sequences (27). The latter enzymes are of interest because members of this MspII family of REases excise a 32–33 bp fragment that includes the methylated bases in a central position and the products lend themselves to high-throughput sequencing approaches. However, they are partially constrained by the sequence specificity of the REases (27).

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**Escherichia coli strains**

C2523 (NEB-Express): flhA2 [lon] ompT gal sulA11 R(mcr-73::miniTn10–fhuA2 [lon] ompT gal sulA11)2 [dem] R(rgbo-210::Tn10-Tet5) endA1 Δ(mcr-c-mrr)114::IS10

C2925 (NEB dam-::dem–); ara-14 leuB6 flhA31 lacY1 tss78 glnV44 galK2 galT22 mcrA dem-6 hisG4 rjF101 R(rgbo-210::Tn10) Tet5 endA1 rpsL136 (StrR) dam13::Tn9 (CamR) xylA-5 mtl-1 thi-1 mcrB1 hsdR2

ER2796 (=DB24): flhA2 Δ (lacZ) r1 glnV44 trp-31 dem-6 his-1 zed-501::Tn10 argG6 rpsL104 dam-16::Kan xyl-7 mtl-2 metR1 mcr-62 Δ (mcrB-hsd-mrr)114

**Methyltransferase cloning**

In total, 16 known and putative MTase genes (Table 1) were amplified from bacterial genomic or recombinant plasmid DNA sources with Phusion-HF DNA polymerase using gene-specific oligonucleotide primers (Supplementary Table S1). The 5’-end oligonucleotides incorporated a PstI site (SbfI site in the case of the gene encoding M.SacI which contains an internal PstI site), followed by the sequence 5’-TTAAGG:3’ (to terminate
and M.EsaLHCI were assessed by restriction with PstI M.EcoKDam, M.EcoKDcm, M.EsaBC1I, M.EsaBC2I M.Tsp509I. Constructs containing genes encoding M.HpaII, M.NspI, M.RsaI, M.SacI, M.Sau3AI and containing M.AatII, M.AluI, M.BstNI, M.CviQI, cognate restriction endonuclease in the cases of constructs was assessed by restriction with PstI reisolated from ER2796 cells and their methylation status all known confirmed by restriction analysis. Plasmids were then used to transform ER2796, also called DB24, a strain that lacks transform ER2796, also called DB24, a strain that lacks

Table 1. MTases subjected to specificity characterization by SMRT DNA sequencing

| No. | Methyltransferase name | Methylation context       | Plasmid size (bp) | No. of sites |
|-----|------------------------|---------------------------|-------------------|-------------|
| 1   | M.CviQI                | GT(m6A)mC                 | 3566              | 3           |
| 2   | M.RsaI                 | GTA(m4C)                  | 3986              | 2           |
| 3   | M.EcoKDam              | G(m6A)TC                  | 3592              | 23          |
| 4   | M.EsaLHCI              | GA(m4C)                   | 3590              | 19          |
| 5   | M.Sau3AI               | GA(m5C)                   | 3995              | 19          |
| 6   | M.AluI                 | AG(m5C)T                  | 4331              | 19          |
| 7   | M.EsaBC1I              | AG(m4C)T                  | 3797              | 18          |
| 8   | M.BstNI                | (m4C)mWG                  | 4142              | 5           |
| 9   | M.EcoKDcm              | (m5C)mWG                  | 4175              | 7           |
| 10  | M.EsaBC2I              | T(m4C)mGA                 | 3566              | 2           |
| 11  | M.NspI                 | R(m5C)mATGY               | 3950              | 3           |
| 12  | M.HpaII                | (m5C)mGG                  | 3833              | 15          |
| 13  | M.SacI                 | GAG(m5C)mTC               | 3936              | 1           |
| 14  | M.Tsp509I              | AATT (m6A; position unknown) | 3863              | 7           |
| 15  | M.AatII                | GACGTC (m6A/m4C; position unknown) | 3750              | 2           |
| 16  | M.BceJI                | Unknown                   | 4760              | Unknown     |

Sample preparation and SMRT sequencing

An aliquot of ~25 ng of plasmid DNA was whole-genome amplified (WGA) using the REPLI-g Midi Kit (Qiagen, Valencia, CA, USA). Amplification factors were typically approximately 1600× (~40 μg output), translating to <0.06% of residual methylated DNA in the control samples. Further trimming of 5% of data from the top and bottom of the IPD distributions (see data analysis section) removed any remaining spurious signal from modified DNA. WGA and native plasmid DNA was sheared to an average size of 300 bp via adaptive focused acoustics (Covaris, Woburn, MA, USA). SMRTbell template sequencing libraries were prepared as previously described (30). Briefly, sheared DNA was end repaired, A-tailed and hairpin adapters with a single T-overhang were ligated. Incompletely formed SMRTbell templates were degraded with a combination of Exonuclease III (New England Biolabs; Ipswich, MA, USA) and Exonuclease VII (USB; Cleveland, OH, USA). Primer was annealed and samples were sequenced on the PacBio RS as previously described (31,32).

Data processing

Reads were processed and mapped to the respective reference sequences for each plasmid using the Basic Local Alignment with Successive Refinement (BLASR) mapper (http://www.pacbiodevnet.com/smrtanalysis/software/blasr) and the Pacific Biosciences SMRT Analysis pipeline (http://www.pacbiodevnet.com/smrtanalysis/software/smrtpipe) using the standard mapping protocol. Interpulse
durations (IPDs) were measured as previously described (28) for all pulses aligned to each position in the reference sequence. For each position, the distribution of IPDs was compared between the native and WGA samples separately for the forward and reverse strands. The comparison was made using baseline-corrected IPD ratios. Baseline correction was applied by dividing the IPD mean for each position by the mean of mean IPDs over all positions in the plasmid, excluding those positions where the known methyltransferase recognition motif was detected and a window of 6 bases in each direction around such positions. This correction was applied to both the native sample IPD mean and the WGA control mean before obtaining the ratio of the two. In addition, 5% of outlier values were trimmed from both sides of the IPD distribution at each position before taking the ratio. IPD ratio plots were visualized using Circos (33).

The uncertainty in the IPD ratio was quantified by calculating the standard error of the mean of the IPD ratio using the delta method:

$$
\tilde{SE} \left( \frac{\mu_1}{\mu_2} \right) \approx \sqrt{\frac{1}{n} \left( \frac{s_1^2 \mu_2^2}{\mu_1^2} + \frac{s_2^2 \mu_1^2}{\mu_2^2} \right)}
$$

where $\mu_1$ and $\mu_2$ are the average IPD values of the native and control, $s_1$ and $s_2$ are their standard deviations and $n$ is the lower sequencing coverage of the two samples. The relationship between this standard error and the sequencing fold coverage is shown in Supplementary Figure S1 for three different methyltransferases (M.EcoKDam, M.EsaLHCI, M.Sau3AI).

RESULTS

SMRT DNA sequencing of plasmids containing MTase-mediated methylation

Plasmids containing cloned DNA-methyltransferase genes were expressed in an E. coli strain that otherwise lacked endogenous methyltransferase activities. The expressed methyltransferase acts to modify a specific recognition sequence by adding a methyl group to individual bases forming m6A, m4C or m5C. The methyltransferase will modify both the genomic and plasmid DNA. Plasmid DNAs containing the modifications were isolated and converted into SMRTbell templates to facilitate sequencing (30). Plasmids were also subjected to whole-genome amplification (WGA) to create a control DNA template lacking any DNA base modifications. Native and control samples were subjected to SMRT DNA sequencing (Figure 1). SMRT DNA Sequencing involves monitoring an individual DNA polymerase while it is replicating the input DNA, employing phospholinked nucleotides with different fluorophores for each of the four bases to produce fluorescence pulses at each incorporation step (29,32). As the DNA polymerase is sensitive to even subtle perturbations in the DNA template, it is possible to detect the presence of a methylated base by analyzing the polymerase kinetics as it moves across the DNA (28). This is manifested by the polymerase slowing down in a predictable manner upon encountering a base modification relative to the speed of copying the same non-modified control sequence. Thus, the time between fluorescent pulses (interpulse duration or IPD) is longer for a DNA template that contains a methylated base than for one that does not (28). One
simple means of visualizing the differences in polymerase kinetics between a native and control sample is to graph the ratio of IPDs (methylated/control) for each position along the DNA template. The extent and magnitude of these kinetic signals is dependent on the nature of the base modification and the local sequence context. Because the polymerase is in contact with the modification for several bases before and after occupying the polymerase active site, the kinetic signal can encompass multiple nucleotide incorporations surrounding the methylated position (28).

We confirmed the activities of the different MTases for all samples by restriction digestion (Figure 2). In all cases, the DNAs were protected completely from restriction digestion by the specific REase that corresponds to the particular MTase expressed on the plasmid, indicating complete methylation at the cognate sites (Figure 2). The validity of using WGA for generating the control data set for reference sequence and kinetics was confirmed by analyzing a portion of the plasmid common to all samples, comparing the kinetics using as the control data either native, unamplified DNA from the plasmid lacking an MTase gene, or whole-genome amplified (WGA) DNA derived from the MTase plasmid (Supplementary Figure S2).

Characterization of MTase specificities

We carried out SMRT sequencing on DNA templates with methylation marks from 16 different bacterial MTases, including enzymes with both known and unknown specificities (Table 1). Plasmid sequences were obtained through applying standard analysis tools (www.pacbio.com/devnet), yielding high fold coverage over the entire DNA template from a single sequencing run (minimum of 300-fold; Supplementary Table S2), and are available at http://pacificbiosciences.com/devnet/files/how-tos/dna-methyltransferase/1.0/index.html. Consensus sequences of each of the plasmids can be found in Supplementary File S1. Results from the kinetic analysis are shown by example in Figure 3 for the two MTases, M.CviQI and M.RsaI, which modify the sequence context 5'-GTAC-3' to impart m6A or m4C, respectively. For each MTase, the left panel shows a global analysis of the IPD ratio data for both strands of the entire plasmid. The right panels show detailed bar graphs of IPD ratios for two representative template positions. The large peaks of increased IPD ratio are present at sites of DNA modification and correspond to the presumed recognition sequences for each bacterial DNA MTase, based on the known specificity of the cognate restriction enzymes. As expected, the SMRT sequencing data from a plasmid lacking an MTase gene was devoid of any methylation-specific kinetic signals (Supplementary Figure 2).
For the sequence context 5'-GATC-3', MTases exist that can impart all three methylation types: M.EcoKDam (m6A), M.EsaLHCI (m4C) and M.Sau3AI (m5C) (Figure 4). For the dam MTase in E. coli (M.EcoKDam), we obtained data consistent with previously observed kinetic signals for m6A (28) showing large IPD ratios at m6A template positions. In addition, we observed a secondary peak at the +5 position relative to the modified base, likely due to the intimate contact of the DNA polymerase with the nascent double-stranded DNA, and its dynamic sensitivity to slight structural perturbations caused by the presence of the methyl group (28,34). Such secondary peaks enhance the ability to detect and discriminate different base modifications.

The third common base modification in bacteria, m5C, is imparted on the 5'-GATC-3' sequence context by the MTase M.Sau3AI (Figure 4C). Whereas the methyl groups in m6A and m4C are directly involved in base pairing, the m5C methyl group is not and is instead positioned in the major groove of the nascent double-stranded DNA which has few direct contacts with the DNA polymerase. As a result, m5C modifications cause more subtle perturbations of the DNA which in turn result in smaller effects on the DNA polymerase dynamics (28). While under the current sequencing conditions, the IPD ratios are therefore smaller and spread across multiple bases surrounding the modification, the majority of m5C positions
Figure 4. MTase specificities determined from SMRT sequencing. The sequence context 5’-GATC-3’ is methylated by (A) M.EcoKDm (m6A), (B) M.EsaLHCl (m4C) and (C) M.Sau3AI (m5C). The left panel shows IPD ratio data for both strands over the entire plasmid, with the inner and outer circles representing the reverse and forward DNA template strands, respectively. Template positions are indicated by the numbered track middle circle, with blue markers denoting occurrences of MTase target sequence contexts. The right panels show IPD ratios for two representative template positions containing the target context (bold letters), the methylated base is highlighted in red. Error bars represent the standard error of the ratio of means and is calculated as described in the ‘Materials and Methods’ section.
could be distinguished from unmodified cytosine based on polymerase kinetics, with each of the MTase target sites showing IPD ratio peaks above background.

**Determination of methylation patterns of DNA MTases with unknown specificities**

We applied our method to determine the methylation patterns of several DNA MTases for which the position and/or type of methylation is not known (Figure 5). The first example, M.Tsp509I, was selected from the category of MTases for which the target sequence context and the type of methylation was available, but the exact site of methylation was undetermined. Taking advantage of the base resolution of methylation detection during SMRT sequencing, we determined that it is the inner adenine base of the sequence context 5'-AATT-3' that was methylated to m6A (Figure 5A), as the IPD ratio peak was observed at this position, as well as the secondary peak at position +5 (see above).

The second example of determining the specificity of an MTase was from the category of MTases where the target sequence context was available; but both the type and position of methylation were unknown. We sequenced a plasmid expressing M.AatII (Figure 5B), known to methylate the target sequence 5'-GACGTC-3' either at m4C or m6A, and determined that this enzyme methylates the adenine, but not the cytosines, again directly visible from the strong signals at the A position and the secondary peak at +5.

The third example was M.BceJII, an MTase for which neither the target sequence, nor the type and position of methylation were known (Figure 5C). This MTase is part of a putative Type III restriction system from *Burkholderia cenocepacia* strain J2315 that is difficult to transform. Type III REases rarely give complete digests making it difficult to deduce their specificity. Since the specificity-defining sequence is in the MTase protein, this means that the cognate MTases have previously been employed to determine specificity, but the approach needed is quite tedious (18). Kinetic signatures obtained from SMRT sequencing the plasmid expressing this Type III MTase were observed at every occurrence of the sequence context 5'-CACAG-3' and at no other positions. For each occurrence, the second adenine position displayed kinetic signals of methylation to m6A (Supplementary Figure S4). Because the target sequence is non-palindromic and does not contain adenosine in the reverse complement, the methylation occurs only on one strand as is typical for Type III MTases (35). The presence of m6A in the 5'-CACAG-3' sequence context was further validated by inhibition of digestion of an overlapping sequence by the methyl-sensitive ScaI restriction endonuclease (Supplementary Figure S5).

**Some MTases display off-target activities**

While the majority of MTases displayed kinetic signals present exclusively at their cognate sites indicating strict context specificities, we observed additional, off-target kinetic signals for some MTases. For example, a global analysis of M.EcoKD Dam modified DNA shows numerous smaller kinetic signals in addition to the 5'-GATC-3' cognate site, suggesting partial methylation at off-target sites (Figure 4A, left panel). These off-target signals were found to encompass sequence contexts differing from the target context by one base (Figure 6). For M.EcoKD Dam, the 5'-GACC-3' off-target context was most affected, followed by detectable signals for 5'-HATC-3' (H = A, C or T) and 5'-GATT-3'. For example, the inset of Figure 6 shows IPD ratio data for the off-target 5'-GACC-3' sequence: the lower average IPD ratio value is suggestive of partial methylation and in this case, methylation only occurs on one strand because there is no adenine in the reverse complementary sequence (5'-GGTC-3'). This example also exhibits a secondary IPD ratio peak at the +5 position which is commonly observed with m6A modifications in similar sequence contexts. The partial methylation of an off-target 5'-GACC-3' site was confirmed by showing ~50% inhibition of Sall cleavage at an overlapping 5'-GTGACC-3' sequence (Supplementary Figure S6).

**DISCUSSION**

Thanks to the availability of a large number of cloned DNA methyltransferase genes that are expressed in an *E. coli* background devoid of other DNA methylation, it has been possible to prepare a series of pure plasmid DNAs each of which carries methylation signatures for just one methyltransferase. Using a few well-characterized methyltransferases as controls, we have been able to confirm their methylation specificity using the SMRT sequencing approach and find that in addition to obtaining interpretable signals for m6A and m5C as reported previously (28), it is also possible to detect m4C. This means that the three common types of methylation typically found in bacterial and archaeal genomes can be unambiguously distinguished. Furthermore, when a suitable number of sites are present, both the type of methylation and a unique recognition sequence for the methyltransferase can be assigned. By applying this same technique to several methyltransferases with either unknown types and/or positions of methylation or in one case, both an unknown type of methylation and an unknown recognition sequence, we have been able to assign specificity to the M.Tsp509I methyltransferase, the M.AatII methyltransferase and a new methyltransferase, M.BceJII, encoded by ORF 3494 in the *B. cenocepacia* genome. The latter is of particular interest because it is part of a Type III RM system and traditionally it has been quite difficult to obtain the recognition sequences for these enzymes. The SMRT approach described here is well-suited for that purpose and if applied at a whole bacterial genome level, should also be suitable to obtain recognition sequences for Type I RM systems, which typically have even been more difficult to determine than the Type III systems.

Since most bacterial genomes contain many RM systems of all types, it seems likely that genome sequencing using the SMRT approach should, in many cases, allow the direct discovery of recognition sequences...
Figure 5. Determination of unknown MTase specificities. Base-resolved MTase target specificities were resolved for (A) M.Tsp509I as 5'-A m6A TT-3', (B) M.AatII as 5'-G m6A CGTC-3' and (C) M.BceJ I as 5'-CAC m6A G-3'. The left panel shows IPD ratio data for both strands over the entire plasmid, with the inner and outer circles representing the reverse and forward DNA template strands, respectively. Template positions are indicated by the numbered track middle circle, with blue markers denoting occurrences of MTase target sequence contexts (if known). The right panels show IPD ratios for two representative template positions containing the target context (bold letters), the methylated base is highlighted in red. Error bars represent the standard error of the ratio of means and is calculated as described in the 'Materials and Methods' section.
that the initiation of DNA replication is not affected if additional methyl groups are present at non-cognate sites. Dam methyltransferase can affect transcription when GATC sites are methylated [reviewed earlier (5)]. The other known function of the Dam methyltransferase in damage repair would not be expected to be affected by off-target methylation. Whereas it is possible that the observed off-target signals are caused by mutation in the MTase genes present as a smaller fraction in the samples, we have not observed such minority species from SMRT sequencing, with a current detection limit of ~2–3% (30).

It should be noted though that for most of the methyltransferases that we studied, the fidelity was actually quite high and relatively few off-target signals were found. This might be viewed as a little surprising since there is no clear biological selection mechanism that would ensure the accuracy of the methyltransferase component of an RM system. This is in contrast to the restriction enzyme itself where there is a strong selection for the cognate site since off-target cleavage known as star activity could easily cause damage to host genomes. In this context, it is worth noting that many restriction enzymes do show unwanted star activity (37) and it would be of considerable interest to check whether the methyltransferases that accompany these promiscuous restriction enzymes show a similar promiscuity in their recognition, thereby protecting the organism against the potentially deleterious action of its restriction enzyme.

We are greatly encouraged by the results of the studies described here, and plan to undertake a more systematic analysis of whole genome sequences, both with a view to examining the levels of DNA methylation in these genomes and possibly assigning specificity to the methyltransferases and correlating it with the predicted methyltransferase genes in the genome. Such studies will also provide the significant quantities of data needed to enable exact quantitation of the degree of partial methylation and to define the precise sequences that lead to off-site methylation. By judicious analysis of methylation patterns, SMRT sequencing could also prove extremely useful in permitting the correct reassembly of individual genomes from complex metagenomic sequences.

**ACCESSION NUMBER**

Genbank accession number JN569339.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online: Supplementary Tables 1 and 2, Supplementary Figures 1–6 and Supplementary File 1.

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