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REVIEW

Prokaryotic DNA methylation and its functional roles

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DNA methylation is known as a universal mechanism of epigenetic regulation in all kingdoms of life. Particularly, given that prokaryotes lack key elements such as histones and nucleosomes that can structurally modify DNA, DNA methylation is considered a major epigenetic regulator in these organisms. However, because DNA methylation studies have focused primarily on eukaryotes, the mechanism of prokaryotic DNA methylation has been less studied than in eukaryotes. DNA methylation in prokaryotes plays an important role in regulating not only the host defense system, but also the cell cycle, gene expression, and virulence that can respond directly to the environment. Recent advances in sequencing techniques capable of detecting methylation signals have allowed for the characterization of prokaryotic genome-wide epigenetic regulation. In this review, we describe representative examples of cellular events regulated by DNA methylation in prokaryotes, from early studies to current applications.

***Keywords*:** DNA methylation, DNA methyltransferase, restriction-modification, prokaryotic epigenetics

Introduction

DNA methylation is catalyzed by methyltransferases (MTases), which transfer methyl groups from S-adenosyl-1-methionine (SAM) (Cheng, 1995) to DNA. This mechanism has been known for decades and allows DNA to incorporate methylated bases in addition to the four canonical bases (Jeltsch, 2002). DNA methylation is a component of the epigenetic system involved in the regulation of cellular events in all three kingdoms of life. This process is also reversible, does not alter the original genetic sequence, and modulates epigenetic functions by regulating protein binding affinity (Beaulaurier *et al.*, 2019; Sánchez-Romero and Casadesús, 2020). Unlike eukaryotes, which possess complex epigenetic regulation mechanisms (e.g., DNA methylation, histone modifications, and nucleosome positioning), bacterial epigenetic regulation is primarily achieved through DNA methylation (Marinus, 1996; Casadesús and Low, 2006). DNA methylation occurs in three forms: C-5 and N-4 cytosine methylation (5mC, 4mC) and N-6 adenine methylation (6mA) (Beaulaurier *et al.*, 2019; Sánchez-Romero and Casadesús, 2020). Among these variants, 5mC is predominantly found in eukaryotes, whereas 6mA and 4mC are mainly found in bacteria.

Historically, DNA methylation has been associated with restriction-modification (RM) systems consisting of MTases and cognate restriction enzymes (REases) (Arber, 1974). The RM system is a defense mechanism used to protect the host genome from the invasion of transposons and viral DNA. This system distinguishes foreign DNA through base modifications and defends its own DNA from restriction enzymes. However, in addition to being a defense mechanism, MTases also regulate cellular events and DNA replication independently from REases (Vasu and Nagaraja, 2013; Sánchez-Romero and Casadesús, 2020).

Detecting methylated bases in bacteria has been a decades-long challenge. Bisulfite genome sequencing for 5mC detection has been primarily used in eukaryotes (Frommer *et al.*, 1992). However, the detection of bacterial DNA methylation has been historically limited because most bacterial DNA methylation occurs in 6mA form. Since the recent development of long-read sequencing technology, including Pacific Biosciences' single-molecule real-time (SMRT) and Oxford Nanopore Technologies' nanopore approaches, the aforementioned technical limitations have started to be overcome, and bacterial DNA methylation can now be directly measured (van Dijk *et al.*, 2018).

Here, we summarize the history and characterization of MTases and highlight the functional role of DNA methylation in bacteria (Fig. 1). Moreover, we discuss recent research on methylation from a microbial ecology perspective.

History of bacterial DNA methylation

The first report of DNA methylation in bacteria was made in 1955 by a study that identified adenine N6 position modifications by methyl groups in *Escherichia coli* under thymine-deficient conditions (Dunn and Smith, 1955). Later, in 1962, DNA MTases were found to be involved in the RM system (Arber and Dussoix, 1962), and research on site-

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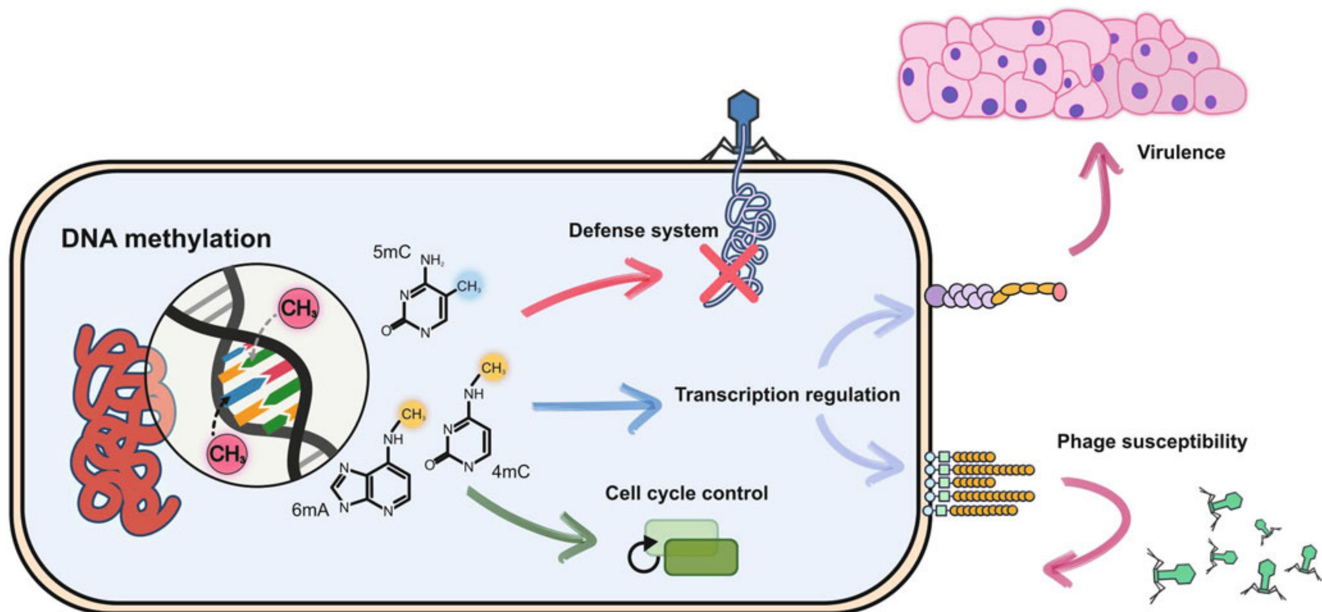

Fig. 1. Overview of the roles of prokaryotic DNA methylation. Prokaryotic DNA methylation consists of three forms (C-5 and N-4 cytosine methylation and N-6 adenine methylation), regulates important cellular events such as defense system, transcription regulation, and cell cycle.

specific DNA REases with MTases has continued to grow ever since. Additionally, 4mC, which has only been primarily identified in prokaryotes, was discovered in *E. coli* via BcnI MTase (CCSGG) in 1983 (Janulaitis *et al.*, 1983). Methylated bases incorporate methyl groups into the major grooves of double-helix DNA structure, thereby regulating DNA-protein interactions. Through this mechanism, methylation enables the codification of additional information without altering the genetic information of the organism, and is therefore considered a new component of the genetic code (Jeltsch, 2002).

General features of DNA methyltransferases

DNA MTases are classified as exocyclic (5mC) and endocyclic (6mA, 4mC, also called N-MTases) according to the location of the methylation in the target bases (Cheng, 1995; Bheemanaik *et al.*, 2006). However, regardless of this classification, all MTases have a similar structure consisting of three domains. Two of the three domains, SAM binding and catalytic domains, are conserved across multiple kingdoms, whereas the target recognition domain (TRD) exhibits sequence variations (Malone *et al.*, 1995). The conserved domain structures and motifs of MTases across kingdoms may indicate that they originate from a common ancestor and have diversified following the insertion and shuffling of the domains (Bujnicki, 2002). Although most bacterial DNA MTases are highly conserved at species, some of MTases are conserved across multiple higher taxonomic scales (Oliveira and Fang, 2020). For example, Dam MTase is found across several genera, such as *Escherichia*, *Salmonella*, and *Yersinia*, belonging to the Gamma proteobacteria. CcrM MTase is prevalent in several genera *Caulobacter*, *Agrobacterium*, and *Sinorhizobium* belonging to Alphaproteobacteria (Wion and Casadesús, 2006).

In eukaryotic species, specific DNA target motifs are methylated at very low levels, which are only 3% of the motifs throughout the genome (Zhu *et al.*, 2018). Unlike eukaryotic DNA MTases, bacterial DNA MTases methylate specific DNA target motifs. Typically, more than 95% of the motifs are methylated by DNA MTases throughout the bacterial genome (i.e., GATC motif in a *E. coli* and CAAAAA motif in a *Clostridium difficile*) (Wion and Casadesús, 2006; Zhu *et al.*, 2018). However, there are also MTases with multiple specific targets (e.g., M.SPR – CCGG or CCWGG) (Bunk *et al.*, 1984) and non-specific target (M.EcoGII – A) systems (Bunk *et al.*, 1984; Murray *et al.*, 2018). These ambiguous target-specific MTases have been found in phage and prophage regions and are likely a mechanism to overcome bacterial defense mechanisms.

DNA methylation in restriction-modification (RM) systems

RM systems were originally considered a bacterial defense mechanism that prevents invasion by phages and other horizontal DNA transfer. The RM system consists of two main elements, MTases and REases, and is controlled by DNA methylation. The MTase maintains the methylation status of a specific sequence in the host genome. For example, when foreign DNA invades, the REase recognizes a lack of methylation, determines that it comes from an exogenous source, and cleaves (Bickle *et al.*, 1978). Although similar in function, RM systems have been classified into four main types in bacteria (types I, II, III, and IV) based on their subunit structure, cleavage site, sequence recognition, and cofactor requirements (Roberts *et al.*, 2003).

Type I systems are multi-subunit systems consisting of three

separate genes: *hsdM* (M, DNA methyltransferase genes), *hsdR* (R, endonuclease genes), and *hsdS* (S, sequence recognition genes) (Loenen *et al.*, 2014). The Type I MTase is composed of two MTases with one S subunit complex (M_2S_1), whereas the REase is composed of two methyltransferases and two endonucleases with one S subunit to form a complex ($R_2M_2S_1$). Each S subunit, containing two TRDs, recognizes a bi-partite motif (e.g., AACNNNNNNGTGC), and the methylation of the Type I RM system occurs in the bipartite motif. However, the cleavage of the DNA sequence by the Type I R subunit occurs up to several kb away from the recognition site because the R subunit attaches to the DNA and then performs ATP-dependent DNA translocation (Ershova *et al.*, 2015).

Type II systems are the best-characterized group of RM systems and are composed of independent MTase and REase enzymes. MTases are activated as monomers, but REases are composed of various complexes ranging from monomers to tetramers (Pingoud *et al.*, 2014). They mostly bind to short 4–8 nt long palindromic motifs (e.g., GANTC), methylating the motif or cleaving DNA inside or nearby the recognition site. However, Type IIG systems, which are combined MTases and REases, recognize non-palindromic sequences (e.g., GCCCAG) and cut outside the DNA binding site (Pingoud *et al.*, 2014). Type III RM systems consist of *mod* and *res* genes encoding sequence recognition (Mod), modification (Mod), and hydrolysis (Res) factors. MTases are composed of two Mod-subunits, and because only Mod-subunits contain a DNA-binding specific domain, REase consists of a complex with a Res subunit and two Mod-subunits (R_1M_2) (Rao *et al.*, 2014). MTases (Mod-dimer) bind to short 4–6 bp long sequences and methylate non-palindromic motifs (e.g., CGAAT). Moreover, REases typically cleave 25–27 bp away from the recognition site due to DNA translocation. Finally, unlike other types of RM systems, the Type IV RM system contains only the REase and is a methylation-dependent restriction system that attacks modified phage DNA. Due to the evolutionary arms race between bacteria and phages, the Type IV RM system has evolved to have low sequence specificity (i.e., unlike other RM systems) to protect unmethylated host cells from a wide range of foreign DNA with various methylation patterns (Loenen and Raleigh, 2014).

Transcriptional regulation by DNA methylation

The bacterial 6mA decreases the DNA thermodynamical stability and changes DNA curvature (Wion and Casadesús, 2006; Low and Casadesús, 2008), and these structural alterations affect their interactions with proteins. The 6mA of Proteobacteria is primarily used as a signal to identify when and where proteins and DNA interact (Messer and Noyer-Weidner, 1988). Protein-DNA interactions lead to substantial changes in gene expression. For example, transcription factors (TFs) and MTases regulate gene expression by competing with each other at specific motif sites that could affect transcription (Lim and Van Oudenaarden, 2007; Oliveira *et al.*, 2020).

Phase variation switches, which regulate population-wide gene expression, are regulated by MTases in bacteria. Representative examples of transcriptional regulation are the pyelonephritis-associated pili (*pap*) operon and *agn43* gene in *E. coli*. For example, the *pap* operon, which encodes pilus adhesins of uropathogenic *E. coli*, can be activated or inactivated through Dam methylation (Hernday *et al.*, 2002). There are six binding sites for global regulator proteins (Leucine responsive protein, Lrp) in the upstream region of *papBA* operon. Two of these sites contain the $GATC^{prox}$ (in site 2) and $GATC^{dist}$ (in site 5) GATC motifs. In the inactivated state (hereinafter referred to as the "OFF state"), Lrp binds to 1–3 sites and interferes with RNA polymerase function, which prevents the transcription of *pap*. This consequently reduces Lrp binding to 4–6 sites and limits DNA methylation at $GATC^{prox}$ after two cycles of replication. However, the $GATC^{dist}$ site that is not affected by Lrp or RNA polymerase undergoes methylation during the replication cycle. Accordingly, Lrp has a higher affinity for $GATC^{prox}$ than $GATC^{dist}$ and creates a feedback loop that prolongs the OFF state. When the ancillary protein PapI is expressed, Lrp is translocated to 4–6 sites, including $GATC^{dist}$, and the switch changes to the ON (i.e., active) state (Hernday *et al.*, 2003). The affinity of PapI/Lrp increases at 4–6 sites rather than 1–3 sites, which leads to the methylation of $GATC^{prox}$. PapB triggers PapI transcription, which produces a positive feedback loop that prolongs the ON state. Therefore, $GATC^{prox}$ methylation and $GATC^{dist}$ nonmethylation signal the ON state.

Unlike the methylation-induced phase variation mentioned above, the importance of phase-variable MTases (also known as phasevarions) has been recently elucidated (Beaulaurier *et al.*, 2019). Spontaneous frameshifting or recombination near coding sequences encoding MTases can lead to phenotypic differences between cells. Phasevarions are genetic systems that can alter genome-wide methylation patterns by specific phase-variable MTases, which consequently affect the expression of a set of genes (Srikhanta *et al.*, 2005). The *mod* gene encoding the site-specific DNA methyltransferase of *Haemophilus influenzae* is known to undergo phase variation due to tetranucleotide repeats within its open reading frame (Srikhanta *et al.*, 2005). Additional cases of phase variation were found in *Mycoplasma pulmonis* (Dybvig *et al.*, 1998), *Streptococcus pneumoniae* (Tettelin *et al.*, 2001), *H. pylori* (de Vries *et al.*, 2002; Srikhanta *et al.*, 2011), and *N. meningitidis* (Gawthorne *et al.*, 2012; Jen *et al.*, 2014; Beaulaurier *et al.*, 2019). These were thought to be important factors controlling the expression of multiple genes throughout the genome, but their phase-variable behavior was only inferred indirectly due to a lack of technology at the time of their characterization. However, the advent of SMRT sequencing technology capable of identifying precise motif-specific methylation has deepened our understanding of previously discovered phase-variable MTases (Srikhanta *et al.*, 2017; Atack *et al.*, 2018).

DNA methylation alters phenotypes to adapt to the environment by either directly regulating gene expression or by modulating the expression of several genes through phase variation in MTases. Particularly, DNA methylation is known to alter the surface properties of bacteria and regulates various genes, structures, and processes related to pathogenesis such as sporulation, adhesins, pili, iron-transport, and lipooligo-

saccharides (Atack *et al*., 2018; Oliveira *et al*., 2020). Oliveira *et al.* (2020) reveal CAAAAAA methylation of *C. difficile* controls sporulation through an integrated analysis of DNA methylation data and genomic and transcriptome data (Oliveira *et al*., 2020). Furthermore, although the exact mechanisms of this phenomenon have not been identified, Park *et al.* (2019) have reported that MTases affect virulence and cell membrane structure of phytopathogenic bacteria *Xanthomonas axonopodis* pv. *glycines*. Additionally, studies have begun to link DNA methylation with multiple antibiotic resistance (Yuan *et al*., 2021). DNA methylation patterns and transcriptome of *Mycobacterium tuberculosis* were also altered by treatment with rifampicin or isoniazid (Chen *et al*., 2018). These findings suggest that genes may be turned on or off through an epigenetic switch via the competition between TFs binding sites and methylation. In this way, DNA methylation occurs in a subset of specific bacterial populations, thereby altering their phenotypes in response to environmental changes (Beaulaurier *et al*., 2019).

DNA methylation through cell cycle progress

Cell-cycle-regulated methyltransferase (CcrM) is an MTase originally found in *Caulobacter*, and its homologs are known to be conserved in Alphaproteobacteria (Reisenauer *et al*., 1999). Prior to the discovery of the exact function of CcrM methylation, the overexpression of CcrM was linked to aberrant morphology and chromosomal replication. These early studies suggest that CcrM has an essential relationship with the cell cycle of Alphaproteobacteria (Zweiger *et al*., 1994; Wright *et al*., 1997).

During its cell cycle, *C. crescentus* divides asymmetrically into stalked and swarmer cells (Marczynski and Shapiro, 2002), changing DNA methylation along with the replication clocks. The *dnaA*, *gcrA*, *ctrA*, and *ccrM* genes are sequentially present from the replication origin (*Cori*) to the replication terminus and are involved in a replication cascade throughout the replication fork progression (Collier *et al*., 2007). Chromosome replication initiates with the binding of the replication initiation protein DnaA, which is transcribed by the full methylation of GANTCs at *Cori*. Given that CcrM MTase is in the terminus region, it is not expressed until chromosomal replication is complete, which leaves the *dnaA* promoter in a hemimethylated state. The hemimethylated state of the *dnaA* promoter inhibits *dnaA* transcription, after which DnaA activates the transcription of *gcrA*. GcrA also activates proteins associated with polarity, motility, and cell division but activates transcription of *ctrA*, which regulates the cell cycle. Finally, CtrA transcribes *ccrM* in the hemimethylated state of both GANTC motifs in the *ccrM* promoter region, which is delayed until the replication fork passes through *ccrM*. GANTCs at *Cori* are methylated by CcrM, which serves as a signal to initiate a new round of DNA replication (Marczynski and Shapiro, 2002).

Unlike in Alphaproteobacteria, DNA hemimethylation in Gammaproteobacteria is transient and regulates specific genes only briefly involved in the replication cycle. Like *Caulobacter*, the *dnaA* gene in *E. coli* is located near the replication origin (*oriC*) and acts as the main DNA replication initiator (Reyes-Lamothe and Sherratt, 2019). DnaA is regulated by Dam MTase, which methylates three GATC motifs at *dnaA* promoters (*dnaA2*). However, in the case of Gammaproteobacteria, the regulation of DNA replication is not simply regulated by MTase alone, but also by an additional protein SeqA, a negative regulator of DNA replication, which also plays a role in sequestration. To initiate chromosomal replication, SeqA, which delays Dam MTase activity, is required to be released from *oriC* and the *dnaA* promoter (Campbell and Kleckner, 1990; Waldminghaus and Skarstad, 2009). Additionally, SeqA binds to the hemimethylated GATC motifs in the daughter *oriC*s, thus inhibiting new replication, and remains in this position throughout the first third of the cell cycle. Also, transient hemimethylation states of daughter strands by SeqA is thought to be a means to relieve the burden of the Dam-dependent recovery system (Low and Casadesús, 2008).

As in the examples above, DNA methylation is clearly involved in the regulation of the cell cycle in bacteria. In both *E. coli* and *Caulobacter*, DNA methylation at *oriC* and *Cori* have a critical role in activating DnaA and signaling the initiation of chromosome replication. Interestingly, an analogous signal such as methylation of GANTC and GATC is used to regulate DNA replication in different classes of bacteria (Low and Casadesús, 2008).

Current sequencing technologies for methylation detection

DNA methylation detection methods have historically been focused on 5mC due to its biological importance in eukaryotes (Robertson, 2005). However, in prokaryotes, adenosine methylation (6mA) is more prevalent than cytosine methylation (4mC, 5mC), and conventional methods have limitations in characterizing these methylated bases. Third-generation sequencing technology such as SMRT and nanopore sequencing are innovative techniques that directly characterize DNA molecules and methylation states without PCR amplification (van Dijk *et al*., 2018). To detect methylated bases, both sequencers use a similar method of comparing the native nucleotide signal to the control signal of methylation-free amplified whole-genome DNA (Flusberg *et al*., 2010; Stoiber *et al*., 2017; Liu *et al*., 2019). Although SMRT sequencing technology has better accuracy in detecting methylation, its detection sensitivity varies by nucleotide bases. On the other hand, nanopore technology can detect any methylated base, however with a relatively lower accuracy than SMRT sequencer.

Several computational binning methods have been developed in metagenome studies to create draft genomes from short assembled metagenomic contigs using sequence composition and contig abundance. The methylation data from the third-generation sequencers is integrated with these binning methods, enabling the separation of draft genomes of species and strains. Specifically, species and strain levels can be identified based on their unique methylated sequence motifs, which are specific to their MTase activities. Additionally, current knowledge on the shared common methylation motifs between mobile genetic elements (MGEs) and their host

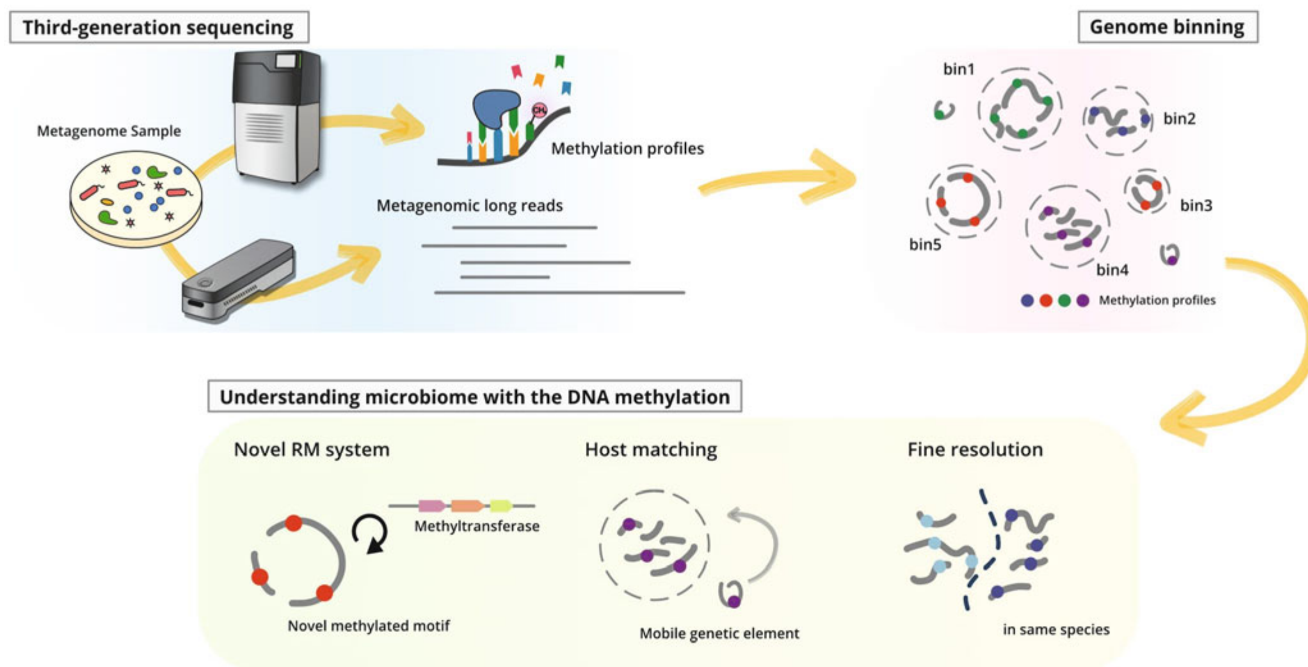

Fig. 2. Strategy for using DNA methylation data in metagenome analysis. Single-molecule, real-time (SMRT) and nanopore sequencing produce long reads containing DNA methylation information for each nucleotide bases. The DNA methylation information from each read is used for genome binning along with the tetranucleotide frequency. Information on the methylation of each genomic bin allows us to discover a novel restriction-modification (RM) system, identify hosts of mobile genetic elements, and differentiate fine-level genome bins.

chromosomes has led to improvements in the strategies used to link MGEs to their host species (Beaulaurier *et al.*, 2018; Tourancheau *et al.*, 2020). Hiraoka *et al.* (2019) used SMRT sequencer to discover novel methylated motifs in uncultured bacteria and archaea. In this study, nineteen draft genomes of mostly uncultivated bacteria and archaea were reconstructed, and nine novel methylated motifs were identified from these genomes. These findings suggest novel methylation systems in uncultured environmental prokaryotes (Hiraoka *et al.*, 2019).

Recent release of Pacific Biosciences' Sequel II sequencer and the development of flow cell chemistry in Oxford Nanopore Technologies enable the sequencing of very long reads with high accuracy (Logsdon *et al.*, 2020). Furthermore, the continuous improvement of methylation detection algorithms will likely contribute to the advancement of bacterial methylome research (Beaulaurier *et al.*, 2019). These innovations reduce genome fragmentation in metagenome assemblies and allow near-complete draft genomes to be recovered directly from the environment. Together, the added methylation data can be used to purify high-resolution genomes at the strain level and to find bacterial host of MGEs, thus helping to better understand complex microbiome (Fig. 2).

Conclusion

The vast majority of DNA methylation studies have so far focused on eukaryotes. However, recent advances in sequencing technologies have enabled the identification of 6mA and 4mC (i.e., the major methylated bases in prokaryotes) at the single nucleotide level. Prokaryotic DNA methylation is not only responsible for defense mechanisms but is also involved in cellular events that regulate the cell cycle, gene expression, and virulence. Additional multi-omics data could reveal the gene expression control system in prokaryotes, and through this, methylation signals might be used to estimate the state in which specific microorganisms respond to the environment. In metagenomic studies, genome-wide methylation data could be used as bacterial strain and MGEs specific epigenetic signals, which will undoubtedly be established as a new strategy for future strain-level microbial ecology studies. This review provides insights into the current state and historical milestones of prokaryotic DNA methylation research.

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Conflict of Interest

We have no conflicts of interest to report.

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