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DNA methylation in bacteria: from the methyl group to the methylome

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Formation of C^5-methyl-cytosine, N^4-methyl-cytosine, and N^6-methyl-adenine in bacterial genomes is postreplicative, and occurs at specific targets. Base methylation can modulate the interaction of DNA-binding proteins with their cognate sites, and controls chromosome replication, correction of DNA mismatches, cell cycle-coupled transcription, and formation of epigenetic lineages by phase variation. During four decades, the roles of DNA methylation in bacterial physiology have been investigated by analyzing the contribution of individual methyl groups or small methyl group clusters to the control of DNA–protein interactions. Nowadays, single-molecule real-time sequencing can analyze the DNA methylation of the entire genome (the 'methylome'). Bacterial methylomes provide a wealth of information on the methylation marks present in bacterial genomes, and may open a new era in bacterial epigenomics.

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Introduction

Base methylation is a DNA modification present in all kingdoms of life including bacteria [1]. C^5-Methyl-cytosine (m5C), the archetypal methylated base in eukaryotic DNA, is also found in bacteria [1,2]. In addition, bacterial genomes contain N^6-methyl-adenine (m6A), which is found in lower eukaryotes but not in vertebrates, and N^4-methyl-cytosine (m4C), which is exclusively bacterial [1–3]. The methyl group of m6A, m4C and m5C protrudes from the major groove of the double helix, which is a typical place for the recognition of DNA motifs by DNA-binding proteins [3]. As a consequence, the methylation state of critical A or C moieties can regulate the interaction between DNA-binding proteins and their cognate DNA sequences [3,4].

Formation of m6A, m4C and m5C is catalyzed by DNA methyltransferases that recognize specific DNA motifs [3,5]. Base methylation involves transfer of a methyl group from S-adenosyl-methionine to DNA [2,3,5]. During DNA replication, nonmethylated nucleotides are incorporated into the newly synthesized strand. Hence, the daughter molecules are 'hemimethylated' (methylated in the template strand only) [2–4]. Hemimethylation can be used as a physiological signal by the bacterial cell, and the duration of the hemimethylated state varies among bacterial taxa (see below).

Restriction-modification systems

The overwhelming majority of DNA methyltransferases described in the literature are part of restriction-modification systems [6–8]. Each restriction-modification system is made of a restriction endonuclease and a DNA (adenine or cytosine) methyltransferase [6]. In most restriction-modification systems, base methylation prevents DNA cleavage by the cognate endonuclease, thus protecting host DNA. However, restriction enzymes that are active on modified DNA have been also described [9]. The traditional view of restriction-modification systems as primitive immune systems that protect bacteria against phages and other invading DNAs has been progressively broadened to accommodate observations that suggest additional roles [10]. An especially relevant observation is that knockout of certain restriction-modification systems alters the gene expression pattern of the cell, which suggests an unsuspected role in epigenetic control of gene expression [10–12]. Despite their contribution to bacterial welfare, restriction-modification systems can also be viewed as addiction modules that promote their own survival [13]. Selfishness may contribute to explain why certain bacterial species contain surprisingly high numbers of restriction-modification systems: between 15 and 20 in *Neisseria gonorrhoeae*, and over 25 in *Helicobacter pylori* [10].

Solitary DNA methyltransferases

'Solitary' DNA methyltransferases are found in many genomes [2,4,14], and probably derive from ancestral restriction-modification systems that lost their restriction enzyme. Restriction-modification systems in which the modification enzyme is functional but the restriction enzyme is inactive are functional equivalents of solitary DNA methyltransferases [15].

A paradigm of solitary DNA methyltransferase is the Dam enzyme of Gamma-proteobacteria, which methylates the adenosine moiety of 5′GATC3′ sites [2,3]. The natural substrate for Dam methylase is the hemimethylated DNA formed during DNA replication [2,16], but the rate of methylation is virtually the same for nonmethylated and hemimethylated DNA [2]. A remarkable property of Dam methylase is its high processivity, which permits methylation of >50 GATC sites without dissociation from DNA. This high processivity contrasts with the distributive methylation reaction performed by DNA methylases of restriction-modification systems [5].

In *Escherichia coli* and its relatives, lack of Dam methylation causes pleiotropic defects, indicative of the existence of multiple DNA–protein interactions under GATC methylation control [2,4,17•,18]. In fact, some of the roles of Dam methylation in the physiology of Gamma-proteobacteria can be inferred from the phenotypes of *dam* mutants. For instance, asynchronic cell division indicates a role in DNA replication and/or chromosome segregation, while increased mutation rate indicates a role in DNA repair [2,4]. In turn, attenuation of *Salmonella enterica dam* mutants upon mice infection reveals the involvement of Dam methylation in the control of virulence functions [4,17•]. Unlike in other enteric bacteria, Dam methylase is essential in *Vibrio cholerae*, and the cause is the need of Dam methylation to initiate replication of chromosome 2 [19].

Another solitary DNA methylase, the cell-cycle regulated (CcrM) methyltransferase of Alpha-proteobacteria, was originally identified in *Caulobacter crescentus* [20]. CcrM may be an essential cell function in certain Alpha-proteobacteria [14]. In *Caulobacter*, it is only essential in rich medium [21]. CcrM methylates adenine in 5′GANTC3′ sites, where N is any nucleotide [4,14]. Despite the similarity of their DNA targets, Dam and CcrM have independent evolutionary origin [4]. Additional, relevant differences are that CcrM has a preference for hemimethylated DNA as a substrate while Dam does not [17•], and that CcrM is not processive [22]. Furthermore, Dam is always present in the cell, while synthesis of CcrM is restricted to a specific period of the cell cycle, when chromosome replication approaches completion [14,23•]. CcrM homologues have been found in soil-dwelling Alpha-proteobacteria such as *Agrobacterium tumefaciens* and *Sinorhizobium meliloti*, and in the animal pathogen *Brucella abortus* [4,14,17•].

Transcriptional regulation by DNA adenine methylation

If a DNA methylase target is embedded in a promoter or a regulatory region, its methylation state can modulate binding of RNA polymerase or transcription factors, thus making transcription responsive to DNA methylation [4,18,24]. Classical examples of transcriptional regulation by DNA methylation involve the adenine methyltransferases Dam and CcrM [14,18]. However, any DNA methyltransferase can potentially control transcription if it happens to methylate a DNA target at a promoter or at a nearby region involved in transcriptional control [10,11••,25•,26] (Table 1).

Studies with Dam and CcrM suggest that DNA methylation-dependent transcriptional controls can be classified into two main types:

(i) Clock-like controls that use the methylation state of DNA (methylation or hemimethylation) as a signal to couple gene expression to a specific stage of the cell cycle [24]. Examples of activation by hemimethylation include the conjugal transfer gene *traJ* and the IS10 transposase gene in Gamma-proteobacteria [27,28], and the cell cycle regulatory gene *ctrA* in Alpha-proteobacteria [23•,29]. Examples of repression

Table 1

Examples of loci under Dam methylation control in Gamma-proteobacteria

Type of control	Locus	Methylation-sensitive protein or protein complex	Number of regulatory GATC sites	Location of GATC site(s)	Position of GATC sites[a]
Clock-like	*tnp(IS10)*[b]	RNA polymerase	1	Promoter	−14
	traJ[c]	Lrp	1	UAS[d]	−52
Switch-like	*papBA*[b]	Lrp	2	UAS	−155, −53
	agn43[b]	OxyR	3	Promoter	+1, + 20, +33
	std[c]	HdfR	3	UAS	−242, −229, −220
	sciH[b]	Fur	3	UAS	Not determined[e]
	gtr[f]	OxyR	4	UAS	−110, −97, −46, −33
	opvAB[c]	OxyR	4	UAS	−174, −124, −101, −51

[a] Each number indicates the position of the G moiety of a GATC on the coding DNA strand.
[b] *E. coli*.
[c] *Salmonella enterica*.
[d] Upstream activating sequence.
[e] GATC sites are in the UAS but their precise location cannot be established because the transcription start point has not been determined.
[f] *S. enterica* bacteriophage P22.

by hemimethylation include the chromosome replication gene *dnaA* in Gamma-proteobacteria [30] and the cell division genes *ftsZ* and *mipZ* in *Caulobacter* [23•,29].

(ii) Switch-like controls that turn off and on gene expression, sometimes in a reversible manner, upon formation of DNA methylation patterns. The latter are combinations of methylated and nonmethylated sites reminiscent of the DNA methylation patterns found in eukaryotic chromosomes [24]. Because active demethylation is not known to occur in bacteria, competition between specific DNA-binding proteins and Dam methylase is the only known mechanism that generates nonmethylation [18]. Hindrance of Dam methylation by competing proteins requires that the processivity of Dam methylase is reduced [24]. This reduction typically occurs at GATC sites that are part of GATC clusters (two or more GATC sites separated by short distances) and contain AT-rich sequences at their boundaries [31,32].

Switch-like controls of gene expression are typically found at phase variation systems [33,34•], and a classical example is the *pap* operon of uropathogenic *E. coli*, which encodes fimbrial adhesins [35]. Because of phase variation, populations of uropathogenic *E. coli* contain a mixture of *pap*-ON and *pap*-OFF cells [35]. The *pap*-ON and *pap*-OFF subpopulations harbor distinct DNA methylation patterns in the *pap* regulatory region, which contains two GATC sites of the reduced processivity type [35]. In the OFF state, $GATC_{prox}$ is nonmethylated and $GATC_{dist}$ is methylated. In the ON state, $GATC_{prox}$ is methylated and $GATC_{dist}$ is nonmethylated (Figure 1). The methylation-blocking protein that creates Dam methylation patterns at the *pap* operon is the global regulator Lrp [36]. The *pap* regulatory region contains six sites for Lrp binding. In the absence of the ancillary factor PapI, Lrp binds the downstream sites, and transcription is repressed (OFF state). Switching to ON occurs in the presence of PapI, which stimulates translocation of Lrp from the proximal binding sites to the distal sites (Figure 1).

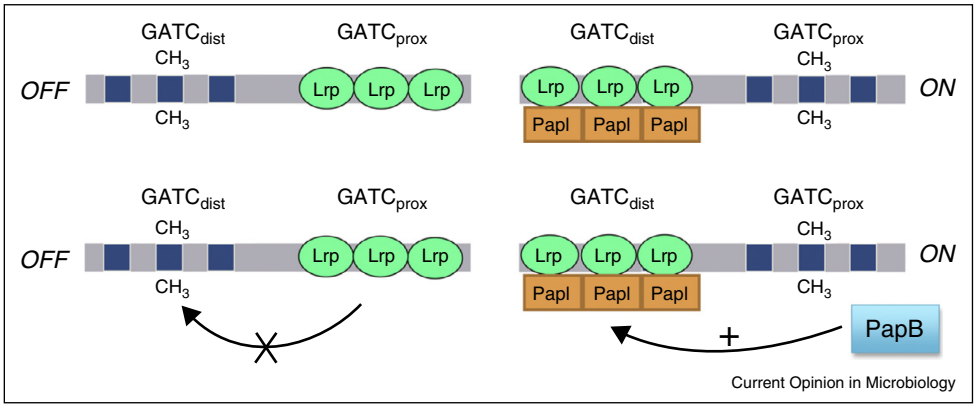
Certain phase variation loci controlled by Dam methylation use DNA-binding regulators other than Lrp. For instance, the adhesin gene *agn43* of *E. coli* [37], the glycotransferase locus *gtr* of bacteriophage P22 [38], certain *gtr* loci of the *Salmonella* chromosome [39], and the *Salmonella opvAB* operon involved in O-antigen phase variation [40] are all controlled by OxyR.

DNA adenine methylation by certain phase-variable type III restriction-modification systems has been found to regulate expression of specific genes, giving rise to a phase-variable regulon or 'phasevarion' [10,25•]. Certain phasevarions conserve their restriction-modification activity; in others, however, the modification gene (*mod*) remains active but the type III restriction enzyme is inactivated by mutation. Phase-variable synthesis of Mod methylase generates two subpopulations of bacterial cells, one of which contains N^6-methyl-adenine in the genome while the other subpopulation does not. As a consequence, each lineage shows a distinct pattern of gene expression which affects DNA methylation-sensitive loci [10,25•].

Roles of DNA adenine methylation in bacterial pathogenesis

The involvement of DNA adenine methylation in bacterial virulence was initially shown in the mouse model of typhoid: the lethal dose 50 (LD_{50}) of a *dam* mutant of *Salmonella enterica* serovar Typhimurium was 10,000-fold

Figure 1



(Top) Dam methylation patterns associated with the OFF and ON states of the *pap* operon of uropathogenic *E. coli*. Nonmethylation (of $GATC_{dist}$ in the OFF state, and of $GATC_{prox}$ in the ON state) is a consequence of Lrp binding. (Bottom) Feedback loops that propagate the OFF and ON states of the *pap* operon. Binding to the downstream sites reduces Lrp affinity for the upstream sites, propagating the OFF state. Synthesis of PapB boosts PapI synthesis, and propagates the ON state.

Table 2

Bacterial pathogens in which DNA methylation plays roles (confirmed or predicted) in virulence

Taxonomic group	DNA methyltransferase	Methylated base	Bacterial species
Alpha-proteobacteria	CcrM	m6A	*Brucella abortus*
Gamma-proteobacteria	Dam	m6A	*Salmonella enterica* *Escherichia coli* O157:H7
	Mod	m6A	*Pasteurella multocida* *Aggregatibacter actinomycetemcomitans* *Klebsiella pneumoniae* *Yersinia enterocolitica* *Aeromonas hydrophila* *Edwardsiella tarda* *Haemophilus influenzae*
Epsilon-proteobacteria	Cj1461	m6A	*Campylobacter jejuni*
	HpyAVIBM	m5C	*Helicobacter pylori*
	Several DNA methyltransferases	m6A	*H. pylori*
Firmicutes	Dam	m6A	*Streptococcus mutans*
Actinobacteria	MamA	m6A	*Mycobacterium tuberculosis*
Beta-proteobacteria	Mod	m6A	*Neisseria gonorrhoeae* *Neisseria meningitidis*

higher than that of the wild type upon oral inoculation [41,42]. This extreme attenuation reflects the pleiotropic defects of *Salmonella dam* mutants, which include reduced colonization capacity, envelope instability, ectopic expression of fimbriae, sensitivity to bile salts, and altered O-antigen chain length in the lipopolysaccharide [17•,43,44].

Virulence-related defects associated with loss of DNA methylation have been reported in other pathogens (Table 2). Relevant examples are *Mycobacterium tuberculosis* [45•], enterohemorrhagic *E. coli* O157:H7 [17•], and the oral cavity pathogens *Streptococcus mutans* [46] and *Aggregatibacter actinomycetemcomitans* [17•]. In bacterial pathogens where DNA methylation is essential, overproduction of DNA methylase provides a method to test the involvement of Dam methylation in pathogenesis [17•]. Among Gamma-proteobacteria, Dam methylase overproducers are attenuated in *V. cholerae*, *Aeromonas hydrophyla*, *Yersinia* spp., and *Pasteurella multocida* [17•]. Among Alpha-proteobacteria, overproduction of CcrM methylase reduces *B. abortus* proliferation inside macrophages [17•].

Roles of DNA cytosine methylation in bacterial physiology

Except for DNA cytosine methylases that are part of restriction-modification systems, the existence of C^5-methyl-cytosine in bacterial genomes has raised enigmatic questions during several decades [2,17•]. Spontaneous deamination of C^5-methyl-cytosine generates thymine and gives rise to T:G mismatches. Even though enteric bacteria possess a repair system that specifically repairs such mismatches, it seemed obvious that formation of C^5-methyl-cytosine had a problematic side [47]. A speculation was that formation of T:G mismatches might be a payoff for unkown physiological benefits of m5C. However, loss of the solitary methyltransferase Dcm did not seem to have phenotypic consequences in *E. coli* [17•], at least under laboratory conditions.

Recent studies, however, suggest that DNA cytosine methylation may have physiological roles including regulation of gene expression. In *H. pylori*, lack of an orphan C^5-methyl-cytosine methyltransferase known as HpyAVIBM alters the expression of genes involved in motility, adhesion, and virulence [48]. The presence of DNA repeats in the *hpyAVIBM* coding sequence raises the possibility of phase-variable expression based on repeat expansion and/or retraction, thus forming a C^5-cytosine phasevarion [48]. In *E. coli*, lack of DNA cytosine methylation has been shown to increase expression of the stress response sigma factor RpoS [49]. In another study, *E. coli dcm* mutants were found to overexpress a membrane transporter involved in ethidium bromide transport [50].

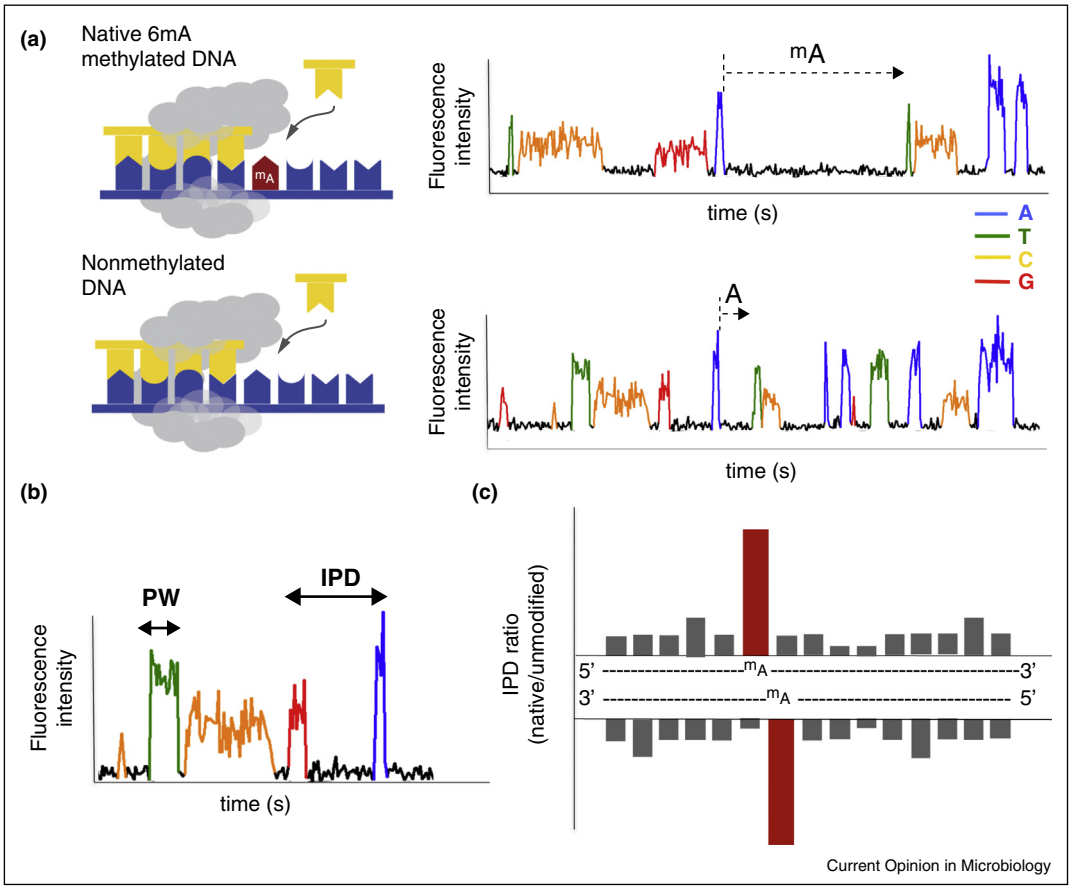
Bacterial methylomes

During decades, a hurdle in the study of bacterial DNA methylation has been the difficulty to detect methylated bases. Detection of C^5-methyl-cytosine by bisulfite genomic sequencing, a gold standard in eukaryotes [51], had limited impact as most investigations of the physiological roles of bacterial DNA methylation dealt with N^6-methyl-adenine [4,17•,18,24]. The last few years, however, have witnessed relevant advances in nucleic acid sequencing technology. For instance, a revolutionary technique that permits the determination of both the sequence of DNA and the methylation state of nucleotides has been developed. This technique, known as single-molecule real-time (SMRT) sequencing [52••], monitors in real time the activity of single DNA polymerase molecules that use

fluorescent nucleotides to synthesize DNA complementary to a template. Addition of a nucleotide is detected as a pulse of fluorescence whose color identifies the nucleotide. The interval between successive pulses (interpulse duration, IPD) is statistically longer if the template contains a methylated base (Figure 2), and the kinetic signatures of C^5-methyl-cytosine, N^4-methyl-cytosine, and N^6-methyl-adenine templates can be distinguished [52••,53]. At a given position, an altered IPD ratio between native (methylated) DNA and PCR-amplified (nonmethylated) DNA identifies a methylated nucleotide in the template [52••,53]. Analysis of PCR-amplified DNA is not necessary if standard IPD values have been previously stored *in silico*. SMRT sequencing solves the problem of N^6-methyl-adenine detection, and also detects N^4-methyl-cytosine. Detection of C^5-methyl-cytosine is also feasible after conversion to C^5-carboxy-cytosine [53].

Analysis of DNA methylation at a single nucleotide resolution deciphers the complete methylation pattern (the 'methylome') of a genome, and can provide a wealth of information. For instance, DNA motifs that contain methylated bases identify the targets of the DNA methyltransferases that are active in the bacterial isolate under study [54]. Putative loci encoding C^5-methyl-cytosine methyltransferase genes, either solitary or components of restriction-modification sytems, can be then identified by bioinformatic analysis. Prediction of m6A and m4C DNA methyltransferases is more difficult, but can be expected to improve as the databases grow [55]. To match DNA methyltransferases with their targets, mutant analysis followed by SMRT sequencing can be performed. An alternative possibility is to clone the putative methyltransferase gene on a plasmid, and to introduce the construct into a heterologous host (e.g., an *E. coli* strain lacking DNA methyltransferases). Subsequent SMRT

Figure 2



Methylome analysis by single molecule real time sequencing. **(a)** Native (methylated) genomic DNA and whole genome-amplified (nonmethylated) DNA are used as templates. The presence of a methylated base (e.g., N^6-methyl-adenine) in the DNA template delays the incorporation of the complementary nucleotide. **(b)** The delay in dNTP incorporation opposite a modified base is detected on a chromatogram as an extended interpulse duration (IPD). The IPD ratio is defined as the ratio between the mean IPD in the native sample and the mean IPD in the control sample. **(c)** At the site of modification, the IPD ratio reveals kinetic variation that exceeds baseline levels. PW (pulse width) is the duration of a fluorescence pulse. IPD (interpulse duration) is the time between successive pulses.

sequencing can identify the DNA methyltransferase target [54]. In certain cases, DNA methyltransferase targets can be inferred from comparison of methylation motifs in closely related genomes [12]. The contribution of SMRT sequencing to the characterization of DNA methyltransferases and their targets can be especially relevant, if not crucial, in bacterial species that harbor multiple restriction-modification systems [56,57].

Like other high throughput technologies, SMRT sequencing provides a 'big picture' which can serve as startpoint for further investigation [26,58]. However, on certain occasions the big picture alone can provide relevant information. An example of this kind was the finding that a phage-encoded restriction-modification system controlled transcription of hundreds of loci in the core genome of *E. coli* O104:H4, a pathogenic strain that caused an outbreak in Germany in 2011 [11••]. The involvement of lysogeny in virulence had no antecedents in *E. coli*, and the profound impact on the host transcriptome made by a phage-encoded DNA methylase was equally unsuspected [11••].

If the DNA methyltransferases active in a bacterial species (or in a given strain of a bacterial species) and their DNA targets are known, SMRT sequencing can also detect hemimethylated and nonmethylated DNA targets [11••,58]. As discussed above, nonmethylated DNA targets at or upstream of bacterial promoters are often hallmarks of transcriptional regulation by DNA methylation [18,24]. Combined with genome-wide analysis of transcription in the wild type and in a DNA methyltransferase mutant, identification of nonmethylated DNA targets can spot transcriptional units under putative DNA methylation control (Figure 2).

The combination of methylome and transcriptome analysis may prompt a conceptual shift in the field of bacterial DNA methylation if the involvement of restriction-modification DNA methylases in gene regulation turns out to be widespread. Epigenetic control of gene expression has been hitherto considered a task of orphan DNA methyltransferases only [4,24], and DNA methylation control has been viewed as the consequence of long co-evolution of a DNA methyltransferase and the host genome [18]. However, the possibility that acquisition of a DNA methyltransferase can cause a sudden change in the transcriptome must be also considered. Because of their diversity [10,55] and their frequent horizontal transfer [59,60], restriction-modification systems may be viewed as promiscuous modules able to generate epigenetic polymorphism. Acquisition of one such module may thus lead to unpredictable gene expression changes, a trial-and-error game like many other evolutionary games. Furthermore, changes in the specificity of DNA methyltransferases may occur frequently, thus creating novel epigenetic patterns of gene expression to be subjected to natural selection [12].

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Cheng X: **DNA modification by methyltransferases**. *Curr Opin Struct Biol* 1995, **5**:4-10.

2. Marinus MG: **Methylation of DNA**. In *Escherichia coli and Salmonella. Cellular and Molecular Biology,* edn 2nd. Edited by Neidhardt FC. Washington, D. C.: ASM Press; 1996:782-791.

3. Jeltsch A: **Beyond Watson and Crick: DNA methylation and molecular enzymology of DNA methyltransferases**. *Chembiochem* 2002, **3**:274-293.

4. Wion D, Casadesus J: **N6-methyl-adenine: an epigenetic signal for DNA–protein interactions**. *Nat Rev Microbiol* 2006, **4**: 183-192.

5. Cheng X: **Structure and function of DNA methyltransferases**. *Annu Rev Biophys Biomol Struct* 1995, **24**:293-318.

6. Loenen WA, Dryden DT, Raleigh EA, Wilson GG, Murray NE: **Highlights of the DNA cutters: a short history of the restriction enzymes**. *Nucleic Acids Res* 2014, **42**:3-19.

7. Loenen WA, Dryden DT, Raleigh EA, Wilson GG: **Type I restriction enzymes and their relatives**. *Nucleic Acids Res* 2014, **42**:20-44.

8. Rao DN, Dryden DT, Bheemanaik S: **Type III restriction-modification enzymes: a historical perspective**. *Nucleic Acids Res* 2014, **42**:45-55.

9. Loenen WA, Raleigh EA: **The other face of restriction: modification-dependent enzymes**. *Nucleic Acids Res* 2014, **42**:56-69.

10. Vasu K, Nagaraja V: **Diverse functions of restriction-modification systems in addition to cellular defense**. *Microbiol Mol Biol Rev* 2013, **77**:53-72.

11. •• Fang G, Munera D, Friedman DI, Mandlik A, Chao MC, Banerjee O, Feng Z, Losic B, Mahajan MC, Jabado OJ *et al.*: **Genome-wide mapping of methylated adenine residues in pathogenic *Escherichia coli* using single-molecule real-time sequencing**. *Nat Biotechnol* 2012, **30**:1232-1239.
Characterization of the methylome of *E. coli* O104:H4, and identification of a prophage-encoded restriction-modification system that altered the host transcriptome.

12. Furuta Y, Namba-Fukuyo H, Shibata TF, Nishiyama T, Shigenobu S, Suzuki Y, Sugano S, Hasebe M, Kobayashi I: **Methylome diversification through changes in DNA methyltransferase sequence specificity**. *PLoS Genet* 2014, **10**:e1004272.

13. Kobayashi I: **Behavior of restriction-modification systems as selfish mobile elements and their impact on genome evolution**. *Nucleic Acids Res* 2001, **29**:3742-3756.

14. Collier J: **Epigenetic regulation of the bacterial cell cycle**. *Curr Opin Microbiol* 2009, **12**:722-729.

15. Fox KL, Dowideit SJ, Erwin AL, Srikhanta YN, Smith AL, Jennings MP: ***Haemophilus influenzae* phasevarions have evolved from type III DNA restriction systems into epigenetic regulators of gene expression**. *Nucleic Acids Res* 2007, **35**:5242-5252.

16. Jeltsch A: **Maintenance of species identity and controlling speciation of bacteria: a new function for restriction/modification systems?** *Gene* 2003, **317**:13-16.

17. • Marinus MG, Casadesus J: **Roles of DNA adenine methylation in host–pathogen interactions: mismatch repair, transcriptional regulation, and more**. *FEMS Microbiol Rev* 2009, **33**:488-503.
A review of the roles of DNA adenine methylation in bacterial physiology, with emphasis on virulence-related functions.

18. Casadesus J, Low D: **Epigenetic gene regulation in the bacterial world**. *Microbiol Mol Biol Rev* 2006, **70**:830-856.

19. Val ME, Kennedy SP, Soler-Bistue AJ, Barbe V, Bouchier C, Ducos-Galand M, Skovgaard O, Mazel D: **Fuse or die: how to survive the loss of Dam in *Vibrio cholerae***. *Mol Microbiol* 2014, **91**:665-678.

20. Stephens C, Reisenauer A, Wright R, Shapiro L: **A cell cycle-regulated bacterial DNA methyltransferase is essential for viability**. *Proc Natl Acad Sci USA* 1996, **93**:1210-1214.

21. Gonzalez D, Collier J: **DNA methylation by CcrM activates the transcription of two genes required for the division of *Caulobacter crescentus***. *Mol Microbiol* 2013, **88**:203-218.

22. Albu RF, Jurkowski TP, Jeltsch A: **The *Caulobacter crescentus* DNA-(adenine-N6)-methyltransferase CcrM methylates DNA in a distributive manner**. *Nucleic Acids Res* 2011, **40**:1708-1716.

23. • Mohapatra SS, Fioravanti A, Biondi EG: **DNA methylation in *Caulobacter* and other Alphaproteobacteria during cell cycle progression**. *Trends Microbiol* 2014, **22**:528-535.
A review of the roles of CcrM methylation in the physiology of Alphaproteobacteria.

24. Low DA, Casadesus J: **Clocks and switches: bacterial gene regulation by DNA adenine methylation**. *Curr Opin Microbiol* 2008, **11**:106-112.

25. • Srikhanta YN, Fox KL, Jennings MP: **The phasevarion: phase variation of type III DNA methyltransferases controls coordinated switching in multiple genes**. *Nat Rev Microbiol* 2010, **8**:196-206.
A review of the physiological roles of phase-variable DNA methyltransferases.

26. Lluch-Senar M, Luong K, Llorens-Rico V, Delgado J, Fang G, Spittle K, Clark TA, Schadt E, Turner SW, Korlach J *et al.*: **Comprehensive methylome characterization of *Mycoplasma genitalium* and *Mycoplasma pneumoniae* at single-base resolution**. *PLoS Genet* 2013, **9**:e1003191.

27. Roberts D, Hoopes BC, McClure WR, Kleckner N: **IS10 transposition is regulated by DNA adenine methylation**. *Cell* 1985, **43**:117-130.

28. Camacho EM, Casadesús J: **Regulation of *traJ* transcription in the *Salmonella* virulence plasmid by strand-specific DNA adenine hemimethylation**. *Mol Microbiol* 2005, **57**:1700-1718.

29. Gonzalez D, Kozdon JB, McAdams HH, Shapiro L, Collier J: **The functions of DNA methylation by CcrM in *Caulobacter crescentus*: a global approach**. *Nucleic Acids Res* 2014, **42**:3720-3735.

30. Kucherer C, Lother H, Kolling R, Schauzu MA, Messer W: **Regulation of transcription of the chromosomal *dnaA* gene of *Escherichia coli***. *Mol Gen Genet* 1986, **205**:115-121.

31. Peterson SN, Reich NO: **GATC flanking sequences regulate Dam activity: evidence for how Dam specificity may influence *pap* expression**. *J Mol Biol* 2006, **355**:459-472.

32. Coffin SR, Reich NO: **Modulation of *Escherichia coli* DNA methyltransferase activity by biologically derived GATC-flanking sequences**. *J Biol Chem* 2008, **283**:20106-20116.

33. van der Woude MW: **Re-examining the role and random nature of phase variation**. *FEMS Microbiol Lett* 2006, **254**:190-197.

34. • van der Woude MW: **Phase variation: how to create and coordinate population diversity**. *Curr Opin Microbiol* 2011, **14**:205-211.
A review of the functions and mechanisms of phase variation in bacteria.

35. van der Woude M, Braaten B, Low D: **Epigenetic phase variation of the *pap* operon in *Escherichia coli***. *Trends Microbiol* 1996, **4**:5-9.

36. Hernday AD, Braaten BA, Low DA: **The mechanism by which DNA adenine methylase and PapI activate the pap epigenetic switch**. *Mol Cell* 2003, **12**:947-957.

37. Haagmans W, van der Woude M: **Phase variation of Ag43 in *Escherichia coli*: Dam-dependent methylation abrogates OxyR binding and OxyR-mediated repression of transcription**. *Mol Microbiol* 2000, **35**:877-887.

38. Broadbent SE, Davies MR, van der Woude MW: **Phase variation controls expression of *Salmonella* lipopolysaccharide modification genes by a DNA methylation-dependent mechanism**. *Mol Microbiol* 2010, **77**:337-353.

39. Davies MR, Broadbent SE, Harris SR, Thomson NR, van der Woude MW: **Horizontally acquired glycosyltransferase operons drive Salmonellae lipopolysaccharide diversity**. *PLoS Genet* 2013, **9**:e1003568.

40. Cota I, Blanc-Potard AB, Casadesus J: ***STM2209-STM2208 (opvAB)*: a phase variation locus of *Salmonella enterica* involved in control of O-antigen chain length**. *PLoS One* 2012, **7**:e36863.

41. Heithoff DM, Sinsheimer RL, Low DA, Mahan MJ: **An essential role for DNA adenine methylation in bacterial virulence [see comments]**. *Science* 1999, **284**:967-970.

42. Garcia-Del Portillo F, Pucciarelli MG, Casadesus J: **DNA adenine methylase mutants of *Salmonella typhimurium* show defects in protein secretion, cell invasion, and M cell cytotoxicity**. *Proc Natl Acad Sci USA* 1999, **96**:11578-11583.

43. Sarnacki SH, Marolda CL, Noto Llana M, Giacomodonato MN, Valvano MA, Cerquetti MC: **Dam methylation controls O-antigen chain length in *Salmonella enterica* serovar Enteritidis by regulating the expression of Wzz protein**. *J Bacteriol* 2009, **191**:6694-6700.

44. Lopez-Garrido J, Casadesus J: **Regulation of *Salmonella enterica* pathogenicity island 1 by DNA adenine methylation**. *Genetics* 2010, **184**:637-649.

45. • Shell SS, Prestwich EG, Baek SH, Shah RR, Sassetti CM, Dedon PC, Fortune SM: **DNA methylation impacts gene expression and ensures hypoxic survival of *Mycobacterium tuberculosis***. *PLoS Pathog* 2013, **9**:e1003419.
A description of bacterial lineage formation associated with DNA methylation in a major human pathogen.

46. Banas JA, Biswas S, Zhu M: **Effects of DNA methylation on expression of virulence genes in *Streptococcus mutans***. *Appl Environ Microbiol* 2011, **77**:7236-7242.

47. Poole A, Penny D, Sjoberg BM: **Confounded cytosine! Tinkering and the evolution of DNA**. *Nat Rev Mol Cell Biol* 2001, **2**:147-151.

48. Kumar R, Mukhopadhyay AK, Ghosh P, Rao DN: **Comparative transcriptomics of *H. pylori* strains AM5, SS1 and their *hpyAVIBM* deletion mutants: possible roles of cytosine methylation**. *PLoS One* 2012, **7**:e42303.

49. Kahramanoglou C, Prieto AI, Khedkar S, Haase B, Gupta A, Benes V, Fraser GM, Luscombe NM, Seshasayee AS: **Genomics of DNA cytosine methylation in *Escherichia coli* reveals its role in stationary phase transcription**. *Nat Commun* 2012, **3**:886.

50. Militello KT, Mandarano AH, Varechtchouk O, Simon RD: **Cytosine DNA methylation influences drug resistance in *Escherichia coli* through increased *sugE* expression**. *FEMS Microbiol Lett* 2013, **350**:100-106.

51. Frommer M, McDonald LE, Millar DS, Collis CM, Watt F, Grigg GW, Molloy PL, Paul CL: **A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands**. *Proc Natl Acad Sci USA* 1992, **89**:1827-1831.

52. •• Flusberg BA, Webster DR, Lee JH, Travers KJ, Olivares EC, Clark TA, Korlach J, Turner SW: **Direct detection of DNA methylation during single-molecule, real-time sequencing**. *Nat Methods* 2010, **7**:461-465.
A comprensive, reader-friendly description of the SMRT technology.

53. Davis BM, Chao MC, Waldor MK: **Entering the era of bacterial epigenomics with single molecule real time DNA sequencing**. *Curr Opin Microbiol* 2013, **16**:192-198.

54. Clark TA, Murray IA, Morgan RD, Kislyuk AO, Spittle KE, Boitano M, Fomenkov A, Roberts RJ, Korlach J: **Characterization of DNA methyltransferase specificities using single-molecule, real-time DNA sequencing**. *Nucleic Acids Res* 2011, **40**:e29.

55. Roberts RJ, Vincze T, Posfai J, Macelis D: **REBASE—a database for DNA restriction and modification: enzymes, genes and genomes**. *Nucleic Acids Res* 2010, **38**:D234-D236.

56. Krebes J, Morgan RD, Bunk B, Sproer C, Luong K, Parusel R, Anton BP, Konig C, Josenhans C, Overmann J *et al.*: **The complex methylome of the human gastric pathogen *Helicobacter pylori***. *Nucleic Acids Res* 2014, **42**:2415-2432.

57. Murray IA, Clark TA, Morgan RD, Boitano M, Anton BP, Luong K, Fomenkov A, Turner SW, Korlach J, Roberts RJ: **The methylomes of six bacteria**. *Nucleic Acids Res* 2012, **42**:4140.

58. Kozdon JB, Melfi MD, Luong K, Clark TA, Boitano M, Wang S, Zhou B, Gonzalez D, Collier J, Turner SW *et al.*: **Global methylation state at base-pair resolution of the *Caulobacter* genome throughout the cell cycle**. *Proc Natl Acad Sci USA* 2013, **110**:E4658-E4667.

59. Xu Q, Morgan RD, Roberts RJ, Blaser MJ: **Identification of type II restriction and modification systems in *Helicobacter pylori* reveals their substantial diversity among strains**. *Proc Natl Acad Sci USA* 2000, **97**:9671-9676.

60. Kobayashi I, Nobusato A, Kobayashi-Takahashi N, Uchiyama I: **Shaping the genome—restriction-modification systems as mobile genetic elements**. *Curr Opin Genet Dev* 1999, **9**:649-656.