N^6-methyl-adenine: an epigenetic signal for DNA–protein interactions

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Abstract | N^6-methyl-adenine is found in the genomes of bacteria, archaea, protists and fungi. Most bacterial DNA adenine methyltransferases are part of restriction–modification systems. Certain groups of Proteobacteria also harbour solitary DNA adenine methyltransferases that provide signals for DNA–protein interactions. In γ-proteobacteria, Dam methylation regulates chromosome replication, nucleoid segregation, DNA repair, transposition of insertion elements and transcription of specific genes. In *Salmonella*, *Haemophilus*, *Yersinia* and *Vibrio* species and in pathogenic *Escherichia coli*, Dam methylation is required for virulence. In α-proteobacteria, CcrM methylation regulates the cell cycle in *Caulobacter*, *Rhizobium* and *Agrobacterium*, and has a role in *Brucella abortus* infection.

Restriction–modification (R–M) system
Bacterial mechanism of defence against invasion by foreign DNA (for example, viruses). They are composed of genes that encode a restriction enzyme and a modification methylase.

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In microbial genomes, the most common DNA modification is post-replicative base methylation. C^5-methyl-cytosine (m5C) and N^6-methyl-adenine (m6A) are found in the genomes of many fungi, bacteria and protists, whereas N^4-methyl-cytosine (m4C) is found only in bacteria[1]. m6A is also present in archaeal DNA[2]. Base modification in bacterial genomes is performed by two classes of DNA methyltransferases: those associated with restriction–modification (R–M) systems[3], and solitary methyltransferases that do not have a restriction-enzyme counterpart. Examples of the latter are the N^6-adenine methyltransferases Dam and CcrM (cell-cycle-regulated methyltransferase), and the C^5-cytosine methyltransferase Dcm[4–7]. In eukaryotes, m5C has roles in gene expression, chromatin organization, genome maintenance and parental imprinting, and has attracted much interest because of its involvement in human disease[8]. By contrast, the functions of the prokaryotic Dcm enzyme remain unknown[4].

Diverse groups of Proteobacteria use m6A as a signal for genome defence, DNA replication and repair, nucleoid segregation, regulation of gene expression, control of transposition, and host–pathogen interactions[4–7] (FIG. 1). Methylation of the amino group of adenine lowers the thermodynamic stability of DNA[9] and alters DNA curvature[10]. Such structural effects can influence DNA–protein interactions, especially for proteins that recognize their cognate DNA-binding sites by both DNA primary sequence and DNA structure[11]. All known functions of m6A in Proteobacteria rely on regulating the interaction between DNA-binding proteins and their cognate DNA sequences. Typically, m6A is used as a signal to indicate when and where a given DNA–protein interaction must occur[12].

DNA adenine methylation and R–M systems

The discovery of R–M systems was the consequence of observations made in the early 1950s on host-controlled variation of bacterial viruses[13,14]. The molecular explanation for this phenomenon came several years later, when it was shown that the growth of bacteriophages in bacteria was restricted by endonucleases that attack unmethylated viral DNA, whereas host DNA is protected by a specific methylation pattern[15]. R–M systems are found in both Bacteria and Archaea and are believed to have evolved to protect bacteria against viruses. m6A is, with m5C and m4C, one of the three methylated bases that protect DNA against endonuclease digestion. Therefore, R–M systems contain both a restriction enzyme and a cognate DNA adenine or cytosine methyltransferase[3].

R–M systems have been classified into three types on the basis of structural features, position of DNA cleavage and cofactor requirements. In types I and III, the DNA adenine or cytosine methyltransferase is part of a multisubunit enzyme that catalyses both restriction and modification[3]. By contrast, type II R–M systems have two separate enzymes: a restriction endonuclease and a DNA adenine (or cytosine) methyltransferase that recognize the same target[3]. In addition to these three types of R–M systems in which adenine or cytosine methylation protects against endonuclease cleavage, restriction systems

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Distributive enzyme
Enzyme that dissociates from its substrate after one round of catalysis.

Processive enzyme
Enzyme that performs multiple cycles of catalysis without dissociating from its substrate.

that are specific for methylated bases have also been described[16]. Therefore, the pattern of adenine methylation extends the coding capacity of DNA to a new function, the distinction between 'self' and 'non-self'[17]. This classic viewpoint, which considers R–M systems as a barrier against foreign DNA invasion, has progressively evolved to include new biological functions and concepts, such as the involvement of R–M systems in genetic exchange[18] and the maintenance of species identity[19]. Other evidence indicates that, in type II R–M systems, the couple made by the methyltransferase and the cognate restriction endonuclease behaves as a 'selfish' element in a host–parasite-type interaction with bacteria[20]. This view originates from the observation that type II R–M genes are often linked with mobile elements and that their loss can cause cell death through restriction cleavage of the genome. This cell death occurs because R–M methyltransferases are distributive and not processive. Therefore, the residual methyltransferase activity is unable to protect all the restriction sites against cleavage by the remaining endonuclease molecules.

The Dam methylase of γ-proteobacteria

The first solitary DNA adenine methyltransferase that was found to provide signals with physiological significance was the Dam enzyme of *Escherichia coli*[4,6,7]. Dam transfers a methyl group from S-adenosyl-methionine to the amino group of the adenine moiety embedded in 5′-GATC-3′ sites[4]. Methylation occurs shortly (but not immediately) after DNA replication; therefore, passage of the replication fork leaves GATC sites transiently hemimethylated. The *E. coli* Dam enzyme methylates both hemimethylated and unmethylated GATC sites with similar efficiency[4]. Based on the organization of amino-acid domains, Dam is classified in the α-group of DNA amino methyltransferases and shares significant identity with *Dpn*IIA, *Mbo*IA and other DNA methyltransferases that are part of R–M systems[6,7]. This relatedness indicates that Dam has evolved from an ancestral R–M system. A difference, however, is that the Dam methylase is highly processive, whereas R–M modification methyltransferases are not[21].

Homologues of the *E. coli* Dam methylase are found in the orders Enterobacteriales, Vibrionales, Aeromonadales, Pasteurellales and Alteromonadales[6]. In *E. coli* and *Salmonella* species, *dam* mutations cause pleiotropic defects but do not impair viability[4,22]. In *Vibrio cholerae*, a *dam* mutation is lethal[23] and can prevent replication initiation on both chromosomes[24]. Dam methylation is also essential in certain strains of *Yersinia*[23].

Chromosome replication and nucleoid segregation. The replication origin (*oriC*) of the *E. coli* chromosome contains 11 GATC sites in 254 bp, a density that is 10-fold higher than expected from a random distribution[4]. Chromosome replication starts when the initiator protein DnaA binds at the *oriC* and separates the two strands of the double helix[25]. DnaA binding at the *oriC* region is only possible if the *oriC* GATCs are methylated; a hemimethylated origin is inactive[4,25].

On DNA replication, the GATC sites of *oriC* are not methylated immediately and remain hemimethylated for up to one-third of the cell cycle[26]. The cause of this delay is that the hemimethylated origin is sequestered by a protein called SeqA. As a consequence, methylation of the newly synthesized strand in the daughter *oriC*s by the Dam methylase is prevented[27,28]. As long as SeqA-mediated hindrance of GATC methylation persists, the *oriC* remains hemimethylated, and the start of a new replication cycle is delayed[29].

Dam methylation also has a role in the regulation of DnaA synthesis. One of the promoters of the *dnaA* gene (*dnaA2*) is only active if its three GATC sites are methylated[30,31]. Methylation of the *dnaA2* promoter is regulated by a sequestration mechanism that is analogous to that operating at *oriC*[25,26]. Because *oriC* and *dnaA* are separated by only 50 kb, both regions can be sequestered almost simultaneously by SeqA[26,32]. In addition to SeqA, a protein of unknown function that binds to hemimethylated DNA has been shown to contribute to *dnaA* repression[33].

Hemimethylation of *oriC* provides a signal for nucleoid segregation. The hemimethylated origins of the two daughter chromosomes bind to segregation-driving proteins that are located in the outer membrane[34,35]. SeqA also has a role in this process, as indicated by the observation that SeqA overproduction inhibits sister-chromosome segregation and cell division[36]. SeqA also binds to hemimethylated GATC sites behind replication forks, and might have a role in nucleoid organization[37–39].

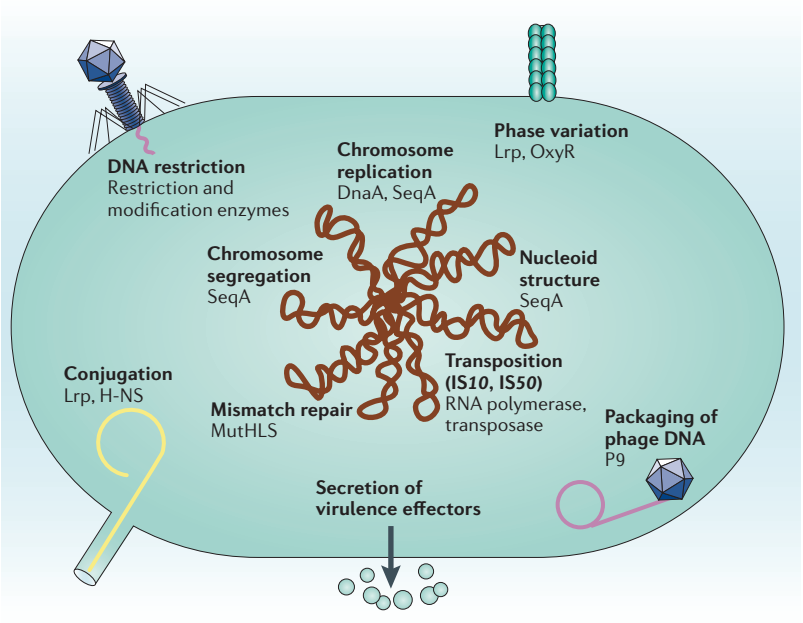

Figure 1 | **Overview of the roles of N⁶-methyl-adenine in enteric bacteria.** N⁶-methyl-adenine (m6A) is involved in many fundamental bacterial cell processes: bacterial defence against bacteriophages and transposons; regulation of chromosome replication, chromosome segregation and reorganization of the nucleoid after DNA replication; DNA-strand discrimination for mismatch repair; regulation of conjugal transfer of plasmids; the packaging of phage DNA into capsids; and the transcriptional regulation of fimbrial operons and other virulence genes. Where known, the methylation-sensitive DNA-binding proteins involved in each process are indicated.

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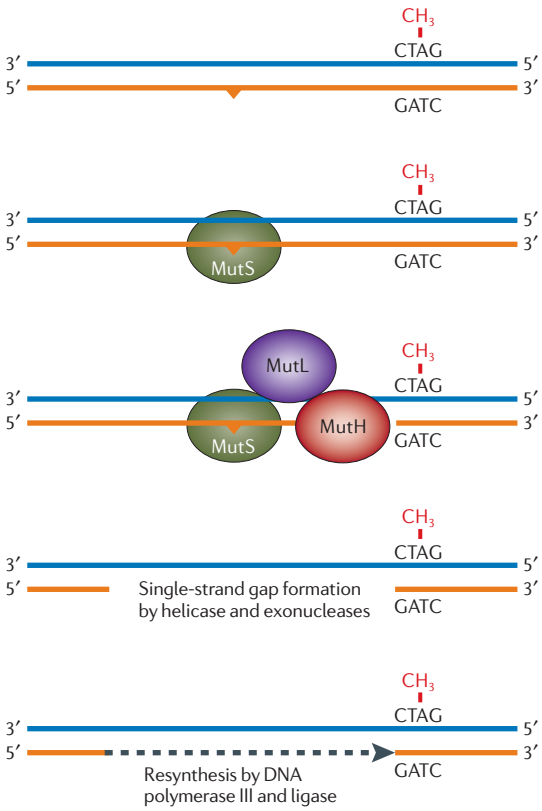

Figure 2 | **Dam-directed mismatch repair.** Assembly of the MutHLS complex at a base mismatch is followed by MutH-mediated cleavage of the newly synthesized strand (shown in orange) at the nearest GATC. Transient GATC hemimethylation of the newly synthesized strand provides the signal for strand discrimination. Depending on the distance, cleavage might require DNA looping (not shown).

Dam-directed mismatch repair. Mismatched base pairs inevitably arise during DNA replication, and their repair requires discrimination between the template strand and the newly synthesized, error-prone strand. This information is provided by the transient lack of adenine methylation in the newly synthesized DNA strand[40]. After recognition of a mismatched base pair by the MutS protein, a complex comprising MutS, MutL and MutH is formed[40] (FIG. 2). The ternary complex assembles at a DNA mismatch, and MutH, which is a GATC-specific endonuclease, cleaves the phosphodiester bond 5′ to the guanine nucleotide in the non-methylated, newly synthesized DNA strand. Transient GATC hemimethylation in the newly synthesized strand therefore provides the signal for strand discrimination by MutH[40]. The resulting ends are substrates for the UvrD helicase and for exonucleases that degrade the daughter strand past the erroneously incorporated nucleotide[40,41]. Resynthesis of the gap by DNA polymerase III and phosphodiester-bond formation by ligase follow[40].

MutH endonuclease is also active on unmethylated GATC duplexes. Therefore, in Dam⁻ strains, assembly of the MutHLS complex at a mismatch can result in MutH-mediated cleavage of GATC sites in both DNA strands[42]. This explains the sensitivity of Dam⁻ mutants to agents that cause mismatches and other DNA lesions recognized by MutS[43–46], as well as the dependence of Dam⁻ mutants on recombination and other DNA-repair functions[4,22]. Another relevant phenotype of Dam⁻ mutants is an elevated frequency of transition mutations, which is consistent with the failure of MutHLS to deal properly with mismatches[4,22]. Interestingly, the overproduction of Dam methylase results in higher mutation rates than its absence[4]. One explanation is that excess Dam methylase shortens the duration of transient hemimethylation of newly replicated DNA molecules, thereby preventing MutH-mediated incisions at hemimethylated GATCs[4]. It must be noted that the level of Dam methylase is tightly adjusted to growth rate[47], probably to permit sufficient (but not excessive) delay in the methylation of newly replicated DNA.

Regulation of transposition. In the bacterial transposons IS*10* and IS*50*, Dam methylation represses transposition by two independent mechanisms. First, in the IS*10* transposase promoter, methylation of a GATC site that overlaps the –10 module hinders binding of RNA polymerase and inhibits transcription of the transposase gene. Passage of the replication fork renders the GATC transiently hemimethylated, and permits transcription[48]. In an analogous fashion, transcription of the IS*50* transposase gene from the P_1 promoter is repressed by methylation of two GATC sites located in the –10 module[49]. Second, in both IS*10* and IS*50*, methylation of GATCs at the ends of the transposon inhibits transposase activity at these ends[48–50]. In IS*10*, only one of the two hemimethylated species is active for transposition[48], an asymmetry that further reduces the frequency of IS*10* transposition and contributes to lowering the mutation rate. An additional advantage of using Dam hemimethylation as a signal is that transposition occurs during DNA replication, and a host cell with two chromosomes is more likely to survive transposition events that affect essential loci. As in every host–pathogen interaction, increased host survival can be viewed as a favourable factor for transposon endurance.

Regulation of phase variation. Phase variation, the reversible generation of variants of surface antigens, is frequent among pathogenic bacteria. Pyelonephritis-associated pili (Pap) of uropathogenic *E. coli* mediate adhesion to the mucosa in the urinary tract[51]. Synthesis of Pap pili is turned 'ON' or 'OFF' by a mechanism that gives rise to two populations: one with pili, the other without. The switch rate from ON to OFF is 100-fold higher than from OFF to ON[51]. Because pili are highly immunogenic, phase variation might contribute to stealth invasion of the urinary tract. Furthermore, reduction of energetically expensive pili synthesis might have selective value for the population[52].

Switching of Pap phase variation is controlled at the transcriptional level by a mechanism that involves Dam methylation and the leucine-responsive regulatory protein, Lrp[52]. The upstream-regulatory sequence of the *papBA*

Transition mutation
A nucleotide substitution that changes a purine to a purine (A↔G) or a pyrimidine to a pymiridine (C↔T).

Leucine-responsive regulatory protein
Global regulator of the bacterial cell that regulates gene expression in response to exogenous leucine and other metabolic signals.

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Adhesin
Bacterial surface protein that facilitates adhesion to host tissues.

Redox-sensitive regulator
Protein that can exist in two different states in response to the redox potential of the cell.

operon contains six binding sites for Lrp (FIG. 3). Two of these sites contain GATC motifs (GATC^{dist}, located in site 5, and GATC^{prox}, located in site 2). In the OFF state, Lrp binds cooperatively and with high affinity to sites 1, 2 and 3, and prevents RNA-polymerase binding and transcription of the *pap* operon. Lrp binding at sites 1–3 reduces the affinity of Lrp for sites 4, 5 and 6. Owing to Lrp binding at sites 1–3, methylation of nascent DNA molecules after passage of the replication fork is prevented, and GATC^{prox} becomes unmethylated after two rounds of replication. By contrast, the GATC^{dist} that is located in the unbound site 5 can undergo a normal cycle of hemimethylation/methylation. The high affinity of Lrp for the unmethylated GATC^{prox}, and its inability to bind a methylated GATC^{dist}, creates a feedback loop that propagates the OFF state. Hence, methylation of GATC^{dist} and unmethylation of GATC^{prox} are hallmarks of the OFF state (FIG. 3).

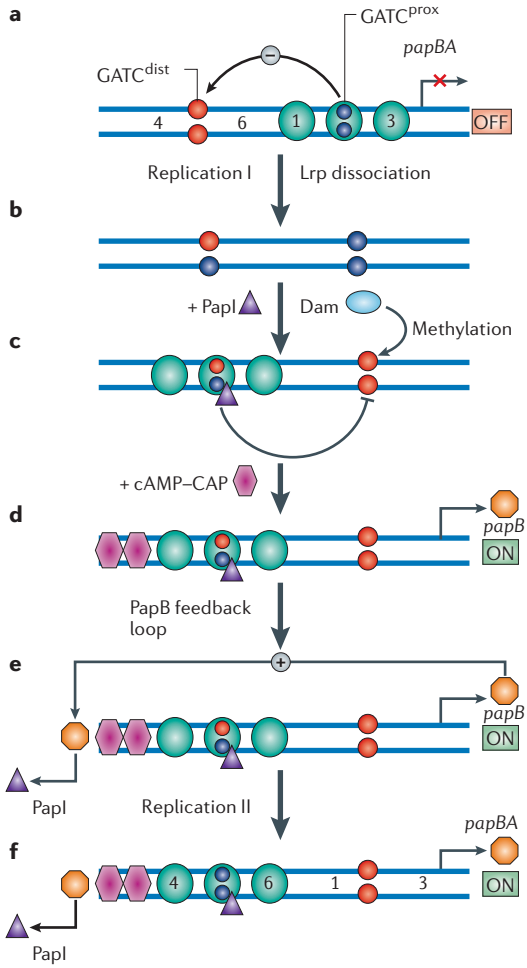

Figure 3 | **Model for switching from phase OFF to phase ON in the *pap* operon of uropathogenic *Escherichia coli*. a** | In the OFF state, Lrp (green oval) binds to sites 1–3 and prevents methylation of GATC^{prox}. Unmethylated GATCs are shown as blue circles, and methylated GATCs are shown as red circles. Binding of Lrp to sites 1–3 reduces the affinity of Lrp for sites 4–6. **b** | Every replication round offers an opportunity for switching if PapI becomes available. **c** | In the presence of PapI, Lrp translocates from sites 1–3 to sites 4–6. Lrp binding to GATC^{dist} prevents methylation of GATC^{dist} on the nascent DNA strand, while the unbound GATC^{prox} undergoes methylation. **d** | If the intracellular concentration of cAMP is high, the global regulator catabolite gene activator protein (CAP) activates *papB* transcription. **e–f** | PapB stimulates *papI* transcription, which creates a positive feedback loop that perpetuates the ON state. Figure modified with permission from REF. 52 © (2002) National Academy of Sciences, USA.

To switch to the ON state, Lrp must be translocated to the cognate binding sites 4–6. One of these binding sites (site 5) contains GATC^{dist}. Translocation requires the ancillary protein PapI, which increases the affinity of Lrp for both sites 4–6 and 1–3, probably by binding to the GATCs in these regions[52,53]. However, the affinity of PapI/Lrp for sites 4–6 is much higher than for sites 1–3, which tends to move PapI/Lrp to sites 4–6 at low PapI levels. Methylation of GATC^{prox} inhibits binding of PapI/Lrp, thereby facilitating movement of PapI/Lrp to sites 4–6 (REF. 53). Therefore, unmethylation of GATC^{dist} and methylation of GATC^{prox} define the pattern of the ON state[52]. One of the *pap* products, PapB, activates *papI* transcription, thereby creating a positive feedback loop that will propagate the ON state[52] (FIG. 3).

The model for *pap* regulation proposed by David Low envisages that switching in both directions requires replication, and that the PapI level is a crucial factor for switching[53]. The PapI level is regulated by hitherto unknown mechanisms, and an appealing hypothesis is that PapI synthesis might be indirectly controlled by the ribosome by regulating PapB expression (D. A. Low, personal communication). Additional regulators of the *pap* operon include the nucleoid protein H-NS[52] and the RimJ *N*-acetyltransferase[54], which repress synthesis of Pap pili at low temperature, the cAMP-dependent regulator CRP[52] and the envelope stress system CpxAR[55].

In *E. coli*, synthesis of Prf and S pili, which are associated with urinary-tract infections, and Afa, K88 and CS31a pili, which are associated with diarrhoeal infections, is also regulated by Dam and Lrp[6]. In *Salmonella enterica*, synthesis of Pef fimbriae is regulated by a phase-variation mechanism that involves Dam, Lrp, the nucleoid protein H-NS and the σ factor RpoS[56]. Therefore, regulation by Dam methylation and Lrp seems to be frequent among fimbrial operons.

In the *E. coli agn43* gene, which encodes a non-fimbrial adhesin that is involved in autoaggregation and biofilm formation, Dam methylation prevents binding of the redox-sensitive regulator OxyR, a repressor of *agn43* transcription, to an operator located downstream of the transcription start site[57,58] (FIG. 4). Dam methylation is therefore an activator of *agn43* transcription. As a consequence, Dam$^-$ mutants are locked in the OFF state, and OxyR$^-$ mutants are locked in the ON state[58,59]. The *agn43* operator contains three GATC sites, and methylation of any two sites can block OxyR binding[57]. In turn, OxyR binding to the *agn43* operator competes with Dam methylase and prevents methylation of the operator[58]. Switching from the

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Conjugal transfer
Transfer of bacterial DNA on cell-to-cell contact.

F sex factor
Plasmid that is present in certain *Escherichia coli* strains that can transfer chromosomal genes, which led to the discovery of bacterial conjugation.

ON to the OFF state requires OxyR binding to a hemimethylated operator after DNA replication, competing with the hemimethylated GATC-binding protein SeqA[60]. Switching from the OFF to the ON state simply requires a decrease in active (oxidized) OxyR, which leads to operator release; after DNA replication, the *agn43* operator is methylated (FIG. 4). In addition to hindrance of repressor binding, Dam methylation has an additional role in the *agn43* promoter: methylation of the upstream GATC site increases transcription initiation, which occurs precisely at the guanine nucleotide of the GATC[61].

Inheritance of Dam methylation patterns. Early studies envisaged that GATC sites remained hemimethylated for a short time after replication, and the occurrence of unmethylated DNA was viewed as a non-physiological condition[4,6]. The discovery of SeqA introduced the notion that the duration of hemimethylation at certain GATCs could be extended by sequestration and concomitant hindrance of Dam methylase activity. However, SeqA-mediated sequestration is temporary, and the sequestered GATC sites are fully methylated before cell division. By contrast, methylation-blocking proteins such as Lrp and OxyR allow the formation of GATC sites that are stably undermethylated, and inherited as such[6].

Heritable methylation patterns like those found in *pap* and *agn43* might not be exceptions. The *E. coli* chromosome contains at least 50 sites that are stably hemimethylated or unmethylated (BOX 1), and a subset of these are located in putative regulatory regions[6]. Interestingly, the distribution of undermethylated GATC sites in the *E. coli* genome varies depending on growth conditions, raising the possibility that transduction of environmental or physiological signals can change the methylation state of certain GATCs[62,63]. DNA demethylases have not been described in bacteria; therefore, active demethylation of GATC sites is unlikely to occur. Current evidence indicates that GATC undermethylation is caused by binding of proteins involved in gene regulation or nucleoid organization[6]. Local differences in the processivity of Dam methylase, which is influenced by the sequence context of GATC sites, might also contribute to GATC undermethylation[64].

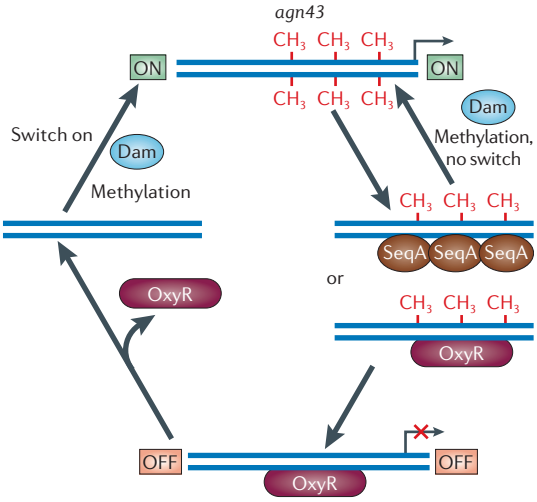

Figure 4 | **Model for regulation of *agn43* transcription.** Methylation of GATC sites in the *agn43* operator prevents binding of the OxyR repressor and defines the ON state. After DNA replication, SeqA and OxyR compete for binding to the hemimethylated operator. SeqA binding is transient and permits GATC methylation, thereby propagating the ON state. By contrast, OxyR binding hinders GATC methylation, and *agn43* is switched OFF in the following replication round. Switching to the ON state occurs when OxyR leaves the operator, permitting GATC methylation. Figure modified with permission from REF. 60 © (2002) Blackwell Publishing.

Regulation of bacterial conjugation. Conjugal transfer of the *Salmonella* virulence plasmid (pSLT) and the F sex factor is repressed by Dam methylation[65,66]. In pSLT, repression of mating by Dam methylation involves two concerted

Box 1 | **Methylation states of GATC sites in the *Escherichia coli* genome**

Methylated
- Found at most GATC sites.
- Signal for activation of *dnaA* transcription and for DnaA binding to the replication origin (*oriC*).

Hemimethylated (transient)
- Found at all GATC sites after DNA replication.
- Signal for Dam-dependent mismatch repair and for activation of cell cycle-coupled promoters (for example, *tnp* IS*10*).
- Signal for SeqA-mediated nucleoid organization.

Hemimethylated (extended)
- Found at *oriC* on sequestration by SeqA.
- Prevents chromosome replication and *dnaA* transcription.
- Signal for nucleoid segregation.

Hemimethylated (stable)
- Created by binding of proteins that prevent Dam methylation in one or more DNA replication rounds.
- An obligate step for switching in *pap* and *agn43*. Also found at specific sites on the chromosome.

Unmethylated
- Created by binding of proteins that prevent methylation in two or more DNA replication rounds. At the *pap* regulatory region, created by Lrp. At the *agn43* operator, created by OxyR. Also found at other chromosomal sites.

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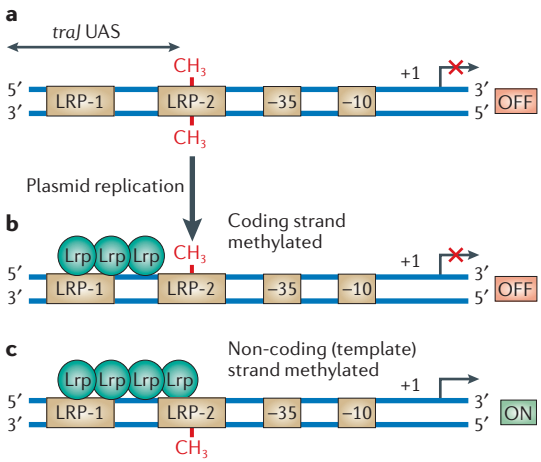

Figure 5 | **Model for regulation of *traJ* transcription in the *Salmonella* virulence plasmid.** The transcriptional activator Lrp binds to two cognate sites in the *traJ* upstream activating sequence (UAS). Lrp binding to the downstream site is inhibited by GATC methylation. As a consequence, *traJ* is not transcribed in a non-replicating plasmid. Passage of the replication fork leaves the *traJ* UAS hemimethylated. However, activation of transcription occurs only in one daughter-plasmid DNA molecule, because methylation of the coding strain does not permit formation of the Lrp–DNA activating complex.

actions: transcriptional activation of the *finP* gene, the product of which is a small RNA that inhibits conjugation[67]; and transcriptional repression of the *traJ* gene, which encodes a transcriptional activator of the transfer operon[68]. At the *finP* promoter, Dam methylation prevents transcriptional repression by the nucleoid protein H-NS[67]. At the *traJ* promoter, Dam methylation prevents binding of Lrp, an activator of *traJ* transcription[68]. The upstream activating sequence (UAS) of *traJ* contains two Lrp-binding sites (FIG. 5), both of which are necessary for transcriptional activation. One site contains a GATC, and its methylation state affects Lrp binding: GATC methylation reduces Lrp binding, and residual Lrp protein bound to the UAS forms a complex that does not permit promoter activation. By contrast, unmethylated GATC and GATC hemimethylation permit Lrp binding and formation of an activating complex near the –35 module[68]. This mechanism might regulate conjugal transfer in response to plasmid replication and the presence of sufficient Lrp[68].

A remarkable trait of *traJ* regulation by Lrp and Dam methylation is that hemimethylated DNA molecules show different properties depending on the location of m6A, in a manner that is reminiscent of IS*10* control[48]. When the *traJ* non-coding strand is methylated, Lrp-mediated activation is permitted; when m6A lies on the *traJ* coding strand, activation by Lrp is hindered[68]. Therefore, newly replicated plasmid molecules are in two different epigenetic states — one state permits Lrp binding and subsequent *traJ* synthesis, whereas the other does not. This epigenetic switch might moderate the activation of conjugal transfer, thereby limiting the metabolic and energetic burden placed on the host by the plasmid conjugation apparatus.

Roles in bacterial virulence. Initial evidence for a relationship between Dam methylation and bacterial virulence was provided by the regulation of adhesin-encoding genes[6,52]. However, the effect of *dam* mutations on the infection of model animals was first reported in *Salmonella enterica*[69–71]. Dam⁻ mutants of *Salmonella* are attenuated in the mouse model[69–71] and present pleiotropic virulence-related defects such as envelope instability with leakage of proteins and release of membrane vesicles[72], ectopic expression of the Dam-repressed fimbrial operon *stdABC*, which results in export of highly immunogenic fimbrial proteins (R. Balbontín *et al.*, unpublished results), reduced secretion of the SipC protein translocase encoded on *Salmonella* pathogenicity island I[70], and increased sensitivity to bile[46,72,73]. The relative contribution of these factors to the avirulence of *Salmonella* Dam⁻ mutants remains to be established, and additional components might be found. Envelope instability is not found in *E. coli* Dam⁻ mutants[72], and this difference might partly explain why Dam methylation is not a major virulence factor in *Shigella flexneri*, a close relative of *E. coli*[74].

Salmonella Dam⁻ mutants persist in the liver, the spleen and the lymph nodes of infected mice[69,70]. This persistence, combined with loss of cytotoxicity to M (microfold) cells[70], favours a strong immune response in the infected animal[71,75]. Use of *dam* mutations, alone or combined with other mutations, might offer unique advantages for the design of *Salmonella*-based live vaccines[75]. An undesirable trait, however, is the increased mutation rate typical of Dam⁻ mutants[22].

Inactivation of Dam methylase in *Haemophilus influenzae* causes defects that are similar to those described in *Salmonella*, including avirulence in an animal model and reduced invasion of epithelial cells[76]. Dam⁻ mutants of *Yersinia pseudotuberculosis* are also attenuated[77].

In bacterial species in which Dam⁻ mutants are not viable (for example, *V. cholerae* and certain strains of *Yersinia*), overproduction of Dam methylase is tolerated and causes virulence attenuation[23]. Dam-overproducing strains of *Y. pseudotuberculosis* and *Yersinia enterocolitica* show increased secretion of Yops (Yersinia outer proteins), a group of virulence proteins that are involved in inhibition of phagocytosis and of proinflammatory-cytokine release[78,79]. Overproduction of *E. coli* Dam methylase has also been shown to attenuate virulence in *Pasteurella multocida*[80].

Transcriptome analysis and proteomic studies have identified *E. coli* and *S. enterica* genes with impaired expression in Dam⁺ and Dam⁻ hosts[38,81]. In *E coli*, there is evidence that Dam methylation regulates metabolic pathways, respiration and motility in response to environmental cues[81]. In *S. enterica*, Dam methylation regulates genes that encode flagellar subunits, Braun lipoprotein and certain fimbriae, as well as genes required for invasion of epithelial cells (R. Balbontín *et al.*, unpublished results). These data fulfil the prediction that Dam methylation regulates the expression of virulence genes in *Salmonella*[69], and raises the possibility that Dam-regulated genes are also found in other pathogens.

Translocase
A protein involved in the translocation of proteins across membranes, and in the integration of proteins into the cytoplasmic membrane.

Salmonella pathogenicity island I
(SPI-1). Gene cluster of ~40 kb, located on centisome 63 in the *Salmonella* chromosome. The products of SPI-1 are necessary for invasion of epithelial cells.

M (microfold) cell
Cell type located in the Peyer's patches of the small intestine. M cells are involved in antigen transport and interact with *Salmonella* and other bacterial pathogens.

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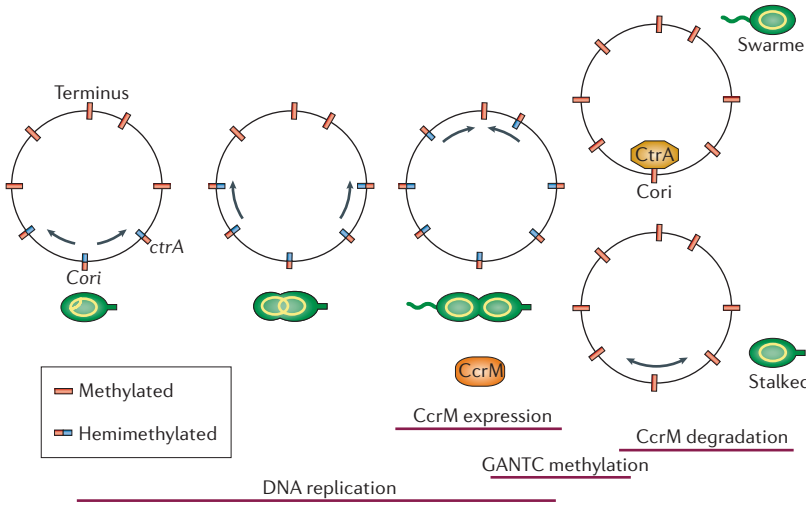

Figure 6 | **Regulation of the *Caulobacter* cell cycle.** In *Caulobacter crescentus*, asymmetrical cell division produces a non-replicating swarmer cell and a replicating stalked cell. In the swarmer cell, CtrA binds to the chromosomal origin of replication (*Cori*) and inhibits DNA-replication initiation, whereas CtrA proteolysis allows DNA replication to occur on the methylated *Cori* in the stalked cell. However, activation of *Cori* also requires full methylation (orange boxes), which is mediated by the cell-cycle-regulated methyltransferase (CcrM). Because CcrM is degraded after cell division, passage of the replication fork is not followed by methylation of the daughter DNA strands, and the origin (and most of the chromosome) is hemimethylated (blue and orange boxes) until the late stages of replication, when there is a burst in CcrM expression. The extent of hemimethylation indicates the distance covered by the replication machinery, and can be viewed as a measure of time. DNA-binding proteins (for example, transcription factors) that recognize hemimethylated sites might permit an ordered sequence of events during DNA synthesis. Figure modified with permission from REF. 5 © (1999) American Society for Microbiology .

Bacteriophage infection. The genomes of certain phages of enteric bacteria have few GATC sites, whereas other phage genomes have a GATC content similar to that found in the bacterial host genome[82,83]. The scarcity of GATC sites in the genomes of virulent phages might protect against cutting by host MutH[83]. On the other hand, certain phages have *dam* genes which might contribute to protection against MutH by ensuring that GATC sites are methylated during the lytic cycle[83].

Dam methylation regulates transcription of the *cre* gene of phage P1 (REF. 84) and the *mom* gene of phage Mu[85]. The *mom* gene encodes a DNA-modification function that generates N^6-carboxy-methyl-adenine[86]. This unusual modified base protects Mu DNA from various host-controlled restriction systems. Transcription of *mom* is regulated by Dam methylation in a manner that is reminiscent of *agn43*: methylation of three closely spaced GATC sites prevents binding of the repressor protein OxyR. In the *mom* promoter, however, the OxyR-binding region is located upstream of the –35 module[85]. OxyR can bind a hemimethylated *mom* promoter, and might limit *mom* expression during lytic phage development[85]. This action might explain why Mom-mediated modification of Mu DNA is more efficient after induction of a lysogen than during an exogenous lytic cycle[85]. Note that DNA methylation of phage DNA is usually incomplete during the lytic cycle, probably because the speed of rolling-circle replication exceeds the capacity of the Dam methylase[85].

Packaging of P1 DNA into capsids proceeds by a processive headful mechanism that uses concatemeric phage DNA molecules produced by rolling-circle replication. Packaging is initiated at the *pac* site, a 162-bp sequence that contains seven GATC sites. Protein 9, the P1 packaging enzyme, can only cut *pac* if most of its GATC sites are methylated[87]. P1 produces its own Dam methylase, and the importance of m6A in P1 infection is indicated by the observation that a P1 Dam^- mutant produces only 5% of the normal phage titre on infection of a Dam^- host. A tentative explanation is that protein 9, which is the product of an early phage gene, binds hemimethylated *pac* sites produced by theta replication and protects them from the host Dam methylase[88]. P1 circular molecules with hemimethylated and unmethylated *pac* sites are therefore produced. In the second stage of replication (rolling circle) P1 Dam methylase, the product of a late gene, is allowed to methylate only one *pac* site per concatemer; the other *pac* sites are protected (but not cut) by protein 9. This mechanism permits headful packaging, and avoids cutting of *pac* sites inside a concatemer[88].

The solitary CcrM methylase of α-proteobacteria

Cell-cycle-regulated methyltransferase (CcrM) was discovered in *Caulobacter crescentus*[89,90]. Unlike Dam, CcrM belongs to the β-group of DNA amino methyltransferases, and shares homology with the *HinfI* methylase of *H. influenzae*[5]. The target for CcrM is 5′-GANTC-3′. Like Dam, CcrM is highly processive; however, unlike Dam, CcrM is more active on hemimethylated DNA than on unmethylated DNA[5]. CcrM methylation is an essential cell function, and participates in the regulation of the *Caulobacter* cell cycle[5,89,90].

CcrM homologues have been found in *Agrobacterium tumefaciens*[5], *Sinorhizobium meliloti* (formerly known as *Rhizobium meliloti*)[5] and *Brucella abortus*[91]. In both *Agrobacterium* and *Sinorhizobium*, CcrM is essential and participates in cell-cycle regulation, indicating that the roles of CcrM methylation might be conserved among α-proteobacteria[5]. CcrM is also essential in *Brucella*, and aberrant CcrM expression impairs the ability of this pathogen to grow inside macrophages[91].

Regulation of the* Caulobacter *cell cycle. *C. crescentus* is a dimorphic bacterium with two different cell types: the replicating stalked cell and the non-replicating swarmer cell[90]. These cells differ in morphology and behaviour: the swarmer cell harbours a flagellum and is motile, whereas the stalked cell is non-motile. Cell division yields one swarmer cell and one stalked cell[90]. CcrM methylase is only produced during the late stage of chromosome replication, which occurs only in the stalked cell[5,90]; the *Caulobacter* chromosome of stalked cells therefore remains hemimethylated until CcrM is produced. Activation of the *Caulobacter* replication origin (*Cori*) requires full methylation; therefore, synthesis of CcrM methylase is a hallmark for completion of one cell cycle, and CcrM-mediated methylation of *Cori* provides a signal for the initiation of the next replication round[5] (FIG. 6).

Lysogen
Bacterial cell that carries a viral genome in a non-infectious, repressed state.

Rolling-circle replication
Mode of DNA replication that uses a circular DNA molecule as a template to produce concatemers of linear DNA molecules.

Headful mechanism
Introduction of DNA into a bacteriophage capsid in such a way that the length of the packaged DNA molecule is determined by the size of the capsid.

Theta replication
Mode of DNA replication that uses a circular DNA molecule as a template to produce two circular DNA molecules.

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Shortly after cell division, CcrM is degraded in both daughter cells[5,90]. In the non-dividing swarmer cell, chromosome replication is prevented by CtrA, a global regulator that has an important role in cell-cycle regulation. CtrA binds to the replication origin, which is methylated, and prevents replication initiation[90]. During swarmer-to-stalked cell differentiation, CtrA is degraded and remains undetectable until chromosome replication has started. Absence of CcrM prevents methylation after DNA replication and leaves the origin (and most of the chromosome) hemimethylated until the late stages of replication, when a burst in CcrM synthesis occurs. Transcription of the *ccrM* gene is activated by CtrA, which progressively accumulates in the stalked cell as chromosome replication progresses[5,90]. However, activation of *ccrM* transcription by CtrA is inhibited by methylation of two GANTC sites located in the leader of the *ccrM* coding sequence[5]. This inhibition might contribute to delay *ccrM* transcription until the replication fork reaches *ccrM*, and might prevent earlier activation by CtrA[5,90].

Synthesis of the key cell-cycle regulator CtrA is also regulated by GANTC methylation. One of the two *ctrA* promoters (P_1) contains a GANTC site near its –35 module[92]. Transcription from P_1 is repressed when the GANTC is methylated. Passage of the replication fork renders the promoter hemimethylated and activates transcription. This mechanism might boost *ctrA* gene transcription in response to replication progression. In turn, CtrA accumulation turns on synthesis of the CcrM methylase as soon as the replication fork reaches the *ccrM* gene[92]. This model is supported by elegant genetic evidence: if the *ctrA* gene is moved to an ectopic position near the replication terminus, *ctrA* transcription from the methylation-sensitive P1 promoter remains repressed, and CtrA accumulates more slowly[92].

m6A in eukaryotic genomes

The many functions performed by m6A in prokaryotic DNA contrast with the paucity of data regarding the role, or even the presence, of m6A in eukaryotes. Indeed, the first attempts to determine the base composition of eukaryotic DNA found m5C as the only methylated base in these genomes[93]. However, there is now accumulating evidence that indicates the presence of m6A in several lower eukaryotes[94]. So far, m6A has been detected in the DNA of *Penicillium chrysogenum*[95], the green alga *Chlamydomonas reinhardtii*[96] and several ciliates such as *Oxytricha fallax*, *Paramecium aurelia*, *Stylonichia mytilius* and *Tetrahymena pyriformis*[94]. A distinct feature of ciliate protozoa is the existence of a nuclear dimorphism that leads to two kinds of nuclei in the same cell: a germline nucleus (micronucleus) and a polyploid somatic nucleus (macronucleus)[97]. The finding that adenine methylation is restricted to macronuclear DNA has lead to the suggestion that m6A could be involved in the control of deletions and chromosome fragmentation that occur during macronucleus formation[94]. Unfortunately, proteins that interact with m6A in lower eukaryotes have not yet been characterized. Adenine DNA methyltransferase genes have also been reported in *Chlorella* viruses[98]. In some cases, these adenine DNA methyltransferases are associated with cognate restriction enzymes[99]. The existence of viral adenine DNA methyltransferases indicates a potential for horizontal gene transfer among unicellular eukaryotes[100]. A systematic study of the phylogenetic distribution of m6A in eukaryotes could determine whether vertical transmission or lateral gene transfer explains the presence of m6A in some protists.

Conclusions and future perspectives

Since the discovery of methylated bases in DNA, studies of DNA methylation have mainly concerned m5C in mammals. Consequently, considerable progress has been made in understanding how DNA methylation affects transcriptional regulation, chromatin structure and genome stability in higher eukaryotes. Less work has been performed on the role of adenine methylation in bacteria. However, an emerging idea is that adenine methylation in bacteria could be the counterpart of cytosine methylation in mammals. This offers an opportunity for the cross-fertilization of ideas between bacterial and mammalian epigenetic research.

The widespread functions of GATC and GANTC methylation in bacteria illustrate both the efficiency and the versatility of m6A as an epigenetic signal for the control of DNA–protein interactions. Methylation patterns at the *pap* operon[51,52] and the *agn43* gene[58] illustrate how *E. coli* lineages can inherit loci in different epigenetic states, a phenomenon reminiscent of genomic imprinting. In turn, differential regulation of IS*10* and *traJ* in sister DNA molecules[48,68] shows that bacterial DNA molecules in different epigenetic states can coexist in the same cell, similar to mammalian X-chromosome inactivation. These examples show that epigenetic phenomena are not restricted to the kingdom Eukaryota, and outline the utility of m6A as an epigenetic signal. Furthermore, the discovery of CcrM indicates that there might be other solitary adenine methyltransferases in bacteria. They can be expected to be identified when new whole genome sequences become available, and their study will probably provide new insights into the biological roles of m6A.

The use of m6A to control DNA–protein interactions avoids the problem created by the high mutability of m5C, which undergoes high frequencies of deamination, giving rise to thymine[101]. This might also explain why, in bacterial species that use m6A as an epigenetic signal, solitary m5C methyltransferases do not seem to have been selected to control crucial DNA–protein interactions. For example, no biological function has yet been found for Dcm in *E. coli*[4]. In higher eukaryotes, unrepaired T:G mismatches give rise to CG→TA transitions, which are known to be involved in many hereditary diseases and cancer[8,102]. Therefore, the use of m5C instead of m6A by eukaryotes to control gene expression might be a less fortunate evolutionary event[103].

The finding that m6A is essential for growth or virulence in certain bacteria[5,6] raises the possibility of using Dam or CcrM inhibitors as antimicrobial agents[64]. However, the use of such alternative antibiotics requires the absence of m6A in the human genome.

Genomic imprinting
Epigenetic mechanism in diploid organisms by which only one allele (maternal or paternal) is expressed.

X-chromosome inactivation
Epigenetic silencing of most genes in one of the two X chromosomes in somatic cells of mammalian females.

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The current opinion that mammalian DNA is devoid of m6A is based upon analyses which have a detection limit of about 0.01%; therefore, the presence of thousands of m6As in the human genome cannot be excluded. Moreover, the recent discovery of an N^6-adenine DNA methyltransferase in wheat coleoptiles[104] might stimulate a re-examination of the presence and the potential roles of m6A in higher eukaryotes.

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Competing interests statement
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