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Roles of DNA adenine methylation in host-pathogen interactions: mismatch repair, transcriptional regulation, and more

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Abstract

The Dam methylase of gamma-proteobacteria and the CcrM methylase of alpha-proteobacteria catalyze an identical reaction (methylation of adenosine moieties using *S*-adenosyl-methionine as methyl donor) at similar DNA targets (GATC and GANTC, respectively). Dam and CcrM are of independent evolutionary origin. Each may have evolved from an ancestral restriction-modification system that lost its restriction component, leaving an "orphan" methylase devoted solely to epigenetic genome modification. Formation of 6-methyladenine lowers the thermodynamic stability of DNA and changes DNA curvature. As a consequence, the methylation state of specific adenosine moieties can affect DNA-protein interactions. Well known examples include binding of the replication initiation complex to the methylated *oriC*, recognition of hemimethylated GATCs in newly replicated DNA by the MutHLS mismatch repair complex, and discrimination of methylation states in promoters and regulatory DNA motifs by RNA polymerase and transcription factors. In recent years, Dam and CcrM have been shown to play roles in host-pathogen interactions. These roles are diverse and only partially understood. Especially intriguing is the evidence that Dam methylation regulates virulence genes in *E. coli*, *Salmonella*, and *Yersinia* at the postranscriptional level.

Keywords

Dam; CcrM; Pathogenic bacteria; Transcription; GATC regulation

Introduction

Postreplicative DNA methylation superimposes on the primary DNA sequence secondary information that has significance for DNA transactions such as transcription, transposition, initiation of chromosome replication, and prevention of mutations by DNA repair (reviewed in Løbner-Olesen *et al*., 2005; Casadesus & Low, 2006; Wion & Casadesus, 2006; Low & Casadesus, 2008). The most common postreplicative base methylations are N^6-methyladenine (6-meA) and 5-methylcytosine, which are found in both prokaryotes and eukaryotes, and N^4-methylcytosine which is restricted to bacteria. The chromosomes of the model gamma-proteobacteria *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (*S. enterica* hereafter) contain about 20,000 6-meA residues which are the products of two distinct methyltransferases. Most adenine methylations occur in the sequence GATC catalyzed by the enzyme DNA adenine methyltransferase (Dam). In *E. coli* about 600 6-meA residues are due

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to the action of M.EcoK, part of a classical type I restriction/modification system. A third adenine methyltransferase, YhdJ, is not produced under normal laboratory conditions, and its role in cellular metabolism remains unknown (Broadbent *et al.*, 2007). The adenine methyltransferases discussed in this article are not part of a restriction/modification system and are often referred to as orphan or solitary methyltransferases. An exception, however, is the modification subunit of a type III DNA restriction system which regulates gene expression in *Haemophilus influenzae* (Fox *et al.*, 2007) (see below).

Another model organism that has been used to study the physiological roles of DNA adenine methylation is *Caulobacter crescentus*, a member of the alpha branch of proteobacteria. In *C. crescentus*, the DNA adenine methyltransferase CcrM (cell cycle-regulated methyltransferase) recognizes and methylates the sequence GANTC, and has a role in cell-cycle regulated events (Marczynski & Shapiro, 2002). CcrM methylation in *C. crescentus* and a few other members of the alpha-proteobacteria are included in this review.

The Dam enzyme is encoded by the *dam* gene, and much of our knowledge about the cellular functions of Dam came from studying *dam* mutants in *E. coli* and *S. enterica*. The properties of these mutants showed that the DNA transactions most affected are Dam-directed mismatch repair, initiation of chromosome replication, and regulation of gene expression. As discussed below, altered gene expression patterns *in* dam mutants impair host-pathogen interactions. In addition to the known examples of transcriptional regulation, there are hints that Dam methylation may also influence gene expression by postranscriptional mechanisms. We list the instances of postranscriptional regulation and speculative models to explain them. This article is a companion to other recently published reviews (Løbner-Olesen *et al.*, 2005; Casadesus & Low, 2006; Wion & Casadesus, 2006; Heusipp *et al.*, 2007; Low & Casadesus, 2008).

Dam methylation

The Dam methyltransferase

The *E. coli* Dam enzyme, which is a monomer in solution, catalyzes transfer of the methyl group from *S*-adenosyl-L-methionine (SAM) to the N^6 position of the adenine residue in GATC sequences, using base flipping to position the base in the enzyme's catalytic site. The natural substrate for the enzyme is hemimethylated DNA, where one strand is methylated and the other is not. This is the configuration of DNA immediately behind the replication fork. Double-stranded DNA is a better methyl acceptor than denatured DNA, and there is little difference in the rate of methylation between unmethylated and hemimethylated DNA (Herman & Modrich, 1982). The enzyme appears to have two SAM binding sites; one is the catalytic site and the other increases specific binding to DNA, probably through an allosteric transformation (Bergerat *et al.*, 1991). Dam is thought to bind the template and to slide processively along the DNA, methylating about 55 GATC sites per binding event (Urig *et al.*, 2002). The atomic structure of Dam complexed with DNA has been solved to 1.89 angstrom resolution in the presence of *S*-adenosyl-homocysteine (a product of the Dam reaction) (Horton *et al.*, 2006). The structure shows both non-specific backbone contacts and specific contacts with the GATC bases. Importantly, the aromatic ring of Y119 intercalates into the DNA between GA and TC, thereby flipping the adenine into the enzyme's active site. The unpaired T residue can adopt an intrahelical or extrahelical position. Four other important contacts are made: K9 to G, L122 and P134 to C and by R124 to T. These and flanking phosphate contacts by conserved residues (R95, N126, N132, and R137) position Dam on the DNA duplex.

There are about 130 molecules of Dam per *E. coli* cell, and this level is optimal to allow a period of time between synthesis of the extending nucleotide chains and methylation of GATC sequences within them (Boye *et al.*, 1992). The actual time between synthesis and methylation can be rapid for plasmid molecules (2–4 sec) (Stancheva *et al.*, 1999) or about 1 min for

chromosomal DNA in slow-growing cells with a doubling time of about 100 min (Campbell & Kleckner, 1988). Increases or decreases in the number of Dam molecules can profoundly alter the physiological properties of the cell (see below). Adenine methylation lowers the thermodynamical stability of DNA and alters DNA curvature, thereby affecting DNA-protein interactions at certain GATC-containing DNA motifs (Wion & Casadesus, 2006). Steric hindrance of protein binding by the methyl group is also conceivable (Wion & Casadesus, 2006).

The cellular level of Dam is regulated mainly by transcription. The *dam* gene transcripts arise from five distinct promoters. The major dam promoter (P_2) is located 3 kb upstream of the gene (Løbner-Olesen *et al.*, 1992), and is regulated by growth rate: the faster the growth rate the greater the level of transcript. This makes sense for Dam as fast growing cells, which contain multiple replication origins, are expected to require a greater concentration of the enzyme than slow growing ones which contain few replication origins. Dam is a substrate for the Lon protease, and there might be regulation of the enzyme level by this mechanism (Calmann & Marinus, 2003).

Dam competes with two other proteins, MutH and SeqA, for hemimethylated GATC substrate sites. These two proteins act before Dam to participate in removal of replication errors (MutH, see below) and to form the compacted and properly supercoiled chromosome structure for the nucleoid (SeqA). Increasing the cellular level of Dam causes a decrease in the amount of hemimethylated DNA, and prevents these two proteins from carrying out their functions, leading to an increased mutation rate and a change in supercoiling of the chromosome, respectively (Herman & Modrich, 1981; Marinus *et al.*, 1984; Løbner-Olesen *et al.*, 2003).

Although Dam methylase is a highly processive enzyme, it may become less processive at GATC sites flanked by specific DNA sequences (Peterson & Reich, 2006). Reduced processivity may allow competition between Dam and specific DNA binding proteins, thus permitting the formation of nonmethylated GATCs. For instance, the *E. coli* chromosome contains about 36 specific, unmethylated *dam* sites (Ringquist & Smith, 1992; Wang & Church, 1992; Hale *et al.*, 1994; Tavazoie & Church, 1998). The number of unmethylated sites in the chromosome varies depending on growth phase and growth rate, suggesting that the proteins which bind to them could be involved in gene expression or in the maintenance of chromosome structure. The unmethylated *dam* sites appear to be mostly (Ringquist & Smith, 1992) or completely (Palmer & Marinus, 1994) modified in strains overproducing Dam, suggesting that the enzyme competes with other DNA binding proteins at these specific sites. Evidence for competition between Dam and other DNA binding proteins at several unmethylated sites has been obtained, as discussed in more detail below (Regulation of Gene Expression). Alternatively or in addition, some GATC sites in DNA structures [e.g., non-B-form DNA such as H-DNA (Parniewski *et al.*, 1990)] are relatively resistant to methylation at the normal cellular level of the enzyme. Palindromic structures containing GATCs are also relatively resistant to Dam methylation (Allers & Leach, 1995).

In addition to the unmethylated GATC sites discussed above, persistent hemimethylated sequences have been detected in the chromosome (Ogden *et al.*, 1988; Campbell & Kleckner, 1990). These are distinct from the transiently hemimethylated GATC sites which occur immediately behind the replication fork due to the time lag between DNA replication and Dam methylation. The persistent hemimethylated sites are discussed in more detail below (Initiation of Chromosome Replication).

Dam-directed mismatch repair

Errors that arise from DNA replication need to be removed from the newly synthesized DNA strand, and not from the parental strand. In *E. coli* and *S. enterica*, this discrimination is

achieved by virtue of the hemimethylated state of DNA behind the replication fork: the newly synthesized DNA is not methylated but the parental strand is methylated (Pukkila *et al.*, 1983). The base mismatch formed by a replication error (e.g., G-T) is recognized and bound by the MutS protein which recruits the MutL protein (a molecular matchmaker) to form a ternary complex with MutH. The latent endonuclease activity of MutH is unmasked by the complex, and the enzyme cleaves the unmethylated strand 5' to the G at a nearby GATC site. MutH is then displaced from the complex by the UvrD helicase. UvrD unwinds DNA and the exposed single strand is degraded by exonucleases until the mismatch is removed. The resultant gap is filled in by DNA polymerase III holoenzyme, and the is nick sealed by DNA ligase. Finally the hemimethylated GATC is symmetrically methylated by Dam (reviewed in Iyer *et al.*, 2006). Since MutH is active on hemimethylated, but not fully methylated DNA, mismatch repair action is confined to the hemimethylated region behind the replication fork.

Among the evidence supporting the above model is that lack of Dam methylation and overproduction of Dam both lead to the same result: an increase in spontaneous mutation frequency (Marinus & Morris, 1974; Herman & Modrich, 1981). Overproduction of Dam leads to premature methylation of new DNA, thereby preventing MutH action if a mismatch is present. In turn, lack of Dam results in loss of strand discrimination, leading to use of the parental strand as template for mismatch repair with a ½ probability.

Single- and double-strand breaks have been detected in the chromosome of *dam* mutants as a consequence of mismatch repair (Marinus & Morris, 1974; Wang & Smith, 1986). Homologous recombination is required to repair the double-strand breaks, and this explains why mutations inactivating homologous recombination are synthetically lethal in a *dam* mutant background (Marinus, 2000).

Initiation of Chromosome Replication

As mentioned above, persistent hemimethylated sites have been detected at the origin of chromosome replication, *oriC*, and the region surrounding it (Campbell & Kleckner, 1990). This region includes the *dnaA* gene which is located 43 kb from *oriC*. DnaA initiates chromosome replication by binding to *oriC* and facilitating duplex opening to load DnaB helicase and DNA polymerase III holoenzyme. The persistence of the hemimethylated state is due to the high density of GATC sequences in *oriC* (11 in 245 bp) and in the promoter region of *dnaA* (8 in 219 bp), providing multiple binding sites for the SeqA protein. The SeqA-induced hemimethylated state in this region of the chromosome lasts for about one-third of the cell cycle (“sequestration”), but the mechanism by which it is relieved is not known. The purpose of sequestration is to prevent re-initiation from *oriC* from occurring more than once per cell cycle. For initiation to occur most efficiently, *oriC* and the *dnaA* promoter region must be fully methylated. This also contributes to ensuring that initiation occurs only once per cell cycle (Braun *et al.*, 1985; Yamaki *et al.*, 1988). In *Salmonella enterica*, SeqA may play replication-related roles similar to those described in *E. coli* (Prieto *et al.*, 2007). In *V. cholerae*, both Dam methylation and SeqA are essential (Julio *et al.*, 2001; Saint-Dic *et al.*, 2008), and SeqA overproduction causes DNA replication arrest (Saint-Dic *et al.*, 2008).

In fast growing *E. coli* or *S. enterica* cells, the time required for chromosome replication exceeds the doubling time. Under such conditions, *E. coli* and *S. enterica* cells contain multiple copies of *oriC* due to initiations that occurred two or three generations ago. These origins fire simultaneously during the cell cycle leading to synchronous initiation, which is thought to be due to the immediate release of DnaA from an origin after initiation (reviewed in Nielsen & Løbner-Olesen, 2008). This release will temporarily increase the DnaA/*oriC* ratio in wild type cells for the remaining fully methylated origins. After initiation, other mechanisms ensure that DnaA is not in the proper conformation for initiation. Among these mechanisms is a reduction in transcription of the *dnaA* gene. Sequestration by SeqA after initiation keeps the *dnaA*

promoter region in a hemimethylated state which reduces transcription initiation because the *dnaA* promoter GATC sequences need to be fully methylated for maximal expression (Braun *et al.*, 1985).

In *E. coli dam* cells there is no sequestration by SeqA; consequently, DnaA can immediately rebind origins after the first initiation event, and initiate a second time when the concentration of the active form of DnaA is high enough. Transcription from the *dnaA* gene continues throughout the cell cycle although at a reduced level. Dam methylation, therefore, is not essential for replication initiation; rather the cell uses methylation to discriminate between old and new origins.

Regulation of Gene Expression

Since the state of GATC sites (methylated, unmethylated, hemimethylated) can affect specific binding of DpnI (cuts methylated DNA), DpnII (cuts unmethylated DNA), Dam, SeqA and MutH, then it is not surprising that the presence of this tetranucleotide in promoter or regulatory sequences can affect gene expression by regulating binding of RNA polymerase or transcriptional regulators. The promoter region of the *dnaA* gene discussed above, for example, is maximally active in the fully methylated state, consistent with its biological role. In contrast, there is evidence that specific protein binding yields about 36 unmethylated GATCs in the *E. coli* chromosome (reviewed in detail by Casadesus & Low, 2006). Nine such GATCs are in the cyclic AMP binding protein (CAP) binding sites preceding the *mtlA*, *cdd*, *flhD*, *gcd*, *ycdZ*, *yffE*, *ppiA*, and *proP* operons (Wang & Church, 1992), suggesting that gene expression might be modulated by Dam methylation through differential CAP binding. Other genes with GATCs that overlap with protein binding sites are: *hrsA*, *kdgT* (Fnr), *pspA*, *yjdG* (IHF), *fep* (Fur), *carA* (CarP, IHF), *agn43* (*flu*) (OxyR), *ppiA* (Lrp, CAP), and *yhiP* (Lrp) (Hale *et al.*, 1994; Tavazoie & Church, 1998). Data supporting specific binding of a regulatory protein either *in vivo* or *in vitro* are only available for a fraction of the genes listed. Additional unmethylated GATC sites were found in the non-coding regions of *rspA*, *ydjL*, *yahM*, *bhsA*, *yjdD*, *yhiP*, *yiaK*, *yidX*, and *yihU/V* genes (Hale *et al.*, 1994; Tavazoie & Church, 1998), although their significance is not known.

Studies on the *pap* operon have provided the most detailed evidence that unmethylated GATCs are involved in transcriptional control (reviewed in Casadesus & Low, 2006). Pyelonephritis-associated pilus (Pap) expression is regulated by a phase variation mechanism in which individual cells either express pili (phase-on) or not (phase-off). When Pap pilus gene expression is in the phase-off state, GATC1028 is fully methylated and GATC1130 is unmethylated. Conversely, in the phase-on state, the methylation state at these two sites is reversed. In a strain overproducing Dam, the transition from phase-off to phase-on is prevented, whereas in a *dam* mutant the opposite transition does not occur. The mechanism of phase variation involves competition between Dam and the transcriptional activators Lrp and PapI. Lrp is required for methylation protection of GATC1130, and both Lrp and PapI are required for protection of GATC1028 (Casadesus & Low, 2006). Other pilus systems also appear to be under Dam control, but they have not been analyzed as deeply as *pap* (Casadesus & Low, 2006). Formation of Dam methylation patterns also regulates the *agn43* gene of *E. coli*, which encodes a non-fimbrial adhesin (Henderson & Owen, 1999; Waldron *et al.*, 2002; Wallecha *et al.*, 2002).

In addition to unmethylated sites, there is also evidence that hemimethylated GATCs can control gene expression. Transposition of Tn*10* is regulated by the methylation state of two specific GATC sites in IS*10* right (Roberts *et al.*, 1985). Overproduction of Dam decreases transposition, whereas it is increased in a *dam* mutant. One of the GATC sites overlaps the -10 region of the transposase (*tnp*) promoter, while the other is near the inner end of IS*10* in the target area for transposase action. In DNA that is not being replicated, these sites are methylated

and inert for transposition. Upon replication, these sites become hemimethylated but only one of the hemimethylated species is activated for transposition. In a wild-type strain, the transposase promoter is only active in the IS*10* species that presents methylation of the transposase coding strand and unmethylation of the non-coding strand. Coupling of transposase synthesis and activity to hemimethylation implies that transposition is repressed for most of the cell cycle, and can only be induced when the element is replicated. The asymmetry imposed at the replication fork means that only one of the two copies of the element can transpose. Hence one copy can remain in place while the other finds an alternative location. Coupling transposition to replication may help to prevent potentially deleterious effects of excessive transposition (Roberts *et al.*, 1985). Other transposons such as Tn*5* and Tn*903* and the insertion element IS*3* also use Dam methylation to control transposition (Curcio & Derbyshire, 2003).

Another case of transcriptional activation by strand-specific hemimethylation has been described in the *traJ* gene of the *S. enterica* virulence plasmid, albeit with the difference that the regulatory GATC is not located in the promoter itself but in an upstream binding site for the transcriptional activator Lrp (Camacho & Casadesus, 2002). Another difference is that the active configuration is opposite to that of IS*10*: methylation of the *traJ* non-coding strand permits Lrp binding and subsequent *traJ* transcription, but methylation of the coding strand does not (Camacho & Casadesus, 2005).

Several *E. coli* promoters have GATC sites in their −10 or −35 regions. These include promoter regions for the *sulA*, *trpS*, *trpR*, *tyrR*, and *glnS* genes, and expression of these genes is increased in *dam* mutants (reviewed by Plumbridge, 1987; Barras & Marinus, 1989; Marinus, 1996). It is not known whether expression of these genes is increased in a hemimethylated configuration, but even if it were the physiological role for coupling their transcription to replication is not obvious.

In the *finP* gene of the *Salmonella* virulence plasmid, which encodes a small regulatory RNA, Dam methylation prevents repression by the nucleoid protein H-NS (Camacho *et al.*, 2005). In contrast, the overlapping *traJ* gene is also repressed by H-NS but in a Dam-independent manner. Protection from H-NS repression is still observed when a GATC that overlaps the −10 module of the *finP* promoter is eliminated by site-directed mutagenesis (Camacho *et al.*, 2005). This observation suggests that the effect of Dam methylation on *finP* transcription is not local but global, perhaps reflecting, among several possibilities, the existence of structural differences between dam^+ and *dam* nucleoids.

Global gene expression analysis comparing wild type and *dam* mutants using microarrays has been performed in *E. coli* and *S. enterica* (Oshima *et al.*, 2002; Løbner-Olesen *et al.*, 2003; Robbins-Manke *et al.*, 2005; Balbontin *et al.*, 2006). The results are difficult to compare given the differences in strain backgrounds, media, arrays, and other experimental conditions, as well as in the goals of the experiments. However, upregulation of SOS gene expression in the *dam* background was detected in each case, and decreased motility in two out of three studies.

Dam methylation also occurs in many bacteriophages that infect enterobacteria. The regulation of phage genes by Dam and the role of Dam methylation in P1 development have been reviewed elsewhere (Marinus, 1996; Wion & Casadesus, 2006).

CcrM methylation

Caulobacter

Caulobacter crescentus, a member of the alpha-proteobacteria, has defined morphological stages in its life cycle. The DNA methyltransferase in this organism is CcrM, which methylates adenine moieties in the sequence GANTC (Marczynski & Shapiro, 2002). Although the DNA

methylation target of CcrM is similar to that of Dam, CcrM belongs to a different family of methyltransferases. In contrast to Dam, CcrM is more active on hemimethylated than unmethylated DNA. However, like Dam, CcrM is highly processive. Unlike Dam in *E. coli* or *S. enterica*, CcrM is an essential function in *Caulobacter*, and is not present at all stages of the life cycle. Both Dam and CcrM are substrates for the Lon protease (Reisenauer *et al.*, 1999).

The life cycle of *Caulobacter* involves differentiation into two cell types, stalked cells and swarmers. Chromosome replication occurs only in stalked cells, and involves the sequential action of three key unstable regulators: DnaA, GcrA, and CtrA (Collier *et al.*, 2007). The genes for these regulators are located sequentially on the chromosome, with *dnaA* closer to the origin of replication (C*ori*) and *ctrA* at the most distal location. The action of these regulators, acting as a transcriptional cascade, is determined by the state of methylation of chromosomal DNA. DnaA initiates chromosome replication at the fully methylated C*ori* in a manner similar to that described in *E. coli*. Since CcrM is not present at this stage, replication produces two hemimethylated daughter DNA molecules during fork progression. As in *E. coli*, expression of the *dnaA* gene, which lies near C*ori*, is attenuated on hemimethylated DNA, thereby reducing the possibility of premature initiation. DnaA also activates transcription of the *gcrA* gene, the product of which controls transcription of replication genes encoding DNA polymerase III holoenzyme, DNA helicase and primase. GcrA in turn activates transcription of the *ctrA* gene, which contains two GANTC sequences in the upstream regulatory region of the promoter and one close to the −35 hexamer (Collier *et al.*, 2006). Again, this promoter is active only when hemimethylated; expression of the gene is, therefore, coordinated with the cell cycle (Reisenauer & Shapiro, 2002). CtrA binds C*ori* to prevent premature initiation. In addition, CtrA activates transcription of the *ftsZ* and *ccrM* genes, and represses transcription of *gcrA*. FtsZ is a key cell division protein and its CtrA-controlled production can couple chromosome replication and cell division. Transcription of the *ccrM* gene occurs only in the hemimethylated state, and is activated by CtrA binding to the upstream regulatory region of *ccrM* (Collier *et al.*, 2007). This arrangement ensures that the concentration of CcrM increases toward the end of the replication cycle. The *ccrM* promoter also contains two GANTC sequences presumably ensuring autoregulation of the gene (Reisenauer, *et al.*, 1999). The production of CcrM is followed by methylation of the daughter chromosomes, which silences the *ctrA* and *ccrM* genes and activates transcription of *dnaA*. CcrM also prepares C*ori* for replication initiation by fully methylating it.

After cell division, the DNA of both cell types is fully methylated (Marczynski & Shapiro, 2002). In the swarmer cell, CtrA remains bound to C*ori* and the CcrM protein is degraded, preventing further methylation and thereby ensuring that the origin is hemimethylated and inert for further initiation. In the stalked cell, however, CtrA is destroyed by proteolysis, allowing initiation to proceed on the fully methylated C*ori* (Marczynski & Shapiro, 2002).

Other bacteria

At least 20 other members of the alpha subdivision of proteobacteria contain CcrM homologs (Reisenauer *et al.*, 1999). In *Agrobacterium tumefaciens*, *Sinorhizobium* (*Rhizobium*) *meliloti*, and *Brucella abortus*, the *ccrM* gene is indeed essential for viability (Wright *et al.*, 1997; Robertson *et al.*, 2000; Kahng & Shapiro, 2001), and the *ccrM* genes from *S. meliloti* and *C. crescentus* are functionally interchangeable (Wright *et al.*, 1997). When overproduced in any of these organisms, CcrM causes defects in cell division, cell morphology and initiation of DNA replication. All the data above suggest that the physiological functions of CcrM in *Caulobacter* might be conserved in these other species.

Roles of DNA adenine methylation in host-pathogen interactions

Evidence for a relationship between Dam methylation and bacterial virulence was first provided by the regulation of adhesin-encoding genes like the *pap* operon of *E. coli* and others (Casadesus & Low, 2006). However, the role of Dam methylation in the infection of model animals was first investigated in *Salmonella enterica*, and later in other pathogens (Heusipp *et al*., 2007). A simple genetic approach was to compare the LD_{50} of a *dam* mutant with that of the wild-type. Additional details about the infection process were provided by examination of animal organs and *in vitro* studies using cell cultures. In bacterial species where DNA adenine methylation is essential, an alternative strategy was to examine the effects of Dam and CcrM methylase overproduction (see below). Although not known to occur in nature, Dam methylase overproduction provides a useful laboratory tool, both to overcome viability problems and to detect cases in which undermethylation is a critical factor for gene expression. However, Dam overproduction in *E. coli* leads to a *seqA* phenotype (Løbner-Olesen *et al.*, 2003). If this is also the case in other organisms, certain phenotypic effects seen upon Dam overproduction might be the result of SeqA deficiency.

Salmonella enterica

Dam methylation is an essential factor in *Salmonella* virulence, and its absence causes severe attenuation in the mouse model: the LD_{50} of a *dam* mutant is 10,000-fold higher than that of the wild type by the oral route, and 1,000-fold higher intraperitoneally (Garcia-del Portillo *et al.*, 1999; Heithoff *et al.*, 1999). Lack of SeqA causes a more modest decrease in *Salmonella* virulence, and attenuation is only observed by the oral route (Prieto *et al*., 2007). In the last few years, a combination of genetic screens, transcriptomic and proteomic analyses, cell culture studies and organ analysis upon mouse infection have provided insights on the causes underlying the extreme attenuation of *Salmonella dam* mutants. To date, the following virulence-related defects have been described:

(i) *Salmonella dam* cells show reduced capacity to interact with the intestinal epithelium, due to impaired epithelial cell invasion (Garcia-del Portillo *et al.*, 1999). The main cause of this defect seems to be inefficient activation of genes in pathogenicity island I (SPI-1) (Balbontin *et al*., 2006). Inefficient SPI-1 expression in *dam* mutants reflects the existence of lowered levels of the main SPI-1 transcriptional activator, HilD. However, Dam-dependent regulation of *hilD* appears to be postranscriptional, and therefore indirect (López-Garrido, J., Casadesus, J., unpublished data).

(ii) Reduced motility, another relevant defect of Salmonella *dam* mutants, may also contribute to inefficient invasion. Transcriptome analysis has shown that *dam* mutants have multiple alterations in the expression of flagellar genes, in a pattern too complex to be readily deciphered or even modeled (Balbontin *et al.*, 2006).

(ii) Lack of Dam methylation causes envelope instability, with release of outer membrane vesicles and leakage of proteins (Pucciarelli *et al.*, 2002). Vesicle release has been tentatively associated with impaired binding of Tol and PAL envelope proteins to peptidoglycan. Protein leakage may also be a side effect of envelope fragility. One factor contributing to envelope instability in *dam* mutants may be reduced transcription of the *lppB* gene, which encodes Braun lipoprotein (Balbontin *et al.*, 2006).

(iii) The *std* fimbrial operon, which is tightly repressed under laboratory conditions, undergoes derepression in *dam* mutants, and the StdA fimbrial protein becomes one of the most abundant proteins detected in cell extracts (Alonso *et al.*, 2005). Ectopic production of Std fimbriae contributes to virulence attenuation in *S. enterica dam* mutants, as indicated by the observation that an *stdA dam* strain outcompetes a *dam* strain during mouse infection (Jakomin *et al.*,

2008). It is possible that massive expression of Std fimbriae in *dam* mutants may interfere with signal exchange between the host and the pathogen, and may additionally overalert the host immune system. Dam methylation probably controls binding of transcriptional regulators to the *std* upstream activating sequence (UAS), which contains a cluster of 3 GATC sites. Genetic screens for *std* regulators have identified the GATC-binding protein SeqA and RosE, an homolog of the arginine repressor ArgR, as repressors of *std* expression (Chessa *et al.*, 2008; Jakomin *et al.*, 2008). In turn, the poorly known HdfR protein, a LysR relative, is an activator of *std* expression in *dam* and *seqA* mutants. Interestingly, derepression of the *std* operon by *dam* and *seqA* mutations occurs in only a fraction of the bacterial culture, suggesting the occurrence of either bistable expression or phase variation (Jakomin *et al.*, 2008).

(iv) *Salmonella dam* mutants are extremely sensitive to bile salts, a defect that may compromise their survival in the hepatobiliary tract (Heithoff *et al.*, 2001; Pucciarelli *et al.*, 2002). The main extracellular niche for *Salmonella* in persistent infections and during chronic carriage is the gall bladder, which contains high concentrations of bile. Because of their envelope defects, *dam* mutants are more sensitive to the detergent activity of bile salts. In addition, lack of DNA strand discrimination for mismatch repair makes *dam* mutants more sensitive to the DNA damaging activity of bile salts (Prieto *et al.*, 2004). The relevance of bile-induced DNA damage during animal infection is illustrated by the ample repertoire of *Salmonella* DNA repair functions required to cope with bile-induced DNA lesions: besides Dam-directed mismatch repair, bile resistance also requires base excision repair, SOS translesion synthesis, and RecB-mediated recombinational repair (Prieto *et al.*, 2006). Although bile salts are weak mutagens, long exposure to high concentrations of bile (e. g., in persistent and chronic infections) might increase genetic polymorphism in *Salmonella* populations. This view is consistent with the high frequency of chromosome rearrangements known to occur in *Salmonella typhi* (Echeita & Usera, 1998) and other host-adapted serovars (Liu & Sanderson, 1995; Liu & Sanderson, 1998).

Enterohemorrhagic Escherichia coli OH157:O7

A critical step during colonization and pathogenesis by enterohemorrhagic *E. coli* is the formation of “pedestals” that result from the accumulation of actin filaments beneath adherent bacteria, elevating them above the surrounding cell surfaces (Hayward *et al.*, 2006). Wild type *E. coli* O157:H7 show relatively poor pedestal formation on cultured mammalian cell lines while deletion of the *dam* gene results in a dramatic increase in both adherence and actin pedestal formation (Campellone *et al.*, 2007). Increases in adherence and pedestal formation *in vitro* correlate with elevated protein levels of intimin, Tir, and another secreted protein, $EspF_U$.

Dam methylation plays an additional role in enterohemorrhagic *E. coli* by controlling production of a virulence factor, Shiga toxin 2 (Stx2) (Murphy *et al.*, 2008). This toxin is encoded by a lambdoid prophage that has a relatively low threshold for induction. During infection, prophage induction may occur in a fraction of the bacterial population, thereby permitting Stx2 release.

Haemophilus influenzae

Certain strains of *Haemophilus influenzae*, a causative agent of respiratory tract infections, require Dam methylation for efficient invasion of both endothelial and epithelial cell lines (Watson *et al.*, 2004). In other strains, however, *dam* mutants are fully invasive. The cause of these strain-specific differences is not known (Watson *et al.*, 2004).

In addition to Dam, *H. influenzae* possesses a DNA methyltransferase (Mod) which is part of a type III restriction-modification system and undergoes phase-variation expression due to

DNA repeat instability. Mod has been shown to regulate a number of *H. influenzae* genes, some positively and others negatively (Srikhanta *et al.*, 2005). Phase variation of Mod expression may cause random switching of genes such as *dnaK*, potentially involved in cell adhesion, *hbpA*, which encodes a heme transport protein, and several genes for surface proteins of unkown function. Phase variation of Mod may thus provide a mechanism for the generation of diversity in *H. influenzae* populations by controlling a phase variable regulon or "phasevarion" (Srikhanta *et al.*, 2005; Fox *et al.*, 2007). Systems of this kind might exist in other bacterial pathogens as *Helicobacter pylori* and *Neisseria meningitidis*.

Pasteurella multocida

In the bovine respiratory pathogen *Pasteurella multocida*, overproduction of Dam methylase causes attenuation in the mouse model, suggesting that Dam methylation may control the expression of virulence genes (Chen *et al.*, 2003). It is not known whether the *Pasteurella* Dam methylase, which is closely related to that of *H. influenzae*, is essential or dispensable.

Actinobacillus actinomycetemcomitans

Synthesis and secretion of leucotoxin, a potential virulence factor related to RTX poreforming hemolysins, is exacerbated in *dam* mutants of the periodontal disease agent *Actinobacillus actinomycetemcomitans*. Furthermore, *A. actinomycetemcomitans dam* mutants show reduced invasion of epithelial cells (Wu *et al.*, 2006).

Klebsiella pneumoniae

In *Klebsiella pneumoniae*, an opportunistic pathogen causing respiratory and urinary tract infections, lack of Dam methylation causes partial attenuation upon intranasal or intraperitoneal inoculation of mice (Mehling *et al.*, 2007). The mild attenuation of *Klebsiella dam* mutants has not been hitherto correlated with altered expression of known virulence genes.

Campylobacter jejuni

Knockout of a putative DNA methyltransferase gene (*cj1461*) in the intestinal pathogen *Campylobacter jejuni* causes reduced motility, aberrant flagellar appearance, and hyperadherence to epithelial cells accompanied by reduced invasion (Kim *et al.*, 2008). Some of these traits are reminiscent of virulence-associated defects previously described in *Salmonella dam* mutants. However, the putative Cj1461 protein shows little homology with Dam methylase, and neither its DNA methylation activity nor its DNA target have been so far determined.

Yersinia enterocolitica

Dam methylation is essential in certain strains of *Yersinia*, and dispensable in others (Julio *et al.*, 2001; Robinson *et al.*, 2005). In *Yersinia* strains in which Dam methylation is essential, Dam methylase overproduction does not impair growth (Julio *et al.*, 2001). However, Dam-overproducing strains are attenuated in the mouse model, and their avirulent phenotype is pleiotropic (Julio *et al.*, 2001; Julio *et al.*, 2002). A relevant defect of *Y. enterocolitica* Dam overproducers is enhanced invasion capacity, probably associated to transcriptional alterations in invasin genes *inv* and *ail*, and to changes in the composition of lipopolysaccharide (LPS) O-antigen (Fälker *et al.*, 2007). The latter phenotype may involve postranscriptional control (see below). Furthermore, *Y. enterocolitica* Dam overproducing-strains show impaired secretion of Yop effector proteins, which become insensitive to Ca^{2+}-mediated control (Julio *et al.*, 2002). The latter defect is associated with enhanced degradation of LcrG, which in turn reflects increased transcription of the gene encoding ClpP protease (Fälker *et al.*, 2005). An additional trait that may contribute to attenuation in Dam-overproducing strains is enhanced bacterial motility (Fälker *et al.*, 2007). Some such defects have been also described in viable

dam mutants of the related species *Y. pseudotuberculosis* and *Y. pestis* (Robinson *et al.*, 2005; Taylor *et al.*, 2005).

Vibrio cholerae

Vibrio cholerae mutants lacking Dam methylase are not viable. However, as described above for *Y. enterocolitica*, Dam overproduction does not impair bacterial growth. *V. cholerae* overproducers of Dam methylase are attenuated in the suckling mouse model, but the causes of attenuation remain to be established (Julio *et al.*, 2001).

Aeromonas hydrophila

Aeromonas, a promiscuous pathogen and humans and animals, requires Dam methylase for viability. However, as in similar cases reported above, investigators have been able to examine the involvement of Dam methylation in pathogenesis by constructing Dam methylase-overproducing strains (Erova *et al.*, 2006). Attenuation was observed upon intraperitoneal infection of mice with a Dam overproducer, and several virulence defects were identified *in vitro*: (i) reduced cytotoxicity associated to type 3 secretion; (ii) reduced motility; and (iii) enhanced cytotoxic and hemolytic activities associated to the Act enterotoxin, which is secreted by a type 2 secretion system. All virulence-related alterations associated to Dam methylase overproduction disappeared when critical amino acids within its DNA methylation motif were eliminated, thereby confirming that DNA adenine methylation is involved in *Aeromonas hydrophila* pathogenesis (Erova *et al.*, 2006).

Brucella abortus

Overproduction of CcrM methylase decreases proliferation of *B. abortus* inside murine macrophages, suggesting that CcrM methylation may play a role in intracellular replication, which is a hallmark of *Brucella* infections (Robertson *et al.*, 2000). Because CcrM is essential in this species, specific inhibitors of the enzyme have been sought and considered as potential antimicrobials (Benkovic *et al.*, 2005).

GATC regulation by DNA adenine methylation: Facts

Very Short Patch (VSP) Repair in *Escherichia coli*

A system that altered the frequency of recombinants by correcting T-G mismatches with repair tracts shorter than 20 bp was discovered during a study of homologous recombination in bacteriophage lambda, and termed “very short patch” (VSP) repair (Lieb, 1983). A hotspot for C to T transitions in the lambda *cI* gene turned out to be located in a DNA cytosine methyltransferase recognition sequence, CCAGG, altering it to CTAGG (Coulondre *et al.*, 1978). Deamination of 5-methylcytosine (5-meC) yields thymine, and thus creates a T-G mismatch. Mutations due to 5-meC deamination occur in stationary phase, but not in exponentially growing bacteria, and the role of VSP repair is to prevent the resulting mutagenic event by restoring C-G pairs prior to DNA replication (Lieb & Bhagwat, 1996; Lieb & Rehmat, 1997). T-G mismatches are recognized and cleaved 5' to the T by the Vsr endonuclease (Hennecke *et al.*, 1991); conventional base excision repair involving DNA polymerase I and DNA ligase then follows (Lieb & Bhagwat, 1996). There is also a requirement for the MutS and MutL proteins of Dam-directed mismatch repair, but their role is uncertain (Bhagwat & Lieb, 2002). The level of Vsr in the wild type is low in logarithmic phase cells, and high in stationary phase cells as expected from the biological rationale for VSP (Macintyre *et al.*, 1999).

The *E. coli vsr* gene is in a transcriptional unit with the *dcm* (DNA cytosine methyltransferase) gene. The 3' end of the *dcm* gene is overlapped by the first six codons of the *vsr* gene, which is in a +1 register relative to *dcm* (Dar & Bhagwat, 1993). Such an overlap is uncommon in

E. coli, and in this case may serve to couple expression of these genes. Both *dcm* and *vsr* appear to be transcribed into a single mRNA, and translation of *vsr* appears to be dependent upon translation of the upstream *dcm* coding sequence (Dar & Bhagwat, 1993). However, western analysis showed that the Vsr level varies with growth rate, while the level of Dcm does not change during the exponential and stationary phases of growth. The mechanism by which this is achieved is not known. The location of the promoter and its mode of regulation are also unknown.

Surprisingly, VSP repair is reduced in *E. coli dam* mutants as measured by an increase in the mutation frequency of CCAGG to CTAGG (Bell & Cupples, 2001). Western blotting indicated that, unlike the wildtype, there was no increase in Vsr level upon entrance into the stationary phase in *dam* cultures. However, the level of Dcm remained unaltered. Since the *vsr* and *dcm* genes are co-transcribed, it was concluded that regulation of Vsr in a *dam* mutant is probably achieved by a postranslational mechanism (Bell & Cupples, 2001). Because the *vsr* mRNA levels in dam^+ and *dam* strains were not determined, an effect on mRNA stability cannot be excluded.

Pedestal formation in enterohemorrhagic E. coli O157:H7

As described in the host-pathogen interactions section above, *dam* mutants of enterohemorrhagic *E. coli* show increased adherence and pedestal formation *in vitro*, which is correlated with elevated protein levels of three effector proteins: intimin, Tir, and $EspF_U$. However, the increased levels of effectors did not result from an increase in mRNA levels as measured by microarrays, northerns and RT-PCR, suggesting a post-transcriptional mechanism of regulation (Campellone *et al.*, 2007). To further investigate the basis of this observation, an *E. coli* O157:H7 *hfq* mutant was constructed, and pedestal formation was as robust as in a *dam* mutant (Brady, M., Leong, J.M., Marinus, M.G., unpublished data). The *hfq* mutant contains an elevated level of Tir (Fenton, A., Marinus, M.G., unpublished data).

LPS composition in Yersinia enterocolitica

Dam overproduction in *Yersinia enterocolitica* causes numerous metabolic alterations, including a change in the composition of LPS O-antigen, which contains increased amounts of lipid A core without O-antigen subunits (Fälker *et al.*, 2007; Heusipp *et al.*, 2007). The O-antigen gene cluster consists of two transcriptional units but the transcript levels in the Dam overproducer, as measured by RT-PCR, of representative genes in each cluster (*ddhA*, *gne* and *rosA*) was unchanged relative to wild type. Thus, the modulation of LPS structure seems to involve an unknown postranscriptional mechanism (Fälker *et al.*, 2007).

Transcription of *S. enterica* pathogenicity island SPI-1

As described in the host-pathogen interactions section above, pathogenicity island SPI-1 is essential for virulence of *S. enterica*. Transcriptomic analyses of *S. enterica* model strain SL1344 and *dam* derivatives showed that transcription of invasion genes in pathogenicity island SPI-1 was decreased in the absence of Dam methylation (Balbontin *et al.*, 2006). In confirmation, using a *lac* transcriptional fusion to one of the SPI-1 genes (*sipC*), beta-galactosidase activity was found to increase when a culture of the wild type entered the stationary phase. In contrast, *dam* mutants showed a low level of expression in both the exponential phase and the starionary phase (Balbontin *et al.*, 2006). Transcriptional data were recapitulated at the protein level: more SipC was found in the wild type than in *dam* mutants. A similar effect of Dam methylation was found for other representative SPI-1 genes, suggesting that the whole island might be under Dam control.

Transcriptional control of pathogenicity island SPI-1 is complex and multi-layered (reviewed by Jones, 2005). Transcription of the *hilA* gene in SPI-1 appears to be central for the expression

of the other genes on the island. Expression of *hilA* is activated by RtsA, HilC and HilD, the latter being the more important. HilD is a member of the AraC/XylS family of transcriptional activators. Expression of *hilD* is in turn modulated by the products of the *csrA* and *csrB* genes, which have opposite effects on transcript levels. CsrA destabilizes specific mRNA molecules by interactions at the ribosome binding site. The *csrB* gene encodes an untranslated RNA that binds CsrA to prevent mRNA degradation. While deletion of the *csrB* gene has only a mild effect on *hilA* transcription and none on invasion, deletion of *csrA* or overexpression of CsrA both reduce *hilA* transcription about 10-fold, and invasion of epithelial cells about 100-fold. CsrA, therefore, appears to have both positive and negative regulatory effects on SPI-1 gene expression.

GATC regulation by DNA adenine methylation: Hypotheses

Proteolysis

Postranscriptional regulation can occur at the level of the message and/or at the protein level. There are many examples of "unstable" regulatory proteins whose concentration is determined by the balance between synthesis and degradation by specific proteases. For example, one model for *E. coli* VSP repair could be that proteolysis is more active on Vsr during logarithmic growth than in the stationary phase. This would imply that a specific protease activity is decreased in cells approaching the stationary phase of growth. In *dam* mutants, this decrease would not occur, leading to continued proteolysis. Similar arguments can be made for the other systems described above. Whatever the mechanism of postranscriptional regulation turns out to be, the key proteins will be subject to proteolysis. even though this may not be the primary regulatory mechanism. For further discussion, however, we will assume that proteolysis is not the primary mechanism of postranscriptional control.

mRNA translation

For some of the examples of postranscriptional regulation listed above (e. g., VSP repair in *E. coli*), mRNA levels have not been determined, leaving open the possibility that mRNA stability is altered in *dam* mutants. In other examples above, the steady-state mRNA levels have been measured and are unchanged. An appealing model for such situations is to invoke translational regulation through the involvement of a small RNA molecule, either an antisense RNA or a small non-coding RNA (sRNA). An example of Dam-mediated regulation was described in the synthesis of FinP, an antisense RNA acting on the *traJ* transcript (Torreblanca *et al.*, 1999; Camacho *et al.*, 2005). Transcription of *finP* is decreased in *dam* mutants (Camacho *et al.*, 2005).. In addition to this example, microarray analysis indicates that many non-coding regions of the *E. coli* chromosome show either increased or decreased transcript levels in a *dam* mutant versus wild type (Campellone *et al.*, 2007). For each example of posttranslational regulation discussed below we offer a hypothesis invoking small RNA molecules.

The *vsr* transcript also includes, and is preceded by, the *dcm* coding sequence. While the Dcm protein is present at the same steady-state level in both exponential and stationary phases, the Vsr protein is induced in stationary phase (Bell & Cupples, 2001). A sRNA could be induced upon entry into stationary phase allowing translation of the *vsr* gene but not affecting the upstream *dcm* sequence. The sRNA could, for instance, unmask a readthrough region of the transcript or stabilize the 3' end. This hypothesis fits with the observation that many sRNAs are induced as cells enter the stationary phase (Majdalani *et al.*, 2005). In *dam* mutants, transcription of the particular sRNA might be downregulated, thereby preventing *vsr* translation.

The EHEC *tir* transcript also encodes the gene (*eae*) encoding intimin but not that for $EspF_U$. Since there are examples of an sRNA molecule binding to two separate messengers, co-

regulation of translation of *espF*$_U$ and *tir* is possible. In this case an sRNA might bind constitutively to the mRNAs to prevent translation (negative regulation). Alternatively, some structural feature of the mRNA might prevent translation, and the sRNA might modify it to allow translation (positive regulation). By a signal currently unknown, transcription of the sRNA gene might be altered when the organism finds itself at the right place in the alimentary tract, allowing translation of the messages. In a *dam* mutant, synthesis of the sRNA would be altered, allowing constitutive translation of the effector messages.

A similar type of model can be proposed for LPS composition changes in *Yersinia*. Overexpression of Dam is known to alter transcription profiles such that genes not normally expressed are activated, and these could include loci for small regulatory RNAs and/or antisense RNAs. Such changes in sRNA or antisense RNA levels might be due to Dam preventing SeqA to access its substrate sites. Hence, it would be interesting to analyze LPS composition in a *Yersinia seqA* mutant.

For pathogenicity island SPI-1, a role for sRNA or antisense RNA could also be invoked for one of the many regulators known to activate transcription of the island. As mentioned above, one of these regulators is a small RNA, *csrB*. Increased *csrB* transcription in a *dam* mutant could affect the level of CsrA, which in turn activates SPI-1 gene expression through HilD. However, preliminary evidence suggests that neither *csrA* nor *csrB* are under Dam methylation control (López-Garrido, J., Casadesus. J., unpublished data).

Most sRNA molecules require Hfq for binding to their cognate mRNAs. If the models above require sRNAs, then the effects of *dam* methylation should be mirrored in an *hfq* mutant. A positive correlation would support the model while a negative one would exclude Hfq-dependent sRNAs but not antisense RNAs.

Practical uses of DNA adenine methylation: vaccines and DNA adenine methylase inhibitors

The strong attenuation of *dam* mutants, combined with their capacity to persist at low levels in animal organs (causing an almost asymptomatic infection), makes *Salmonella dam* strains appropriate to be used as live vaccines. In fact, *dam* mutants of *S. enterica* have been shown to elicit immune responses in chickens and calves with paramount efficiency (Dueger *et al.*, 2001; Heithoff *et al.*, 2001; Dueger *et al.*, 2003). An oral, live *dam* vaccine has been also described in *Haemophilus influenzae* (Watson *et al.*, 2004). Interestingly, a viable *dam* mutant of *Yersinia pseudotuberculosis* was found to protect mice against infection by the wild type, and also to cross-protect against plague (Robinson *et al.*, 2005). An alternative and efficient strategy for the design of live vaccines against *Y. pseudotuberculosis* is Dam overproduction (Julio *et al.*, 2002).

A negative trait that may hamper use of *dam* mutants as live vaccines for humans is hypermutation, caused by lack of DNA strand discrimination for mismatch repair. In *Salmonella*, lack of Dam methylation increases spontaneous mutation rates 10–15 fold (Torreblanca & Casadesus, 1996). Dam overproduction does not provide a solution to overcome this problem: early studies in *E. coli dam* mutants showed that Dam overproduction increases mutation rates well above Dam absence (Marinus *et al.*, 1984). Similar observations have been made in *Salmonella*: expression of Dam methylase from a multicopy plasmid increases the spontaneous mutation rate over 400-fold (Torreblanca & Casadesus, 1996). Increased mutation rates might, however, allow the use of *dam* vaccines in livestock animals, if animal health regulations permit.

Attenuation of *dam* strains in a variety of human pathogens has also raised the possibility of using Dam or CcrM methylase inhibitors as antibacterial drugs (Benkovic *et al.*, 2005; Mashhoon *et al.*, 2006). In alpha-proteobacteria, such drugs would be bactericidal. In *Salmonella* and other pathogens in which Dam methylation is not essential, Dam inhibitors could be expected to attenuate virulence by transforming wild-type bacteria into phenocopies of *dam* mutants. Because Dam methylation is a dispensable function in enteric bacteria, inhibitors specifically targeted at Dam methylase should be harmless for the normal intestinal flora. A drug of this kind should be also harmless for the host, because adenine methylation is rare, if not absent, in mammalian cells (Ratel *et al.*, 2006).

Whatever the fate of Dam-based vaccines and Dam inhibitors in *Salmonella* and other pathogens, neither strategy can be envisaged as universally valid. Enterohemorrhagic *E. coli* *dam* mutants show increased production of both actin pedestals and Shiga toxin (Campellone *et al*., 2007; Murphy *et al*., 2008). They are therefore potentially more virulent, and useless as live vaccines. In turn, administration of a Dam-inhibiting drug to infected animals might increase enterohemorrhagic *E. coli* virulence. An additional potential problem is that a Dam inhibitor might increase spontaneous mutation rate in the intestinal flora.

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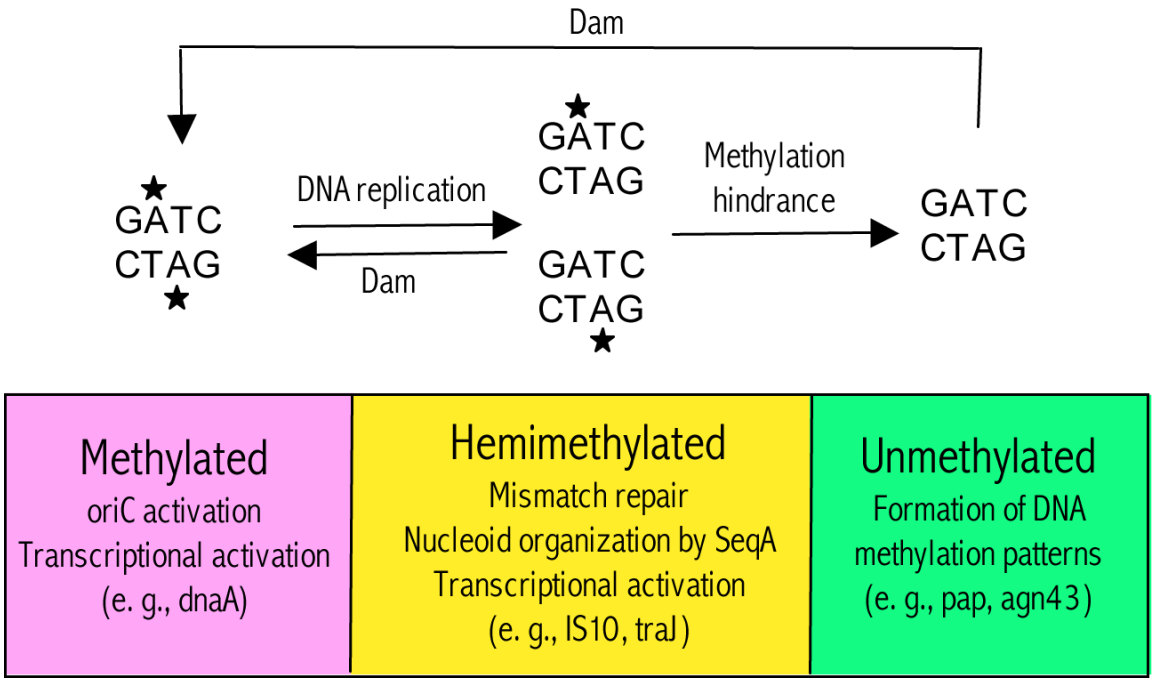

Figure 1.
States of GATC site methylation in gamma-proteobacteria. DNA replication generates hemimethylated GATC sites, usually short-lived since Dam methylation occurs shortly after synthesis of the daughter DNA strand. At certain GATC sites, however, the default methylation-hemimethylation cycle associated with DNA replication can be skewed by binding of proteins that prevent DNA methylase activity. Such binding can merely delay methylation or prevent it beyond cell division, thereby permitting daughter cells to inherit the hemimethylated state if methylation hindrance persists. Replication of hemimethylated GATC sites produces unmethylated DNA, generating DNA methylation patterns like those occurring in eukaryotic cells.

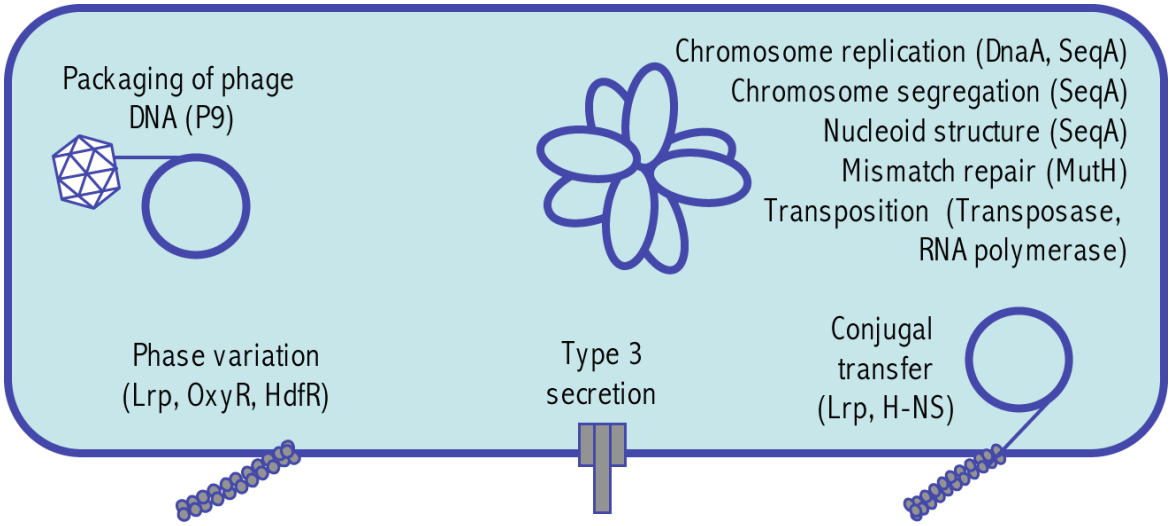

Figure 2.
Overview of the roles of 6-meA in enteric bacteria. When known, the methylation-sensitive DNA-binding proteins involved in each process are also indicated.

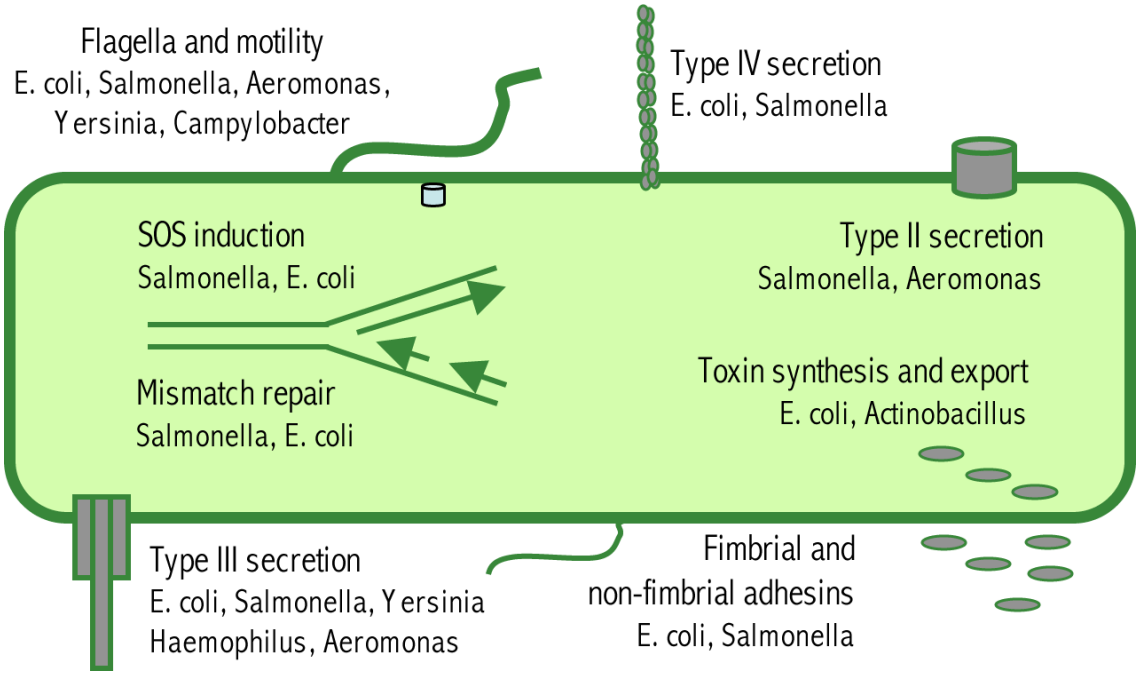

Figure 3.
Cell functions under Dam methylation control in bacterial pathogens.

Table 1

Examples of transcriptional regulation by Dam methylation

Gene	Location of regulatory GATC(s)[a]	Protein(s) involved	Active state
agn43	Operator	SeqA, OxyR	Methylated
dnaA	Promoter	SeqA, DnaA	Methylated
finP	Unknown	H-NS	Methylated
IS*10*	Promoter	RNA polymerase	Hemimethylated
mom	UAS	OxyR	Hemimethylated
pap	UAS	Lrp, PapI	Methylated/unmethylated pattern
std	UAS	SeqA, HdfR	Umethylated
traJ	UAS	Lrp	Hemimethylated

[a] "Operator" is used in the classical Jacob & Monod sense, to describe a regulatory sequence for repressor binding. "UAS" is an acronym for "upstream activating sequence".

Table 2

Examples of transcriptional regulation by CcrM methylation

Gene	Location of regulatory GANTC(s)	Protein(s) involved	Active state
dnaA	Promoter	Unknown	Methylated
ctrA	Promoter	GcrA	Hemimethylated
ccrM	Leader	CtrA	Hemimethylated

Table 3

Virulence-related defects of *Salmonella dam* mutants

Stage of infection	Virulence defect	Tentative cause
Intestinal	Bile sensitivity	Envelope instability
		MutHLS-induced DNA breakage
	Deficient invasion of epithelial cells	Reduced SPI-1 expression
	Reduced colonization of the caecum	Ectopic synthesis of Std fimbriae
Systemic	Sensitivity to hydrogen peroxide	MutHLS-induced DNA breakage
	Reduced colonization of lymph nodes, liver and spleen	Impaired expression of *spv* operon products
	Reduced spleen colonization	Ectopic synthesis of Std fimbriae