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DNA Methylation

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Abstract

The DNA of *E. coli* contains 19,120 6-methyladenines and 12,045 5-methylcytosines in addition to the four regular bases and these are formed by the postreplicative action of three DNA methyltransferases. The majority of the methylated bases are formed by the Dam and Dcm methyltransferases encoded by the *dam* (DNA adenine methyltransferase) and *dcm* (DNA cytosine methyltransferase) genes. Although not essential, Dam methylation is important for strand discrimination during repair of replication errors, controlling the frequency of initiation of chromosome replication at *oriC*, and regulation of transcription initiation at promoters containing GATC sequences. In contrast, there is no known function for Dcm methylation although Dcm recognition sites constitute sequence motifs for Very Short Patch repair of T/G base mismatches. In certain bacteria (e.g., *Vibrio cholerae*, *Caulobacter crescentus*) adenine methylation is essential and in *C. crescentus*, it is important for temporal gene expression which, in turn, is required for coordinating chromosome initiation, replication and division. In practical terms, Dam and Dcm methylation can inhibit restriction enzyme cleavage; decrease transformation frequency in certain bacteria; decrease the stability of short direct repeats; are necessary for site-directed mutagenesis; and to probe eukaryotic structure and function.

INTRODUCTION

DNA methylation in bacteria is most often thought of in its role to protect DNA from restriction endonucleases. In addition to this role, however, studies in *Escherichia coli*, *Salmonella enterica* serovar Typhimurium (referred to as *S. enterica* hereafter) and *Caulobacter crescentus* have shown that methylated bases have other biological functions. In these cases, the methylated bases are not part of a restriction/modification system and the enzymes that produce them are often referred to as orphan or solitary DNA methyltransferases. The postreplicative DNA methylation produced by these enzymes superimposes on the primary DNA sequence secondary information that has significance for DNA transactions such as transcription, transposition, initiation of chromosome replication, mRNA utilization and prevention of mutations by DNA repair. These alterations are brought about in two ways, the first being simply a change in the steady-state level of the methyltransferase either up or down from normal. The second mechanism is through the

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configuration of the nucleotide sequence subject to methylation; it can exist as symmetrically methylated, unmethylated or two possible hemi-methylated arrangements. The details about the changes in DNA transactions through alteration of methyltransferase levels or state of methylation sequences form the bulk of this review.

This review is an updated version of that which appeared in the last print edition of *Escherichia coli* and *Salmonella*: Cellular and Molecular Biology [1]. Several reviews of DNA methylation have appeared recently that are considered complementary to this article [2–5].

METHYLATED BASES IN DNA

The DNA of *E. coli* K-12 contains two modified bases; 6-methyl-adenine (6-meAde, Fig. 1) and 5-methylcytosine (5-meCyt, Fig. 1). About 1.5% of all adenines (19,120 in GATC) and 0.75% of all cytosines (12,045 in CCWGG) in the chromosome are methylated, and the modifications occur in specific sequences resulting from the action of three DNA methyltransferases (Table 1). The EcoK adenine methyltransferase, which is encoded by the *hsd* (host specificity) genes, is part of the classical EcoK restriction/modification system described in detail in EcoSal chapter 4.4.6. The gene for a fourth methyltransferase, YhdJ, is not expressed under laboratory growth conditions [6].

The Dam (DNA adenine methyltransferase) enzyme, which modifies GATC sequences, forms over 99% of the 6-meAde in *E. coli* DNA, since strains lacking this enzyme contain only the contribution expected from the EcoK enzyme [7, 8]. The Dcm (DNA cytosine methyltransferase) protein, methylating CC(A/T)GG sites, is responsible for all the 5-meCyt in DNA, since none of this modified base can be detected in cells deleted for the *dcm* gene (2). A *dam dcm hsdS* mutant contains no detectable modified bases in DNA, indicating that such bases are not essential for *E. coli* viability [8].

Although *dam* gene function is not essential for viability of *E. coli* in a wild-type background, it is required in recombination-deficient mutants such as *recA*, *ruvABC*, etc. [9]. The reason for this is explained below, in the *dam*-directed mismatch repair section. In contrast, in bacteria such as *Vibrio cholerae*, Dam methylation is an essential function although the reason for it has yet to be determined [10, 11].

Distribution of GATC Sequences in Chromosomal DNA

Analyses of *E. coli* DNA sequences [12–14] have indicated the following details about the GATC tetranucleotide. (i) It is represented, on average, once every 243 nucleotides, which is close to the 1/256 expected in a random base sequence. (ii) It is present at a higher than expected frequency in numerous chromosomal locations (e.g., *dnaA*, *rpsP*, *metL*, *malP*, *rplS*, *xylB*, *gltX*, and *guaBA*) in addition to *oriC*. The significance, if any, of this clustering is unknown for all these genes except *dnaA* and *oriC* (see Initiation of Chromosome Replication below). (iii) It is found more frequently in translated regions than in non-coding or non-translated regions, which is consistent with more frequent mismatch repair surveillance. In particular, rRNA- and tRNA-encoding genes exhibited the lowest GATC content of all genes examined. This deficiency may be correlated with unwanted

palindromic secondary structures. (iv) Finally, the GATC tetranucleotide is never separated from another GATC sequence by more than 2 kb. This allows for *dam*-directed mismatch repair to occur over the whole genome, since it is less efficient at distances greater than 2 kb [15]. (See “DNA Mismatch Repair,” doi:10.1128/ecosalplus.7.2.5.)

The statistical data described above give the frequency of GATC sites in chromosomal DNA. These sites, however, can be present in unmethylated, hemimethylated, or fully methylated configurations. All GATC sites appear to be methylated in chromosomal and plasmid DNA isolated from *E. coli* by conventional standard methods and using restriction enzymes such as DpnI and DpnII to monitor methylation status. DpnI cleaves only at methylated sites, DpnII cleaves only at unmethylated sites, and Sau3AI (or BfuCI) will cut regardless of methylation status. Neither DpnI nor DpnII digests hemimethylated sequences [16, 17]. Techniques such as pulse-field gel electrophoresis of digested DNA and specific end-labeling procedures [18–21], however, indicate that the *E. coli* chromosome contains about 36 specific, unmethylated *dam* sites. The number and intensity of unmethylated sites in the chromosome vary depending on growth phase and growth rate, suggesting that the proteins which bind to them could be involved in gene expression or maintaining chromosome structure. The unmethylated *dam* sites appear to be mostly [18] or completely [22] modified in strains overproducing Dam, suggesting that the enzyme competes with other DNA binding proteins at these specific sites. Alternatively, at some GATC sites the increased Dam concentration may allow for modification of DNA structures (e.g., non-B-form DNA such as H-DNA [23]) relatively resistant to methylation at the normal cellular level of the enzyme. Palindromic structures containing GATCs are also relatively resistant to Dam methylation [24]. Alteration in helix stability by DNA methylation can be detected by abnormal migration of DNA fragments in denaturing gels [25]. Evidence for competition between Dam and other DNA binding proteins at several unmethylated sites has been obtained. These findings indicate that these sites are involved in regulation of gene expression, and they are discussed in more detail in that section below (Regulation of Gene Expression).

In addition to the unmethylated GATC sites discussed above, persistent hemimethylated sequences have been detected in the chromosome [26, 27]. These are distinct from the hemimethylated GATC sites which occur transiently immediately behind the replication fork due to the time lag in modifying new Dam methylation sites. The persistent hemimethylated sites are discussed in more detail below (Initiation of Chromosome Replication).

Single-molecule real-time deep DNA sequencing can identify modified bases and can, therefore, assign if a modified base in a particular motif is in a methylated, hemi-methylated or unmethylated state. In the pathogenic *E. coli* strain C227-11, 89.2%, 9.7% and 1.1% of GATC modifications were in methylated, hemi-methylated or unmethylated configurations [28]. Of 23 unmethylated sites tested, all were in non-coding regions and were near genes encoding the phosphotransferase system.

The results from *E. coli* strain C227-11, indicate that there is little “off-target” methylation by Dam at its normal cellular concentration. Single-molecule real-time deep DNA

sequencing was also used to demonstrate that when Dam is overproduced from a multicopy plasmid, substantial methylation of A in GACC sequences occurs, as well as lower, but significant, modification in AATC, CATC and TATC sequences [29]. In vivo results derived from Dam overproduction should be treated with caution not only because of off-target GATC methylation, but also due to interference with SeqA (see “Initiation of chromosome replication”) and MutH action on hemi-methylated DNA (see “*dam*-directed mismatch repair”).

Dam methylation and DNA structure

Methylation of GATC sites can influence DNA structure because the methyl group protrudes into the major groove leading to duplex destabilization [30, 31]. Alteration in helix stability by DNA methylation can be detected by abnormal migration of DNA fragments in denaturing gels [43]. Dam methylation increases curvature of GATC-containing DNA as determined by electrophoretic mobility shift assays [52, 171]. Non-B-form DNA such as H-DNA [165]) and palindromic structures containing GATCs are relatively resistant to methylation at the normal cellular level of Dam methyltransferase [3]. Whether changes in curvature, especially in regions of high GATC content such as *oriC*, promote binding of proteins, such as SeqA, or play a role in origin function is not known. The destabilization of a methylated 13-bp duplex oligonucleotide is the basis for a fluorescence-based assay for Dam methyltransferase activity [32].

Distribution of CC(A/T)GG Sequences in Chromosomal DNA

Analyses of the Dcm recognition sequences, CCTGG and its complement CCAGG, indicated that these occur at a higher than expected frequency: every 385 bp instead of every 512 bp as predicted from random sequence [13]. As discussed below (VSP Repair), in stationary phase cells, the Dcm sequences are constantly subjected to cycles of deamination of 5-meCyt followed by repair of the resulting T-G mismatch and subsequent remethylation by Dcm. This cycling prevents the accumulation of C to T mutational changes in the chromosome although statistical data suggests such a drift has occurred albeit at a low frequency [33, 34].

The state of methylation at *dcm* sites can be monitored by digestion with EcoRII, which cuts only if the sequence is unmethylated, and BstNI, which cleaves regardless of methylation status (Table 1). As is the case with *dam* sites, a small but undetermined number of unmethylated *dcm* sites have been detected in *E. coli* chromosomal DNA [18]. One assumes that there must also be hemimethylated *dcm* sites in chromosomal DNA, but their existence has not yet been demonstrated.

The investigation of unmethylated and hemimethylated *dam* and *dcm* sites in chromosomes has demonstrated the utility of this approach to identify regions of the chromosome with interesting biological features such as sequences that are bound by proteins which have regulatory functions. These kinds of studies should allow a functional dissection of the *E. coli* chromosome to be integrated with DNA sequence information.

Methylated *dam* and *dcm* Sequences in DNA of Other Bacteria and Phages

Methylated *dam* and *dcm* sites are found in most enterobacteria and the *E. coli dam* gene DNA hybridizes under stringent conditions to the DNA of other enterobacteria [35]. Methylated *dam* sites have also been detected in various gram-positive and gram-negative bacteria as well as in some archaeobacteria [22]. Methylated *dcm* sites are found only in members of the Enterobacteriaceae (M. Lieb, personal communication). Strains with a *dcm* gene also contain the *vsr* gene (see VSP Repair).

There are many bacterial species (and all eukaryotes) which do not contain methylated *dam* and/or *dcm* sites and presumably have other unknown mechanisms to substitute for Dam and Dcm functions.

The GATC tetranucleotide in the genomes of several bacteriophages is present at lower than the expected frequency [14, 36]. The reason for this is not known although protection against host MutH endonuclease is often cited as a reason [37]. This seems very unlikely as phages such as fd, lambda and T7, which have undermethylated *dam* sites plaque on *dam* (*mutH*⁺) mutants with the same efficiency as wildtype [38]. Furthermore, the extent of methylation of the *dam* sequence in phages is also low. The probable reason for this is that methylation does not keep up with phage replication. For example, in phage lambda DNA, only about half the GATCs are methylated when propagated in wildtype bacteria but almost all are in wildtype bacteria over-expressing Dam [39].

Single-molecule real-time deep DNA sequencing has been used to analyze the “methylome” of *Geobacter metallireducens*, *Chromohalobacter salexigens*, *Vibrio breoganii*, *Bacillus cereus*, and two strains of *Campylobacter jejuni* [40]. The technique identified several known and new Type I, II and III restriction/modification motifs as well as methylated GATC sequences in *C. salexigens* and *Vibrio breoganii*, presumably produced by Dam homologs. This sequencing technology shows great promise in detecting modified bases in genomes although the detection of 5-methylcytosine was not possible for these studies, although it will be in the future (R. J. Roberts, New England Biolabs).

DNA METHYLATION GENES

The *hsd* genes which specify the EcoK methyltransferase map at 99 min on the genetic map and 4,615 kb on the physical map. The corresponding map locations for the *dam* gene are 76 min and 3,536 kb, those for the *dcm* gene are 44 min and 2,042 kb and the *yhdJ* gene at 73 min and 3,410 kb. The DNA methylation genes are thus unlinked to one another. All *dam* mutations, except one, are in a single complementation group and are recessive [41]. The exceptional mutation is very leaky, making it difficult to assign conclusively within the same complementation group. These genetic data suggest that no other functional *dam* methylation genes exist in *E. coli*. In the annotated *E. coli* genome, there is the *damX* gene which has no effect or relationship with *dam* methylation. This gene (b3388) was originally designated *urf74.3* in the literature because its function was, and remains, unknown [42]. The origin or reason for the designation *damX* is unknown.

The *dam* Gene

The 834-bp *dam* gene is part of a transcriptional unit containing at least four genes [42, 43] and perhaps six or seven [44]. The locations of promoters and a transcriptional terminator which affect *dam* are shown in Fig. 2. Each promoter has been cloned individually, and the order of promoter strength is P2 > P1 > P3 > P4 > P5 [45]. Insertion of the *cat* (chloramphenicol acetyltransferase) coding sequence into the *aroK* gene reduces transcription across the *dam* gene by 70%, and a mini-Tn10 insertion in *urf74.3* (*damX*; b3388) does so by 90% [43]. These data indicate that promoters P1 and P2 (situated about 3.5 kb upstream of *dam*) and P3 (located 2 kb upstream) are the most important for *dam* gene transcription. Promoters P1 through P4 all show the typical RNA polymerase sigma-70 recognition sequences.

Only promoter P2 has thus far been shown to be regulated [46]. This promoter is growth rate regulated by a mechanism distinct from that used for rRNA and tRNA gene promoters [45]. Transcription initiation from P2 is not affected by the stringent response, ribosomal feedback, or the level of Fis protein, all of which affect growth rate-dependent rRNA and tRNA promoters ([47]. Conversely, mutations in the *cde* (constitutive *dam* gene expression) gene, located at 15 min on the genetic map and 670 kb on the physical map, abolish growth rate regulation of the *dam* P2, but not rRNA growth rate-dependent, promoters [45]. The *cde* gene was subsequently shown to be identical to *lipB* [48], but the connection between lipoic acid biosynthesis and *dam* gene regulation remains unknown.

The rationale for growth rate regulation of the *dam* gene may be to correlate Dam levels with the amount of hemimethylated DNA close to the replication fork and at *oriC*. Cells growing with different doubling times synthesize DNA at different rates. To maintain the optimal level of hemimethylated DNA, the amount of Dam must be adjusted accordingly [45]. If *dam* gene expression were not regulated, too much or too little Dam would result in increased mutagenesis, asynchronous initiation of chromosomes, and alteration of the frequency of transposition.

Overproduction of Dam in *E. coli* alters the wildtype phenotype depending on the level of overproduction. At low level overproduction (about 10-fold), Dam out competes SeqA for GATC binding sites to produce a *seqA* phenocopy causing alterations in global gene transcription and chromosome initiation synchrony [49] (See Initiation of Chromosome Replication). Greater than 10-fold overproduction leads Dam to out compete the MutH protein for GATC sites as well resulting in a mutator phenotype due to inhibition of DNA mismatch repair [50, 51] (See *dam*-Directed Mismatch Repair). As described above, Dam overproduction leads to off-target methylation of GACC, AATC, CATC and TATC sequences [29].

Phage genomes often have an open reading frame encoding a putative Dam or Dam-like protein with signature motifs. For example, phage P1 and the T-even phages encode their own Dam methyltransferases which are expressed at some stage during their cell cycle. On the other hand, the lambda-like prophages in *E. coli* O157:H7 all have a putative *dam* gene which is not expressed under normal laboratory growth conditions. These are not pseudogenes because the *E. coli* O157:H7 VT2-Sa prophage derived *dam* homologue can be

expressed to methylate GATC sequences [52]. When the host *dam* gene is eliminated from the chromosome of *E. coli* O157:H7, there is no detectable chromosomal GATC methylation indicating repression of the prophage *dam* genes [53]. The role of phage-encoded Dam in the life cycle remains obscure as is also the case for *Vibrio* phages [54].

The *dcm* Gene

The 1,419-bp *dcm* gene is overlapped at its 3' end by the first six codons of the *vsr* gene, which is in a +1 register relative to *dcm* [55]. Such an overlap is uncommon in *E. coli* and in this case may serve to link the expression of these genes. Both genes appear to be transcribed into a single mRNA, and translation of *vsr* appears to be dependent upon translation of the upstream *dcm* coding sequence [55]. The mechanism by which this is achieved is not known. The location of the promoter(s) and its mode of regulation are also unknown. The possibility of growth rate regulation of *dcm* gene expression has not been tested. There is no obvious phenotype associated with the under- or overproduction of Dcm.

The *yhdJ* Gene

The *yhdJ* gene comprises 885 bp and is predicted to produce a protein of 294 amino acids with a predicted molecular weight of 33,397 Da. Annotation of the gene predicted that the protein contains conserved methyltransferase motifs for S-adenosyl-L-methionine (SAM) binding and catalysis. The order of these domains places YhdJ in the beta group of methyltransferases, along with CcrM and its homologs. *E. coli* YhdJ shares identity with the adenine methyltransferases M.AvaIII (55.8%) and CcrM from *C. crescentus* (34.3%). CcrM (cell cycle-regulated methyltransferase), is found in species of the alpha subdivision while Dam is present in the gamma subdivision of proteobacteria [2, 3]. Where it has been examined, CcrM is essential for viability, whereas Dam and its homologs are only essential in a few species [56]. In both *E. coli* and *S. enterica*, however, the *yhdJ* gene can be deleted without loss of viability and without any obvious phenotype [6]. In addition expression of the gene is below the level of detection in both *E. coli* and *S. enterica* under normal laboratory conditions of cultivation [6]. Nothing is known why this is so or how gene expression is regulated.

DNA METHYLTRANSFERASES

DNA methyltransferases transfer the methyl group from S-adenosyl-L-methionine (SAM) to specific residues in double-stranded DNA. Dam methyltransferase flips out the adenine residue from the DNA and modifies it [57, 58] and it is probable that the same basic mechanism is used for other *E. coli* methyltransferases. In *E. coli*, the substrate for Dam is GATC in hemimethylated DNA behind the replication fork. That is, the parental strand is methylated and methyl transfer occurs only onto the GATCs in the newly synthesized unmethylated strand.

Dam

The methylation of specific GATC sites in DNA of exponentially growing cells is rapid, occurring within the minimum time (about 1 min) allowed by the sensitivity of the method [26] for chromosomal DNA and 2–4 sec on plasmid DNA [59]. An already mentioned

exception to this involves GATC sites in *oriC* and the *dnaA* promoter, which remain hemimethylated longer than other sites; this is due to the binding of SeqA (discussed in Initiation of Chromosome Replication).

Dam has been purified 3,000-fold and is a single polypeptide chain of 278 amino acids with an apparent molecular size of 32 kDa [60]. It has an S_w20 , of 2.8S and a Stokes radius of 2.4 nm and exists in solution as a monomer. The enzyme has a turnover number of 19 methyl transfers per min (but see below) and an apparent K_m of 3.6 nM for DNA. 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. Dam transfers one methyl group per DNA binding event even when binding a fully unmethylated site (but see below).

Dam has been suggested to have two SAM-binding sites: a catalytic site, and one which increases specific binding to DNA perhaps as a result of an allosteric change in the protein [61]. DNA binding and/or methyl transfer is influenced by flanking sequence; the optimal sequence is 5'-GGGGATCAAG-3' [62]. Dam is thought to bind the template and to slide processively along the DNA searching for substrate sequences [63, 64]. Further evidence for a sliding mechanism was the demonstration that in each binding event about 55 methylated GATC sites are formed before the enzyme dissociates from DNA [65](cf. previous paragraph and [60]). Processivity, however, is very dependent on sequence context [62]. An unmethylated GATC and its complement in duplex DNA are modified on both strands before the enzyme dissociates and this has been termed "intrasite processivity." Intrasite methylation occurs only when GATCs are clustered and not separated by more than 400 bp [66].

In fast-growing bacteria there are 130 Dam molecules per cell in K-12 strains and 100 molecules per cell in B strains [67]. Each of these molecules would need to transfer 39 methyl groups per min to methylate all available GATC sites in a cell with a doubling time of 30 min. Dam is a substrate for the Lon protease [68] suggesting another possible regulatory mechanism in addition to growth rate-dependent transcriptional control [46].

The Dam enzyme of *E. coli* is part of a family of methyltransferases that share nine amino acid sequence motifs [69] including the highly conserved -DPPY- that appears to be involved in SAM binding. Close relatives include the Dam proteins of phages P1, T1, T2 and T4, EcoRV, FokI, MboI, NlaIII and DpnII. The evolution of Dam appears to be a recent acquisition along with SeqA and MutH [2]. As shown in Fig. 3, Dam is present in one clade of bacteria which consists of the orders *Enterobacteriales*, *Vibrionales*, *Aeromonadales*, *Pasteurellales*, and *Alteromonadales*. Members of this clade share the following features. First, the *dam* gene is organized in an operon with *aroK* and *aroB*. Second, they have homologues to SeqA and MutH which have the same hemimethylated substrate as Dam. Third, they have separated the replication initiator gene (*dnaA*) from the origin of replication (*oriC*). Fourth, GATC sites are approximately 10-fold over-represented in their *oriC* and *dnaA* promoters. The phylogenetic distribution of Dam and its association with SeqA, MutH and other proteins has also been described using a different Web resource [70].

The atomic structure of Dam complexed with DNA has been solved to 1.89 Å resolution in the presence of S-adenosyl-homocysteine [58]. 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.

An altered specificity Dam mutant has been obtained by extensive mutagenesis of the *dam* gene [71]. The variant methylates GATT, but not GATC sequences but its specific activity is only about 20% that of the parental enzyme. The alteration in specificity was facilitated by converting amino acids R124 and P134, which contact C and T respectively, to serine. Further studies along these lines may allow for conversion of GATC specificity to the off-target sequences, especially CACC.

Selective inhibitors of Dam have been sought for use as potential therapeutics because humans do not produce this enzyme. A high-throughput small molecule screen identified several lead compounds including those which appear to bind specifically to the allosteric sites of Dam [72]. Some compounds showed greater than 400-fold selectivity for Dam compared to murine DNA cytosine methyltransferase (Dnmt1). Dam activity can also be specifically inhibited by cyclic peptides (e.g., SGWYVRNM) at IC₅₀s of 50–150 μM, concentrations which do not inhibit HhaI methyltransferase [73]. The mechanism of inhibition is not yet known. Several S-adenosyl-methionine analogues can inhibit Dam methyltransferase activity in the approximately 10 μM range [74].

Dcm

The purification and biochemical properties of the Dcm protein have not been reported, but from the DNA sequence, a 472-amino-acid protein of 53,465 kDa should be produced. Protein sequence comparisons indicate that, like other 5-meCyt methyltransferases, Dcm contains 10 conserved motifs including a Pro-Cys motif [75]. The cysteine residue is essential for catalysis but not for DNA binding, suggesting a mechanism of methyl transfer (Fig. 4) similar to that for thymidylate synthase [76], i.e., attack of the C-6 of cytosine by cysteine 177 of Dcm to activate the C-5 position for methylation [77, 78].

YhdJ

In *E. coli* over-expressing YhdJ, genomic DNA is protected from cleavage by NsiI while wild-type cellular DNA is not protected [6]. Similarly, partially purified YhdJ is able to protect DNA from NsiI cleavage *in vitro*. *In vivo* analysis of DNA isolated from the over-producing strain showed that the methylation occurred at the second or 3' adenine in the NsiI recognition sequence, 5'-ATGCAT-3'. It is likely that this is, or at least contains, the YhdJ recognition sequence. CcrM methyltransferases play a key role in the initiation of chromosome replication but YhdJ over-production does not affect synchronization in *dnaC2(Ts)* bacteria [6]. This property together with its being a non-essential gene makes YhdJ distinct from other members of the CcrM family of methyltransferases.

BIOLOGICAL FUNCTION FOR 5-meCYT

To identify the biological role for cytosine methylation, mutant strains lacking this modified base in DNA were isolated [79, 80]. Unfortunately, no obvious phenotype has yet been found associated with the *dcm* mutations. In discussing possible functions for 5-meCyt, it is worth noting that in contrast to *E. coli* K-12, *E. coli* B lacks the *dcm* gene.

dcm Mutations

The most widely used *dcm* allele, *dcm-6* [80], is defective in both methylation and VSP repair (see below) and shows mutational changes in codons 26 and 45 compared to wild type [55]. The polar effect of the nonsense codon (TGA) at position 45 in *dcm* would most easily explain the effect on *vsr*. Mutations *dcm-9* and *dcm-10* are also *Vsr*⁻ but *dcm-1*, *dcm-4* and *dcm-7* are *Vsr*⁺ (M. Lieb, personal communication).

In addition to *dcm-6*, the *mec* mutant allele of *dcm* [79] has been frequently employed, although the location of the mutation in the gene is not known. Two large deletions which remove *dcm* and additional genes have been shown to lack Dcm methylation [41, 81]. A site-directed deletion of the gene (JW1944) and its replacement with the kanamycin coding sequence is available from the Keio collection (<http://ecoli.naist.jp>).

VSP Repair

Spontaneous mutational hotspots for *amber* nonsense mutations occur in the *lacI* gene at the 5-meCyt residue in the Dcm recognition site CCAGG, altering it to CTAGG [82]. A similar result was obtained in the *cI* gene of phage lambda in growing bacteria [83] but the mutation frequencies at these hotspots were severely reduced in stationary phase bacteria [84]. When these *amber* mutations were used in genetic crosses, anomalous recombination frequencies were obtained [83] which led to the discovery of a very short patch (VSP) repair system correcting T-G mismatches in Dcm recognition sequences (Fig. 5) [85, 86].

Such T-G mismatches can occur in non-replicating DNA by the deamination of 5-meCyt. This reaction is analogous to the deamination of cytosine to form a uracil-guanine mismatch, which is a substrate for uracil-N-glycosylase. In a similar manner, the T-G mismatch is a substrate for the strand- and sequence-specific *Vsr* endonuclease, followed by conventional DNA polymerase I-dependent excision repair [87] and finally by DNA ligase. VSP repair can thus be viewed as counteracting the potential mutagenic effects of 5-meCyt deamination (Fig. 5). As expected, in *dcm* mutants no mutational hotspots are detectable [82, 88].

The T-G mismatch resulting from cytosine deamination should also be recognized by the MutS protein of the *dam*-directed mismatch repair (MMR) system. This indeed is the case but surprisingly, it was found that inactivating MutS or its partner MutL reduced VSP repair by an order of magnitude [88]. This decrease may be related to the relative amounts of *Vsr* and the Mut proteins in logarithmic and stationary phase cells. In logarithmic phase cells, *Vsr* is not detectable while the Mut proteins are present at their normal concentration. In stationary phase cells, however, the endonuclease MutH protein (partners with MutS and MutL—see *dam*-Directed Mismatch Repair) concentration decreases three-fold [89] while the *Vsr* concentration is at its maximal [90]. In other words, during logarithmic growth, the

Mut proteins are maximally efficient when replication errors are most likely while in stationary phase when DNA synthesis is minimal and 5-meCyt deaminations accumulate, VSP is most active.

One possible explanation for these results, is that MutS and MutL partner with MutH in growing cells but preferentially with Vsr in non-growing cells (both MutH and Vsr are endonucleases) [86]. Support for this idea is that Vsr interacts with MutL in a two-hybrid assay [91, 92]. One model for MutS translocation suggests that it forms loops [93] such that MutS and MutL are at the base of the loop and the mismatch is at the apex. In this case, the mismatch could be bound by another molecule of MutS in growing cells but in non-growing cells could be bound by Vsr [86]. An alternative model is that MutS and MutL enhance Vsr binding at the mismatch through an alteration of DNA secondary structure. Such a model was proposed based on the crystal structure of Vsr [94].

VSP repair is reduced in *dam* mutants and correlates with a reduction in the level of Vsr but not Dcm [95]. Since the *dcm* and *vsr* genes are co-transcribed, the regulation of *vsr* in is probably posttranslational. Discussion of this issue will be continued in the Post-Transcriptional Regulation section.

Protection from Restriction Endonucleases

Unmethylated *dcm* sites are substrates for the EcoRII restriction endonuclease [79], suggesting that one function may be protection of DNA from group N plasmids which produce these restriction endonucleases [13]. Upon transfer such plasmids into a naive cell, however, the cognate modification enzyme is produced before the restriction protein thereby affording protection even in the absence of Dcm. A role for Dcm in protection from restriction enzymes, therefore, seems problematic and in addition since some *E. coli* strains (e.g., strain B) do not possess the *dcm* gene it should have been eliminated from the biosphere by this model. It is of interest that the EcoRII methyltransferase shows about 70% amino acid sequence similarity with Dcm. Both enzymes methylate the same DNA sequence, and the function of M.EcoRII is known: it protects DNA from cleavage by EcoRII.

BIOLOGICAL FUNCTIONS FOR 6-meADE

Mutant strains lacking DNA adenine methylation were isolated in order to identify the role of this methylated base in cell metabolism. Unlike the *dcm* mutants, there are several phenotypic traits associated with *dam* mutants which have helped to define the multiple roles of 6-meAde in DNA metabolism.

dam Mutations

The most commonly used *dam* mutant alleles are *dam-3*, *dam-4*, *dam-13::Tn9* (chloramphenicol resistance), and *dam-16::Kan^R* (Table 2). The mutational changes in *dam-3* (Gly13Asp) and *dam-4* (Gly12Glu) are surprising because these are not critical amino acids for DNA contacts in the co-crystal structure (see above). However, Gly12 makes a backbone phosphate contact between G and A in GATC and so the introduction of

a negative charge might decrease enzyme binding. This explanation may also apply to Gly13.

Insertion and deletion alleles of *dam* have been isolated and characterized in *Salmonella enterica*. In general these have the same phenotypic properties as the *E. coli* mutants [96]. Other *dam* mutants were isolated on the basis of altered frameshift mutagenesis and the *dam-1* allele confers properties similar to those of *dam* mutant alleles in *E. coli* [97].

The *E. coli dam* mutants exhibit a variety of phenotypic traits and other properties (Table 3). The bewildering array of differences compared to the wild type suggests that *dam* methylation and the level of Dam itself have multiple functions in the cell. These functions are correlated with three DNA transactions: DNA mismatch repair, regulation of gene expression and initiation of chromosome replication. For these transactions which are described in detail below, the amount of hemimethylated DNA trailing the replication fork is critical. Decreasing or increasing the level of hemimethylated DNA by using a Dam-overproducing plasmid or a *dam* mutant, respectively, profoundly alters the function involved.

***dam*-Directed Mismatch Repair (see also module 7.2.5, “DNA Mismatch Repair”)**

The most direct and convincing evidence for the involvement of *dam* methylation in mismatch repair comes from the use of *in vitro*-constructed heteroduplexes of phage lambda DNA [39]. Heteroduplexes containing a mismatched base pair were constructed with one strand methylated, both strands methylated, or neither strand methylated. The unmethylated strand was preferentially repaired in heteroduplexes containing one methylated and one unmethylated strand. If neither strand was methylated, repair occurred equally on both strands. No repair was observed when both strands were fully methylated [39]. These results indicate that the function of Dam methylation is to impart strand selectivity and that the role of the repair system in the wild type is to remove replication errors in the newly synthesized undermethylated DNA strand trailing the replication fork (Fig. 6). The errors are base mismatches or deletion/insertions of up to four nucleotides [98]. In *dam* mutants where strand discrimination is lost, mutations are introduced into the parental strand 50% of the time, thereby explaining the mutator phenotype. It has been repeatedly observed, however, that the mutation rate in *dam* bacteria is much less than 50% that of *mutS*, *mutL*, or *mutH* cells [99, 100]. This result has been interpreted to indicate that mismatch repair in *dam* cells may frequently lead to a lethal outcome [101]. As expected, the mutation spectrum of *dam* and *mut* strains is identical with AT to GC and GC to AT transitions and frameshift mutations predominating [95, 102–105].

Further evidence for the role of Dam in strand discrimination is that *dam* mutants and wild-type cells overproducing Dam show a mutator phenotype [50, 51]. Unlike the wild type, where repair is confined to the newly replicated strand, *dam* mutants have lost strand discrimination and in addition to correcting mismatched bases in the new strand, mutations are introduced into the parental strand using the newly synthesized mutant strand as template. In Dam-overproducing cells, the high concentration of Dam greatly reduces the transient lifetime of hemimethylated GATCs in newly replicated DNA, thereby preventing mismatch repair and so the mutation rate increases.

Mismatch repair in *Escherichia coli* has two functions: correction of replication errors behind the replication fork and prevention of recombination between similar but not identical DNA sequences (“antirecombination”). To correct replication errors, the MutS protein binds to mismatches in DNA and recruits MutL and MutH resulting in activation of the latent endonuclease activity of MutH to produce a nick in the newly-synthesized unmethylated DNA strand 5' to the G in a nearby GATC sequence. The UvrD helicase loads on the nicked DNA in a MutS- and MutL-dependent manner and begins to unwind single-strand DNA either in the 5' to 3' direction or the 3' to 5' direction depending on the orientation of the mismatch to the GATC sequence. The single-stranded DNA is digested either by ExoI, ExoVII or ExoX in the 3' to 5' direction or the RecJ or ExoVII in the 5' to 3' direction. The resultant gap is filled by the action of DNA polymerase III, and after the action of ligase the duplex DNA is methylated by Dam. Fully methylated DNA is not a substrate for mismatch correction.

The loss of directionality of mismatch repair in a *dam* mutant must be responsible for the formation of DNA single and double-strand breaks by mismatch repair [7, 106, 107]. The increase in the number of single-strand breaks in *dam lig* cells compared to *dam* bacteria indicates that most of the nicks created by MutH can be sealed by ligase [41].

The double-strand breaks created by mismatch repair are the basis for many of the phenotypes associated with *dam* listed in Table 3, such as inviability with inactive recombination genes, induction of prophages, induced SOS response, etc. The inviability of *dam* cells with *recA*, *recBCD*, *ruvABC* and *priA* indicates that double-stranded ends are formed and that the RecBCD pathway is required to repair mismatch repair-induced double-strand breaks [9]. There are two possible ways in which these breaks could be formed. First, a replication fork encountering a nick created by MutH would collapse according the model proposed by Kuzminov [108] and shown in Fig. 7A. This model requires that the mismatch repair system recognizes some kind of endogenous damage ahead of the replication fork. Wyrzykowski and Volkert [109] reported that base pairs containing oxidative lesions are subject to mismatch repair. Since these could occur ahead of an oncoming fork in *dam* cells, it might encounter the gap intermediate made during the mismatch repair reaction and lead to fork collapse. The RecBCD pathway would then restore the fork and the PriA pathway would reload the DnaB helicase followed by DNA polymerase III holoenzyme (“replication restart”).

In the second model, MutH nicks the GATC on the 5' side of the G on both strands creating a double-strand break in non-replicating DNA (Fig. 7B). The same recombination proteins would be required to effect repair of the double-strand break with the exception of PriA as there should be no necessity for replication restart. However, if the double-strand break occurs immediately behind the fork (Fig. 7C) where mismatch repair normally acts, there might be fork disruption requiring replication restart. At present there is physical evidence for double-strand breaks in *dam recBC* cells but not in *dam recBC mut* bacteria [106, 107, 110]. The *recBC* mutation is necessary to stabilize double-stranded ends although neutral single-cell electrophoresis allowed visualization of double-strand breaks in a *dam* cell in the presence of RecBCD (Fig. 8) [110]. However, whether these breaks are dependent on replication (which would favor the first model) is not yet known.

It might be expected that the presence of double-strand breaks in *dam* cells is the likely explanation for the high basal level of the SOS response and induction of prophages. The SOS level in a population of *dam* cells is heterogeneous as measured by *recA::gfp* fusions [111] and this is probably a reflection of the stochastic nature of the SOS inducing signal. A *dam* *lexA* (Ind⁻) mutant is viable if RecA and RuvAB are supplied in *trans* from multicopy plasmids suggesting that these are the only necessary contribution from the SOS system [9]. Surprisingly, elimination of MutH, MutL, or MutS by mutation in *dam* cells does not reduce SOS regulon expression [112], indicating that an additional inducing signal must be generated although the nature of it is unknown but might be connected to asynchronous initiation of chromosome replication in *dam* bacteria (Fig. 7D) (see Regulation of Gene Expression).

Initiation of Chromosome Replication

Newly replicated DNA is hemimethylated and bound by SeqA—Replication of the fully methylated chromosome generates a transient wave of hemimethylated GATC sites (methylated on the parental strand, but not the daughter strand) behind the replication fork. Re-methylation of most chromosomal GATC sites takes place within 2–4 seconds after passage of the replication fork [59].

In the chromosomal replication origin, *oriC*, and the *dnaA* gene promoter, hemimethylated DNA persists for a large part of the cell cycle [26]. This is likely the result of the high density of GATC sites in these regions. There are 11 GATC sequences within the 245 bp minimal *oriC*, and eight GATC sequences within a 219 bp region covering *dnaA* promoters P1 and P2, which provides multiple binding sites for the SeqA protein [113]. SeqA consist of two functional domains. The N-terminus is responsible for oligomerization and the C-terminus for DNA binding [114, 115]. SeqA exists as a homodimer in solution [116, 117] and each dimer binds a pair of hemimethylated GATC sites separated by up to 31 bp [118, 119]. The SeqA protein also binds fully methylated DNA albeit with lower affinity [120]. It was proposed that dimers of SeqA are capable of oligomerizing into a left-handed helical structure (filament) with the hemimethylated DNA wrapped around it [116] thereby introducing negative supercoils [121]. The formation of a SeqA filament on hemimethylated *oriC* enables SeqA to bind hemimethylated GATC sequences more tightly than the monomeric Dam enzyme and this may explain why *oriC* is sequestered for an extended period [116]. Support for this hypothesis comes from mutations that disrupt the SeqA dimer-dimer interface and hence prevents filament formation. Such mutations lead to a *seqA* mutant phenotype, despite the mutant protein retaining the ability to bind pairs of hemimethylated GATC's as a dimer [116, 122]. Evidence also suggests that the DnaA protein assists SeqA in keeping *oriC* hemimethylated for a long time [123].

A number of chromosomal hemimethylated GATC sites in addition to *oriC* and the *dnaA* promoter are substrates for SeqA. Localization studies show that SeqA is localized in discrete foci dependent on ongoing replication [124, 125]. The foci most likely represent multimers of SeqA bound to hemimethylated DNA behind the replication fork [125, 126]. Cytological studies of SeqA foci further suggest that replication forks originating from the same origin are organized into replication factories [127].

Methylation and initiation synchrony—In fast growing cells the time required to replicate the bacterial chromosome exceeds the culture doubling time, and initiation of replication may take place one, two or even three generations prior to cell birth. Consequently, cells are born with replicating chromosomes and containing multiple origins of replication. Initiation of replication in such cells takes place only once at each origin within a short time interval of the cell cycle leading to synchronous initiation [128]. Cells therefore contain mainly 2^n replication origins (where $n = 0, 1, 2, 3$, etc.).

Dam methylation, despite facilitating duplex opening in the *oriC* region [129], is not essential for initiation of replication. Rather, methylation of origin GATC sites are instrumental in maintaining initiation synchrony, because it allows for a discrimination between an old (uninitiated) origin and one that has recently been initiated.

Initiation at the first origin within a single cell is assumed to be set by binding of DnaA to one or more low-affinity sites within *oriC* (see below). The DnaA protein is furthermore assumed to be released from an origin upon initiation [130]. Following initiation, the two nascent and hemimethylated origins are rapidly bound by the SeqA protein, preferentially to two positions on either side of the IHF site [131] to prevent open complex formation [132, 133]. This sequestration renders the origin inaccessible to DnaA for about one third of a generation time and prevents immediate reinitiation [26, 113]. Consequently, the DnaA protein released from the first initiated origin in a cell will momentarily increase the DnaA/*oriC* ratio for the remaining fully methylated, i.e., “old” origins and their initiation will follow in a cascade-like manner (“the initiation cascade” [130]).

In *dam* mutant cells the situation is different. When replication has initiated at one of the cellular origins, the two newly synthesized origins are unmethylated, and will not be sequestered. They will therefore immediately re-bind the DnaA protein liberated at the initiation event, and bring the DnaA/origin ratio below the threshold level. This will prevent the occurrence of the initiation cascade. Because only a single origin is initiated at a particular time in the *dam* cells, the decrease in DnaA/origin ratio will not be as marked as in the wild-type cells, leading to a shorter time period for the build-up of initiation potential (accumulation of DnaA protein) for the next initiation. Consequently initiations in *dam* mutant cells are spread over the entire cell cycle, i.e. they are asynchronous [130].

In the absence of SeqA [113] or in the presence of high levels of Dam methyltransferase [134] origins are rapidly re-methylated, and re-initiations at the same origin occurs frequently, resulting in an asynchrony phenotype. This is also the case for origins that cannot be sequestered due to mutations in four or eight *oriC* GATC sites [135]. Density shift sedimentation analysis corroborates that the minimal inter-replication time, i.e., the time between two successive initiations from the same origin, is reduced only a few minutes in sequestration-deficient cells to about 0.6 generation relative to wild-type cells [136].

Cells where the initiation interval is extended, for example by oversupply of DnaA, also initiate asynchronously, and it was concluded that asynchronous initiation occurs when the initiation period is longer than origin sequestration [137, 138]. Asynchronous cells fail to discriminate between an old non-initiated origin from a recently initiated origin, and

consequently origins compete for initiation factors and are randomly selected for initiation. This results in replication incompatibility, best demonstrated by the inability of these cells to stably maintain minichromosomes [138–140]. Amino acid starvation of *E. coli* cells results in induction of the “stringent response” mediated by guanosine tetra- and pentaphosphates. A consequence of this induction is the inhibition of chromosome replication initiation at *oriC*. This inhibition does not occur in *seqA* or *dam* mutant cells, suggesting that SeqA and Dam are required [141].

Methylation and once-per-cell cycle initiation of replication—In wild-type cells, initiations from the same *oriC* are separated by the culture doubling time on an average [136]. Sequestration of newly replicated hemimethylated origins is, despite immediate blocking of re-initiation, not sufficient to maintain once-per-cell cycle initiation at *oriC*. Sequestration lasts only about 1/3 of a generation time but provides a time interval during which other mechanisms operate to lower the intracellular concentration or the activity of the DnaA protein for initiation to occur one mass doubling later (for reviews see [142, 143]).

In *E. coli* DnaA is bound to the strong origin binding sites R1, R2 and R4 throughout the cell cycle [144]. Replication initiation commences with further binding of DnaA to the weaker recognition sites within the origin—that is, R2, R3, and R5, which are indifferent to the nucleotide-bound status of DnaA—and to I2, I3, tau1 and tau2 as well as 6-mer sites in the AT-rich region that are specific for DnaA^{ATP} [145–147]. The I1 box has been reported to bind DnaA associated with both ATP and ADP [145] or DnaA^{ATP} only [146]. With the help of accessory proteins IHF, HU and DiaA [148, 149], a larger DnaA-DNA nucleoprotein complex is formed at *oriC* [150, 151] where both DnaA^{ATP} and DnaA^{ADP} participate [152–155]. Because many of the weaker DnaA binding sites are dispensable for proper timing and synchrony of initiation [156], it is thought that the DnaA filament is mainly anchored at high affinity binding sites such as R1 and R4. The DnaA-DNA complex promotes duplex opening in an adjacent AT-rich region. This open complex is stabilized by the binding of DnaA^{ATP} to specific 6 bp sequences in the single-stranded region [147]. The participation of DnaA^{ATP} in formation of the pre-RC complex and in stabilizing the open complex may explain why this configuration of the protein is limiting for initiation *in vivo* [157, 158]. Subsequently, the DnaA protein recruits the hexameric DnaB helicase associated with ATP-bound DnaC as a DnaB₆DnaC₆ complex to the single-stranded region of the open complex. DnaC loads the DnaB helicase on the open complex to promote further duplex opening to form the pre-RC stage. During this process, ATP is hydrolyzed and DnaC is released, and the transition to replication proceeds by the loading of two or three DNA polymerase III holoenzymes at the origin [159].

During the period where *oriC* is sequestered, the availability of active ATP-bound DnaA protein is lowered. Several mechanisms contribute to lowering both the amount and activity of DnaA available for initiation.

Because the *dnaA* gene is close to *oriC*, it is replicated soon after initiation. The *dnaA* gene promoter is, like *oriC*, rich in GATC sites and consequently sequestered and transcriptionally inactive for approximately the same fraction of the cell cycle as the origin [26]. The absence of de novo DnaA synthesis during sequestration where cell growth

continues, contributes to lowering the amount of DnaA available for initiation when sequestration ends [160].

Another mechanism that contributes to lowering the amount of available DnaA while sequestration of the hemimethylated *oriC* persists is the generation of new DnaA binding sites outside the origin by replication. These serve to titrate DnaA protein away from *oriC* [161]. The most prominent of these DnaA binding sites, *datA*, is located about 470 kb away from *oriC* and replicated within the period of origin sequestration [162]. The *datA* region can bind several hundred molecules of DnaA [162]. Because *datA* contains five R-type DnaA boxes, it is believed to bind both DnaA^{ATP} and DnaA^{ADP} equally well although this has never been determined experimentally. Like sequestration of the *dnaA* promoter, titration of DnaA to non-*oriC* binding sites serves to lower the amount of the protein available for initiation.

Finally, the activity of DnaA is reduced by conversion of active DnaA^{ATP} to inactive DnaA^{ADP} by hydrolysis [163]. Two processes are described that contribute to this inactivation. One of these is a process called RIDA (Regulatory Inactivation of DnaA), which converts active DnaA^{ATP} to inactive DnaA^{ADP} by hydrolysis [163]. RIDA activity involves two proteins: The DnaA-related protein Hda [164] and the DNA-loaded beta clamp of the DNA polymerase III holoenzyme. In RIDA, the beta-subunit of Pol III forms a complex with the Hda and DnaA proteins, stimulating the ATPase activity of the latter to promote conversion of DnaA^{ATP} to the inactive DnaA^{ADP} [165]. RIDA is dependent on DNA-loaded beta-clamps, i.e., ongoing replication, and therefore the RIDA process is accelerated by an initiation event. However, because initiation of replication requires only a fraction of the DnaA protein to be ATP bound [152, 153] and because, newly synthesized DnaA protein is mainly ATP bound, an efficient turn-off of replication by RIDA is dependent on a period without de novo DnaA protein synthesis. This is provided by sequestration of the hemimethylated *dnaA* promoter.

A second mechanism for conversion of DnaA^{ATP} to DnaA^{ADP} involves the *datA* region in a process called DDAH (*datA*-dependent DnaA-ATP hydrolysis) [166]. DnaA^{ATP} binds to the DnaA-boxes in *datA* in an Integration Host Factor (IHF)-dependent manner, and the *datA*-DnaA-IHF complex promotes DnaA^{ATP} hydrolysis. This may seem contradictory as IHF is known to stimulate initiation of replication when it is bound to *oriC* prior to initiation [167, 168], but *datA* is bound after initiation has occurred. In this way the DDAH process assist RIDA in turning off unwanted initiations [166].

Origin sequestration-deficient cells are not able to maintain once-per-cell cycle initiation, despite being proficient in *dnaA* promoter sequestration, *datA* duplication and RIDA [113, 134, 136]. Therefore a period of time, where the origin cannot be initiated, is necessary to ensure a decrease in DnaA^{ATP} to a level that does not permit re-initiation in the same cell cycle. This time period is provided by the sequestration of hemimethylated origin. At the end of sequestration, when origins become available for re-initiation, the level of DnaA^{ATP} is below the threshold for initiation, and a period of growth is necessary to accumulate sufficient amounts of DnaA^{ATP} protein for the next round of initiation. This cell cycle-

dependent variation in free DnaA^{ATP} within the cell represents the biological clock responsible for the constant inter-initiation period.

Methylation and nucleoid organization—The second function of SeqA *in vivo* is the organization of nascent nucleoids behind the replication fork. SeqA was found to bind numerous sites across the *E. coli* chromosome [169, 170] and fast growing *E. coli* cells contain multiple replication forks. Because SeqA binds new replication forks more readily than old ones [170], there is a preference for SeqA binding to origin proximal parts of the chromosome under these conditions. This may explain, or at least contribute to, the earlier observations that SeqA was found to bind the region surrounding the replication terminus, i.e., the *ter* macrodomain, poorly [169, 171, 172].

SeqA dimers bound to distant pairs of hemimethylated GATC sites can, concurrent with replication, organize nascent daughter chromosomes into nucleoid domains [116, 173] which may require also the ability of SeqA to generate positive supercoils [174]. Lack of SeqA leads to increased negative supercoiling [175]. SeqA is also involved in proper chromosome segregation as *seqA* mutants, SeqA overproducers and cells with non-sequesterable origins show gross defects in nucleoid positioning within the cell [135, 176, 177].

Two models have been proposed for segregation of newly replicated DNA as it leaves the replication factory. In the *extrusion-capture* model [178, 179], replication of DNA from a stationary factory pushes newly replicated DNA outwards. It has been proposed that SeqA could organize and channel hemimethylated DNA towards the positions of the new nucleoid [126, 179]). In the *sister chromosome cohesion* model, the newly replicated DNA is held together until late in the replication cycle. Upon release from cohesion, origins move rapidly from a central position towards the cell poles [180–182]. There is discrepancy in the literature regarding SeqA's role in cohesion. In one study SeqA dimers were found not to bind separate DNA molecules [183] indicating that they act strictly in *cis* and therefore presumably are not involved in cohesion of sister chromosomes. Furthermore, cohesion between *oriC* sister copies was observed in *dam* mutant cells *in vivo*, and this argues against a role for Dam/SeqA in cohesion [184]. Sister chromosome cohesion, therefore, could result from catenation of daughter chromosomes behind the replication fork [185]. Interestingly, SeqA protein bound to hemimethylated DNA may play a role in decatenation as it interacts with, and stimulates, the decatenating activity of topoisomerase IV [122]. A recent report indicates SeqA dependent co-localization of newly replicated origins, especially during fast growth. In this study SeqA was capable of pairing newly replicated origins *in vitro* [186].

The role of DNA methylation for chromosome replication in other bacteria: The gamma-proteobacterium *Vibrio cholerae* is a close relative of *E. coli*. *V. cholerae* has its genome divided between two chromosomes. The origin of the larger chromosome I, *oriCI*, resembles *E. coli oriC* with respect to the positions of the DnaA binding sites, AT-rich region, GATC sites, etc., whereas *oriCII* of the smaller chromosome II, is different. *oriCII* carries a number of 11-bp or 12-bp iterons to which the chromosome II specific initiator protein, RctB, binds. Each of these iterons contains a GATC site [11]. Newly replicated *oriCI* and *oriCII* remain hemimethylated for an extended period when compared to other

sites on the chromosome [187, 188]. Deletion of the *seqA* gene has opposite consequences for sequestration of *oriCI* and *oriCII*. SeqA deficiency results in a shortened sequestration period of *oriCII*, similar to what is observed for *oriC* in *E. coli*, but is unexpectedly long for *oriCI* [188]. SeqA deficiency resulted in overreplication of both chromosomes [188], whereas SeqA overproduction inhibits replication of both chromosomes [187].

Minichromosomes having either *oriCI* or *oriCII* replicate autonomously in wild-type *E. coli* cells but fail to transform *dam* mutants [11]. For *oriCI*, this indicates either an inability to replicate in the absence of Dam methylation or that incompatibility exists between the chromosomal *oriC* and the extrachromosomal *oriCI*, as previously described for *E. coli* minichromosomes in Dam-deficient cells [189]. In two independent studies, the *oriC* region of the *E. coli* chromosome was replaced with the *V. cholerae oriCI* region. In one study the *dam* gene was found to be dispensable for growth [188] whereas the other study found *dam* to be dispensable only if the overall level of negative supercoiling was increased [190]. This suggests that the genome organization around the origin may influence function and that care should be taken when analyzing origin function in a non-native context. However, in a *V. cholerae* strain carrying a fusion between chromosome I and II and with *oriCI* as sole replication origin, the *dam* gene could be deleted, which clearly shows that Dam methylation is not required for *oriCI* replication or any other processes essential for growth [191].

It is therefore conceivable that the inviability of *V. cholerae dam* mutants [10] results only from a failure to initiate replication at *oriCII*, which agrees well with the observation that *oriCII* minichromosomes transform and replicate as extrachromosomal elements in wild-type *E. coli* cells, but both fail to do so in *dam* mutant cells [11]. The initiation failure may result from difficulties in unwinding the unmethylated *oriCII* [25, 129].

The alpha-proteobacterium *Caulobacter crescentus* is dimorphic. Cell division results in a stalk cell and a swarmer cell. Initiation of replication takes place in the stalk cell immediately after cell division, whereas it is delayed in the swarmer cell until it differentiates into a stalk cell. The chromosomal replication origin covers about 1000 bp and contains five GANTC sequences; the substrate for the essential CcrM enzyme (Cell cycle-regulated DNA Methyltransferase) that methylates the A residue [192–194] (See “DNA Methylation in Caulobacter,” below). DnaA mediated replication initiation takes place on fully methylated DNA and overproduction of CcrM leads to additional initiations, suggesting that these are normally prevented from occurring on hemimethylated origins [193]. It is not clear why methylation of the origin is required for activity but this may result from altered origin structure or to facilitate origin opening or both [25, 129]. Newly replicated origins remain hemimethylated for most of the cell cycle because the CcrM enzyme is only present in late pre-divisional swarmer cells and after DNA replication is complete [193].

The *dnaA* gene of *C. crescentus* is also regulated by CcrM methylation. The *dnaA* gene is close to *oriC* and becomes hemimethylated at about the same time as the origin. When CcrM is present, the *dnaA* promoter becomes remethylated at about the same time as *oriC*, and because the fully methylated *dnaA* promoter is more active than its hemimethylated counterpart, the DnaA protein accumulates in swarmer cells to trigger a new round of

initiation in the swarmer to stalk cell transition [195]. For reviews on *C. crescentus* chromosome replication, see [56, 196].

Regulation of Gene Expression

Since the state of GATC methylation (methylated, unmethylated, hemi-methylated) can affect specific binding of DpnI, DpnII, Sau3A (BfuCI), Dam, SeqA and MutH, then the presence of this tetranucleotide in promoter or regulatory sequences should also affect gene expression through inhibition or promotion of repressor or activator binding.

Regulation at methylated GATC sites—The P2 promoter region of the *dnaA* gene discussed above, for example, is fully active only in the completely methylated state, consistent with its biological role (25). Currently, this is the only example of this type of regulation.

Regulation at unmethylated GATC sites—In contrast to the lone *dnaA* example above, there is evidence that specific protein binding results in 36 unmethylated GATCs in the *E. coli* chromosome [19]. Nine of these are in the cyclic AMP binding protein (CAP)-binding sites preceding the *mtlA*, *cdd*, *flhD*, *gcd*, *ycdZ*, *yffE*, *ppiA*, *proP*, and *srl* (*gut*) operons [19], suggesting modulation of gene expression 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) [20, 21]. In only a few instances are there data to support specific binding of a regulatory protein either *in vivo* or *in vitro*. The level of methylation of the carbamoyl phosphate synthase (*carA*) gene promoter GATC was dependent upon cultural conditions; more methylation was detected when arginine and pyrimidines were present than in their absence, suggesting a possible regulatory effect. Protection of the *yhiP* GATC by Lrp was dependent on the presence of leucine [21].

Additional unmethylated GATC sites were found in the non-coding regions of *rspA*, *ydjL*, *yahM*, *bhsA*, *yjdD*, *yhiP*, *yiaK*, *yidX*, and *yihU/V* genes [20, 21] although their significance is not known. For all operons containing unmethylated GATCs it would be interesting to determine whether their level of expression changes in cells overproducing Dam. If so, it would suggest that methylation of the sites is important for regulating operon expression.

Studies on the *pap* operon have provided the most detailed evidence that the unmethylated GATCs are involved in controlling gene expression (reviewed in [197]). Pylonephritis-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 (Fig. 10). Conversely, in the phase-on state, the methylation state at these two sites is reversed. In a strain overproducing Dam, the transition of phase-off to phase-on is prevented, whereas in a *dam* mutant the opposite transition does not occur. The mechanism of phase variation is that Dam competes with the transcriptional activators Lrp and PapI such that Lrp is required for protection of GATC1130 and both Lrp and PapI are required for methylation protection of GATC1028 [197]. Other pilus systems also appear to be Dam controlled, although the evidence is not as complete as for *pap* [197].

Another example of proteins binding at specific GATCs leading to phase variation, is the *agn43* (antigen 43 or *flu*) gene of *E. coli* which encodes an autotransporter protein that causes cells to aggregate and form precipitates in culture media [198]. The gene has three -GATC- sites in its promoter region and their methylation leads to full transcription initiation [199, 200]. During replication of the gene, the hemimethylated DNA can be bound by Dam or SeqA or OxyR (Fig. 11). Dam binding to the GATCs allows continued transcription and while SeqA binding is transient and favors its eventual replacement by Dam. OxyR binding, however, is tighter than SeqA and can exclude Dam so that after a second round of replication a fully unmethylated promoter region with bound OxyR is created and gene transcription cannot occur [201].

Regulation at hemimethylated GATC sites—In addition to unmethylated sites, there is also evidence that hemimethylated GATC sites are important to control gene expression. The transposition frequency of Tn10 is directly related to the cellular concentration of Dam acting at two specific GATC sites in IS10 right [202]. 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 (*tsp*) promoter, while the other is near the inner end of IS10 in the target area for transposase action. In DNA that is not being replicated these sites are methylated and inert for transposition (Fig. 12). Upon replication, these sites become hemimethylated but only one of these is activated for transposition. The transposase promoter, in a wild-type strain, is active only in the configuration of methylated transposase coding strand and unmethylated non-coding strand.

The coupling of transposase activation and action to hemimethylation means that transposition is repressed for most of the cell cycle but 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. The coupling to replication helps to prevent the potentially deleterious effects of excessive transposition [202]. Other transposons such as Tn5 and Tn903 and the insertion element IS3 also use Dam methylation to control transposition [203].

Several *E. coli* promoters have GATC sites in either the -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 compared to wild type (reviewed in references [204–206]). It is not known whether expression of these genes is increased in a hemimethylated configuration. However, even if it were the physiological role for a coupling of transcription of these genes to replication is not obvious. For *trpR*, one possibility is that since the *trpR* gene lies between *oriC* and the *trp* operon, a transient boost in *trpR* transcription might provide the increase in Trp repressor concentration needed when duplication of the operon occurs. However, subsequent experiments did not support this idea [207].

Microarray studies—Global gene expression comparing wildtype and *dam* mutants using microarrays has been measured in *E. coli* and *S. enterica* [49, 110, 208, 209]. The results are difficult to compare because different strain backgrounds, media, arrays and other experimental conditions were varied as well as the goals of the experiments. However, the

up-regulation of SOS gene expression in the *dam* background was detected in each case and decreased motility in two studies. In addition, Oshima et al. [208] and Robbins-Manke et al. [110] confirmed the increase in transcription of several SOS genes by RT-PCR.

Oshima et al. [208] found that in addition to increased SOS regulon expression, genes involved in aerobic respiration, amino-acid and nucleotide metabolism were expressed at higher levels in the *dam* strain. Decreased signal was found for genes in anaerobic respiration, flagellar biosynthesis, chemotaxis and motility. Decreased motility of the *dam* mutant was demonstrated on motility plates. The increase or decrease in expression depended on the level of aeration. These results were interpreted in terms of altered binding of Fnr (fumarate nitrate reduction) and CRP (catabolite activator protein) in the promoter regions of affected genes. In addition to the array data, an increase in the steady-state level of a number of proteins was detected in *dam* cells by 2D-gel electrophoresis including YeaF (cell envelope), G3P1 (glyceraldehyde-3-phosphate dehydrogenase A) and GroEL (heat shock response) while a decreased level was noted for Pgk (phosphoglycerate kinase), SodA (superoxide dismutase) and G3P1 (under low aerobic conditions). Further filtering of these data has been reported [210].

The goal of the array experiments conducted by Løbner-Olesen et al. [49] was to compare transcription profiles in *dam*, *seqA* and Dam-overproducing wildtype cells. The striking result was that the profiles for the *seqA* mutant and the Dam-overproducing wildtype cells were very similar (Fig. 9). This result suggests that SeqA binds to the hemi-methylated region behind the fork but is prevented from doing so when Dam is present at a high concentration to reduce the amount of hemimethylated DNA. Since the absence of SeqA increases the superhelicity of chromosomal DNA [175], transcription can initiate at promoters not active at the physiological superhelical density of wildtype cells. There were few known genes in the *dam* strain, other than those belonging to the SOS regulon that showed significant differences in expression levels compared to wildtype. One of these was *dnaA* whose transcription level was moderately reduced confirming previous data obtained with *lac* fusions [211].

Robbins-Manke et al. [110] investigated the transcription profiles of wildtype, *dam*, *mutS* and *dam mutS* cells as well as measuring the number of DNA double-strand breaks in each strain. As expected, there were many more double-strand breaks in the *dam* mutant compared to the wildtype, *mutS* and *dam mutS* bacteria. The array data indicated that the SOS regulon was expressed at a low level in wild-type cells but was at a higher level in the *dam* mutant. The *dam mutS* strain, however, still showed almost as much SOS regulon expression as the *dam* mutant and significantly more than the control *mutS* strain confirming a previous result showing elevated SOS expression in *dam mut* strains using *recA::lac* or *sulA::lac* fusions [112]. Expression of the SOS regulon genes, therefore, does not seem to correlate with mismatch repair-induced double-strand breaks in a *dam* background. Consequently, there must be a DNA substrate other than the processing of MutHLS-induced double-strand breaks that serves as an inducing signal (presumably RecA-covered single-stranded DNA) in *dam* cells although its nature remains unknown. One possibility, however, relates to the asynchronous initiation of replication in *dam* cells - if there are two initiation events close together perhaps the forks are so closely spaced that they run into each other

producing replication fork collapse (Fig. 7D). The exposed double-stranded end becomes a substrate for RecBCD exonuclease which, when encountering a Chi site generates RecA-loaded single-stranded DNA thereby producing an SOS signal.

There were many genes showing increased or decreased expression in the *dam* background in the Robbins-Manke data set but a more restrictive filtering eliminated most of these except for the SOS genes and a few genes involved in translation [212].

Dam methylation affects virulence in *S. enterica* and some other bacteria (See Bacterial Virulence). A microarray analysis of wildtype and *dam* mutant transcripts in this strain identified increased SOS regulon expression in the *dam* strain just as in *E. coli* [209]. Invasion gene transcript levels in pathogenicity island SPI-1 were decreased in *dam* cells while those in the *std* fimbrial operon were increased. Altered expression patterns were found for certain flagellar (*fliCD*) and chemotaxis (*cheR*, STM3216) genes and for the *lppB* lipoprotein gene. The decreased expression of *fli* and *che* genes probably accounts for decreased motility on motility plates. The transfer operon (*tra*) genes of the conjugative virulence plasmid, pSLT, showed increased expression confirming previous *lac* fusion data (See Bacterial Virulence). All these changes in transcription probably help to explain the reduced motility, accumulation of Std protein in membranes and supernatants, envelope instability, and reduced virulence associated with the *dam* mutant. This study lays the foundation for further investigation into the complex regulation of the pathogenicity island and flagella genes.

Methylation-dependent gene expression and *dam* phenotypes—In Table 3, the properties of a *dam* mutant which can be explained by methylation-dependent gene expression include increased transposition by transposons and altered expression of chromosomal and plasmid genes. Since *E. coli dam* cells are viable, it follows that there are no essential genes whose expression are solely dependent on Dam methylation. Rather, Dam methylation can be viewed in the context of fine tuning basal levels of gene expression either by acting directly at GATCs in regulatory sequences or indirectly by affecting nucleoid structure or acting as a timing switch or in an epigenetic fashion.

Methylation-dependent gene expression in bacteriophage—Examples of gene expression modulated by Dam methylation have also been described in bacteriophage systems. The expression of the *mom* gene of bacteriophage Mu was found to be influenced by Dam methylation [213]. The *mom* gene, which is expressed late in the phage life cycle, encodes a modification enzyme which converts adenine to N6-carboxy-methyl adenine [214, 215]. The *mom* gene is non-essential but the mutant has a restricted host range suggesting that Mom's action helps protect the phage DNA from host restriction systems [216]. Dam methylation prevents binding of the *E. coli* OxyR regulatory protein to a 43-bp region upstream of the phage Mu *mom* gene which contains three GATCs [213]. Although it was demonstrated that OxyR binding *in vitro* occurred on unmethylated (but not methylated) DNA substrates, it is probable that binding occurs at hemimethylated sites *in vivo* [217]. Once bound, the OxyR repressor prevents Dam from methylating the three critical GATC sites and prevents transcription initiation, perhaps by interfering with the action of the transactivating C protein on RNA polymerase.

Cre is a bacteriophage P1 site-specific recombinase involved in the formation of covalently-closed circular DNA upon infection of the host. One of the *cre* promoters contains two GATCs in its -35 hexamer and its transcription is repressed by Dam [218]. The biological significance of this regulation is not known.

Control of Phage P1 DNA Packaging into Virions

Phage P1 encodes a *dam* gene which produces a Dam protein that is related to that of the *E. coli* host. P1 *dam* mutant phage growth is normal on a wild-type host but severely restricted on a *dam* mutant host [219]. The requirement for Dam by phage P1 is related to the mechanism of DNA encapsidation. Packaging begins at a fixed site (*pac*) on a concatemer and proceeds by a headful mechanism such that greater-than-genome-length units are packaged. This results in terminal redundancy (same DNA sequence at both ends), which allows the linear genome to circularize by recombination upon infection of the next host.

The *pac* cleavage site is flanked by seven GATCs in a region of 162 bp. *In vitro* and *in vivo* experiments indicate that cleavage of both strands occurs only on fully Dam methylated *pac* regions [219]. Since the phage requires concatemeric DNA for packaging and because the phage proteins necessary for *pac* cleavage are synthesized early in the life cycle, there must be a regulatory mechanism to prevent methylation prior to the initiation of packaging. Furthermore, it is necessary that only one or two *pac* sites in the concatemer are cleaved and that the remainder are protected from cleavage to ensure proper encapsidation. Presumably this occurs by the competitive binding to GATCs of some phage or host protein. Since the phage enzyme(s) responsible for cleavage (“pacase”) can bind to hemimethylated *pac* sites but cannot cleave, it may be that pacase itself prevents methylation [219].

Bacterial Virulence

dam mutants of *S. enterica* have attenuated virulence in the mouse model with the 50% lethal dose 10,000-fold or 1,000-fold higher than wildtype for the oral and intraperitoneal routes of administration respectively [220, 221]. Animals infected with the attenuated *dam* strain are resistant to super-infection by the wildtype offering the possibility of a vaccine [222–228]). There are several deficiencies associated with *dam* mutants that could contribute to attenuate virulence: decreased adhesion/invasion, reduced motility, envelope instability and sensitivity to bile (reviewed in [4, 229]). These deficiencies are probably due to altered gene expression as discussed above (Control of Gene Expression) except for bile sensitivity. The combination of these factors especially envelope instability are probably responsible for the high efficiency immune response in animals infected with *dam* bacteria. The bile sensitivity of *S. enterica dam* cells is due to killing by the *dam*-directed mismatch repair system probably by the formation of DNA double-strand breaks [230, 231]. At present it is not known what DNA modification produced by bile salts is recognized by the *dam*-directed mismatch repair system.

Overproduction of Dam also attenuates virulence of *S. enterica* [221]. This could be due to changing expression of genes that are normally unmethylated or by producing *seqA* phenocopies which change global transcription by altering the supercoiling of DNA [49] or by off-target methylation [29]. A *S. enterica seqA* mutant shows decreased virulence by the

oral (but not intraperitoneal) route of administration and this is explained by the mutant's increased sensitivity to bile salts [232].

Dam is an important component of virulence in certain pathogenic bacteria such as *Salmonella enterica*, *Haemophilus influenzae*, *Actinobacillus actinomycetemcomitans*, *Klebsiella pneumoniae*, enterohemorrhagic *E. coli* O157:H7, *Campylobacter jejuni*, *Yersinia enterocolitica*, *Brucella abortus*, *Vibrio cholerae*, *Pasteurella multocida*, *Yersinia pseudotuberculosis*, and *Aeromonas hydrophila* (reviewed in references [229, 233]). The effects of methylation on virulence have been studied by either constructing a *dam* mutant or overproducing Dam in these organisms. Although a detailed discussion of these studies is beyond the scope of this review, it is interesting that a Dam-mediated regulatory effect on the type III secretion system of *Yersinia enterocolitica* occurs through Clp-dependent proteolysis [234]. Type III secretion systems are widespread in pathogenic gram-negative bacteria.

Subsequent to the publications of the reviews ([229], [233]) discussing the bacteria listed above, Dam has been found to be involved in the pathogenesis of *Edwardsiella tarda*, a fish pathogen important for aquaculture [235], and in *Streptococcus mutans*, a dental pathogen [236]. In both cases, a *dam* mutant was constructed and caused significant attenuation of overall virulence or altered expression of genes with cariogenic potential.

Type VI secretion systems (T6SS) of Gram-negative bacteria are responsible for bacterial killing and/or virulence toward different host cells. Both Fur (ferric iron uptake global transcriptional repressor) and Dam regulate the *sciI* (T6SS) gene cluster as an epigenetic switch in enteroaggregative *E. coli* [237].

An additional virulence-associated phenotype influenced by Dam is conjugal transfer of the F and F-like plasmids including the pSLT virulence plasmid of *S. enterica* [96]. The latter plasmid bears the 7.8 kb *spv* region required for proliferation in the reticuloendothelial system and several other genes that may play roles in other stages of the infection process. The presence of the plasmid, which is self-transmissible, may impart an expanded host range on bacteria carrying it. Conjugal transfer in F and F-like plasmids is derepressed in *dam* mutants of both *S. enterica* and *E. coli* due to increased expression of the *tra* (transfer) operon. The increased transcription is mediated through effects on the regulatory genes, *traJ* and *finP* [238]. Dam methylation has opposite effects on these genes; transcription of *traJ* is increased in a *dam* mutant but transcription of *finP*, a small RNA that antagonizes *traJ* expression, is decreased. The interplay between these effects accounts for the level of *tra* operon expression [239]. The expression of the *traJ* gene is affected by Dam methylation like the *pap* gene and like the coupling of IS10 transposition to the replication fork [240]. Lrp binding to one of two GATCs in the promoter region determines the level of *traJ* expression and in the hemimethylated state binding to the non-coding strand is higher than the coding strand. This suggests that, like Tn10 transposition, expression of *traJ* is coupled to replication and is active on only one of the two hemimethylated configurations and only one of the daughter plasmids will be activated for transfer [239]. The effect of Dam methylation on *finP* transcription appears to be mediated through binding of the nucleoid protein H-NS, not at the promoter but as a global effect reminiscent of altered transcription

in wild-type cells overexpressing Dam leading to a *seqA* phenocopy and altered global transcription [49].

Inactivation of the *dam* gene is not lethal in *E. coli* K-12 but *dam* mutants of enterohemorrhagic *E. coli* O157:H7 strain EDL933 cannot be isolated [241]. The reason for this is due to a lambda-like prophage, 933W, which encodes the genes for the production of Shiga toxin. Genetic studies showed that EDL933 *dam* mutants can be obtained only in cells cured of this prophage or in cells with inactive *dam*-directed mismatch repair. Given the demonstration that spontaneous induction of lambda prophage is increased in *E. coli* K-12 *dam* cells (Table 3) via increased SOS regulon expression [7], a similar model was proposed for EDL933 and prophage 933W [241]. The mechanism whereby prophage induction occurs in almost every cell inactivated for Dam remains to be determined. Increased excision of the defective prophage ST64B from a *dam* mutant of *Salmonella enterica* is also due to enhanced SOS regulon expression [242]. In this case, however, there was also a direct effect on transcription of genes putatively involved in phage induction due to the presence of *dam* sites in the regulatory region of these genes. The conclusion that loss of *dam* leads to inviability of EDL933 through prophage induction is a caution in studies where ability to delete a particular gene is often used to determine if it is essential or not to the viability of the organism. In this case, Dam does not perform an essential function but the cells die due to an indirect cause.

Post-transcriptional Regulation

A critical step during colonization and pathogenesis of enterohemorrhagic *E. coli* O157:H7 is the formation of “pedestals” that result from the accumulation of actin filaments beneath adherent bacteria elevating them above the surrounding cell surfaces [243]. Both intimate adhesion and actin pedestal formation result from the transfer of *E. coli* secreted proteins into the host cell where they interact with mammalian signaling molecules that control actin assembly. One of the secreted proteins is Tir (translocated intimin receptor) which is delivered to the cell membrane where it serves as a receptor for intimin, an adhesin on the outside of the bacterial cell. Wildtype *E. coli* O157:H7 show relatively poor pedestal formation on cultured mammalian cell lines.

Deletion of the *dam* gene of *E. coli* O157:H7 results in a dramatic increase in adherence and actin pedestal formation on cultured human cells compared to wild type [53]. Increases in adherence and pedestal formation *in vitro* correlated with elevated protein levels of intimin, Tir, and another secreted protein, EspF_U. Consistent with its capacity for vigorous interaction with mammalian cells *in vitro* and in contrast to *dam* mutants of several other enteric pathogens, the *dam* mutant of *E. coli* O157:H7 was capable of robust colonization of the intestines of infected animals [53].

The elevated protein levels of intimin and Tir did not result from an increase in mRNA levels as measured by microarrays and RT-PCR suggesting a post-transcriptional mechanism of regulation [53]. 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 (Marinus, M.G., unpublished data). One model to account for this

observation is that translation of *tir* message is controlled, in part, by a small regulatory RNA that requires Hfq chaperone activity to bind the message. In an *hfq* mutant, no small RNA-mRNA binding is possible and translation of *tir* message is not impeded. In a *dam* mutant, however, transcription of the small RNA is decreased relative to wildtype leading to increased translation of *tir* message. This model is currently being tested.

The *vsr* gene is part of the “very small patch” (VSP) repair system discussed above (VSP Repair) and the efficiency of this system is reduced in *dam* bacteria [95]. The level of Vsr in wildtype is low in logarithmic phase cells and higher in stationary phase cells but in a *dam* mutant there is much less Vsr in stationary phase. Although the *vsr* mRNA level was not determined in wildtype and the *dam* mutant, the authors concluded that the reduction of Vsr was due to a post-transcriptional mechanism in the *dam* mutant [95]. Although the actual mechanism remains unknown, the involvement of a small regulatory RNA in *vsr* translation could explain the observations where its level is altered in the *dam* mutant by transcription and its action could be to alter either the rate of translation or message stability.

Dam overproduction in *Yersinia enterocolitica* imparts a hyper-invasive phenotype and results in many changes in cellular metabolism [244]. Among these is a change in the composition of lipopolysaccharide (LPS) O-antigen status where increased amounts of lipid A core, without O-antigen subunits, was observed. 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. The modulation of LPS structure is, therefore, due to an unknown posttranscriptional mechanism and undoubtedly contributes to the hyper-invasive phenotype.

DNA METHYLATION IN CAULOBACTER

The emphasis in this review has been on DNA methylation in *E. coli* and *S. enterica*, both members of the gamma-Proteobacteria. However, it is instructive to briefly review DNA methylation in *Caulobacter crescentus*, a member of the alpha-Proteobacteria which has defined morphological stages. The DNA methyltransferase in this organism is CcrM which methylates adenine in the sequence 5'-GANTC-3' [56] in a distributive manner [245]. Unlike Dam, CcrM is essential for the life of the organism, is cell cycle regulated [246], and is not present at all stages of the life cycle. The life cycle consists of two cell types, stalked cells and swimmers. Chromosome replication occurs only in the stalked cell which has methylated DNA and involves the sequential action of three key unstable regulators: DnaA, GcrA and CtrA (Fig. 13[195]). The genes for these regulators are located sequentially on the chromosome with *dnaA* nearest the origin of replication (*Cori*) and *ctrA* the most distal. 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 *Cori* in a manner similar to that in *E. coli*. Since CcrM is not present at this stage, replication produces two hemimethylated daughter molecules during fork progression. As in *E. coli*, expression of the *dnaA* gene, which lies near *Cori*, 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 the transcription of

replication genes encoding DNA polymerase III holoenzyme, DNA helicase and primase. GcrA in turn activates transcription of the *ctrA* gene the promoter of which contains two 5'-GANTC-3' sequences in the upstream regulatory region and one is close to the -35 hexamer. This promoter also is active only when hemi-methylated and the expression of the gene is, therefore, co-coordinated with the cell cycle. CtrA is able to bind *Cori* to prevent premature initiation as well as activating transcription of the *ftsZ* and *ccrM* genes and repressing transcription from the *gcrA* gene. FtsZ is a key cell division protein and its production by CtrA enables a coupling between chromosome replication and cell division. Transcription of the *ccrM* gene occurs only in the hemi-methylated state and is activated by CtrA which binds to the upstream regulatory region of *ccrM*. This arrangement ensures that the concentration of CcrM increases toward the end of the replication cycle. The *ccrM* promoter also contains two 5'-GANTC-3' sequences presumably ensuring auto-regulation of the gene. The production of CcrM is followed by methylation of the daughter chromosomes which silences the *ctrA* and *ccrM* genes and activates transcription of *dnaA* as well as preparing *Cori* for initiation by fully methylating it.

After cell division the DNA of both cell types is fully methylated. In the swarmer cell, CtrA remains bound to *Cori* and the CcrM protein is degraded preventing further methylation thereby ensuring the origin is hemi-methylated and inert for further initiation. In the stalked cell, however, CtrA is destroyed by proteolysis allowing initiation to proceed on a fully methylated *Cori*.

PRACTICAL APPLICATIONS

Information about particular *E. coli* K-12 *dam* and *dcm* strains and suggestions about their handling and storage as well as plasmids expressing these genes can be found at <http://users.umassmed.edu/martin.marinus/dstrains.html>. A review dedicated to this subject that contains detailed protocols is also available [247].

Cleavage at *dam* and *dcm* Sites

The most frequent use of methylation-deficient strains is for the propagation of DNA molecules lacking either or both *dam* and *dcm* methylated sites [22]. This allows for the digestion by various endonucleases which have recognition sites overlapping methylation sequences. A list of such endonucleases can be found in most catalogs of restriction enzyme suppliers. A list of *dam* and *dcm* strains containing various genetic markers has been published [22] and is available from the *E. coli* Genetic Stock Center (<http://cgsc.biology.yale.edu/>).

An alternative use for Dam is the generation of specific cleavage sites. For example, the action of Dam on a specific DNA molecule will prevent the action of ClaI at overlapping *dam* sites (ATCGATC) but not at non-overlapping sites (ATCGATG/A/T). Hemimethylated GATC sites can be detected using restriction enzymes such as HphI, MboII and TaqI which cleave only one of the two replicated substrate sequences [26].

DNA Sequencing Procedures

DNA prepared in a *dcm* mutant avoids loss of bands corresponding to 5-mCyt by the Maxam-Gilbert chemical sequencing procedure. Methylation of adenine can be shown directly by automated dye terminator sequencing since the modification leads to an increase in the signal of the complementary thymine [248, 249].

Increased Transformation Efficiency

Shuttle-vector plasmid DNAs isolated from wild-type *E. coli* transform *Streptomyces lividans* at a very low frequency. Transformation efficiencies are increased 400- to 10,000-fold when the vector DNAs are prepared from a *dam dcm* mutant [250]. Similar results have been obtained with various *Bacillus* and *Paracoccus* species.

Propagation of Repeated Sequences

An array of 27 direct repeats consisting of 24-bp units in a plasmid was stably propagated in *dam* cells but not wild-type, *recA*, or mismatch repair-defective strains [251]. It is possible that SeqA binds to the 24 bp units (there are 2 GATCs per unit) and destabilizes them in wild-type but not in the *dam* cells where SeqA may not bind efficiently [251]. A 7 kb mutant version of the *lac* operon was constructed and its expansion into an amplified array was studied. Expansion occurred at an increased rate in a *dam* mutant [252].

Site-Directed Mutagenesis

dam-directed mismatch repair can reduce the yield of mutants in site-directed mutagenesis by removal of the desired mutated base. This can be avoided by preparing the template strand in a *dam* mutant, followed by annealing of the mutagenic primer, extension by polymerase and transformation of the appropriate *dam* strain [253]. Alternatively, if plasmid DNA is prepared from wildtype *E. coli*, the methylated template strand can be digested with DpnI after extension of the mutagenic primer by polymerase. DNA fragments produced by PCR should be unmethylated, and after transformation into a wild-type strain the mutant yield should not be affected [39].

Low-Level SOS-Inducing Agents

Many mutagenic and carcinogenic agents induce the SOS system of *E. coli*. Some mutagenic agents (e.g., 2-aminopurine) are too weak to elicit an SOS response in normal *E. coli* cells but do so in *dam* bacteria [254]. This method has been used to screen a large number of compounds [255].

Probing Chromosome Structure and Function

Expression of the cloned *E. coli dam* gene in organisms that do not have Dam methylation can be used as a probe for chromosome structure and function provided methylation is not lethal. The regions which are methylated can be identified by their susceptibility to restriction endonucleases which cleave methylated GATCs. For example, this technique has been used in yeast, where expressed genes tend to be Dam methylated to a greater extent than repressed genes in cells containing the *dam* plasmid [256].

The *in vivo* targets of chromatin proteins can be determined by tethering Dam to a specific protein and subsequently Dam methylation will occur at locations where the protein binds [257]. This technique was successfully applied in *Drosophila* cell cultures and whole flies using the GAL4 protein. Interestingly, even when about 50 % of total genomic GATCs are methylated, there seems to be little effect on fly development.

Another method using a fusion of the protein of interest with a mutant Dam (K9A) allows the protein to bind to target sites and methylate nearby GATCs. Immunoprecipitation is used to isolate DNA fragments that are methylated which can then be used for high-throughput sequencing, microarrays, etc. [258].

***Salmonella dam* Mutants as Live Vaccine Strains**

Salmonellosis is a major problem in livestock management as is contamination of consumer meat products by these bacteria. The prophylactic and therapeutic use of antibiotics has been used for a number of years to control salmonellosis but the emergence of multi-drug resistant variants has necessitated other approaches. Vaccination is a proven prophylactic method to prevent disease and the use of *Salmonella dam* mutants for this purpose seems promising. Following on the original demonstration of significant protection by *dam* mutants from superinfection by wildtype strains in a murine model [220, 221], homologous and heterologous protection has also been demonstrated in avian [222, 224] and bovine models [223, 225, 226].

Dam Methyltransferase Inhibitors

An alternative strategy to control salmonellosis could be the use of a drug to inhibit Dam methyltransferase in *Salmonella* in the digestive tract of livestock thereby reducing virulence. Lead compounds that inhibit the enzyme *in vitro* have already been described [72, 73]. A potential problem, however, is that such agents could increase the virulence of related bacteria such as *E. coli* O157:H7 by stimulating the formation of Shiga toxin [53].

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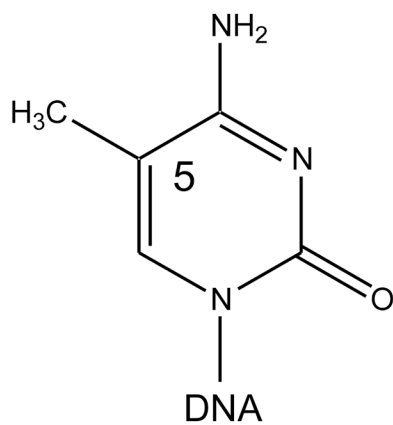
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5-methylcytosine

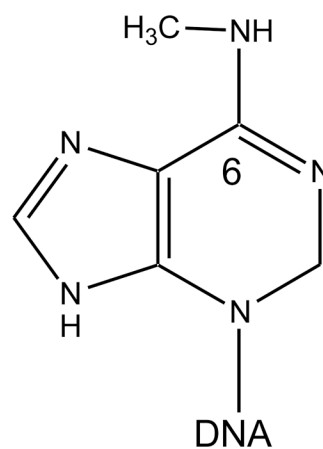
N⁶-methyladenine

FIGURE 1.
Structures of 5-methylcytosine and N⁶-methyladenine.

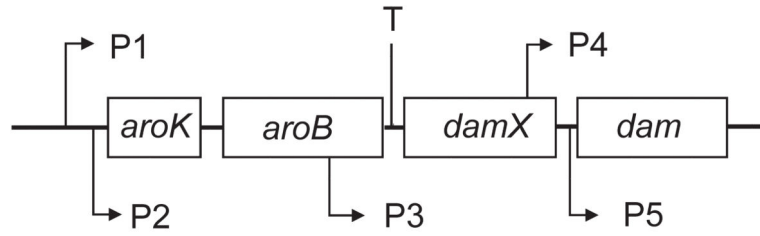


FIGURE 2.

Organization of the *dam* transcriptional unit. The locations of promoters P1 through P5 are indicated, as is the transcription terminator (T) at the end of *aroB*. The major and growth-rate regulated promoter P2 is located 3.2 kb upstream of the *dam* gene.

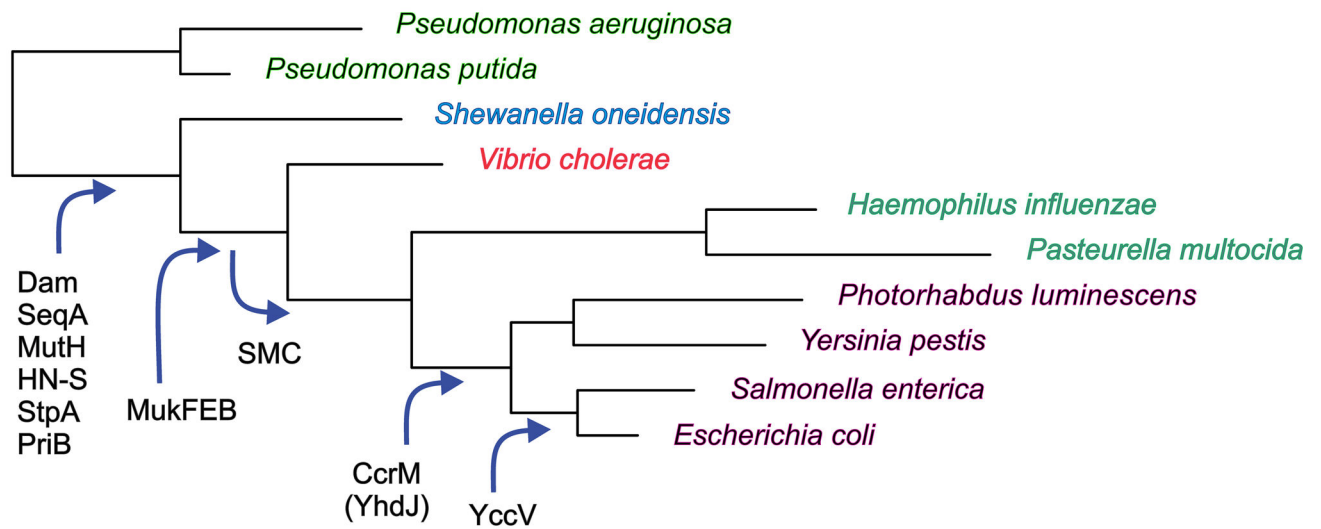
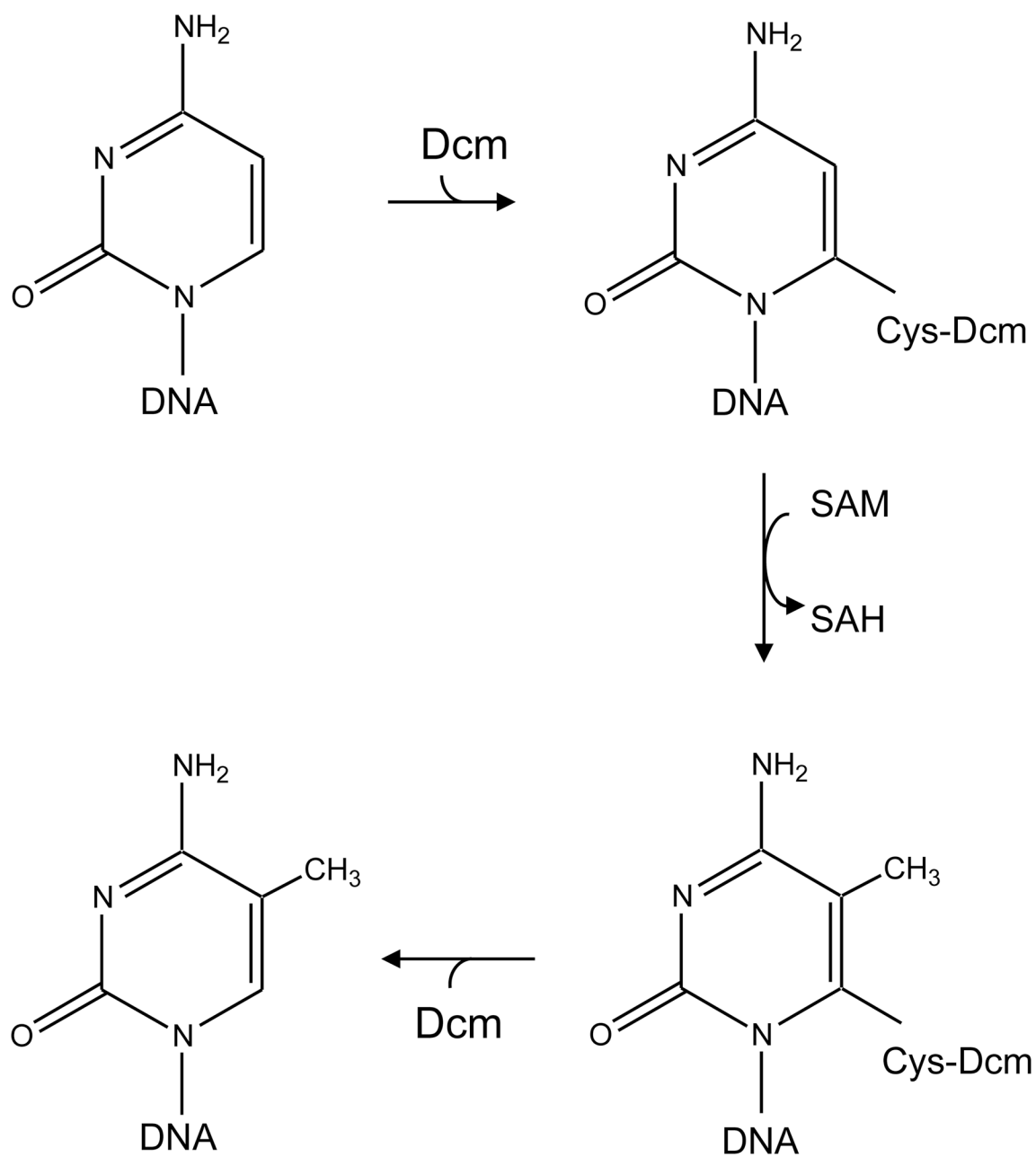
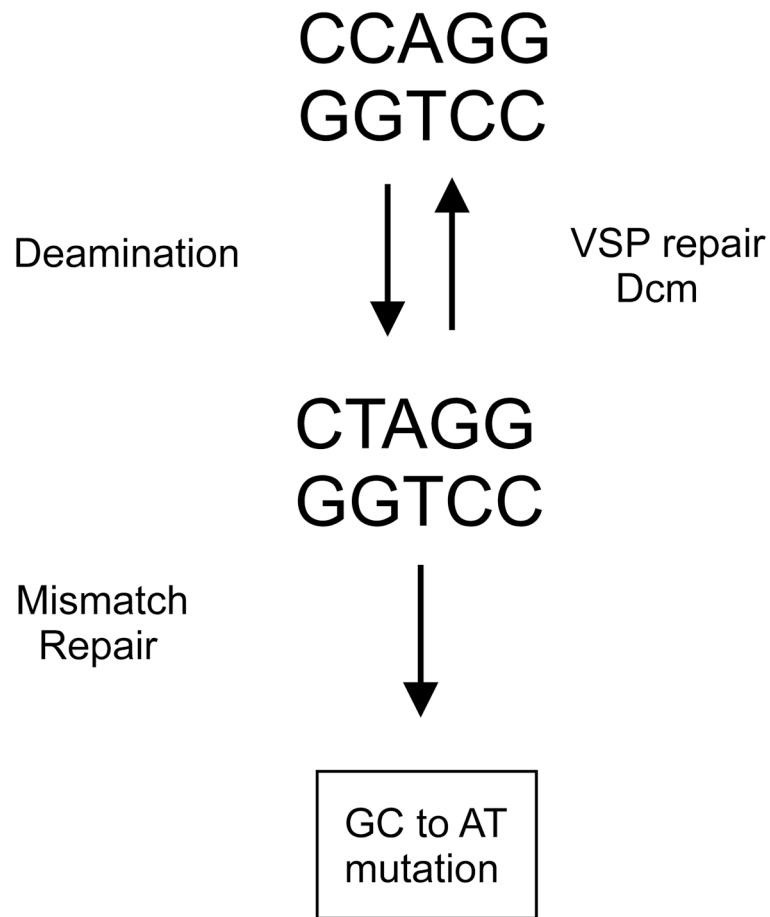


FIGURE 3.

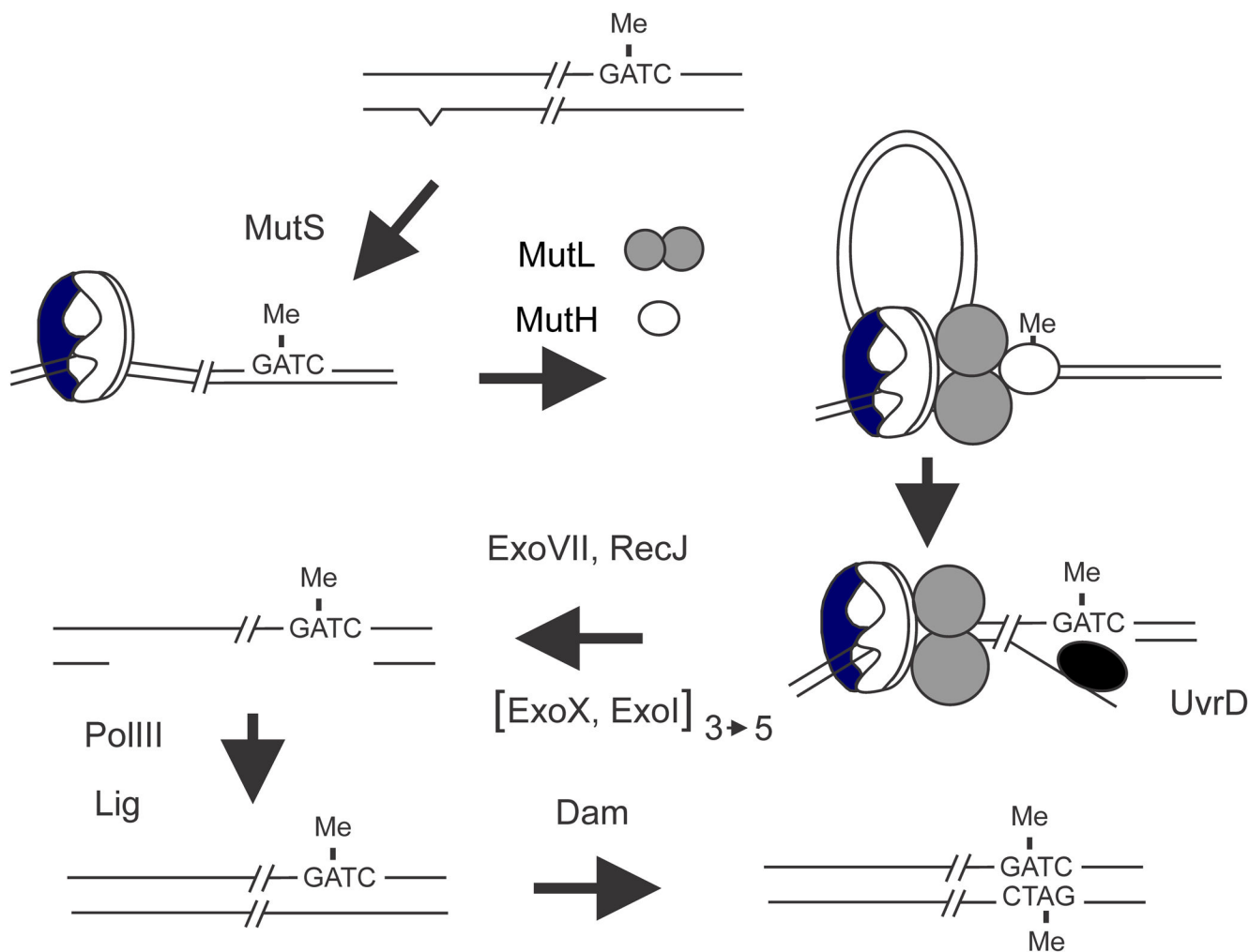
Phylogeny of the Dam clade. TBLASTN searching was used with 4311 *E. coli* proteins against selected genomes with and without the *dam* gene. The genes encoding SeqA, MutH, HN-S, PriB and 75 other proteins were found in all selected genomes with the *dam* gene and in none of the genomes lacking it. Reproduced from [2] with permission from Elsevier Ltd.

**FIGURE 4.**

Catalytic mechanism of methyl group transfer. Nucleophilic attack by cysteine-177 of Dcm at the C-6 position of cytosine leads to the formation of a covalent Dcm-DNA intermediate. This leads to activation of the C-5 position and transfer of the methyl group from SAM. The S-adenosyl-homocysteine (SAH) and the methylated cytosine are released from the covalent intermediate.

**FIGURE 5.**

Deamination, repair and mutagenesis at a Dcm recognition site. Deamination of 5-meCyt in duplex DNA produces a T-G mismatch which is a substrate for Vsr endonuclease. After removal of the T residue, DNA polymerase I and DNA ligase reactions restore the original sequence which is re-methylated by Dcm. Failure to repair before DNA replication or if the MthLS mismatch system acts on the mismatch before Vsr will produce a GC to AT mutation.

**FIGURE 6.**

Dam-directed mismatch repair in *E. coli*. The top of the figure shows DNA immediately behind the replication fork in which the “old” top strand is methylated and the “new” strand is not and also contains a base mismatch (carat) created as a replication error. The mismatch is recognized and bound by MutS followed by recruitment of MutL and MutH to form a ternary complex. The formation of this complex is thought to involve DNA looping to bring the mismatch and a GATC sequence in close proximity but the details are unclear. In the ternary complex the latent nuclease activity of MutH is activated and it cleaves the new unmethylated strand 5' to the GATC sequence. The nick created by MutH serves as an entry site for the UvrD helicase which unwinds the DNA exposing single-stranded DNA which is digested by one or more of the following exonucleases: RecJ, ExoVII, ExoX or ExoI. The exonuclease(s) used depends on the relative orientation of the mismatch to the GATC sequence; in the figure the direction of UvrD unwinding is 5' to 3' and so either ExoVII or RecJ or both are needed. If the mismatch was to the “right” of the GATC sequence, UvrD would unwind in the 3' to 5' direction and ExoX and/or ExoI would digest the single-stranded DNA. The gap created by nuclease digestion removes the mismatched base and is

filled in by DNA polymerase III. The resulting nick is closed by DNA ligase and eventual Dam methylation precludes any further repair.

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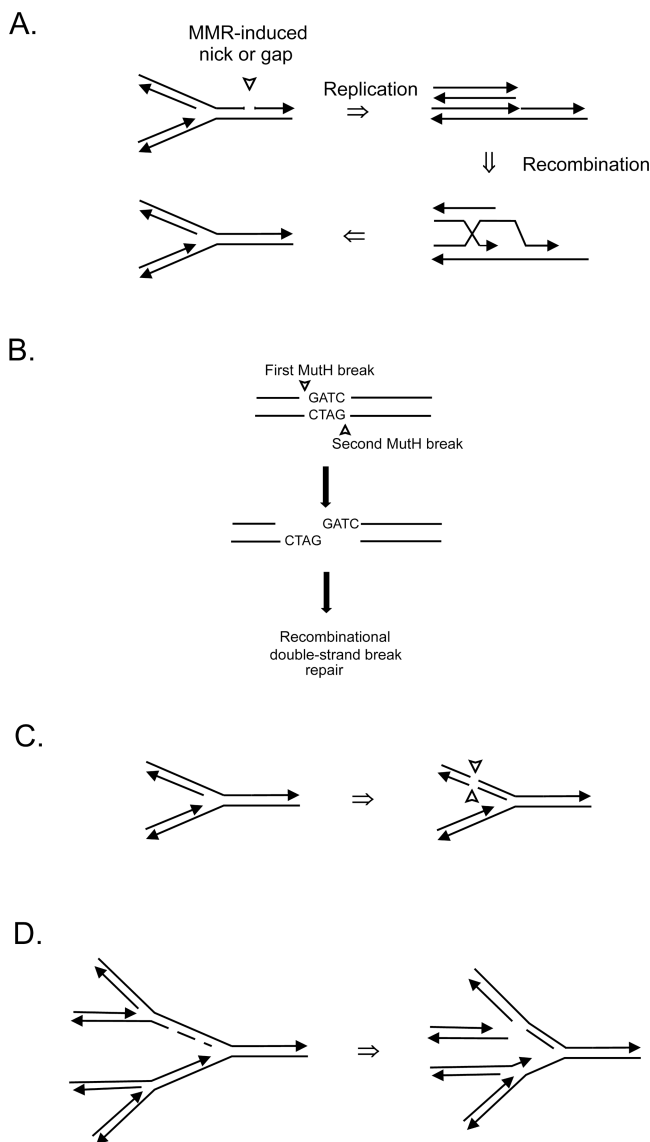


FIGURE 7. Models illustrating double-strand break formation in a *dam* mutant. (A) A replication fork encounters a mismatch repair (MMR) intermediate of a nick or gap on one strand leading to replication fork collapse. The MMR intermediate could arise from the processing of endogenous DNA damage or from repair of a replication error from the previous replication. Recombination between daughter chromosomal arms can restore the fork which can then be loaded with the DnaB helicase and DNA polymerase III holoenzyme. (B) MutH nicking on opposite sides of the same GATC in non-replicating DNA produces a double-strand break which can be repaired using a sister chromosome. (C) MMR processing of a replication error either by action at the same GATC as in panel B or by overlapping excision tracts from GATCs on opposite strands producing a double-strand break that can be repaired using the daughter strands as template. (D) Mismatch repair-independent double-strand break formation. Asynchronous initiation of chromosome replication in a *dam* mutant could lead

to two initiation events close together resulting in two closely spaced forks on each chromosomal arm. If the second fork catches up to the first, replication fork collapse occurs. The exposed double-stranded end becomes a substrate for RecBCD exonuclease which, when encountering a Chi site, loads RecA on single-stranded DNA thereby generating an SOS inducing signal.

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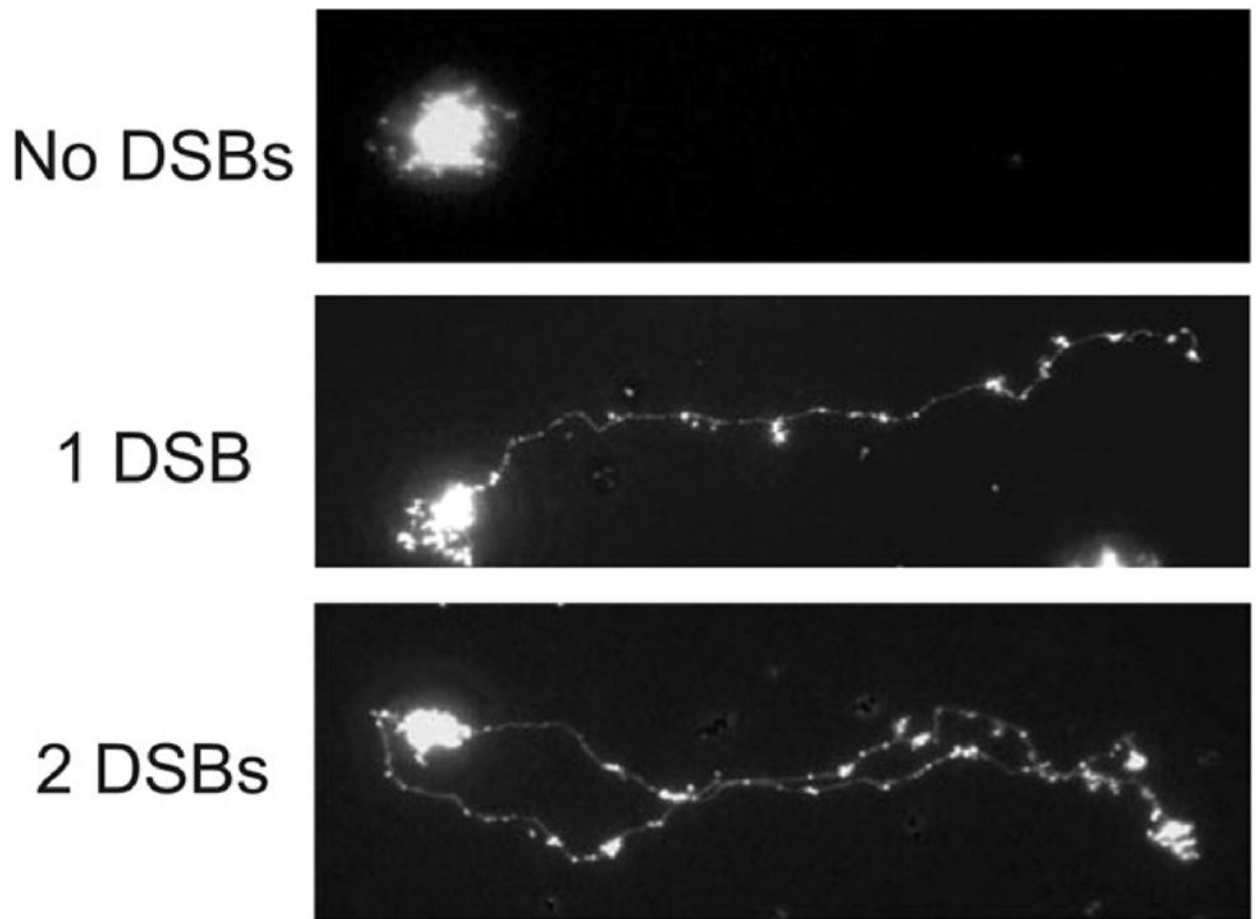
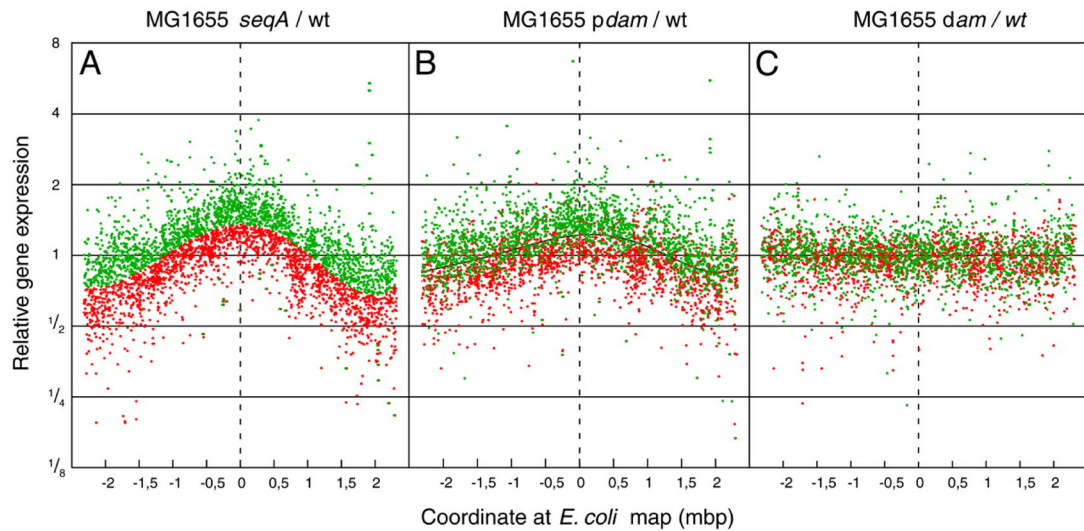
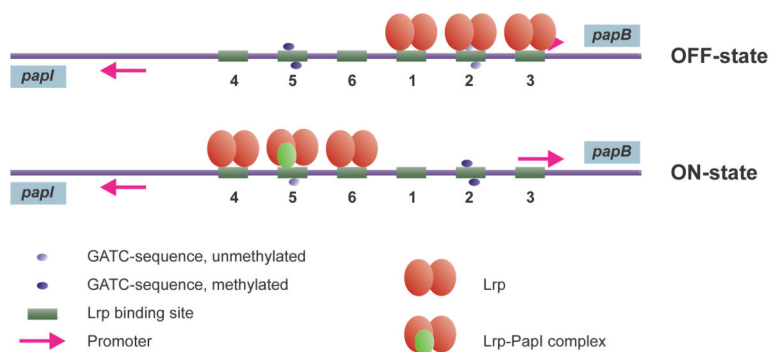


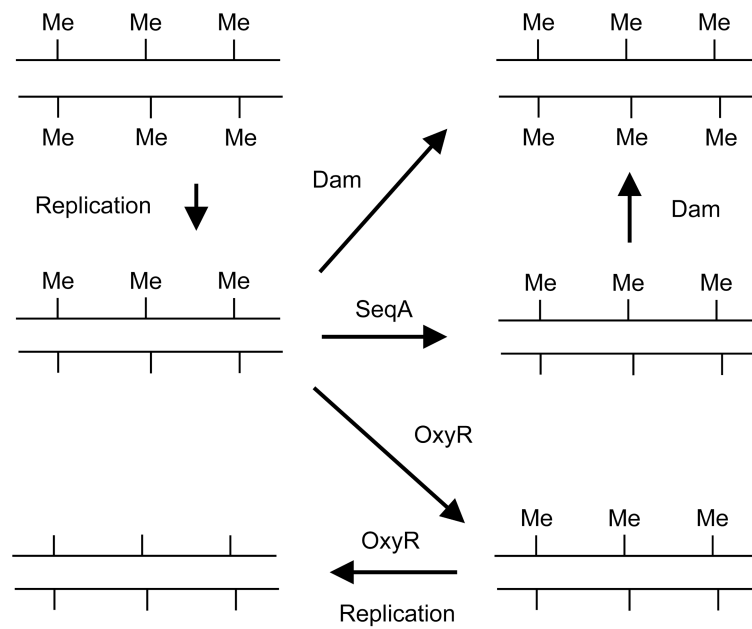
FIGURE 8. Double-strand breaks in an *E. coli dam* mutant detected by single-cell microgel electrophoresis showing disrupted cells with 0, 1 or 2 double-strand breaks. Reproduced from with permission from [110] copyright (2005) American Society for Microbiology.

**FIGURE 9.**

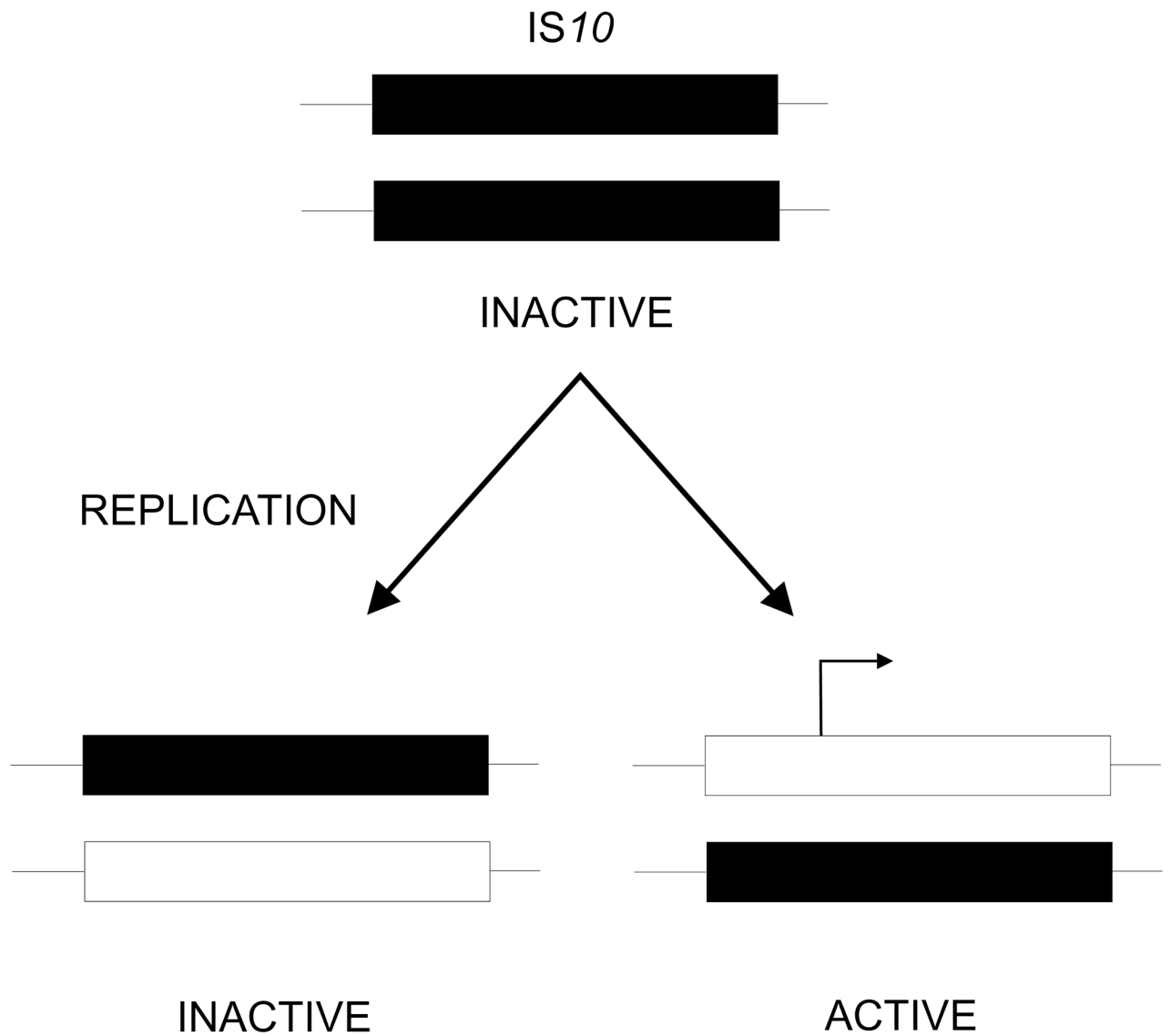
Expression of individual genes as a function of position on the chromosome. All genes for which a significant signal was obtained were plotted relative to wild type as a function of position along the chromosome. The chromosome is linearized at a position directly opposite *oriC*. The replication origin has position 0 on the abscissa. (A) MG1655 *seqA*. (B) MG1655_pTP166 (Dam overproducer). (C) MG1655 *dam-13::Tn9*. Trendlines for the gene expression data are presented in A and B. All points above the trendline in panel A, i.e., genes that were derepressed in the *seqA* mutant are plotted as green dots, and all genes that were repressed in the *seqA* mutant as red dots. Expression data from individual genes in panels B and C have the same color assignment as in panel A (red and green dots). Reproduced from with permission from [49] and Copyright (2007) National Academy of Sciences, U.S.A.

**FIGURE 10.**

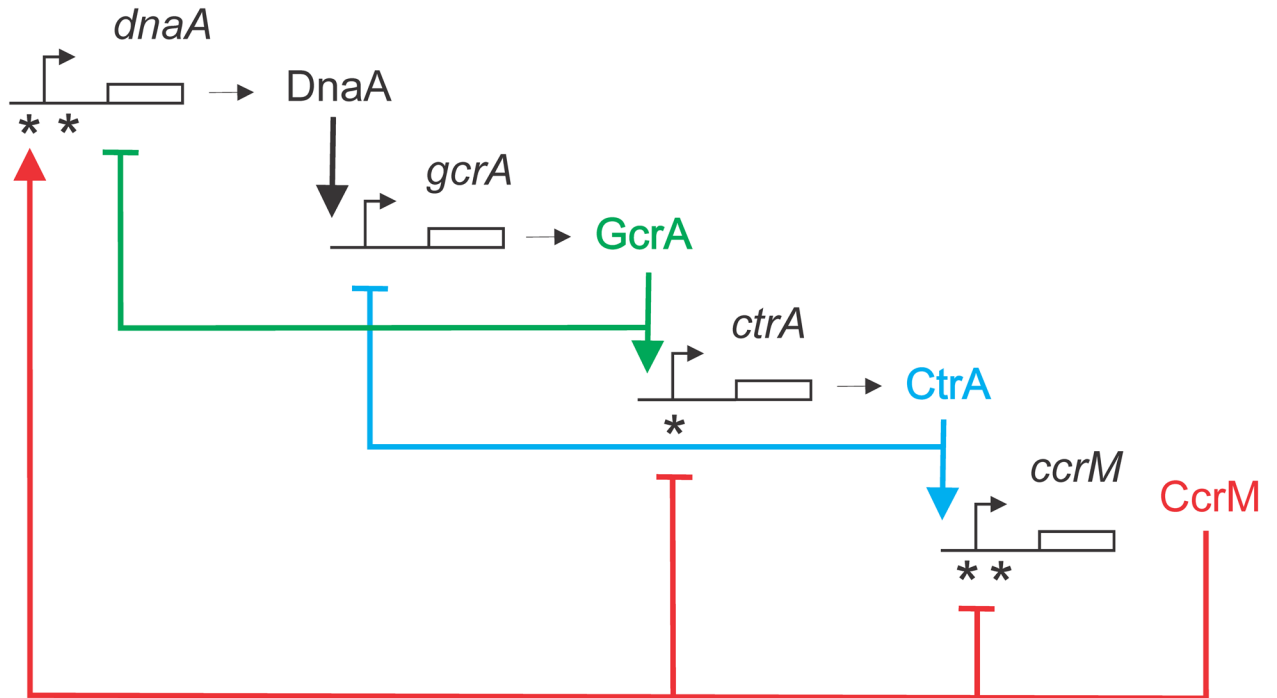
Switching at the *pap* promoter region. Lrp binds co-operatively to either Lrp binding sites 1–3 (in the OFF-state, non-piliated) or to the Lrp binding sites 4–6 (ON-state, piliated). Lrp binding site 3 overlaps the *papB* promoter and Lrp binding to site 3 inhibits transcription. Lrp binding sites 2 and 5 overlaps with GATC sites and Lrp binding to either site prevents methylation of that site by DamMT. Lrp binding to sites 1–3 mutually excludes binding to sites 4–6. When in OFF-state, each DNA replication produce one hemimethylated GATC site (in Lrp site 5) and one unmethylated GATC site (in Lrp site 2) and dissociate Lrp from its binding sites. The OFF-state is preserved by rebinding of Lrp to the same binding sites around the unmethylated Lrp binding site. A shift from phase OFF to ON may occur if PapI mediates Lrp binding to the hemimethylated Lrp site 5, followed by Lrp binding to sites 4 and 6. The shift is further stabilized by full methylation of site 2 by DamMT and conversion of the hemimethylated site 5 to unmethylated by subsequent DNA replications. This figure is adapted from [275] with permission from Cell Press.

**FIGURE 11.**

Regulation of *agn43* gene transcription in *E. coli*. The promoter region of the *agn43* gene contains three GATC sequences which must be methylated (Me) for the gene to be expressed. Replication of the gene will cause transient hemimethylation allowing one of three proteins can bind this DNA. Dam action will methylated the GATCs on the new strand thereby preserving expression of the gene. SeqA can also bind but is easily displaced by Dam resulting in methylation and continued gene expression. OxyR binding prevents Dam action and after a second round of replication the GATCs are unmethylated and transcription is prevented. This modified figure is reproduced with permission from [276] copyright (2002) John Wiley and Sons Ltd.

**FIGURE 12.**

Activation of the *tsp* promoter in IS10. In wildtype bacteria both strands of IS10 are methylated (black rectangles). Upon replication, two hemimethylated forms are produced but only that with the methylated coding strand actively transcribes the *tsp* gene and moves to a new location while the inactive IS10 remains.

**FIGURE 13.**

Regulatory cascade in the *Caulobacter* cell cycle. The genes for *dnaA*, *gcrA*, *ctrA* and *ccrM* are shown together with their respective products. The genes are shown in the sequence they are replicated on the chromosome. Asterisks indicate CcrM recognition sites (GANTC). In addition to the genes shown in this figure, DnaA, GrcA and CtrA control about 40, 50 and 95 other genes respectively. Figure modified with permission from [195] and Copyright (2007) National Academy of Sciences, U.S.A.

TABLE 1

DNA methyltransferases in *E. coli* K-12

Gene(s)	Modification methyltransferase	Recognition sequence ^a	Number in genome ^b	Restriction endonucleases ^c
<i>hsdSM</i>	M.EcoK	-AAC(N)GTCG-	595	EcoKI
<i>dam</i>	Dam	-GATC-	19,120	DpnI, DpnII, Sau3A
<i>dcm</i>	Dcm	-CCWGG-	12,045	EcoRII, BstNI
<i>yhdJ</i>	YhdJ	-ATGCAT-	839	NsiI

^aThe base in boldface is modified.

^bAll M.EcoK sites are methylated. For Dam and Dcm, most sites are methylated but this number also includes hemimethylated and unmethylated sites. YhdJ methylation is below the limit of detection at this sequence.

^cRestriction enzymes recognize the same sequence as the methyltransferases. EcoKI cuts only if the sequence is unmethylated; DpnI cleaves only if the sequence is methylated; Sau3A cuts regardless of the state of methylation. EcoRII cleaves only at unmethylated sites and BstNI cutting is not affected by methylation.

TABLE 2

E. coli K-12 *dam* alleles

Allele	Type of mutation	Location (nt)	Reference
<i>dam-3</i>	GC to AT transition, GGC (gly13) to GAC (asp)	3,513,898	[80]
<i>dam-4</i>	GC to AT transition, GGG (gly12) to GAG (glu)	3,513,901	[259]
<i>dam-13::Tn9</i>	Insertion of Tn9	3,513,725 – 3,513,726	[260]
<i>dam-16::Kan</i>	Replacement of coding sequence	3,513,240 – 3,513,773	[261]
JW3350 (Kan ^R)	Replacement of coding sequence	3,513,102 – 3,513,933	Keio collection [262]
<i>dam-12::Mu</i>	Insertion of MudIII301(Ap, <i>lac</i>)	nd	[260]
<i>dam-18::lacZ</i>	<i>lacZ</i> fusion to <i>dam</i> at BamHI site at normal chromosomal location	3,513,628	[45]
<i>dam-19::Kan</i>	Insertion of EZ::TN<KAN-2>	nd	[263]

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TABLE 3

Altered physiological properties of a *dam* mutant [1]

Reduced Dam activity <i>in vivo</i> and <i>in vitro</i> , leading to a reduction of N-methyladenine in GATC sequences in DNA [80]
A mutator phenotype [7]
A hyper-recombination phenotype [259]
Alleviation of EcoK restriction [264]
Increased number of single-strand DNA strand breaks in <i>dam lig</i> (DNA ligase) cells [41]
Increased number of double-strand breaks in a <i>dam recBC</i> strain [107]
Increased sensitivity to UV light and certain chemicals [7, 109, 230, 255, 265–267]
Increased drug-induced mutagenesis [97]
Derepression of certain genes in the SOS regulon [110, 268]
Increased spontaneous induction of lysogenic phages [38, 241]
Inviability of <i>dam</i> mutant cells with mutations in <i>recA</i> , <i>recB</i> , <i>recC</i> , <i>lexA</i> , <i>polA</i> , <i>priA</i> or <i>ruv</i> [7, 107, 112, 268]
Increased precise excision and transposition of Tn10 and other transposons [269]
Altered expression of certain chromosomal and non-chromosomal genes such as <i>trpS</i> , <i>sulA</i> , <i>glnS</i> , <i>mom</i> , <i>dnaA</i> , <i>pap</i> , <i>traJ</i> , <i>finP</i> , and <i>tsp</i> [4, 202, 205, 206]
Suppression of some <i>dam</i> phenotypes by second-site mutation in <i>mutS</i> , <i>mutH</i> , and <i>mutL</i> [99, 100]
Control of phage P1 DNA packaging into virions [219]
Asynchronous initiation of chromosome DNA replication [134]
Failure to support the growth of plasmids containing the <i>E. coli</i> origin (<i>oriC</i>) of chromosomal replication [270, 271] or the phage P1 <i>ori</i> [272] or those with the RepI replication protein [273]
Failure of Dam methylated plasmids to transform <i>dam</i> mutants at high efficiency [16]

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