Chapter 12

Roles of Methylation and Sequestration in the Mechanisms of DNA Replication in some Members of the Enterobacteriaceae Family

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Additional information is available at the end of the chapter

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1. Introduction

When growing cells divide, they need to copy their genetic material and distribute it to ensure that each daughter cell receives one copy. This is a challenging task especially when the enormous length of the DNA compared to the cell size is considered. During DNA replication, organization of the chromosomes is even more demanding, since replication forks continuously produce new DNA. This DNA contains all the information required to build the cells and tissues of a prokaryotic or an eukaryotic organism. The exact replication of this information in any species assures its genetic continuity from generation to generation and is critical to the normal development of an individual. The information stored in DNA is arranged in hereditary units known as genes that control the identifiable traits of an organism. Discovery of the structure of DNA and subsequent elucidation of how DNA directs synthesis of RNA, which then directs assembly of proteins -the so-called central dogma - were monumental achievements that marked the early days of molecular biology. However, the simplified representation of the central dogma as DNA → RNA → protein does not reflect the role of proteins in the synthesis of nucleic acids. Moreover, proteins are largely responsible for regulating DNA replication and gene expression, the entire process whereby the information encoded in DNA is decoded into the proteins that characterize various cell types. Two of these proteins are the DNA adenine methyltransferase (Dam) and the DNA-Binding Protein (SeqA).

1.1. The Dam methyltransferase

Methylation of DNA by the Dam methyltransferase provides an epigenetic signal that influences and regulates numerous physiological processes in the bacterial cell, including chromosome replication, mismatch repair, transposition, and transcription. A growing number of reports ascribed a role to DNA adenine methylation in regulating the mechanisms of DNA replication in diverse pathogens like in *Salmonella* Typhimurium and *Escherichia coli*, …), suggesting that DNA methylation may be a widespread and versatile regulator of this process. The Dam enzyme catalyzes the transfer of a methyl group from S -adenosyl-L-methionine (SAM) to the N^6 position of the adenine residue in GATC sequences, using base flipping to position the base in the enzyme's catalytic site (Figure 1).

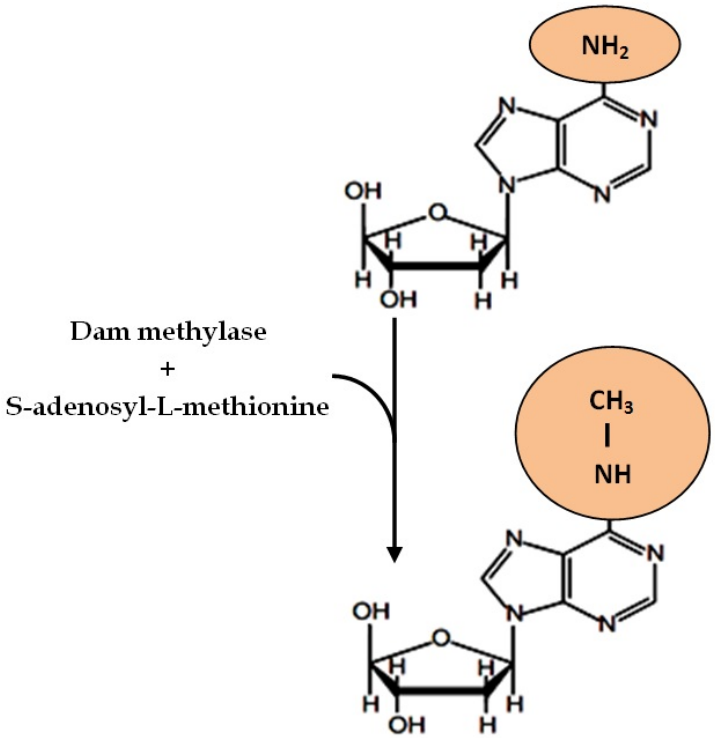

Figure 1. Dam catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to the N^6 position of adenine.

The natural substrate for the enzyme is hemimethylated DNA, where one strand is methylated and the other is not. This is the configuration of DNA immediately behind the replication fork. Double stranded DNA is a better methyl receiver than denatured DNA, and there is little difference in the rate of methylation between unmethylated and hemimethylated DNA [28]. The Dam enzyme appears to have two SAM binding sites; one is the catalytic site

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and the other increases specific binding to DNA, probably through an allosteric transformation [13]. Dam is thought to bind the template and to slide processing along the DNA, methylating about 55 GATC sites per binding event [60]. There are about 130 molecules of Dam per *Escherichia coli* cell, and this is considered optimal because it allows a period of time between the synthesis of the extending nucleotide chains and the methylation of the GATC sequences within them [15]. The cellular level of Dam is regulated mainly by transcription; any increases or decreases in the number of Dam molecules can profoundly alter the physiological properties of the cell.

Dam competes with two other proteins, MutH and SeqA, for hemimethylated GATC substrate sites. These two proteins act before Dam to participate in the removal of replication errors (MutH) and to form the compact and properly super coiled chromosome structure for the nucleoid (SeqA). Increasing the cellular level of Dam causes a decrease in the amount of hemimethylated DNA, and prevents these two proteins from carrying out their functions, leading to an increased mutation rate and a change in the super coiling of the chromosome, respectively [27; 42; 40]. Although Dam methylase is a highly processing enzyme, it may become less processing at GATC sites, flanked by specific DNA sequences [50]. Reduced rate of processing may allow for a competition between Dam and specific DNA- binding proteins, thus permitting the formation of non-methylated GATCs which depended on the growth phase and the growth rate, suggesting that the proteins that bind to them could be involved in gene expression or in the maintenance of chromosome structure. The unmethylated dam sites appear to be mostly [53] or completely [49] modified in strains overproducing Dam, suggesting that the enzyme competes with other DNA - binding proteins at these specific sites. In addition to the unmethylated GATC sites discussed above, persistent hemimethylated sequences have been detected in the chromosome [48; 18]. These are distinct from the transiently hemimethylated GATC sites that occur immediately behind the replication fork due to the time lag between DNA replication and Dam methylation.

1.2. The SeqA protein

SeqA protein was discovered in some prokaryotes as a protein involved in the methylation / hemimethylation cycle of DNA replication [41]. This protein regulates the activation of the chromosome replication origin [41]. Experiments have shown that SeqA has a high affinity to hemimethylated as compared to fully methylated DNA. It binds specifically to GATC sequences which are methylated on the Adenine of the old strand but not on the new strand. Such hemimethylated DNA is produced by progression of the replication forks and lasts until Dam methyltransferase methylates the new strand (Figure 2). It is therefore believed that a region of hemimethylated DNA covered by SeqA follows the replication fork.

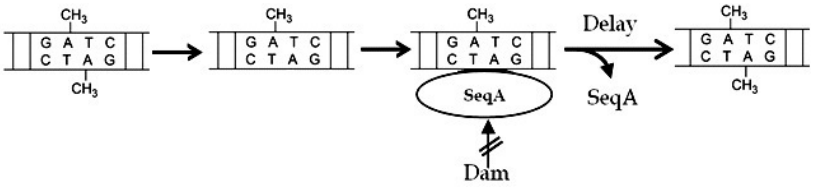

Figure 2. Helically phased GATC sites can be bound by SeqA when they are in the hemimethylated state. Binding of SeqA inhibits Dam methylation, maintaining the hemimethylated state for a portion of the cell cycle. Dissociation of SeqA allows Dam to methylate the hemimethylated DNA, thus generating fully methylated DNA.

Proper chromosome segregation also requires SeqA [5]. Furthermore, SeqA trails the DNA replication fork and may contribute to nucleic organization in newly replicated DNA [17; 37; 40; 67]. Aside from its roles in chromosome replication and nucleic segregation, SeqA is known to regulate the transcription of certain genes. In bacteriophage lambda, SeqA activates the p_R promoter in a GATC methylation-dependent fashion. SeqA also acts as a transcriptional co-activator by facilitating binding of the *c*II transcription factor to the lambda p_I and p_{aQ} promoters. Competition between SeqA and the OxyR repressor for hemimethylated GATC sites has been shown to regulate phase variation in the *Escherichia coli agn43* gene [51]. These examples raised the possibility that SeqA binding to critical GATC sites may likewise regulate the expression of prokaryotic genes like in *Escherichia coli* and *Salmonella*, which are members of the *Enterobacteriaceae* family.

1.3. The competition of Dam methylase and SeqA for GATC sites

Newly synthesized hemimethylated DNA is also a target for Dam methylase. In fact, Dam and SeqA have been suggested to be in competition for hemimethylated DNA [34]. Experiments with unsynchronized cells indicate that the sequestration period becomes shorter upon Dam over-expression [15; 57]. SeqA binding is largely limited to hemimethylated DNA, and the action of Dam will, therefore, transform DNA into a non-target for SeqA. This protein was able to bind DNA despite Dam overproduction. Recent findings showed that SeqA bound to DNA was not actively dissociated by Dam methylase [34]. The same study showed that the SeqA protein spontaneously dissociated from bound DNA after some minutes *in vitro* and that re-binding to the same site was inhibited by methylation [34]. We reasoned that *in vivo* system, the effect of Dam overproduction on SeqA re-binding should increase with increasing distance from replication forks towards the origin of chromosomal replication. This is because the longer a SeqA molecule was bound to the DNA the more likely is its dissociation. Such an effect might be too small to be observed by visual comparison of SeqA binding patterns. This shows that Dam and SeqA are in continuous competition for GATC sites *in vivo* with SeqA being the considerably stronger competitor. Since SeqA has been shown to bind better to DNA regions with more densely packed GATC sites, we speculated that such regions would allow SeqA to be better in competing against Dam than do regions with fewer GATC sites. Recent data indicate that differences in GATC density have only minor impact on the competition of SeqA against Dam methyltransferase [32].

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2. DNA replication

The initiation of chromosomal replication occurs only once during the cell cycle in both prokaryotes and eukaryotes. This initiation is the first and tightly controlled step of a DNA synthesis. Because much of what is known about the regulation of the initiation of bacterial chromosomal replication comes from studies of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, this review focuses mainly on regulatory mechanisms in these species.

In prokaryotic cells, DNA replication and segregation are not temporally separated processes. Some evidence suggests that newly synthesized DNA is continuously segregated to opposite cellular positions [45; 52]. Other work indicates that some parts of segregation may be more abrupt and domain specific [7; 21]. Coordination of DNA replication and chromosome segregation is complicated by the ability of growing with overlapping replication cycles [19; 55]. Whereas during slow growth, chromosomes are replicated in a simple pattern with only one pair of forks; replication during fast growth occurs with one pair of old and two pairs of new forks on one chromosome. (Forks are considered to be 'old forks' as soon as new forks appear at initiation.) Depending on the exact conditions, a cell can have four copies of the multi-fork chromosome and a total of 24 replication forks per cell [22; 44]. How the cell meets the obvious need for efficient organization during such extensive replication is largely unknown. However, the SeqA protein is one of the strongest candidates to contribute [41; 61]. Loss of SeqA leads to severe growth impairment during the rapid but not slow growth [16]. Biochemical studies established that SeqA binding is specific to the sequence GATC with high preference for hemimethylated over fully methylated DNA [10; 58; 11; 34]. Hemimethylation occurs at newly replicated GATC sites which have not yet been re-methylated by the Dam methylase. A transient hemimethylation after the passage of the replication fork was found in an analysis of 10 individual GATC sites [18]. Similarly, transient binding of SeqA was detected at seven genomic sites with multiple GATC sequences [67]. Multiple DNA- bound SeqA dimers can oligomerize to form a higher order structure [25; 47]. The above findings suggested that a SeqA complex follows the replication forks, potentially in a tread milling fashion, growing at the leading end and diminishing at the tailing end. The reduction of the SeqA bound region at the most replisome-distant GATCs would come about through the activity of Dam which turns these sites into non-targets for SeqA by its methylation activity. The process described above is called DNA sequestration.

3. DNA methylation by Dam protein

As mentioned above, the Dam methyltransferase of *Enterobacteriaceae* methylates adenine at the N^6 position in GATC sequences. Methylation of DNA has multiple consequences concerning bacterial physiology including the regulation of chromosome replication, DNA segregation, mismatch repair, transposition, and transcriptional regulation. The molecular basis for the pleiotropic phenotypes associated with Dam is the differential methylation of DNA resulting in an altered affinity of regulatory DNA-binding proteins. Regulatory proteins

might preferentially bind to non -methylated DNA, thereby blocking methylation by Dam, while other proteins bind with high affinity only to hemimethylated or fully methylated DNA [65]. Therefore, it is not surprising that Dam has an impact on pathogenesis, virulence gene expression, influences DNA replication [2] and many other processes [4] in *Salmonella* microorganisms. Dam-overproducing (Dam^{OP}) as well as *dam* mutant strains have been used to assess the role of DNA methylation in DNA replication. By using these strains it is possible to alter the methylation pattern in regulatory regions of genes, thereby changing the binding affinity for regulatory proteins. Although Dam^{OP} does not reflect a physiologically relevant condition, as the Dam levels have been found to remain basically constant in the cell, it is a functional tool to analyze the effects of changes in DNA methylation patterns on gene expression. Strategies of this kind have been successfully used for decades in both eukaryotes and prokaryotes to perturb gene regulation for experimental purposes.

4. DNA sequestration by SeqA protein

Replication of the bacterial chromosomal DNA initiates only once, at a specific region known as the origin of chromosomal replication (*oriC)*, by the initiator protein DnaA. This protein interacts specifically with 9-bp non-palindromic sequences (DnaA boxes) that exists at *oriC*. To ensure that initiation at an origin occurs only once per cell cycle, specific mechanisms exist to control chromosomal replication. In one mechanism, the SeqA protein that is tightly bound to hemimethylated DNA by a mechanism known as sequestration and which recognizes GATC sequences overrepresented within *oriC* and prefers binding to hemimethylated over fully or unmethylated *oriC*.

The chromosomal DNA is methylated at adenine residues in GATC sequences by Dam methylase. Following passage of the DNA replication fork, GATC sites methylated on the top and bottom strands in a mother cell (denoted as fully methylated) are converted into two hemimethylated DNA duplexes: one methylated on the top strand and non-methylated on the bottom strand and one methylated on the bottom strand and non-methylated on the top strand due to semi-conservative replication. Most GATC sites are rapidly re-methylated by the enzyme Dam methylase and exist in the hemimethylated state for only a fraction of the cell cycle (Figure 3).

Exceptions to this are the DNA replication origin of *Escherichia coli* and *Salmonella typhimurium*, the dnaA promoter, and possibly additional GATC sites in the chromosome which bind SeqA. SeqA preferentially binds to clusters of two or more hemimethylated GATC sites spaced one to two helical turns apart (Figure 4).

In the case of *oriC*, sequestration delays re-methylation and prevents binding of the DnaA protein, which controls the initiation of DNA replication. At other sites, binding of SeqA tetramers to hemimethylated GATC sites may organize nucleic domains. Notably, the transcription profile of a SeqA mutant was found to be similar to that of a Dam overproducer strain. Based on this observation, a model was developed in which Dam and SeqA compete

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for binding to hemimethylated for binding to hemimethylated DNA generated at the replication fork.

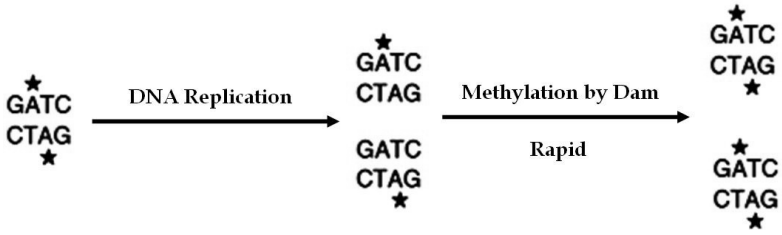

Figure 3. The vast majority of chromosomal GATC sites are fully methylated until DNA replication generates two hemimethylated species, one methylated on the top strand and one methylated on the bottom strand. Within a short time after replication (less than 5 minutes), Dam methylates the nonmethylated GATC site, regenerating a fully methylated GATC site.

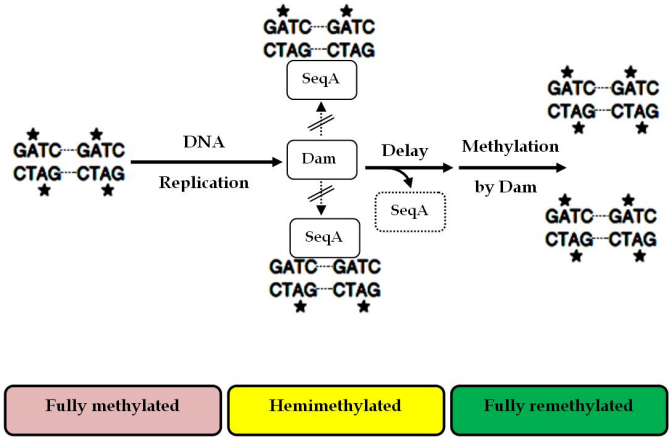

Figure 4. Two or more helically phased GATC sites can be bound by SeqA when they are in the hemimethylated state. Binding of SeqA inhibits Dam methylation, maintaining the hemimethylated state for a portion of the cell cycle. Dissociation of SeqA allows Dam to methylate the hemimethylated DNAs, generating fully methylated DNA.

4.1. Binding of SeqA to Hemimethylated GATC Sequences

As mentioned above, the adenine residues of GATC sequences are methylated on their 6 amino group by Dam methyltransferase [23; 18]. Upon replication, the GATC sequences on the newly replicated strand remain transiently unmethylated, leading to a hemimethylated state of the DNA duplex. The new strand is subsequently methylated by Dam, and the du-

plex becomes methylated on both strands. The initiation of chromosome replication at the origin of chromosomal replication *oriC*, which contains repeated GATC sequences, is tightly controlled [43; 38]. Once initiation is fired, reinitiation from the newly formed *oriC* is prevented by an *oriC* sequestration process affected by the binding of SeqA protein to the newly replicated, hemi-methylated origin [41; 34]. The hemimethylated state of the replicated *oriC* is maintained for about one-third of the cell cycle, whereas it persists in other chromosomal regions for at most 2 minutes. Further, the asynchronous and overinitiation of chromosomal replication characteristic of *seqA* mutants indicates that SeqA is a negative modulator of chromosomal initiation at *oriC* [41, 16].

Topoisomerase IV is essential for the de-catenation and segregation of replicated chromosomes at cell division [35; 36; 1; 68; 29]. Together with DNA gyrase, it also removes the positive super coils that accumulate in front of replication forks and growing mRNA transcripts. SeqA has been shown to promote the relaxation and the de-catenation activity of topoisomerase IV [33]. This appears to result from a specific interaction between topoisomerase IV and SeqA. Besides the asynchrony and overinitiation of chromosomal replication, *seqA* mutants have an aberrant nucleic structure, an increased frequency of abnormal segregation, and increased negative superhelicity of chromosomal and plasmid DNA [41; 30; 63; 64]. These findings suggest that interaction with SeqA is required for proper functioning of topoisomerase IV *in vivo*. In addition, SeqA functions as a transcriptional regulator of the bacteriophage *pR* promoter [59].

The C-terminal region of SeqA interacts via hydrogen bonds and van der Waals contacts with the major groove of DNA, with the hemimethylated A-T base pair and also with the surrounding bases and DNA backbone. The Nuclear Magnetic Resonance (NMR) structure of hemimethylated GATC revealed that it has an unusual backbone structure and a remarkably narrow major groove and suggested that this peculiar structural feature might contribute to recognition of hemimethylated GATC sites by SeqA protein [6]. To form a stable SeqA-DNA complex in the presence of competitor DNA, one SeqA tetramer binds to each of two hemimethylated GATC sequences [26] that are up to 31 bases apart on the DNA [17]. The sequential binding of SeqA tetramers to hemimethylated sites leads to the formation of higher order complexes [26]. Further, the binding of SeqA proteins to at least six adjacent hemimethylated sites induces the aggregation of free proteins onto the bound proteins, thus implying cooperative interaction between the SeqA proteins.

4.2. Effects of *seqA* disruption on DNA replication

As we said before, following the replication fork progression and the nascent strand synthesis, the daughter DNA becomes hemimethylated. SeqA protein binds to the hemimethylated GATC sequences (hemi-sites) and performs various roles to control the cell cycle progression. Immediately after the initiation of replication SeqA binds to the replicated *oriC* and sequesters it from remethylation and re-initiation of replication at the replicated *oriC*. SeqA tracks replication forks as a multiprotein complex and contributes to the maintenance of su-

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perhelicity and de-catenation of daughter chromosomes through the stimulation of topoisomerase IV and results in a synchronous replication.

When rounds of replication are allowed to run to completion, the number of chromosomes per cell is 2n (n = 0, 1, 2, 3, etc). When initiations are asynchronous, as in *dnaA* (Ts) initiation mutants at the permissive temperature and in the *Escherichia coli dam* mutant [14; 56], the presence of a different number of chromosome equivalents (three, five, six, etc.) was detected by flow cytometry. The presence of cells containing a number of chromosomes different from 2n suggests that the *seqA* mutant has a defect in the synchrony of replication initiation. Wild type and *seqA* mutant of *Salmonella enterica* serovar Typhimurium growing exponentially in glucose–casamino acid medium were treated with rifampicin and cephalexin, which block initiation of replication and cell division respectively. Wild-type cells initiated replication synchronously (number of chromosomes per cell is 2n). The appearance of cells with chromosome numbers other than 2n indicates a moderate asynchrony of initiation.

So, the flow cytometer analysis of a *seqA* mutants has shown that replication initiation is asynchronous and can occur throughout the cell cycle, not only at the normal cell age for initiation. The most likely reason for this asynchrony phenotype is that secondary initiations occurred at newly replicated origins in *seqA* mutants, due to lack of sequestration and inadequate methylation. We showed that the initiation synchrony was dependent on intact GATC methylation sites.

This loss of synchrony affected culture growth rates and cell size distributions only slightly and suggest that *seqA* mutants have a slight defect in synchronizing replication initiation. All these results suggest that DNA sequestration plays a role in preventing the occurrence of multiple initiations at a single origin in the same replication cycle. However, using flow cytometry, we found that the asynchrony of initiation, which is one of the phenotypes of the *seqA* mutation, was returned to almost normal in a *seqA* null mutant harbouring the wild-type *seqA* gene under the control of a *tac-* promoter.

The OFF to ON phase rate was reduced in a *seqA* mutant, but much of this effect could be accounted for by a reduction in the Dam / DNA ratio caused by increased asynchronous initiation of DNA replication that occurs in the absence of SeqA, which normally sequesters *oriC* and plays a critical role in timing of DNA replication [8].

5. Membrane sequestration hemimethylated of *oriC*

The coordination of the synchronization of the replication initiation, the activation of the DnaA protein at *oriC*, and the cellular cycle suggested the existence of a very narrow interaction between the bacterial membrane and the SeqA protein [39]. Early studies demonstrated that membranes are capable of binding to hemimethylated *oriC in vitro* and *in vivo*, but not to fully methylated or unmethylated *oriC* [48]. While they are sequestered at the membrane, the recently replicated origins are unavailable for re-initiation and are protected from meth-

ylation by Dam methylase for an extended period. The origins remain sequestered until conditions in the cell are no longer in a state supportive for initiation (Figure 5).

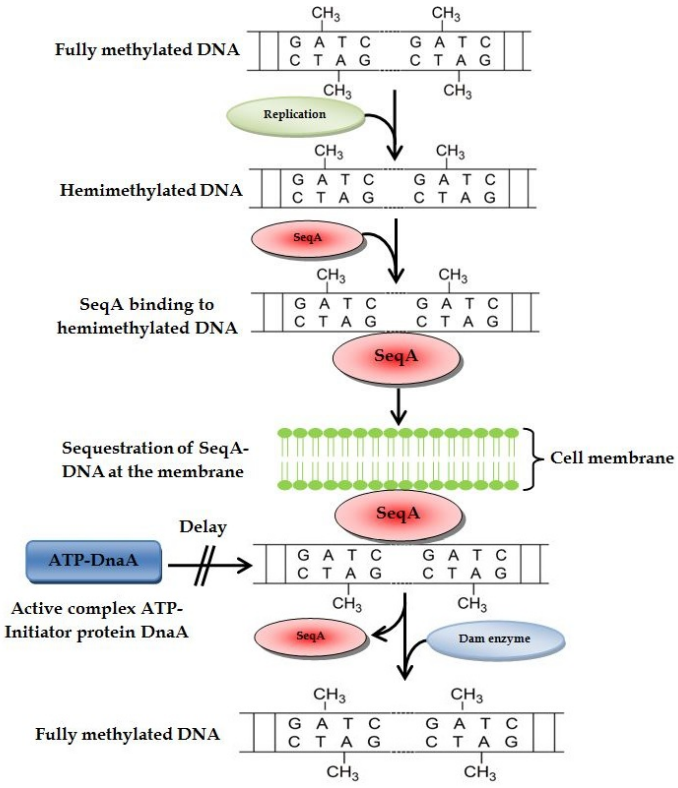

Figure 5. Membrane sequestration of recently replicated origins [4].

Prior to initiation of DNA replication, Dam methylase sites are fully methylated. Immediately following replication, the newly synthesized strand is unmethylated, and the resulting hemimethylated origin is sequestered at the lipid bilayer of membrane by SeqA. This is not accessible to replicatively active ATP–DnaA. After approximately one-third of the cell cycle, the sequestered origin is released and methylated by Dam methylase. At this point in the cell cycle, the levels of ATP–DnaA are not sufficient to catalyze a new round of replication. As such, sequestration serves as a mechanism to prevent secondary initiations. Subsequent work identified SeqA protein to be an essential factor in the *oriC* sequestration. Even though the first steps of SeqA purification involve liberating SeqA from the membrane fraction of cell lysates by treatment with high concentrations of salt and sonication, the primary sequence for SeqA protein does not suggest any obvious membrane-associating domains. This is supported by the crystal structure of the C-terminal DNA-binding domain, and by biochemical studies that show that the N-terminal domain serves in the aggregation of SeqA

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protein into functional homotetramers [24]. Yet, there is some evidence that SeqA has an association with membranes [20; 62]. The original data that newly replicated, hemimethylated origins are sequestered at the membrane hold true. Whether the membrane sequestration of *oriC* occurs directly through the SeqA protein or through an as yet unidentified factor remains unclear.

6. Cooperation between "Dam" and "SeqA" in DNA Replication

In *Escherichia coli*, persistent hemimethylated sites have been detected at the origin of chromosome replication, *oriC*, and the region surrounding it [18]. This region includes the dnaA gene, which is located 43 kb from *oriC*. DnaA initiates chromosome replication by binding to *oriC* and facilitating duplex opening to load DnaB helicase and DNA polymerase III holoenzyme. The persistence of the hemimethylated state is due to the high density of GATC sequences in *oriC* (11 in 245 bp) and in the promoter region of *dnaA* (8 in 219 bp), providing multiple binding sites for the SeqA protein. The SeqA induced hemimethylated state in this region of the chromosome lasts for about one-third of the cell cycle (sequestration), but the mechanism by which it is relieved is not known. The purpose of sequestration is to prevent re-initiation from *oriC* from occurring more than once per cell cycle. For initiation to occur most efficiently, *oriC* and the *dnaA* promoter region must be fully methylated. This also contributes to ensuring that initiation occurs only once per cell cycle [9; 66]. In *Salmonella* Typhimurium, SeqA may play replication-related roles similar to those described in *Escherichia coli* [51]. In *Vibrio cholerae*, both Dam methylation and SeqA are essential [31; 54], and SeqA overproduction causes DNA replication arrest [54].

In fast-growing *Escherichia coli* or *Salmonella* Typhimurium cells, the time required for chromosome replication exceeds the doubling time. Under such conditions, *Escherichia coli* and *Salmonella* Typhimurium cells contain multiple copies of *oriC* due to initiations that occurred two or three generations ago. These origins fire simultaneously during the cell cycle, leading to synchronous initiation, which is thought to be due to the immediate release of DnaA from an origin after initiation [46]. This release will temporarily increase the DnaA / *oriC* ratio in wild-type cells for the remaining fully methylated origins. After initiation, other mechanisms ensure that DnaA is not in the proper conformation for initiation. Among these mechanisms is a reduction in the transcription of the *dnaA* gene. Sequestration by SeqA after initiation keeps the *dnaA* promoter region in a hemimethylated state, which reduces transcription initiation because the *dnaA* promoter GATC sequences need to be fully methylated for maximal expression [9].

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

7. Conclusion

In the *Enterobacteriaceae* family (*Salmonella* Typhimurium, *Escherichia coli*,…), DNA methylation and sequestration modulate a variety of processes such as DNA replication and transcription of certain genes. Deletion of the *dam* and / or *seqA* genes produces a variety of phenotypes ranging from replication asynchrony to virulence attenuation, indicating multiple functions for Dam and SeqA proteins in modulating gene expression, proper chromosome segregation, initiation of chromosome replication, and nucleic stabilization. Given these multiple roles, it is not surprising that these mutations are highly pleiotropic. However, the lack of Dam and / or SeqA proteins does not impair viability. Bacterial mutant strains are more sensitive to these mutations than the wild type which shows the inverse. In addition, no great difference between the mutants of *Salmonella* Typhimurium and those of some *Enterobacterial* species such as *Escherichia coli* was observed with replication asynchrony. In conclusion, the role of Dam and SeqA in the prokaryotic cellular processes such as the DNA replication is clear. So it may rely on their capacity as a global regulator of the gene expression during bacterial life, *in vitro*, in a similar manner as it does *in vivo*.

8. Future research

Our knowledge on the effects of Dam and SeqA proteins in *Enterobacteriaceae* family (*Salmonella* Typhimurium, *Escherichia coli*,…) has considerably improved in the last decade. This fundamental research has several implications that will prove to be useful for the development of novel therapeutic approaches. But, to date, therapeutic applications are still in their early experimental phases, but several recent studies provide promising results for future clinical developments. Over the last few years, many studies have demonstrated that *Escherichia coli* and *Salmonella typhimurium seqA* and / or *dam* mutants exhibit asynchronous DNA replication and are highly attenuated for virulence in mice and have been proposed as live vaccines. These results prove that these proteins might have a role in regulating virulence. In addition, future research must focus on the study of the decreasing virulence and the proteomic and enzymatic activities of a *seqA* and / or *dam* mutant strains. So these perspectives can be useful to more fully understand the significance of the results obtained above. Of special interests are: firstly, the growing list of genes governed by DNA methylation and sequestration in bacterial pathogens ; secondly, the finding of novel genes regulated by Dam and SeqA proteins using high throughput analysis, and, thirdly, the evidence that these proteins may regulate the expression of many unidentified genes involved in the DNA replication.

Finally, the way in which Dam and SeqA participate clearly in the DNA replication is a critical question that deserves further investigation in the near future, and may be research studies will have to identify explanations.

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