Review Article

Dam and its role in pathogenicity of *Salmonella enterica*

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Abstract

Dam methylation is an essential factor involved in the virulence of an increasing number of bacterial pathogens including *Salmonella enterica*. Lack of Dam methylation causes severe attenuation in animal models. It has been proposed that dysregulation of Dam activity is potentially a general strategy for the generation of vaccines against bacterial pathogens. In this review, we focus our attention on the role of methylation by Dam protein in regulating bacterial gene expression and virulence in *Salmonella enterica*.

Keywords: DNA adenine methylase, Pathogenesis, *Salmonella enterica*

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Introduction

DNA methylation takes place throughout the living world, including in bacteria, plants, and mammals. DNA methylation occurs at the C-5 or N-4 positions of cytosine and at the N-6 position of adenine and is catalyzed by enzymes known as DNA methyltransferases (MTases) [1,2]. All MTases use S-adenosyl methionine as a methyl donor.

DNA methylation has historically been associated with DNA restriction modification systems. These systems are considered to be important in protecting cells from foreign DNAs such as transposons and bacteriophages [3,4,5]. Restriction modification systems contain a DNA methylase that protects host DNA sequences from restriction with their cognate restriction enzymes, which digest unmodified foreign DNAs. Certain MTases, including DNA cytosine MTase (Dcm), which methylates the C-5 position of cytosine in CC(A/T)GG sequences; DNA adenine methylase (Dam), which methylates N-6 of adenine in GATC sequences; and cell cycle regulated methylase (CcrM), which methylates the N-6 adenine of GAnTC, do not have cognate restriction enzymes associated with them [6].

Methylation of DNA has numerous consequences for 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, thus blocking methylation by Dam, while other proteins bind with high affinity only to hemimethylated or fully methylated DNA [7].

Dam homologues are widespread among enteric bacteria, including *Escherichia coli*, *Salmonella* spp., *Serratia marcescens*, *Yersinia* spp., and *Vibrio cholerae*, but are also present in disparate genera, including *Neisseria*, among others. Dam is a vital protein in *Vibrio cholerae* and *Yersinia pseudotuberculosis* [8,9]; however, Dam methylation is not essential for the viability of *E. coli* or *Salmonella* spp. [10]. On the other hand, methylation by Dam is fundamental in *Salmonella* virulence, and its absence causes severe attenuation in animals (mice, chickens, cattle) [11,14]. Following oral immunization with *dam Salmonella*, mice infected with 10,000 times the LD_{50} of the wild type strain were highly protected and exhibited reduced proliferation in systemic tissues compared with the wild type strain. However, *dam* mutants are able to persist in the liver, spleen, and lymph nodes of infected mice [11,12].

Moreover, *dam* mutants of *Salmonella enterica* serovar Typhimurium exhibit virulence related

defects such as impaired expression and secretion of invasion proteins [11], reduced cytotoxicity on M cells [11], inefficient colonization of Peyer's patches and mesenteric lymph nodes [11,12], sensitivity to components of innate immunity [15,16,17], envelope instability [18]; changes in LPS expression [Sarnacki *et al.*, unpublished data] and probably additional defects still to be discovered since the pleiotropic effects of Dam methylation. In this review, we focus our attention on the role of Dam methylation in regulating bacterial gene expression and virulence in *S. enterica*.

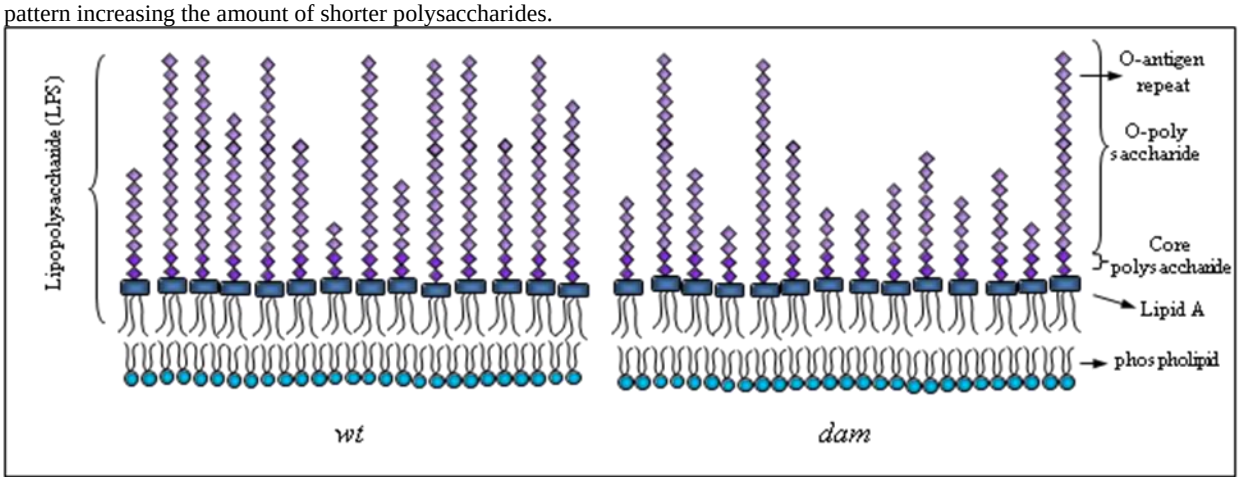
Dam and the regulation of LPS synthesis

The lipopolysaccharide (LPS) is a key component of the outer membrane of gram-negative bacteria that contributes to the stability and permeability-barrier properties of this membrane. LPS is located on the outer leaflet of the outer membrane and consists of three regions: O antigen polysaccharide, core oligosaccharide, and lipid A [19]. The biogenesis of LPS is a complex process involving various steps that occur at the plasma membrane followed by the translocation of LPS molecules to the outer membrane [19,20]. On the periplasmic side of the plasma membrane, translocated subunits polymerize to a certain length, unique to each O antigen, by the concerted functions of Wzy (O-antigen polymerase) and Wzz (O-antigen chain length regulator). The polysaccharide is ultimately ligated to the lipid A-core oligosaccharide. We reported earlier that a *Salmonella enterica* serovar Enteritidis mutant expressing a truncated Dam protein [17,21] fails to agglutinate with anti O9 serum [22]. More recent studies demonstrated that the Dam protein of *S.* Enteritidis has an effect on the determination of O antigen polysaccharide chain length [Sarnacki *et al.*, unpublished data] (Figure 1). We found that Wzz protein is three-fold lower in the *Salmonella dam* mutant than in the parental strain; moreover, *wzz* gene expression is downregulated in the mutant suggesting that a functional Dam is required for adequate levels of *wzz* transcription. Distinct two-component regulatory systems, PmrA/PmrB and RcsC/YojN/RcsB, are known to regulate *wzz* gene expression independently of each other [23]. Therefore, it is plausible that Dam could regulate *wzz* gene expression either directly or by affecting the expression of *pmrA* and *rcsB* genes. Thus, our work uncovers *rcsB* and *wzz* as new targets regulated by Dam methylation and demonstrates that Dam has an effect on the determination of O antigen polysaccharide chain length [Sarnacki *et al*,. unpublished data]. This phenomenon is not unique to *Salmonella* as we also found that the lack of Dam affects the LPS pattern in *E. coli*, increasing the amount of shorter polysaccharides and modifying the size of the banding pattern of the O antigen region [Sarnacki *et al.*, unpublished data]. Furthermore, overproduction of Dam in *Yersinia enterocolitica* results in an increased number of rough LPS molecules [24]. These findings suggest that Dam methylation plays a general role in the O antigen LPS expression in enteric bacteria and could affect not only the expression of bacterial surface proteins but also the expression of surface polysaccharides.

Dam and the regulation of fimbrial genes

The *S.* Typhimurium genome contains a large repertoire of putative fimbrial operons, including the *std* operon, that remains poorly characterized because these operons are not expressed under laboratory conditions [25,26]. On the contrary, Std fimbriae are synthesized during infection and play different roles in virulence. Humphries *et al.* [26,27] observed that mice infected with *S.* Typhimurium seroconvert to StdA, the major protein of Std fimbriae detected upon infection of bovine ileal loops, which provides indirect evidence for *in vivo* expression of the *std* operon. Deletion of *std* reduces intestinal persistence of *S.* Typhimurium in the murine model. These data suggest that Std fimbriae contribute to intestinal colonization by mediating attachment to the intestinal mucosa [28]. However, the underlying mechanisms have not been explored. Dam methylation has previously been implicated in controlling the expression of fimbriae in *E. coli* [29,30] and *S.* Typhimurium [31]. Balbontin *et al.* [32] demonstrated that transcription of the *std* operon is repressed by Dam methylation in *S.* Typhimurium. They observed that *stdA* and *stdB* genes showed the highest levels of Dam dependent regulation among other genes. *dam* mutants of *S.* Typhimurium grown under laboratory conditions express the *std* fimbrial operon, which is tightly repressed in the wild type strain [33]. Chessa *et al.* [34] confirmed and extended this observation by showing that mutations in *dam* resulted in the expression of StdA filaments on the bacterial cell surface. The synthesis of Std fimbriae has been shown to be silenced by *S.* Typhimurium during static growth in LB medium, whereas it is up regulated upon infection of bovine ligated loops [26].

Figure 1. Scheme of the outer membrane of wild type (wt) and *dam Salmonella* strains. The lack of Dam affects the LPS pattern increasing the amount of shorter polysaccharides.



Jakomin *et al*. [35] suggest that Dam methylation regulates *std* expression at the transcriptional level. Jakomin *et al*. [35] and Chessa *et al.* [34] demonstrated that SeqA (GATC binding protein) and RosE are repressors of the *std* operon and that HdfR protein (a member of the LysR family of transcriptional regulators) [36] is an activator of *std* expression whose activity could be antagonized by Dam methylation and SeqA. However, the mechanisms underlying SeqA, RosE, and HdfR mediated regulation of the *std* operon remain to be investigated.

Overexpression of Std fimbriae and release to the extracellular medium contributes to attenuation in *Salmonella dam* mutants by activating the host immune system. It has been suggested that the ectopic fimbrial expression in *dam* mutants may interfere with signal exchange between the host and the pathogen and may overstimulate the host immune system [37].

Dam and bacterial structure stability

Pucciarelli *et al.* [18] demonstrated that the deficiency of Dam methylase causes envelope defects in *S. enterica* [18]. This alteration was denoted by the accumulation of membrane vesicles in the supernatant of stationary cultures of *dam* mutants [18]. The release of membrane material to the extracellular medium correlated with a high protein content in extracts prepared from culture supernatants of *dam* mutants. The lack of Dam methylation in *S. enterica* results in impaired association of membrane proteins to peptidoglycan required for maintenance of envelope integrity, such as OmpA, TolB, peptidoglycan-associated lipoprotein (Pal), and Braun's murein lipoprotein (lppB). Surprisingly, *E. coli dam* mutants do not show an equivalent phenotype of envelope instability [18].

These observations suggest that Dam methylation participate directly or indirectly in maintenance of *Salmonella* envelope integrity. In this way, Balbontín *et al.* [32] demonstrated a decreased synthesis of the *lppB* gene in *Salmonella dam* mutants. This LppB protein is involved in maintaining envelope integrity [18]. Release of membrane vesicles and leakage of proteins may contribute to the virulence attenuation of *S. enterica dam* mutants.

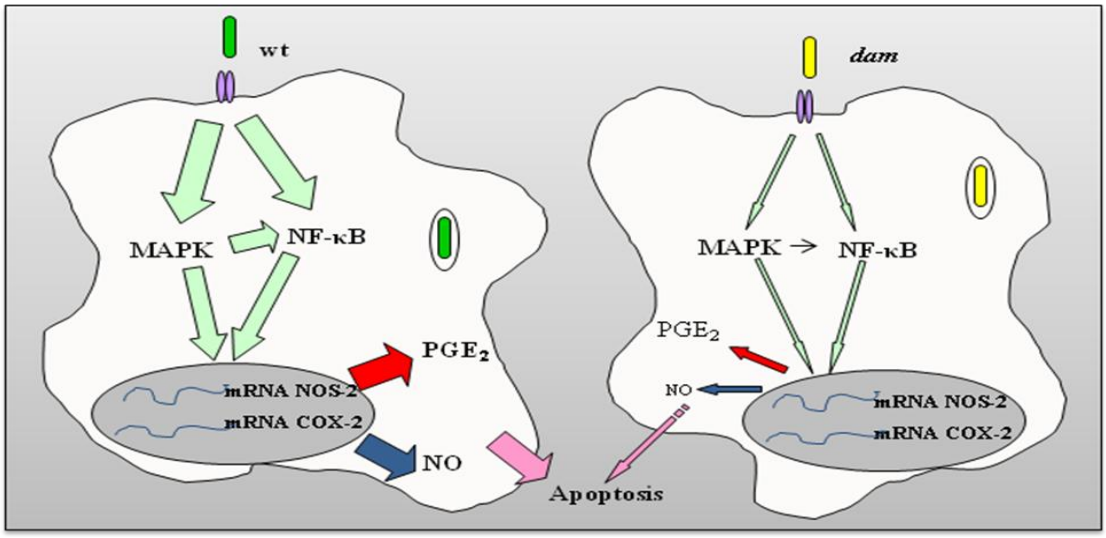
Another relevant defect of *Salmonella dam* mutants that is linked to envelope instability is an increased sensitivity to components of innate immunity including antimicrobial peptides (defensins NP-1 and bactinecin) [15], detergents (bile salts, deoxycholate) [15,17,18,21], and mediators of oxidative damage (H_2O_2) [15,21]. Envelope instability may also lead to an increased release of bacterial antigens.

In summary, the envelope defects, the enhanced sensitivity to innate immune molecules together with massive fimbrial expression on the cell surface discussed above, may contribute to the attenuation of virulence in *S. enterica dam* mutants in animals. Enhanced release of membrane vesicles through infected cells and tissues might cause overstimulation of the host immune response. This ability of *S. enterica dam* mutants to induce strong immune responses in infected animals has been effectively applied to the design of vaccines [11,12,15].

Dam and the secretion of effector proteins

S. enterica employ a type III secretion system (TTSS) to translocate virulence determinants, called effector proteins, from the bacterial cytoplasm into

Figure 2. *S. Typhimurium dam* mutants induce an attenuated inflammatory response in macrophages RAW 264.7.



Dam methylation could participate in the activation of both signalling cascades, MAPK and NFkB, in the inflammatory response induced by *Salmonella* spp. Then, the expression of both NOS-2 and COX-2 and subsequently the production of NO and PGE_2 is significantly reduced in cells infected with *dam* strains with respect to the wild type (wt). In the same way, apoptosis in macrophages treated with *Salmonella dam* mutant is diminished.

the host cell cytoplasm thus increasing their pathogenic potential and the outcome of infection. TTSS involves the coordinated expression of approximately 20 proteins and is therefore regulated by complex mechanisms.

Effects of Dam on TTSS have been observed in *S.* Typhimurium, although the basic regulatory mechanisms have not been elucidated. Several authors observed that both bacteria-associated and secreted proteins are affected by the loss of Dam regulation [11,15,18,38]. A report by Balbontín *et al*. [32] provided evidence that Dam methylation regulates the invasion genes of the pathogenicity island 1 (SPI-1); they proposed a correlation between specific alterations of gene expression and certain virulence defects of *Salmonella dam* mutants. The need for Dam methylation to activate the expression of invasion genes could explain the reduced secretion of SPI-1 effectors such as SipA, SipB and SipC reported earlier [11] and the impaired invasion of epithelial cells [11,38].

Recently, in our laboratory, we demonstrated that Dam methylation regulates synthesis and secretion of the *Salmonella* virulence effector SopA [38]. This finding extends to an effector located outside SPI-1 (and secreted via the SPI-1 TTSS) described in previous reports, indicating that Dam methylation regulates *Salmonella* SPI-1. We observed a significant reduction of SopA synthesis and secretion under SPI-1 conditions in *dam* mutants of *S.* Typhimurium. Interestingly, *dam* mutants expressed SopA under noninducing conditions (28ºC), whereas no synthesis of the effector was observed in the wild type or the complemented strain at this temperature. These results suggest that lack of Dam relaxes the temperature regulation of SopA synthesis. Similar results were observed *in vitro* by SipA, SopB, SopD, and SopE2 effectors of *S.* Typhimurium *dam* strains [46]. These observations are in agreement with previous data obtained from *Yersinia* spp., showing that Dam overproduction leads to the expression and secretion of Yop virulence proteins under nonpermissive conditions [8,40]. These authors demonstrated that Dam overproduction disrupts both thermal and calcium regulation of YopE synthesis and relaxes the thermal (but not calcium) dependence of YopE secretion. Moreover, we observed *in vivo* that intracellular *Salmonella dam* mutants synthesize SopA at lower levels than the wild type strain [38].

In summary, lack of Dam methylation causes an atypical expression of many genes, and these changes have been shown to attenuate the virulence of several pathogens, including *Salmonella* spp., and to confer protective immune responses in vaccinated animals [7]. Dysregulation of Dam activity could lead to ectopic gene expression and the concomitant elaboration of an expanded repertoire of antigens. In addition, the persistence of *dam* mutant vaccines of *Salmonella* spp. in lymphoid tissues, such us Peyer's patches [11,15], may provide a stable source of antigens in sufficient quantity and duration for the transition to the development of potent adaptive immune responses [13,41]. This hypothesis is supported by work with *Salmonella*, wherein loss of

Dam function results in a number of changes in the bacterial physiology. *dam* mutants appear to express *in vitro* a number of genes that are normally only produced *in vivo* during the initiation and progression of bacterial infection [12,15,42]. Such patterns of altered expression and secretion may contribute to attenuated virulence and robust immunity observed in vaccinated animals.

Dam and the inflammatory response in macrophages

The ability of *Salmonella dam* mutants to activate the signalling pathways of the inflammatory response has been investigated in RAW 264.7 cells [43] (Figure 2). The expression of NOS-2 and COX-2, and subsequently the production of NO and PGE_2, is significantly reduced in macrophages infected with *S.* Enteritidis *dam* strains [51]. These results are compatible with studies showing that mice infected with *S.* Typhimurium lacking the Dam protein display reduced induction of NOS-2 and INF-γ compared to wild type infected mice [44,45]. The regulation of NOS-2 and COX-2 expression depend on the activation of NF-κB and alternative signalling pathways such as those involving the ERK1/2 MAP kinases and the family of p38 MAP kinases [46]. At early time points after macrophage infection with *Salmonella dam* mutants, the translocation of p65 to the nucleus is notably impaired and the amount of phosphorylated p44, p42 and p38 is clearly reduced [43]. Taken together, these results suggest that *Salmonella* activation of both signalling cascades is a mechanism that requires Dam protein participation. How *Salmonella* Dam protein participates in the activation of macrophage signaling pathways is not fully understood. During the course of cell invasion, LPS activates cellular signaling pathways leading to changes in the expression of various genes, including those for inflammatory mediators. It is well documented that LPS triggers, among others, the MAPKs and NF-κB pathways in macrophages through the activation of TLR4. Interestingly, we have found that the LPS of the *S.* Enteritidis *dam* mutant shows a high proportion of short polysaccharide chains [Sarnacki *et al.*, unpublished data]. Whether this defective LPS contributes to the attenuated inflammatory response of *dam* mutant-infected macrophages is currently being investigated. Bacterial molecules other than LPS induce macrophage inflammatory response [47]. It has been suggested that *Salmonella* effector proteins such as SopB, SopE2, and certain invasion proteins may be involved in regulating the inflammatory response of infected cells [47,48]. Therefore, it would be likely that the failure of *dam* mutants to induce a whole macrophage inflammatory response correlates with a defective expression of one or more effector proteins; previous works support this hypothesis [11,32]. Moreover, *in vitro* studies using epitope-tagged (3xFLAG) strains of *S.* Typhimurium [49] showed that expression and secretion of several TTSS effector proteins are decreased in *S.* Typhimurium *dam* mutants [38, Giacomodonato *et al.*, unpublished data]. Therefore, the attenuated inflammatory response induced by *dam* mutants could be explained in part to a down-regulated expression/secretion of *Salmonella* effector proteins.

Conclusions

It is clear that Dam methylation is an essential factor involved in the virulence of an increasing number of bacterial pathogens including *S. enterica*. In the near future, research studies will have to identify genes and proteins that are differentially expressed in response to methylation by Dam, together with a careful characterization of regulatory mechanism which mediate these effects in different organisms. This information will contribute to elucidate the different functions of Dam methylation in the intricate regulatory networks underlying bacterial pathogenesis. Finally, the way in which Dam methylation participates in the control and coordination of virulence genes expression is a critical question that deserves further investigation.

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