DNA methylation in mycobacteria: Absence of methylation at GATC (Dam) and CCA/TGG (Dcm) sequences

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Abstract

The presence of 6-methyladenine and 5-methylcytosine at Dam (GATC) and Dcm (CCA/TGG) sites in DNA of mycobacterial species was investigated using isoschizomer restriction enzymes. In all species examined, Dam and Dcm recognition sequences were not methylated indicating the absence of these methyltransferases. On the other hand, high performance liquid chromatographic analysis of genomic DNA from *Mycobacterium smegmatis* and *Mycobacterium tuberculosis* showed significant levels of 6-methyladenine and 5-methylcytosine suggesting the presence of DNA methyltransferases other than Dam and Dcm. Occurrence of methylation was also established by a sensitive genetic assay.

Keywords: **Mycobacteria; DNA methylation; *dam; dcm***

1. Introduction

Tuberculosis has emerged as a major killer disease worldwide. The appearance of multiple drug-resistant clinical isolates of Mycobacterium *tuberculosis* has resulted in renewed interest in the study of biology of mycobacteria. With the application of new genetics, many important biological processes in mycobacteria are being studied [1].

DNA methylation in biological systems influences important cellular functions [2–4]. Methylation of DNA is brought about by DNA methyltransferases which transfer the methyl group from S-adenosylmethionine to specific residues in double-stranded DNA. The methyltransferases in prokaryotes are either part of the host restriction-modification system or independent methylases such as Dam and Dcm. Dam methylase, the product of *dam*, recognises the sequence GATC and methylates adenine at the N6 position [2,5], whereas Dcm methylase, the product of *dcm*, recognises CCA/TGG and adds a methyl group to the internal cytosine at the **C5** position [6]. The Dam methylase mutants exhibit a wide range of phenotypes as a consequence of their effect on mismatch repair, replication reinitiation, transposition, positive and negative regulation of gene expression and packaging of viral DNAs [2,3,7,8]. More recently Dam methylation has been implicated in virulence gene expression [9]. No information is available on Dam/Dcm methylation in the genus mycobacteria. Determination of methylation content and

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a systematic study of methylases in mycobacteria is important considering the biological relevance. Our results indicate the total absence of both Dam and Dcm methyltransferase activities in all the mycobacterial species tested. However presence of methylation at other sites is established by HPLC analysis of genomic DNA and by a powerful genetic screen.

2. Materials and methods

2.1. Bacterial strains, *plasmids* and media

E. coli strain AP1-200-9 was a gift from A. Pieckarowicz [10] and was grown in Luria Bertani broth (LB) supplemented with 1.5 (w/v) of agar. When necessary these media were supplemented with ampicillin, 100 μg/ml and 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal), E. coli strains K704 (F-*rgl*A *rgl*B met-gal-sup11 $r_k^+ m_k^+$), GM119 (F-dcm-6 dam-3 *lac*Z yl *met*1 *gal*K2 *gal*T22 *tx*78 *sup*F44 (*thi*/*tin*A31 mthl), and E. coli DH10B obtained from BRL was used for routine transformations. Mycobacterium smegmatis strains SN2 and mc^{2}6/1-2, and M. tuberculosis H37Ra and H37Rv were used in the present study. *M*. smegmatis mc^{2}6/1-2 and pBAK14 plasmid [11] were gifts from D. Young, UK.

2.2. Preparation of genomic DNA

The DNA from different mycobacteria listed in Table 1 was prepared essentially as described by Srivastava et al. [12].

Table 1
Content of methylated bases in DNAs of different mycobacteria

	mol% 6-methyladenine	mol% 5-methylcytosine
M. smegmatis	0.71± 0.07	0.31± 0.05
M. tuberculosis H37Ra	0.55± 0.07	0.28± 0.03
M. tuberculosis H37Rv	0.45± 0.07	0.57± 0.02

Mycobacterial genomic DNA was subjected to enzyme digestion as described in Materials and methods and analysed by HPLC for detection of modified bases. The values given represent the means ± S.D. for 3 determinations.

2.3. Bacterial transformation

The shuttle plasmid pBAK14 was transformed into different E. coli strains by the calcium chloride method [13] and electroporated into M. smegmatis mc^2 6/1-2 [11]. Plasmid DNA was isolated from E. coli and M. smegmatis strains by alkaline lysis followed by purification on cesium chloride-ethidium bromide density gradients [13].

2.4. Restriction enzyme digestion and electrophoresis

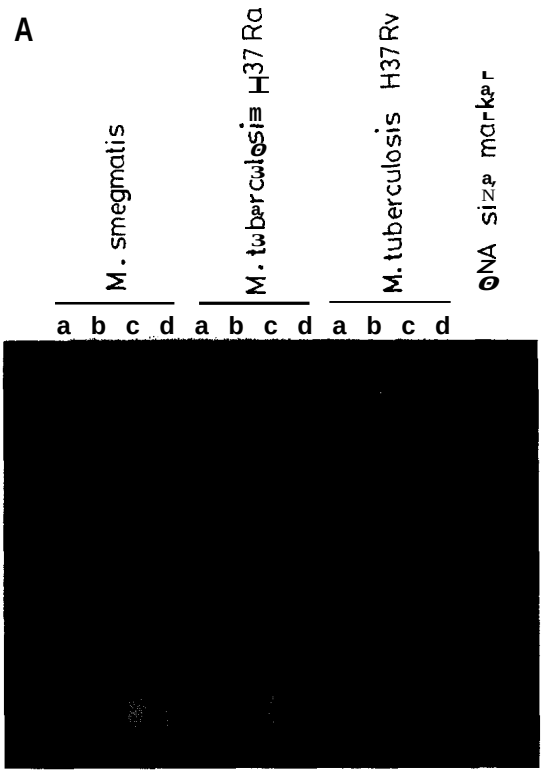
2 μg of genomic DNA was preincubated at 4°C along with 8 units of the appropriate restriction enzymes and gently mixed using a rotary shaker before incubating at the optimum temperature for 3 h. The digested samples were analysed on 0.7% agarose gels. Similarly, 2 μg of plasmid pBAK14 DNA was incubated with 8 units of the enzymes and the digestions were analysed on 4% acrylamide gels. Genomic DNA isolated from E. coli strains served as controls for Dam and Dcm methylation and to optimize cleavage conditions.

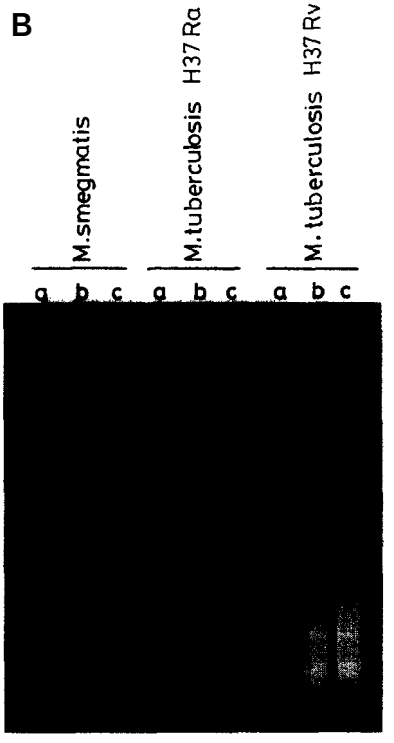
2.5. High performance liquid chromatography analysis

5 μg of genomic DNA was digested with Nuclease P1 for analysis of nucleotides. In order to release nucleosides, 5 μg of DNA was digested with Nuclease P1 and Calf intestinal phosphatase (CIP) or snake venom phosphodiesterase and CIP. The samples were extracted with phenol-chloroform and then analysed by high performance liquid chromatography using RP-18 column equilibrated with 50 mM potassium phosphate buffer pH 5.9 (buffer A). After injection of the samples, the column was washed with 5 ml of buffer A and eluted with a linear gradient of buffer B (buffer A + 50% (v/v) methanol) at a flow rate of 1 ml/min. Absorbance was monitored at 260 nm. The content of methylated bases was determined as described by Eick et al. [14].

2.6. Genetic screen for methylation

E. coli strain AP1-200-9 constructed by Piekarowicz et al. [10] was used to detect in vivo





methylation. This strain of E. coli carries *mcr*A, *mcr*B and mrr temperature sensitive mutations and *lac*Z gene fused to the SOS inducible *din*D promoter of *E. coli.* In the presence of DNA methylating activity, these cells exhibit SOS response as a result of DNA damage caused by the expression of the methyl-directed restriction system at the permissive temperature (30°C). This, in turn, activates the *din*D promoter-*lac*Z fusion, and transformants appear as blue colonies on LB agar plates supplemented with X-gai. In brief, genomic DNA libraries of *M. smegmatis*, *M.* tuberculosis H37Ra and H37Rv were constructed by partial digestion of respective genomic DNA with restriction endonucleases *Bam*HI or *Pst*I and ligating the resulting fragments (size ranging from 1–10 kb) into the corresponding sites of plasmid pUC19. The ligation mixture was transformed into competent *E. coli* AP1-200-9, spread onto LB plates containing ampicillin (100 μg/ml) and X-gal(40 μg/ml), incubated at 42°C for 6–8 h and then shifted to 30°C. The growth was monitored to score for the appearance of blue colonies at 30°C as a result of expression of *lac*Z.

3. Results

A simple approach to determine Dam or Dcm methylation is to examine the susceptibility of genomic DNA to isoschizomer restriction enzymes which show differential cleavage specificity depending on the methylation status of DNA. Enzymes *Sau*3AI, *Nde*II and *Dpn*I all recognise GATC sequence; *Nde*II cleaves when adenine in this sequence is not methylated, while *Dpn*I can restrict only when it is methylated; *Sau*3AI cleaves DNA irrespective of methylation. Similarly *dcm*-modified sites, $C^{m}CA/TGG$, are refractile to *Eco*RII cleavage while the isoschizomer *Bst*NI cuts at both meth-

Fig. 1. Analysis of genomic DNA for Dam (A) and Dcm (B) methylation. A: 2 μg of DNA was incubated with *Dpn*I (lane b); *Nde*II (lane c); *Sau*3AI (lane d) and analysed on 0.7% agarose gel. Lane a: DNA incubated under similar conditions without enzyme. B DNA was digested with *Bst*NI (lane b); *Eco*RII (lane c) and resolved on 0.7% agarose gel. Lane a: Undigested DNA.

ylated and unmethylated sites. The results of genomic DNA digestions with GATC sequence-specific enzymes are presented in Fig. 1A. M. ***smegmatis,*** M. ***tuberculosis*** H37Ra and H37Rv DNA were refractile to cleavage by DpnI but were digested readily with *Sau*3AI and ***Nde*II,** suggesting the absence of Dam methylation. A similar analysis was carried out using enzymes ***BstNI*** and ***EcoRII*** to probe methylation at Dcm sites. The results presented in Fig. 1B show an absence of Dcm methylation since both ***Bst*NI** and ***EcoRII*** digested genomic DNA from all three sources.

The size range of the DNA fragments (< 500 bp) generated in the above experiments rule out the possibility of partial cleavage. However, trace amounts of methylation may go undetected in such analyses of the total genome. Hence, studies were extended to the shuttle plasmid pBAK14 [11]. This plasmid was transformed into ***dam*** + ***dcm*** + (K704) and ***dam*** − ***dcm*** − (GM119) ***E. coli*** strains and electroporated into M. ***smegmatis*** $mc^2 6/1$-2. The plasmids isolated were subjected to isoschizomer restriction analysis. Representative data are shown in Fig. 2A,B. The pattern of digestion with *Dpn*I, ***NdeII*** and ***Sau*3AI** of pBAK14 isolated from M. ***smegmatis*** was identical to that of the plasmid isolated from ***dam*** − ***E. coli;*** the DNAs were completely digested to yield all the expected fragments with enzymes ***Nde*II** and ***Sau*3AI** but were refractile to *Dpn*I cleavage (Fig. 2A). Similar analysis for methylation at CCA/TGG sequences in pBAK14 DNA is shown in Fig. 2B. Complete digestion of the plasmid isolated from M. ***smegmatis*** with ***EcoRII*** was observed (Fig. 2B). These results augment the data obtained with genomic DNA digestions and confirm the absence of Dam and Dcm methylation in M. ***smegmatis*** and ***M. tuberculosis.***

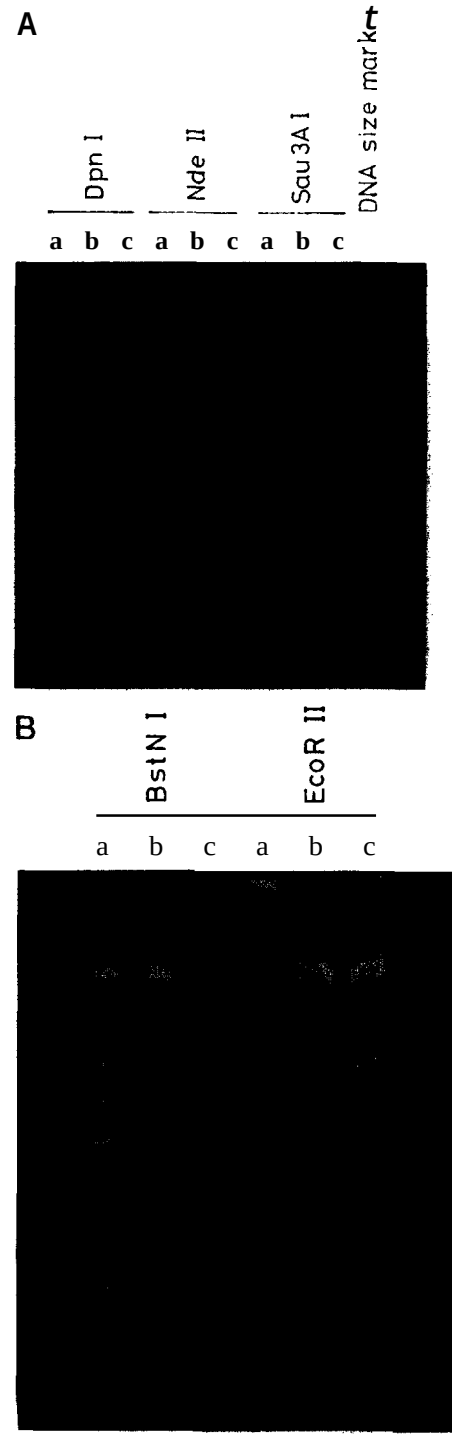


Fig. 2. Restriction digestion of pBAK14 for determination of Dam (A) and Dcm (B) methylation. A 2 μg of pBAK14 DNA isolated from *dam*+ *E. coli* (lane a); *dam*− *E. coli* (lane b); *M. smegmatis* (lane c) was digested with the indicated enzymes. B: 2 μg of pBAK14 DNA isolated from *dcm*+ *E. coli* (lane a); *dcm* − *E. coli* (lane b); *M. smegmatis* (lane c) was digested with *Bst*NI and EcoRII. The DNA fragments were separated on 4% polyacrylamide gel.

Unlike in the members of Enterobacteriaceae, in which Dam methylation is widespread, only few species of ***Bacillus*** and ***Borrelia*** exhibit this activity. We therefore extended the methylation-discriminating isoschizomer analysis to other mycobacterial species such as M. ***avium,*** M. ***phlei, M. gastri, M. terrae, M. uaccae, M. xenopi, M. fortuitum***, ***M. scrofulaceum***, ***M. chelonei abscessus, M. chelonei chelonei, M. nonchromogenicum,*** and ***M. thermoresistibile*** to detect Dam and Dcm methylation. None of the species tested showed Dam and Dcm methylation.

The observation that 'housekeeping' methytransferase activities are absent in mycobacteria led us to examine the content of methylated DNA in these organisms. M. ***smegmatis*** and ***M. tuberculosis*** DNA was subjected to enzyme digestion and the released nucleotides or nucleosides were analysed by HPLC. The mean values of several experiments are presented in Table 1. Both the species showed substantial levels of 6-methyladenine and 5-methylcytosine. The results are in agreement with earlier observations [12]. Furthermore, the existence of methylation in the genomes was confirmed by employing a sensitive genetic screen. The assay exploits induction of SOS response upon DNA damage caused by restriction of DNA by methyl directed restriction system [10]. Thus, when mycobacterial genomic library is transformed into E. ***coli*** AP1-200-9, the methyltransferase containing clones would be a target for ***mcr*ABC** and ***mrr*** system. The damaged DNA in these clones in turn, would elicit SOS response, detected by the appearance of blue colonies as a result of induction of ***din*D-*lac*Z** fusion introduced into the chromosome of the strain. The details of the procedure is given in the Materials and methods section and the results are presented in Table 2. The ***lac*Z** expression in few colonies confirm the occurrence of genomic methylation.

Table 2
In vivo assay for methylation [a]

Genomic library	No. of transformants at 42°C	No. of blue colonies at 30"
1. *M. smegmatis*		
Experiment 1 [b]	3050	3
Experiment 2 [c]	4890	6
2. *M. tuberculosis* H37Ra		
Experiment 1 [b]	3888	4
Experiment 2 [c]	12880	3
3. *M. tuberculosis* H37Rv		
Experiment 1 [b]	2680	8

[a] Genomic library was transformed into *E. coli* AP1-200-9. No blue colonies were obtained in transformations with plasmid pUC19 alone.
[b] *Bam*HI library.
[c] *Pst*I library.

4. Discussion

The results presented in this paper show that the Dam and Dcm methyltransferase activities which, in E. ***coli,*** are responsible for much of the observed DNA methylation, are absent in all the mycobacterial species tested. Since the number of species analysed is not small, it is likely that the absence of Dam and Dcm-specific methylation is characteristic of mycobacteria. Although ***dam*** and ***dcm*** are widely distributed in Enterobacteriaceae, a number of genera belonging to different classes of bacteria are devoid of these genes. ***Bacillus*** constitutes an interesting genus, wherein two species, ***B. popilliae*** and ***B. lentimorbus***, and one strain of ***B. brevis*** (ATCC 9999) possess Dam methylation, while other species tested ***B. cereus, B. licheniformis***, ***B. megaterium, B. pumilis*** and ***B. sphaericus*** were all ***dam*$^-$/*dcm*$^-$** [14]. Similarly in the genus ***Borrelia, B. coriaceae, B. duttonii, B. hermsii, B. turicate, B. parkeri,*** and only three out of the 22 strains of ***B. burgdorferi*** exhibited Dam activity [16]. Bacteria lacking the ***dam*** gene may possess alternate routes for mismatch repair and may operate other mechanisms to regulate replication reinitiation. These alternate strategies could involve novel mechanisms or could still depend on methylation events directed by methyltransferases other than Dam and Dcm. While there is evidence for other mismatch correction routes in some organisms [17], the role of methylation cannot be ruled out in others in view of the presence of methylated bases in the genomes of several bacteria. Significant amounts of 6-methyladenine and/or 5-methylcytosine have been detected in ***B. subtilis*** 168, ***B. breuis,*** A. ***tumifaciens*** and ***S. aureus*** [18].

With the exception of B. ***brevis*** ATCC 9999, where GATC sequences are methylated, others have no detectable Dam and Dcm methylation [15,19–21]. This has led to the suggestion that enzymes of a different specificity but which function similar to the Dam and Dcm methylases may exist in bacteria [20]. The amount of methylation in mycobacteria (Table 1) is comparable to that in E. ***coli*** [2,18] suggesting that methyltransferases other than cognate ones of restriction-modification systems may also be present in mycobacteria.

In the present study, different amounts of 5-methylcytosine were detected between avirulent and virulent strains of M. ***tuberculosis*** (Table 1) although the amount of adenine methylation was approximately the same. These results, the lack of Dcm activity and the presence of 5-methylcytosine in varying amounts, suggest an intriguing possibility of a link between differential methylation and virulence. The recent observation that expression of virulence genes in uropathogenic E. ***coli*** is linked to variation in methylation status of the two Dam sites in the regulatory region of the pap gene cluster may provide a precedent [9]. The cloning and characterization of methyltransferase genes presently underway in our laboratory is an attempt to address this important question.

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