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The effect of methylation on DNA replication in *Salmonella enterica* serovar typhimurium

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Abstract

The DNA adenine methylase of *Salmonella typhimurium* methylates adenine at GATC sequences. Strains deficient in this methylase are not well transformed by methylated plasmids, but unmethylated plasmids transform them at high frequencies. Hemimethylated daughter molecules accumulate after the transformation of dam$^-$ strains with fully methylated plasmids, suggesting that hemimethylation prevents DNA replication. It will also be shown that plasmids isolated from dam$^-$ bacteria are hemimethylated by restriction enzyme digestion. These results may explain why newly formed daughter molecules are not substrates for immediate reinitiation of DNA replication in dam$^-$ bacteria. ***To cite this article: A. Aloui et al., C. R. Biologies 330 (2007).***

Keywords: Dam methylase; *S. typhimurium*; Methylation; Unmethylation; DNA replication

1. Introduction

The biological functions of DNA adenine methylation have been widely investigated in *Salmonella typhimurium*. Mutants lacking DNA adenine methylase show increased spontaneous mutations, moderated SOS induction, enhancement of duplication segregation, inviability of dam *recA* and dam *recB* mutants, suppression of the inviability of the dam *recA* and dam *recB* combinations by mutations that eliminate mismatch repair [1]. *S. typhimunum* dam mutants do not show increased UV sensitivity, suggesting that methyldirected mismatch repair does not play a role in repairing UV-induced DNA damage. *S. typhimunum* dam *recJ* mutants are viable, suggesting that the *Salmonella RecJ* function does not help repair DNA strand breaks formed in the absence of Dam methylation [1]. Plasmid-encoded fimbriae (*Pef*) expressed by *S. typhimurium* mediate adhesion to mouse intestinal epithelium, whose production is an example of methylation-dependent gene regulation [2]. Finally, DNA adenine methyltransferase modulates *Salmonella* virulence in many species, like nematodes and mice [3].

The N^6-methyladenine (6mA) sites are present in the homologous replication origins of five enterobacterial species such as *E. coli*, *S. typhimurium*, etc. [4], suggesting a conserved function for 6mA in bacterial replication. A positive role for dam methylation in *oriC*

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replication is supported by the experimental finding that in vitro DNA synthesis is reduced with unmethylated *oriC* templates such as pOC42 [1,5,6]. The greatly reduced transformation frequencies of *S. typhimurium* strains lacking the Dam methylase (dam$^-$) by *oriC* plasmids pOC42 has also been interpreted as an evidence of a stimulatory role of methylation in *oriC* replication [5,6]. In addition, plasmid pBR322 [7] transforms dam$^-$ strains at reduced frequencies [5,6], although the effect is not as pronounced as with *oriC* plasmids pOC42.

The poor transformation of dam$^-$ strains by methylated plasmids, either carrying the *oriC* origin or not, suggests that the absence of dam methylation can inhibit replication. These results indicate that this origin is not able to function adequately without methylation. The inability of dam$^-$ strains to methylate unmethylated molecules could explain their reduced transformation efficiencies. Transformation inhibition of dam$^-$ strains is shown to be completely reversed when unmethylated plasmids are used and when hemimethylated plasmids accumulate after one round of replication of fully methylated plasmids in dam$^-$ strains. These findings suggest that hemimethylation may play a key role in controlling replication in vivo, providing a mechanism for preventing reinitiation on newly formed daughter molecules.

2. Experimental procedures

2.1. Bacterial strains and plasmids

Table 1 lists plasmids and the genotypes of all strains used in this study. All of the *Salmonella typhimurium* strains originated from Spain.

2.2. Bacterial culture and transformation

The *S. typhimurium* strains used in this study are listed in Table 1. Bacteria were grown routinely at 37 °C in an 8 g l^{-1} fresh sterile liquid medium (Pronadisa, Hispanlab), and aerated by shaking for one night. When necessary, liquid medium was supplemented with ampicillin and tetracycline. Media were solidified by the addition of agar (15 g l^{-1}). Transformation by the calcium chloride method routinely led to 40 ng of plasmid DNA per 200 µl of competent cells [8,9]. Each transformation result in Table 2 is the average of at least two experiments performed with at least two independent isolates of every plasmid type. Competent cells were grown in a 2-fold 8 g l^{-1} sterile liquid medium (Pronadisa, Hispanlab), and transformants were selected on ampicillin and/or tetracycline plates. Plating for antibiotic resistance was performed at the following antibiotic concentrations: ampicillin at 50 mg l^{-1} and tetracycline at 15 mg l^{-1} [10].

2.3. DNA manipulations

Plasmid DNA was purified by the alkaline minipreparation procedure [11]. The DNA concentration was determined by ultraviolet spectrophotometry or by comparison with known standards after gel electrophoresis. The presence or absence of dam methylation was routinely assayed by digestion with *DpnI*. Unmethylated and hemimethylated DNA were found to completely resist *DpnI* digestion, as observed on ethidium bromide-stained gels. Digest was done in a 10-fold concentrated REACT® 4 tampon (invitrogen): [10 mM Tris-HCl (pH 7.4); 400 mM NaCl, 0.1 mM EDTA; 1 mM DTT, 200 µg ml^{-1} BSA; 50% (v/v) glycerol and 0.1% (w/v) TRITON® X-100], and results were analyzed by 0.8% agarose gel electrophoresis.

3. Results

3.1. Transformation with methylated and unmethylated plasmids

Three different plasmids were compared for their ability to transform various *S. typhimurium* strains: plasmid pBR322, plasmid pUC18, and the 6kb plasmid pOC42, which contains the 2kb *PstI oriC* fragment

Table 1
Bacterial strains and plasmids used in this study

Strain or plasmid	Size (Kb)	Relevant genotype or alternate designation	Reference
S. typhimurium strains			
SL1344		Wild type	[19]
SV1610		SL1344 dam-228::Mud*J* Km^r	[19]
Plasmids			
pBR322	4.4	Ap^r Tc^r	[3]
pUC18	2.69	Ap^r	This work
pOC42	6	Tc^r	[1]

Table 2
Results of transformation by the methylated and unmethylated forms of different plasmids

Strain	Number of transformants with pBR322 when	
	methylated	unmethylated
SL1344 (WT)	$_{1}331 \times 10^{5}$	$_{2}139 \times 10^{5}$
SV1610 (dam$^-$)	$_{1'}700$	$_{2'}79 \times 10^{4}$
Strain	Number of transformants with pUC18 when	
	methylated	unmethylated
SL1344 (WT)	$_{3}242 \times 10^{5}$	$_{4}195 \times 10^{5}$
SV1610 (dam$^-$)	$_{3'}1080$	$_{4'}400 \times 10^{3}$
Strain	Number of transformants with pOC42 when	
	methylated	unmethylated
SL1344 (WT)	$_{5}46 \times 10^{5}$	$_{6}50 \times 10^{5}$
SV1610 (dam$^-$)	$_{5'}3$	$_{6'}34 \times 10^{2}$

Table 3
Relative transformation frequencies of various *S. typhimurium* strains by methylated and unmethylated plasmids

Bacterial strains: *S. typhimurium*		
Plasmid	dam$^+$	dam$^-$
pBR322	2.38	8.86×10^{-5}
pUC18	1.24	1.80×10^{-3}
pOC42	0.92	8.8×10^{-2}

The Tf ratios were calculated for each batch of competent cells when transformed with unmethylated and methylated plasmids. The average results of two or more experiments are presented above.

cloned into the *PstI* site of pBR322 [12–14]. The results of transformation by the methylated and unmethylated forms of these plasmids for each strain are presented in Table 2. The frequency of transformation (Tf) was determined in dam$^+$ (SL1344) and dam$^-$ (SV1610) *S. typhimurium* strains. The results are presented as Tf ratios (methylated versus unmethylated transforming DNA) in Table 3.

Table 2 shows that these three plasmids transform dam$^+$ *S. typhimurium* equally well, whether methylated or not (approximately 10^{-5} CFU for each plasmid: values 1 to 6). However, the dam$^-$ strains are not transformed nearly as well by the methylated forms of the three plasmids (values: 2′, 4′ and 6′) compared to their unmethylated counterparts (values: 1′, 3′ and 5′). Table 3 illustrates that the Tf ratios in dam$^-$ bacteria are inferior to those in dam$^+$ bacteria. The values are approximately 10^{-5} for pBR322, 10^{-3} for pUC18 and 10^{-2} for pOC42. Different plasmids were isolated from transformants of each strain and found to be methylated for dam$^+$ and unmethylated or hemimethylated for dam$^-$, by digestion with restriction enzyme (see Experimental Procedures). Because SL1344 and SV1610 are isogenic except for their *dam* loci, the difference in their transformation frequencies must be solely due to the *dam* gene. The absolute level transformation of SL1344 and SV1610 are nearly identical for unmethylated plasmids, except pOC42. This plasmid contains the replication origin of the *Escherichia coli* chromosome (*oriC*), and it is characterized by an elevated number of GATC sequences that are methylated at position N^6 of adenine by the Dam methylase [6,15,16]. This observation has led to an interesting suggestion that transient hemimethylation of these sequences after the initiation of DNA synthesis could make the origin inactive for further initiations. These results are in marked contrast to those obtained by Russell and Zinder [17] with plasmid pSC101. This plasmid transforms dam$^+$ and dam$^-$ *E. coli* at approximately the same frequency, whether methylated or not. Therefore, the decreased transformation of dam$^-$ strains by methylated plasmids is not a general property of all replicons. Because pSC101 has a total of 27 dam sites [18], whereas pOC42 has 33 (22 in pBR322 plasmid and 11 in *oriC* fragment), it must be the particular characteristics of certain dam sites, and not merely their number that affects transformation (at least some of the dam sites responsible for the poor transformation of dam$^-$ strains must therefore lie in the 2kb *oriC* fragment of pOC42).

3.2. Hemimethylated plasmids accumulate in dam$^-$ *S. typhimurium*

The poor transformation of dam$^-$ strains by pBR322, pUC18 and especially by the pOC42 plasmid is a property of fully methylated DNA, but not of unmethylated DNA. In addition, fully methylated or unmethylated DNA can be replicated normally in dam$^+$. In this work, it is shown that dam$^-$ strains should be capable of replicating unmethylated plasmids normally, but

fully methylated plasmids only once, producing two hemimethylated daughter molecules that do not replicate again, because they are not reactivated by Dam methylase. A second round of replication would produce two hemimethylated and two completely unmethylated daughter molecules. We show that fully methylated plasmids are eventually converted to hemimethylated daughter molecules in dam$^-$ *S. typhimurium* and that most of the DNA is resistant to *DpnI* and does not replicate when introduced into dam$^-$ strains.

Competent cells of dam$^+$ and dam$^-$ were transformed with methylated and unmethylated plasmids, and DNA was extracted and analyzed by digestion with restriction enzymes.

Fig. 1 shows the result of such an experiment performed with pBR322. After transformation, plasmid DNA was isolated from different strains and was digested with *DpnI*, which leaves both unmethylated and hemimethylated DNA. After restriction endonuclease digestion, DNA fragments were separated in horizontal 0.8% agarose gel.

Sensitivity to digestion by restriction enzyme *DpnI* indicates that both DNA strands are fully methylated (Fig. 1, lanes 1b and 2b). The intensities of the bands in the two lanes (3b and 4b, Fig. 1) are similar to those in 3a and 4a (Fig. 1), indicating that 4b is completely unmethylated and that 3b shows hemimethylated molecules (made with one methylated strand and one unmethylated strand) that resist digestion by *DpnI*. Thus, fully methylated pBR322 can replicate one round in dam$^-$ bacteria, and the absence of a second round of replication from this methylated plasmid confirms that hemimethylated DNA does not replicate.

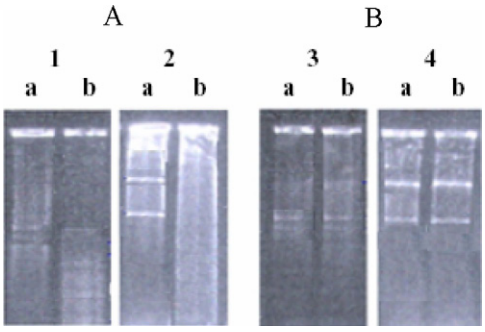

Fig. 1. *DpnI* restriction endonuclease analysis of pBR322 DNA isolated from dam$^+$ (**A**) and dam$^-$ (**B**) strains: lanes **a** were the controls without *DpnI*, lanes **b** were digested with *DpnI*.

4. Discussion

Plasmid pOC42 with the *E. coli* chromosomal replication origin transform dam$^-$ *S. typhimurium* poorly. This result is very similar to that obtained by Russell and Zinder [17], Smith et al. [5], and Messer et al. [6]. It is shown that the greater number of GATC sequences in pOC42 and the particular characteristics of certain dam sites may explain the greater magnitude of the inhibition of transformation observed compared to pUC18 and pBR322. This inhibition of transformation is completely reversed when the transforming DNA is not methylated (Table 2), so it cannot be due to the absence of methylation in dam$^-$ strains. Rather, it must be due to an inhibitory effect of methyl groups present in the transforming DNA. Methylated or unmethylated plasmids transform dam$^+$ strains efficiently (Fig. 2.A.1 and 2), so only using methylated DNA in a dam$^-$ strain results in poor transformation. After putting in dam$^-$ bacteria, fully methylated plasmids replicate one round and are thereby converted into hemimethylated daughter molecules (Fig. 2.B.2), which are refractory to further replication when compared to their unmethylated counterparts [19]. This result agrees with that of Russell and Zinder [17], who found that hemimethylated plasmid (plasmid heteroduplexes with one methylated and one unmethy-

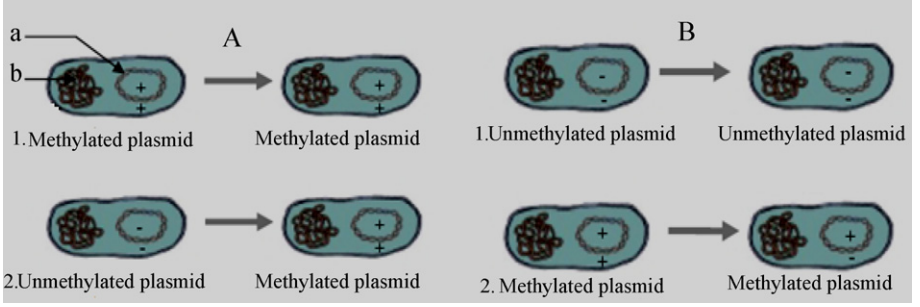

Fig. 2. DNA states in different bacterial strains. (**a**) Transforming plasmid (++: methylated, −−: unmethylated, −+: hemimethylated). (**b**) Genomic DNA. →: After replication. **A**: dam$^+$ strains transformed with: fully methylated plasmid (**A.1**), unmethylated plasmid (**A.2**). **B**: dam$^-$ strains transformed with: unmethylated plasmid (**B.1**), fully methylated plasmid (**B.2**).

lated strand, constructed in vitro) transforms GM2199 (dam^-) at 1/50 the frequency of GM30 (dam^+). Thus, the poor transformation of dam^- strains by methylated plasmids can be explained by the accumulation of hemimethylated molecules, which cannot replicate. Therefore, like fully methylated DNA, hemimethylated DNA transforms dam^- strains poorly. Dam^+ cells can be transformed because methylation of unmethylated DNA creates first hemimethylated and then fully methylated templates for replication (Fig. 2.A.2). Since unmethylated plasmids never become hemimethylated in dam^- strains, they can replicate and transform efficiently (Fig. 2.B.1). The finding that unmethylated plasmids transform dam^- strains at high frequencies conflicts with the results of Smith et al. [5], who found that unmethylated plasmids transformed dam^- *E. coli* strains at lower efficiencies than their methylated counterparts do. However, as was also observed in this study, Messer et al. [6] found that unmethylated plasmids containing the pMB1 replication origin transform dam^- *E. coli* strains better than methylated plasmids. The reason for these differences is unclear. In conclusion, methylation may play a key role in the regulation of DNA replication in vivo, and generally in eukaryotic and prokaryotic cellular processes [20].

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References

[1] P. Hughes, F.Z.S. Houssaini, P. Forterre, M. Kohiyama, *In vitro* replication of a dam methylated and non-methylated oriC plasmid, J. Mol. Biol. 176 (1984) 155–159.

[2] B. Nicholson, D. Low, DNA methylation-dependent regulation of Pef expression in *Salmonella typhimurium*, Mol. Microbiol. 35 (4) (2000) 728–742.

[3] J.P. Oza, J.B. Yeh, N.O. Reich, DNA methylation modulates *Salmonella enterica* serovar *Typhimurium* virulence in *Caenorhabditis elegans*, FEMS Microbiol. Lett. 245 (1) (2005) 53–59.

[4] J.W. Zyskind, J.M. Cleary, W.S.A. Brusilow, N.E. Harding, D.W. Smith, Chromosomal replication origin for the marine *dacterium vibrio harveyi* functions in *Escherichia coli*, Proc. Natl Acad. Sci. USA 80 (1983) 1164–1168.

[5] D.W. Smith, A.M. Garland, G. Herman, E.S. Enns, T.A. Baker, J.W. Zyskind, Importance of state of methylation of *oriC* GATC sites in initiation of DNA replication in *Escherichia coli*, EMBO J. 4 (1985) 1319–1326.

[6] W. Messer, U. Bellekes, H. Lother, Effect of dam methylation on the activity of the *E. coli* replication origin, *oriC*, EMBO J. 4 (1985) 1327–1332.

[7] F. Bolivar, R.L. Rodriguez, P.J. Greene, M.C. Betlach, H.L. Heynecker, H.W. Boyer, J.H. Corsa, S. Falkow, Construction and characterization of new multipurpose cloning vehicles. II. A multipurpose cloning system, Gene 2 (1977) 95–113.

[8] S.N. Cohen, A.C.Y. Chang, H. Leshie, Non-chromosomal antibiotic resistance in bacteria: Genetic transformation of *E. coli* by R-factor DNA, Proc. Natl Acad. Sci. USA (1972) 2110–2114.

[9] T. Maniatis, E.F. Fritsher, J. Sambrook, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 1982.

[10] D. Hanahan, Studies on transformation of *Escherichia coli* with plasmids, J. Mol. Biol. 166 (1983) 557–580.

[11] H.C. Birnboim, J. Doly, A rapid alkaline extraction procedure for screening recombinant plasmid DNA, Nucleic Acids Res. 7 (1979) 1513–1523.

[12] K. Von Meyenburg, F.G. Hansen, E. Riise, H.E.N. Bergmans, M. Meijer, W. Messer, Origin of replication *oriC*, of the *Escherichia coli* chromosome: genetic mapping and minichromosome replication, Cold Spring Harbor Symp. Quant. Biol. 43 (1979) 121–128.

[13] W. Messer, B. Hermann, M. Meijer, S. Hall, Structure of the *E. coli* replication origin *oriC* sequence required for maintenance, establishment and bidirectional replication, Symp. Mol. Cell Biol. ICN–UCLA 19 (1980) 161–169.

[14] A. Landoulsi, P. Hughes, R. Kern, M. Kohiyama, Dam methylation and the initiation of DNA replication on *oriC* plasmids, Mol. Gen. Genet. 216 (1989) 217–223.

[15] K. Sugimoto, A. Oka, M. Sugisaki, M. Takanami, A. Nishimura, Y. Yasuda, Y. Hirota, Nucleotide sequence of *Escherichia coli* K-12 replication origin, Proc. Natl Acad. Sci. USA 76 (1979) 575–579.

[16] M. Meijer, E. Beck, F.G. Hansen, H.E.N. Birgams, W. Messer, K. Von Meyenburg, H. Schaller, Nucleotide sequence of the origin of replication of *Escherichia coli* K-12 chromosome, Proc. Natl Acad. Sci. USA 76 (1979) 580–584.

[17] D.W. Russell, N.D. Zinder, Hemimethylation prevents DNA replication in *E. coli*, Cell 50 (1987) 1071–1079.

[18] A. Bernardi, F. Bernardi, Complete sequence of pSC101, Nucl. Acids Res. 12 (1984) 9415–9426.

[19] A. Landoulsi, A. Malki, R. Kern, M. Kohiyama, P. Hughes, The *E. coli* cell surface specifically prevents the initiation of DNA replication at *oriC* on hemimethylated DNA templates, Cell 63 (1990) 1053–1060.

[20] M. Szyf, L'enzyme qui contrôle le silence des gènes, La recherche de l'actualité des sciences 324 (1999) 56–62.

[21] F.G.P. Portillo, M.G. Pucciarelli, J. Casadesus, DNA adenine methylase mutants of *Salmonella typhimurium* show defects in protein secretion, cell invasio n, and M cell cytotoxicity, Proc. Natl Acad. Sci. USA 96 (1999) 11158–11578.