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Cloning and Preliminary Characterization of a GATC-specific β_2-class DNA:m^{6}A methyltransferase Encoded by Transposon Tn*1549* from *Enterococcus* spp.

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Abstract

A recent study revealed a subfamily of N6-adenine (m^{6}A) methyltransferases that comprises a few functionally studied eukaryotic members acting on mRNA and prokaryotic members acting on DNA as well as numerous uncharacterized open reading frames. Here, we report cloning and functional characterization of a prokaryotic member of this family encoded by transposon Tn*1549* from *Enterococcus* spp.

Key words: DNA methyltransferase, sequence specificity

DNA of prokaryotic and eukaryotic organisms and their viruses is often modified by methylation, carried out by S-adenosyl-L-methionine (AdoMet)-dependent DNA methyltransferases (MTases). In Eukaryota DNA methylation plays a role in crucial regulatory processes, such as regulation of gene expression, embryonic development, genomic imprinting, and carcinogenesis (reviewed by Scarano *et al.*, 2005). Most DNA MTases in prokaryota belong to the restriction-modification (RM) systems, where they serve to protect the host genome against the cleavage by a cognate restriction endonuclease (REase). However, some prokaryotic MTases (here termed "solitary") are not associated with REases and are involved in processes distinct from restriction, such as DNA mismatch repair, regulation of gene expression, and control of timing of DNA replication (reviews: Dryden, 1999; Noyer-Weidner and Trautner, 1993). The best studied solitary MTase is Dam of *Escherichia coli*, which specifically methylates adenine residues within the palindromic sequence GATC, to yield N^6-methyladenine (m^{6}A) (Herman and Modrich, 1982). It is noteworthy that GATC methylation serves as a regulator of gene expression including virulence factors. Accordingly, the Dam activity was found to be necessary for both *in vitro* and *in vivo* virulence of numerous pathogenic bacteria (Chen *et al.*, 2003; Heithoff *et al.*, 1999; Watson *et al.*, 2004).

All known DNA MTases are homologous (*i.e.* they evolved from one common ancestor by accumulating divergent mutations) but their sequences are strongly divergent. One of the characteristic features of DNA MTases is the variability of the linear arrangements of nine common motifs (I–VIII and X). According to the possible linear arrangements of conserved and variable regions, DNA MTases were subdivided into 6 classes: α, β, γ, δ, ε and ζ (Malone *et al.*, 1995). The majority of DNA MTases fall into the classes α, β, and γ (Bujnicki, 2002; Malone *et al.*, 1995). Recently, a subfamily of β-class enzymes was identified that include structurally unusual DNA:m^{6}A MTases M.MunI and M.AvaI as well as m^{6}A MTases acting on mRNA (homologs of the MT-A70 protein) (Bujnicki *et al.*, 2002; Matveyev *et al.*, 2001). These enzymes

Abbreviations: RM, restriction-modification; MTase, methyltransferase; REase, restriction endonuclease; TRD, target recognition domain; m^{6}A, N6-methyladenine; m^{4}C, N4-methylcytosine; m^{5}C, C5-methylcytosine; AdoMet, *S*-adenosyl-L-methionine

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lack the large variable region involved in the target sequence recognition, which is present between motifs VIII and X in the "orthodox" β-class MTases (Bujnicki *et al.*, 2002). For clarity, this subclass of MTases will be referred to hereafter as β_2, as proposed earlier (Matveyev *et al.*, 2001).

Phylogenetic analyses and structure prediction suggested that M.MunI and M.AvaV are related to mRNA MTases more closely than to any other known DNA MTases. Interestingly, these two bacterial MTases are functionally dissimilar: M.MunI is a part of a restriction-modification system (Siksnys *et al.*, 1994) while M.AvaV is a solitary MTase (Matveyev *et al.*, 2001). Their specificity is also different: M.MunI methylates the second adenine in the hexanucleotide CAATTG while M.AvaV methylates the GATC tetranucleotide (*i.e.* exhibits the Dam specificity). Thus, a comprehensive characterization of this intriguing subfamily of DNA MTases requires functional characterization of other prokaryotic members.

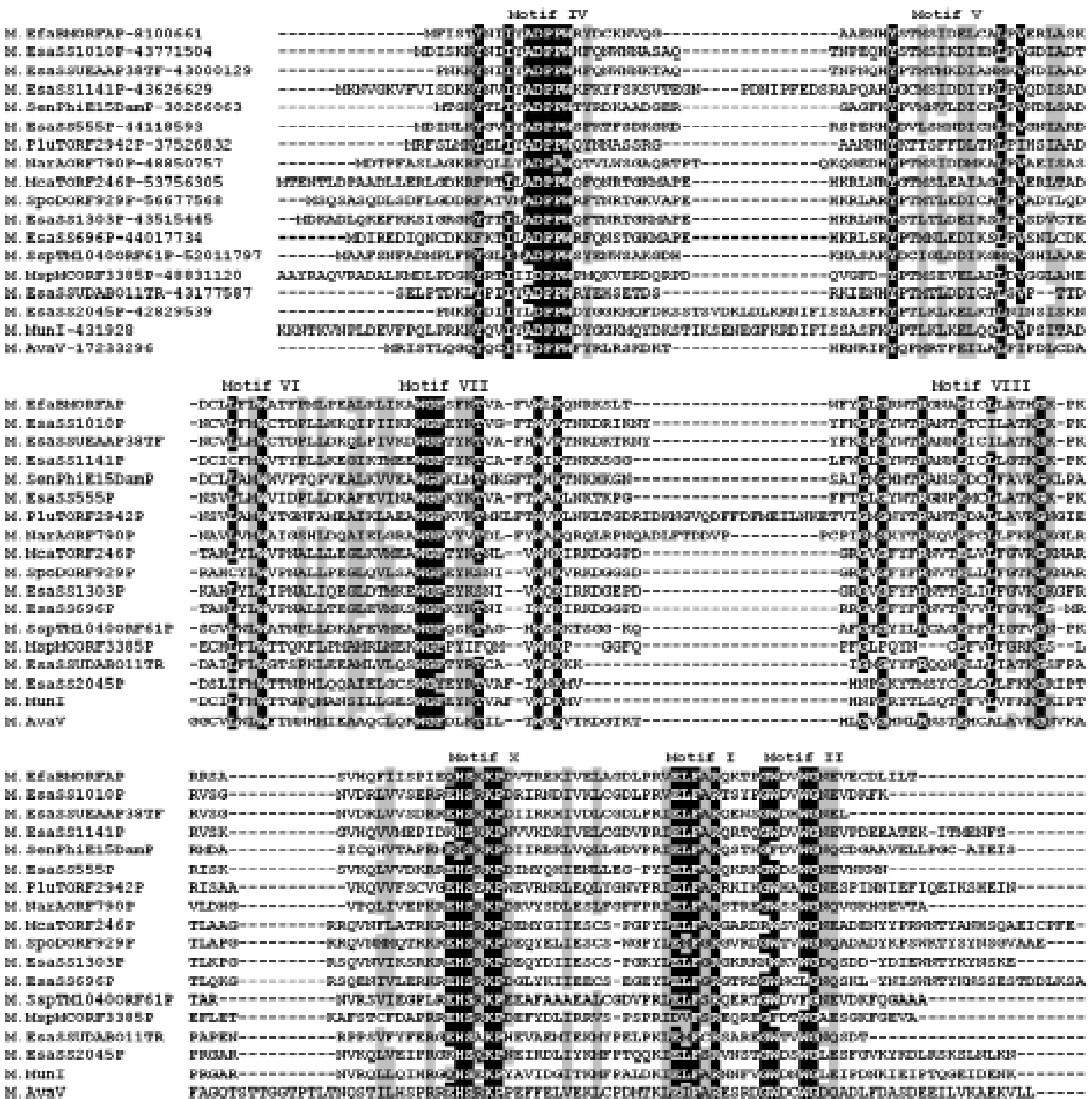

Fig. 1. Sequence alignment of the M.MunI/M.AvaV/M.EfaBMDam family of prokaryotic β_2-class MTases, including many uncharacterized members

Protein names follow the REBASE format, and in the first panel include the Gene Identification numbers (NCBI). N-terminal extensions of M.MunI (15 aa) and MspMCORF3385P (210 aa, including a predicted ParB-like nuclease domain) have been omitted for clarity. Dashes represent insertions or deletions. Identical and conservatively substituted residues are shown on dark background (black and grey, respectively). Conserved motifs (Malone *et al.*, 1995) are indicated; it is noteworthy that motifs III and IX are missing.

Among the proteins closely related to M.MunI and M.AvaI we found an uncharacterized open reading frame (ORF18; dubbed M.EfaBMORFAP) in REBASE (Roberts *et al.*, 2005) encoded by transposon Tn*1549*, which confers VanB-type resistance in *Enterococcus* spp. (Garnier *et al.*, 2000); GenBank Acc.no. AF192329. Neither phylogenetic studies reported earlier (Bujnicki *et al.*, 2002) nor the detailed comparison of sequence similarities between the ORF18 protein and M.MunI or M.AvaI (Figure 1) could reveal whether ORF18 may share sequence specificity with any of these MTases. Therefore, we cloned the corresponding gene and tested its activity *in vivo*.

DNA manipulations, general techniques, and standard reactions were done according to protocols described for *E. coli* (Sambrook *et al.*, 2002) or following recommendation of the enzyme's suppliers. The plasmid expressing the putative M.EfaBMORFAP MTase was constructed as follows: the 444 bp region encompassing open reading frame 18 was amplified by PCR using Tn*1549* DNA from *Enterococcus faecalis* 268–10 and the following primers: Primer Ofr18Nde:
5'-GAAGGAGATATA*CAT**ATG***TTGTTTATTTCAACGTACAACATC-3' corresponds to the 5' terminal part of the *orf18* gene and possesses an optimized Shine-Dalgarno sequence (underlined), the NdeI site (*italic*) with translation codon ATG (**bold**). Primer Orf18Mun:
5'-*CAATTGC***TAC**GTCAGGATAAGGTCACATTCCAC-3' is complementary to the 3' terminal part of the *orf18* gene and possesses a stop codon (**bold**) and the MunI restriction site (*italic*). The amplified blunt-ended PCR fragment was inserted in the SmaI and Ecl136II cleaved pBluescript KSII(+) [Stratagene] generating plasmid pEfaORF18KS carrying the *orf18* gene cloned in-frame with the β-galactosidase promoter of the pBluescript vector. The nucleotide sequence of *orf18* was verified.

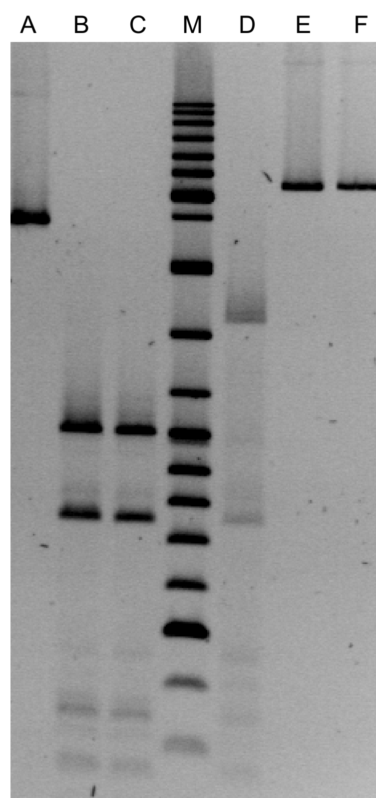

Fig. 2. Cleavage of pEfaORF18KS and pBluescript DNA isolated from *E. coli* GM2163 cells.

Aliquotes of 0.4 μg DNA were digested in 20 μl reaction volumes with 10 u (25-fold excess) of enzymes in buffers recommended by the manufacturers for 8 hrs at 37°C. Lane A: non-digested DNA of pBluescript; lane B: pBluescript digested with MboI; lane C: pBluescript digested with Bsp143I; lane D: pEfaORF18KS digested with Bsp143I; lane E: pEfaORF18KS digested with MboI; lane F: non-digested DNA of pEfaORF18KS; lane M DNA Ladder Mix (GeneRuler™ – Fermentas).

Plasmids encoding an active DNA MTase are methylated *in vivo* and hence resistant to digestion by the corresponding restriction endonuclease, while the DNA from cells lacking the MTase activity are completely cleaved. The degree of resistance of the plasmid DNA to digestion by the restriction enzyme can be used as an indicator of the relative *in vivo* activity of the MTase clones. For this assay, pBluescript plasmid carrying *orf18* gene and the empty vector pBluescript were grown in *E. coli* GM2163 (dam dcm) cells. Plasmid DNA from overnight cultures was prepared using DNA minipreparation kits [Sigma] according to the instruction of the supplier. The purified DNA was tested for sensitivity to cleavage by several restriction enzymes (including MunI and MboI) known to be inhibited by activity of m^{6}A MTases (data not shown). pEfaORF18KS DNA was completely digested with all enzymes used except MboI. The digestion reaction for pEfaORF18KS was repeated using isoschizomeric restriction enzymes MboI and Bsp143I, which differ in their sensitivity to Dam methylation. The cleavage by Bsp143I is not affected by adenine methylation in the 5'GATC3' sequence, whereas MboI depends on by this modification (Hermann and Jeltsch, 2003; McClelland *et al.*, 1994).

The results presented in Figure 2 show that plasmid pEfaORF18KS DNA was totally resistant to cleavage by MboI but sensitive to Bsp143I. On the other hand, the pBluescript vector DNA was cleaved by MboI and Bsp143I, indicating that the cloned insert of pEfaORF18KS exerts the GATC-specific DNA:m^{6}A MTase activity.

In this study, we have cloned and characterized the *in vivo* activity of M.EfaBMORFAP a M.MunI/M.AvaV homolog from *Enterococcus* spp. transposon Tn*1549*. Preliminary studies suggest that the product of *orf18* encodes a DNA:m^{6}A MTase with a Dam-like sequence specificity (GATC) – *i.e.* like M.AvaV and unlike M.MunI. Therefore, we suggest to rename M.EfaBMORFAP as M.EfaBMDam. It will be interesting to determine the function and sequence specificity of other members of the M.MunI/M.AvaV/M.EfaBMDam subfamily. The identification of a Dam-like MTase on mobile genetic element Tn*1549*, which confers antibiotic resistance in clinical isolates of *Enterococcus* spp., suggests that this important determinant of bacterial virulence may be transmitted by horizontal gene transfer. It remains to be determined if M.EfaBMDam may

be essential for the virulence of *Enterococcus* spp. In the light of the fact that Dam MTases are considered as potential drug targets it is important to note that MTases from different classes, such as the "orthodox" Dam from *E. coli* and T-even phages – α class (Herman and Modrich, 1982), Dam MTases from phages such as HP1, VT-2 or T1 (Bujnicki *et al.*, 2001; Piekarowicz and Bujnicki, 1999; Radlińska and Bujnicki, 2001) – γ class and M.EfaBMDam and M.AvaV – β2 class (Matveyev *et al.*, 2001) are structurally very divergent, and therefore may require the development of different specific inhibitors.

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