Cloning and Preliminary Characterization of a GATC-specific β_2 -class DNA:m⁶A methyltransferase Encoded by Transposon Tn1549 from *Enterococcus* spp.

MONIKA RADLIŃSKA^{1,*}, ANDRZEJ PIEKAROWICZ¹, MARC GALIMAND² and JANUSZ M. BUJNICKI³

 ¹ Institute of Microbiology, University of Warsaw, ul. Miecznikowa 1, 02-096 Warszawa, Poland
² Unité des Agents Antibactériens, Institut Pasteur, 75724 Paris Cedex 15, France
³ Laboratory of Bioinformatics and Protein Engineering, International Institute of Molecular and Cell Biology, Trojdena 4, 02-109 Warsaw, Poland

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Abstract

A recent study revealed a subfamily of N6-adenine (m⁶A) 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.

K e y w o r d s: 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 N⁶-methyladenine (m⁶A) (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:m⁶A MTases M.MunI and M.AvaI as well as m⁶A 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; m⁶A, N6-methyladenine; m⁴C, N4-methylcytosine; m⁵C, C5-methylcytosine; AdoMet, *S*-adenosyl-L-methionine

^{*} to whom correspondence should be addressed, e-mail: m.radlinska@biol.uw.edu.pl

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.

			Motif	IV		Notif V	
M. REARHORFAP-91006	61	MFIST	NUTRE	RYDCKNV09	AAENH	STMRIDELCARPER	LASE
M.Esa551010F-43771	504	MDISKR	AT DOUGHN	MECHINERASAQ	THERON	STREEKDING	IADT
M. ESASSUEAAP397F-4	3000129	PURP	IN LUCENCE OF	HPOSTORIKTAQ	THE PROPERTY OF	PENTHEDIAMERIND	TAAD
M.EsaSS1141P-43626	629	MKNVSKVFVISDKK	arviny bioga	KERTESKSVTESN	-PENIPPEDERAPOAH	GCMSIDDIYN#PAOD	TSAD
M.SenPhiE15DemP-30	266063		TLUCTON	TYPEKAADGER	GAGEN	FV66VLDICB-FV9D	LSAD
H. Boass5555P-441185	9.3		or view was also	SFREESDRORD	BSEEKH	OVLANDECHARKON	LARD
M. PluTORF2942P-37526832			STATE STREET	COTHINSSES	AANON	STISFILTS FINS	IAAD
M. NazAORF790P-48850757		MOTPFASLASER	OLLYND PR	OTVINSOLORTPT	OKOORDH	PTHREDEMKARPAR	TRAS
M.McaTORF246P-53756305		MTENTLOPANDLLERLODKRY	and a second	OFOURTORIAPE		OTHER EALAS PARE	URAD
M. SpoDORF929P-56677568		MSQSASQDLSDFLGDDRF	ATTOMOTOR	RETHRICKVAPE	KKRLAR	PINTLEDICACPEAD	агдр
N. E64551303P-43515445			TTULARES	OFTHREWAFE		STLTLDEIRSCHÖSD	TOTE
M.EsaSS696P-44017734		MDIREDIQNCDKN7	KTUL/9033	PEQUISTORMAPE	HKRLSR	PTMALEDIKSPASN	LCDK
N.SeptH104008F61F-52011797		HAAFENFADMPLFP	KALI MARKET	SYNDHSAKODS	KNASAK	рстопрыхамодан	LAAR
M.MspHCORF3385P-48831120		AATRAQVPADALFHDLPDGR	RT III ORN	FINGRVERDORPD	QVGFD-	PTMSEVELAD DAGG	LAHE
M. EsaSSUDAB011TR-43177587		SELPTDKL	(PICTORNAL)	RYERSETDS	RELENH	PTHTLDDICN-SAP-	-TTD
M.E68382045F-42829539		PRKE	DISTREE	DYCCHARGEDRESTSVD	KLULKENIFISSASFK	PTEREKELNDRINS	ISER
M.MunI-431928		KENTEVNPLDEVFPQLPRKEV	SOVER SERVICE	DYGGKHOYDKSTIKSE	NEGFERDIFISSASFE	PTLELKELOO DAPS	ITAD
M.AvaV-17233296		WRISTLQ9Q	QCHIIBRE	FYRLRSEDET	HENRIP	OPERTPETLA SPIPE	LCDA
	Motif	VI Hotif VI	I			Notif VIII	
M.Efashorfap	-DCLOPLZ	TFPHEFEALFLIKA 2000 SFR	WA-FVCLD	QNBRSLT		UPIDRUATIONATHE	R-PR
M.EsaSS1010P	-BCVPTHS	TOPLISKQI PIIKKSPARYK	evo-srz@vie	THEORIENY		WE APPOTCILATE.	8-19K
N.ESASSUEAAP38TF	-BCARTES	TOPLEOROLPIVED.007796	EVA-FHOVO	INEDSTRINT	TFREPS:	WIGARD SICILATED	R-BK
N.Esa551141P	-DCICPH2	TTELLKEOINTHERODATYN	CA-F52IR	TNNK500	Theorem	WINNEFICIATE	R-BK
M. SenPhiElSDamP	-DCLEARCH	VPTOPVEALKVVEA/16 KLMP	nksptehe	THERE GR	GGIA2	INTERNET DOTTAVRE	KLPA
N.Esa3.5555P	-NSVELHE	TIDELLOKAFEVIKAZESKYK	iva-ftead	TNELKEBG	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	MUSHIPMOLATKE	R-BK
M. PluFORF2942F	-HEVGAH?	TORIFAHEAIXLARA (P) SOUR	POIT LA	LNELPSDRIDENSVQDF	FORMETLARETVICES	TT SAMTSDARLAVES	NGIE
M. HazAORF790P	-HAVEVHE	TOSHLOQATELOSA (ILVYV	EL-FYEAD	ORGERPHOADEPTDOVP	PCPTD-63	CONTRACTOR PARTY	ESILR.
M.McaTORF246P	-TABATES	PROXITIE GEOLOGIES (PARTIES	STLVenes	IREDGGED	CDS/Vej	TRUEVENCY TOVEN	993AR
N. SpoDORF929P	-BAHCYLE	PERLEPERLOVISATE YES	NIVEHP	VRIDGGSD		TERMANDELLA FORTES	DOIAR
M.EsaSS1303P	-RAREYIN	PHALIQEGLOTMREADGEVES	NIVAQO	IREDGEPD		TERRETEXLICTOVER	BOFR
M.Esa55696P	TABUTLY	FUALLY EGLEVERS (99) ATE	911-1220	IRIDGGPD	RDS-V91	THREE DAY OF DAYS	S-MR
M.Septel04008F61F	- SCVENEZ.	THELTOKY PARKY OF NOR	NGH28B	KT503-KQ	APRTS)	ITDCV05540104A3	B-BK
M. HSPHOURF3385P	ECHIPTED	TURF DETERMINENTS NATER FT 15	Ver Veru	r Gary	- STRIFT	IN OF VITURAL	See 15
N. KENSSUBARCITTE	-DALUPPLES	VITAL ALL CAME OF CONTRACT OF		к.н	1 K 100	THEODISLIPTICTOR	
M.Esa552045P	-DSLIPHS	THPHLOGAIELOCSARTEYN	VAF-18BB	BIV	HRPSP	TINSTOLOLIPKE	BILPT.
DI DIGNI	-DCIDETING	TOPUNANSI LIGESTINGETIN		019		CITESOTOF VIOLAGE	
H. Avav	accounts.	TUURNERAQCEON	arr1505	VIRDOINT	HERVER	OUT OF THE	BIVKA
		Mat		Martie 1	Motor TT		
M RESERVERS	0023	CINOR TELEVISION	The second	ing and and an installed	and the second second second second		
M. ReadS1010P	RV9G	SUDREAVSERSIS	STAR TRUD	IVELOGDLPRW 46 aA27	AV PRODUCTION NEW DOCT N.		
M. KARRUKARRAFT	RV20		CODITING	IVDLCODLPRIMADO	ENCORPORATION OF URL		
M ReadS1141P	BASE	CONCORD OF PTODIES	NAMES AND	TURLOODVPD TROPAGO	DENTED IN A SECOND FOR S	TEK-TIMENES	
M.SenFhiElSDanF	RHDA		VEDITRES	LVOLLSDVPR D34 LAZO	THE FLY PROCESSA	ELLFOC-AIEIS	
M. Beass5555P	BISE	SVEOLVVDKR BARD	NAND THEY GET	IENGLEG-PYD-MeanO	KRING DOOD NEVNOON		
M. PluTORF2942P	BISAA	VROVVESCVORINE	MANEVROR	LEOLIGINVERLANAUR	THEFTAL	EFICEIKSHEIN	
M. NarACEF790F	VLDB0	VPOLIVEPEREN	SEDEVYSE	LESLFOFFFLEMMAN	TREADSSOUNDVORHOR	WTA	
M.McaTORF246F	TLANG	REVELATERNOLD	NADELC' GI	IESCS-POPYLANAAAA	ARDROSVOP BEADERY	PENETYARESOARIC	PFE-
M. SDODORF929P	TLAPS	RECVERNCTREARING	ASDEOYEL	IESCS-W3PYLOPIOPS	VREBETVERRADADYN	PSWETTSTREEVAAE	
N.Esa551303P	TL&PO	RSQVEVIESEEBARS	UN-DEGYDI	IESCS-POKYLANAOUG	KRUGIZEV ZD DO SDD - YI	IENSTYKYNSKE	
M. Enn59596P	TLONG	ROCENTVLERIERIE	NAPOLYKI .	IEECS-EGEVI-00-6993	TREEPINCLESS SHL -YI	LINNTYINSSEPTDD	LKSA
M. SSpTM104008F61F	TAR	NVRSVIEGPLENKY	W.F.F.AFAA	ARALOGDVPRIMACOO	ERTS/DVT SNEVDSFOR	AAA	
M. MaphCORF3385P	EFLET	FAFSTCFDAPER-IFS	STORFTOL .	IPRVS-PSPPIDV SEE	PERFECTORALSOUPO		
M. ReasSUDAB0117R	PAPEN	RPPSUFYFERGERED	CENEVARIA	TERMYPELPKI, MACOR	ARES TVILLOSDT		
N.E5552045F	PROME	NVKQLWEIPBORGHE	AMELICIA	IYKHYPTQQKI <mark>MA480</mark> V	NETWOOS NELESFOVKS	WELSEKSLELKH	
M.HunI	PRIMAR	NVROLLOINEGONE	REYAVIDG	TTHE PALDET MEASE	NPVER DOCTOR STREET	RIPTOGRIDENK	
M. AvaV	FAGQIST	ROTPTLINGSTILKSPER <mark>ERE</mark>	E PEFFEL/	VERICPONTRIZICASE	SRDSHDCARDOADLYDA	SDEEILVKAEKVLL-	

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 Tn1549, 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'-<u>GAAGGAGA</u>TATA*CATATG*TTGTTTATTTCAACGTACAACATC-3' corresponds to the 5' terminal part of the *orf18* gene and possesses an optimized Shine-Dalgarno sequence (<u>underlined</u>), 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 bluntended 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.

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



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

Aliquotes of 0.4 μ g DNA were digested in 20 μ l 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 Mbol; lane C: pBluescript digested with Bsp1431; lane D: pEfaORF18KS digested with Bsp1431; lane E: pEfaORF18KS digested with Mbol; lane F: non-digested DNA of pEfaORF18KS; lane M DNA Ladder Mix (Carebalart).

der Mix (GeneRulerTM – Fermentas).

for sensitivity to cleavage by several restriction enzymes (including MunI and MboI) known to be inhibited by activity of m⁶A 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:m⁶A 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 Tn1549. Preliminary studies suggest that the product of *orf18* encodes a DNA:m⁶A 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 Tn1549, 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 – β 2 class (Matveyev *et al.*, 2001) are structurally very divergent, and therefore may require the development of different specific inhibitors.

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