Single-Strand Specific, Plasmid Borne DNA Methyltransferases M.BceJIII and M.EcoGIX Regulate Plasmid and Single-Stranded Phage Replication.

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The role of prokaryotic DNA methyltransferases within restriction-modification systems has been well established, while the functional role of the many orphan DNA methyltransferases is still far from clear. Two examples of Dam- and Dcm- orphan DNA methyltransferases have been extensively studied and functional roles in mismatch DNA repair, DNA replication and phase Variation of protein expression have been established (1). We took advantage of the recently developed platform for single-molecule real time sequencing by Pacific Biosciences to investigate DNA methyltransferase specificity. The analysis of total DNA from two pathogenic strains of *B. cenocepacia* J2315 and *E.coli* O104:H4 genomic DNA has revealed the presence of two unusual methyltransferases not previously characterized (2, 3). Both are plasmid-encoded by ORFs in pBCA072 for *B. cenocepacia* J2315 and pESBL for *E.coli* O104:H4. They both result in single-stranded,almost non-specific ^{m6}A modification, within the motif SAB (where S = C or G and B = C, G or T). This methylation is partial and only detected on plasmid DNA. We have called these enzymes M.BceJIII and M.EcoGIX respectively. A set of genetic and biochemical experiments suggested that the activity of these enzymes is associated with plasmid replication and depended on the origin of replication. While ColEI and p15 origins support plasmid modification, the pSC101 origin does not. Moreover, we demonstrated that these enzymes work as a complex with DNA polymerase Iduring plasmid replication and may modify the lagging strand. It is possible they control plasmid and phage replication by discriminating DNA polymerase I-dependent and non-dependent plasmids origins. We suggest that the base flipping inherent to DNA modification may allow the methylase to perform a DNA helicase function and thereby help to control the rate of DNA polymerization to prevent excessive recombination.

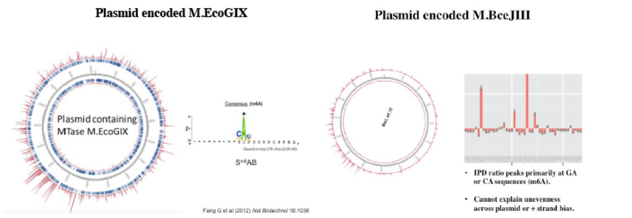

Fig.1 Circle plot of m6A modifications detected across SAB motif on a plasmids carrying M.EcoGIX or M.BceJIII under control of Plac or Ptet promoters respectfully in methylation negative *E.coli* strain ER2796

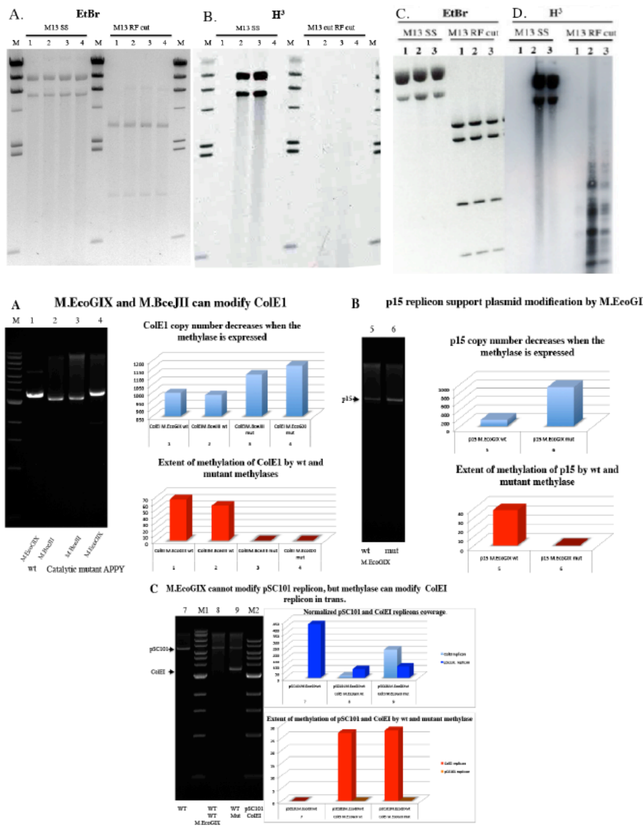

Fig.6 **Effect of origins of replication on plasmid methylation and plasmids copy numbers**. Wild type and APPA catalytic mutant alleles of M.EcoGIX andM.BceJIII have been cloned into ColEI (Panel A), p15 (Panel B) and pSC101 (PanelC) replicons . Plasmids have been transformed in methylase negative strain ER2796 purified and converted to 2kb SMRT libraries by standard PacBio protocol. The copy number of plasmid DNA in each case was estimated by normalization of plasmid mapped reads coverage divided on the level of chromosome mapped reads coverage on a SMRT re-sequencing protocol (blue boxes). Epigenetic modification as a % of S(m6A)B modified motifs was measured as kinetic variations (KVs)in the nucleotide incorporation rates and were deduced from the KV data by SMRT motif and modification analysis protocol (red boxes). Each plasmid preps have been digested with BamHI endonuclease and analyzedby agarose gel electrophoresis (gel panel).

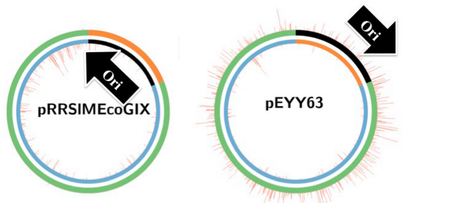

Fig.2. Inverting ColEI origin of replication in plasmids carrying M.EcoGIX allele switches. Plasmid stand m6A modification. Green color indicates (-) strand, blue color indicates (+) strand, ColEI origin of replication highlight in orange color and the arrows indicating direction of plasmid replication.

Fig.5 Methyltransferase activity of *in vitro* synthesized 6xHis-tagged Xa linked MTase polypeptides from PurExpress system (Panel A- ethidium bromide staining and B- H^3 fluorography) and *in vivo* synthesized proteins from T7 express *E.coli* imidazole elution fraction from Ni-NTA beads (Panel C-ethidium bromide staining and D- H^3 fluorography). pSAPv6 vector (1), M.BceJIII wt (2), M.EcoGIX wt (3) and M.EcoIX catalytic mutant (4). M13mp18 RFI forms were digested with NdeI (Panel A and B) or NdeI-BamHI (Panel C and D) after methylation reaction. H^3 radiolabeled markers were produced by modification of l HindIII digested DNA with H^{3}SAM and M.EcoGII.

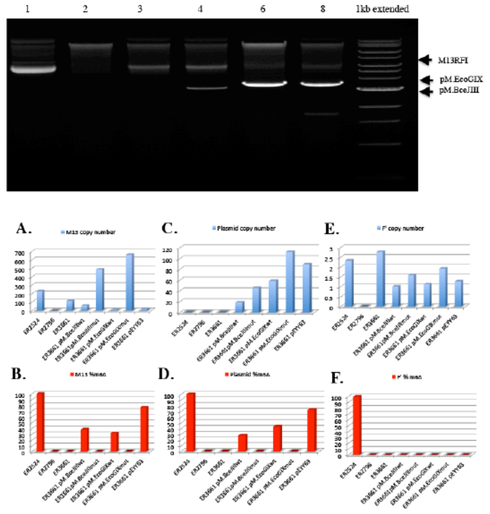

Fig.7. Effect of M.BceJIII and M.EcoGIX MTases expression on replication of replicative forms of M13 (A), plasmids carrying MTase alleles (C), F'factor (E) and modification (B-F) in ER3661 *E.coli* strain. Agarose gel electophoresis of M13 RFI forms (1-8) and plasmids carrying MTase wild type alleles of M.BceJIII (4), M.EcoGIX in pRRSlac (6) and pEYY63 (7) from ER2524 (1), ER2796 (2) and ER3661(3-8) *E.coli* strains.

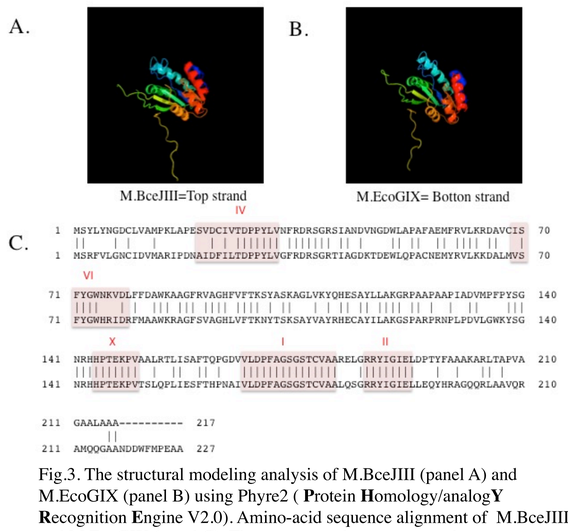

Fig.3. The structural modeling analysis of M.BceJIII (panel A) and M.EcoGIX (panel B) using Phyre2 (**P**rotein **H**omology/analog**Y** **R**ecognition **E**ngine V2.0). Amino-acid sequence alignment of M.BceJIII and M.EcoGIX (panel C). The roman numbers correspondent to conserved motifs in m6A DNA methylases.

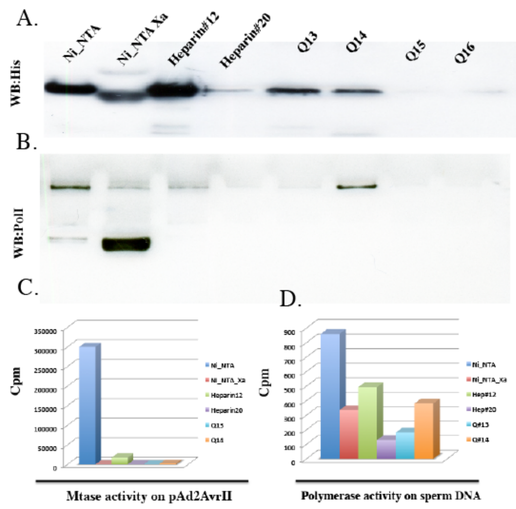

Fig.8. Purification and activity of 6xHisXaM.BceJIII polypeptide from a different column fractions. The presence of MTase in column fractions were monitored by MTase activity (Panel C) or by western blotting with anti- 6xHis mouse antibodies (Panel A). The co-purification of PolAI protein in column fractions was also monitored by polymerase activity (Panel D) and by western blotting with anti- PolAI rabbit polyclonal antibodies (Panel B).

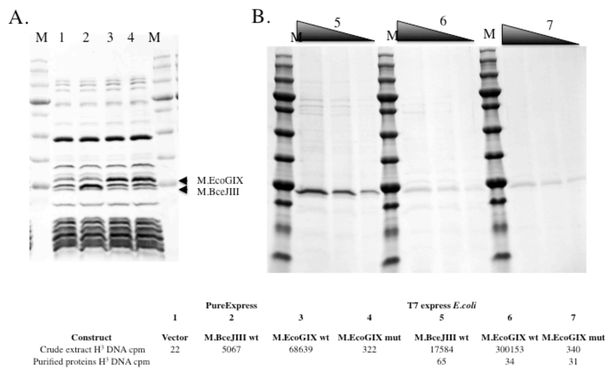

Fig.4. PurExpress (Panel A) vector (1) and 6xHis-tagged Xa linked variants of M.BceJIII wt (2), M.EcoGIX wt (3) and M.EcoGIX catalytic APPA mutant (4). The same variants of MTases were purified from T7express *E.coli* (Panel B) including M.BceJIII wt (5), M.EcoGIX wt (6) and M.EcoGIX catalytic APPA mutant (7). Activity was measured with H^3-SAM in a presence of pSAPV6 constructs as a substrate during protein synthesis in PurExpress or in crude extracts and purified fractions from T7express *E.coli* in a presence of single-stranded M13mp18 DNAs. Proteins expressed in T7 express *E.coli* strain lost activity after three steps of protein

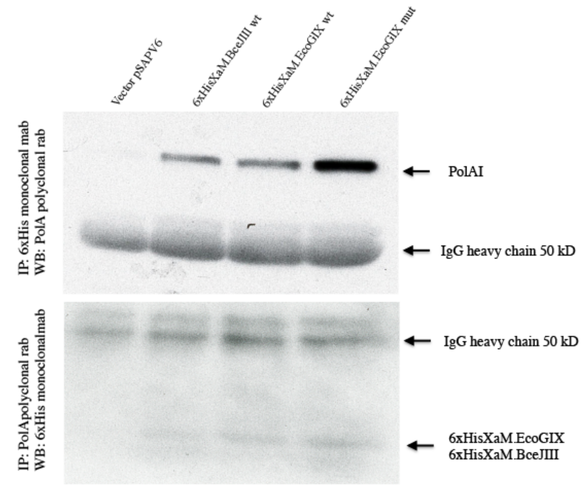

Fig.9. The His-tagged wild type Mtases M.BceJIII and M.EcoGIX and mutant variants have been expressed in ER3081 strain under 80uM IPTG induced cultures. The protein-protein interactions between Mtase and DNA polymerase I complexes were identified using immunoprecipitation (IP) assays of 6xHis epitope-tagged MTase polypeptides with anti-His-tagged monoclonal antibodies followed by western blot visualization with anti-PolAI polyclonal rabbit antibodies (1:5000) or vise versa with IP against PolA with anti-PolAI polyclonal rabbit antibodies followed by western blot visualization with anti-His-tagged monoclonal mouse antibodies (1:1000). The IP complexes were collected on mixture of protein G and protein A magnetic beads followed by detection with the HRP western blot detection system (Cell Signaling Technology, MA).

Reference

(1) Epigenetic Gene Regulation in the Bacterial World. (2006) Casadesús J. and Low D. *Microbiol. Mol. Biol. Rev.* 70(3): 830-856.
(2) Genome-wide mapping of methylated adenine residues in pathogenic *Escherichia coli* using single-molecule real-time sequencing. (2012) Fang, G., Munera,D., Friedman, D. I., Mandlik, A., Chao, M.C., Banerjee, O., Feng, Z., Losic,B., Mahajan, M.C., Jabado, O. J., Deikus, G., Clark, T.A., Luong, K., Murray, I.A., Davis, B.M., Keren-Paz, A., Chess, A., Roberts, R.J., Korlach, J., Turner, S.W., Kumar, V., Waldor, M.K., Schadt, E.E. *Nat Biotechnol*., 30(12):1232-1239.
(3) Molecular dissection of the methylome of *Burkholderia cenocepacia* J2315 (2013). Fomenkov, A., Clark, T., Spittle, K., Anton, B.M., Vincze, T., Korlach, J., Roberts, J.R. FEBS J (compilation of abstracts of FEBS congress) 280, 72-73.