DNA Methylation in Lysogens of Pathogenic *Burkholderia* spp. Requires Prophage Induction and Is Restricted to Excised Phage DNA

M. J. Smith[1] and J. A. Jeddeloh[1,2]*

Orion Genomics, St. Louis, Missouri,[1] and Bacteriology Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland[2]

***Burkholderia mallei*-specific phage ΦE125 encodes DNA methyltransferases in both the lysogenic and replication modules within its genome. Characterization of DNA methylation in recombinant systems, specifically in ΦE125 lysogenic strains of *B. mallei* and *Burkholderia thailandensis*, revealed that, upon induction, cytosine methylation was targeted specifically to the phage episome but not the phage provirus or the host chromosome.**

The prototypic DNA methylation model system is prokaryotic restriction and modification. Host-encoded DNA methyltransferase (DNA MTase) enzymes modify a particular consensus sequence, thereby protecting the host genome from the cognate restriction endonuclease's destruction of unmodified invading viral genomes (13). Recently, DNA methylation in prokaryotes has also been implicated in developmental gene regulation and virulence functions of many pathogens (2, 9, 12, 16). In vitro DNA MTases modify all sites. However, in vivo, some sites remain unmodified (8, 12). Similarly, eukaryotic genomes maintain specific methylation patterns, though their characterized MTases display little site specificity in vitro (1). In any model system, little is known about the regulatory decisions governing modification timing or site selection (1, 5).

Burkholderia thailandensis is a nonpathogenic, gram-negative soil bacterium closely related to the human pathogens *Burkholderia pseudomallei* and *Burkholderia mallei* (3, 4). In a recent report, we described ΦE125, a phage in the *Siphoviridae* (λ-supergroup) family, produced by *B. thailandensis* E125 (22). Of particular interest was the discovery of two DNA MTase genes in both the lysogenic and replication modules of the phage genome. The gene for the DNA MTase located in the lysogenic module (the gp27 gene) encoded an enzyme with homology to MTases that modify the N^6 position of adenine (N^6-methyladenine [m6A]). The replication module-encoded methylase (gp56) was most similar to enzymes that modify the N^4 position of cytosine (N^4-methylcytosine [m4C]). Genomic DNA isolated from the *B. mallei* ΦE125 lysogenic strain BML10, but not *B. thailandensis* E125, contained both m4C and m6A. The expression of the gp27 gene in *Escherichia coli* demonstrated that this gene encodes an m6A MTase. However, the putative MTase gp56 did not display activity in the recombinant system. The present report extends these initial observations and provides a more in-depth analysis of ΦE125 methylation. The cytosine methylation present in the ΦE125 genome after replication in *B. mallei* was restricted to the phage genome. Furthermore, the phage-specific cytosine mod-

ification observed was correlated with the presence of cytoplasmic extrachromosomal phage genomes. The association between phage induction and DNA methylation suggests that there may be an epigenetic aspect to ΦE125 replication.

The focus of this work was the determination of the contributions of MTases to the m4C detected in the host and phage genomes (22). Recent work by Jeltsch and coworkers demonstrated that m6A MTases are also able to methylate cytosine bases, generating m4C (10). Our previous analysis of gp27 in a recombinant system indicated that it had no m4C MTase activity (22). Table 1 outlines the strains, plasmids, and oligonucleotides used. Hypothesizing that host factors may be important for gp56 gene activity, we cloned the MTase genes into the broad-host-range expression plasmid pBHR1 and mobilized them into *B. mallei* ATCC 23344 according to the method described previously by DeShazer et al. (6). A previously described immunoblot analysis (22) was performed with both types of resultant transconjugate clones, and a data set similar to that in our previous report was obtained (data not shown). Recombinant expression of the gp27 gene in *B. mallei* provided m6A to the *B. mallei* chromosome, whereas no effect of gp56 gene expression was detected (22). A BLASTX analysis of the *B. mallei* ATCC 23344 genome indicated that it contained neither DNA m6A MTases nor m4C MTases (data not shown; http://www.tigr.org/tdb/mdb/mdbinprogress.html). Therefore, the only source of these methylases was an integrated *B. thailandensis* provirus; by methylase convention, gp27 was renamed M.BtaI and gp56 was renamed M.BtaII. Expression of the gene in *B. mallei* was not sufficient to obtain M.BtaII activity. Perhaps other phage gene functions were also required, as both BML10 and ΦE125 clearly contained m4C and the *B. mallei* host genome did not.

Next, the extent of m4C methylation present in both the phage and lysogenic strain genomes was determined by McrBC restriction analysis. McrBC detects the methylation of cytosine bases at either position 4 or position 5. McrBC cleaves only DNA methylated at Pu^{m}C dinucleotides, as long as there are two methylated PuC's within 20 bp to 4 kb of each other (7, 14, 15, 19–21). Genomic DNAs from control *B. thailandensis* isolates E27, E125, ATCC 23344, and BML10 and from ΦE125 plate lysate recovered from *B. mallei* were digested with HindIII

* Corresponding author. Present address: Orion Genomics, 4041 Forest Park, St. Louis, MO 63108. Phone: (314) 615-6382. Fax: (314) 615-6975. E-mail: jjeddeloh@oriongenomics.com.

TABLE 1. Strains, plasmids, and oligonucleotides

Strain, plasmid, or oligonucleotide	Description	Reference(s) and/or source
Strains		
E. coli		
TOP10	Cloning strain	Invitrogen
SM10	Mobilizing, *tra* (Km^r)	17
DB24	Lacks all genomic DNA methylation	11
B. thailandensis	Nonpathogenic soil saprophyte	
E27		3, 18, 23
E125		
B. mallei	Soleploid and human pathogen	
ATCC 23344	Human disease isolate; Cm^s Km^s	24; ATCC
BML10	ΦE125-resistant ATCC 23344	22
Plasmids		
pCR2.1	Topoisomerase-mediated cloning, Ap^r Km^r	Invitrogen
pAM1	pCR2.1 *lacZ-gp27* methylase; Ap^r Km^r	22
pCM1	pCR2.1 *lacZ-gp56* methylase; Ap^r Km^r	22
pBHR1	Broad-host-range, mobilizing and expression (*tra oriT* Cm^r Km^r)	MoBiTec
pAM15	pBHR1: p*cat-gp27*; Cm^s Km^r	This study
pAM17	pBHR1: p*cat-gp27*; Cm^s Km^r	This study
pCM18	pBHR1: p*cat-gp56*; Cm^s Km^r	This study
pCM38	pBHR1: p*cat-gp56*; Cm^s Km^r	This study
pDW18	pBluescript KS containing 12,983-bp HindIII fragment from ΦE125; Ap^r	22
Oligonucleotides		
attP F1	GCACGCTGAAGAGTGAGGATG	This study
attP R1	CATACGAGCCAAGGCCCTAAC	This study
16S 5′		3
16S 3′		3
B.th_rDNA F1	CCGGCCTCAACCTGGCAACTGC	This study
B.th_rDNA R1	GAGCCGACATCGAGGTGCCAAACA	This study

alone or HindIII plus McrBC. Southern blot analysis was performed with labeled plate lysate-recovered ΦE125 as a probe (Fig. 1A). Because *B. thailandensis* E27 does not harbor ΦE125, and because immunoblot analysis of its genomic DNA detected no modification (m4C or m6A), it was chosen as a negative control (22). However, E27 genomic DNA was further digested by added McrBC (Fig. 1B), indicating that its genome contained m5C (i.e., m4C that was antibody negative and McrBC positive). The phage genome was extensively digested by added McrBC, indicating cytosine methylation (Fig. 1B, lane 11). Interestingly, the results from the E125 and BML10 lysogens were different. The provirus present in the uninduced E125 genome was not methylated (the HindIII digestion pattern matches the digestion pattern of HindIII plus McrBC [Fig. 1B, compare lanes 5 and 6]). Although the phage present in BML10 was modified, it appeared to be less modified than purified phage genomes alone (Fig. 1B, compare lanes 7 and 8 to lanes 10 and 11). Notice that the mode of the ethidium bromide fluorescence appears to suggest that the genomic DNA from E125 was actually overloaded relative to that from BML10 (Fig. 1B, compare lanes 5 and 6 to lanes 7 and 8). Conversely, the intensity of the phage-specific restriction fragment hybridization was more abundant in the sample from BML10 (Fig. 1A, compare lanes 5 and 6 to lanes 7 and 8). Furthermore, it appears that the genomic HindIII junction fragments are of a different stoichiometry than that of phage-

derived internal HindIII fragments present in BML10 (Fig. 1A). E125, on the other hand, displayed fragments of similar intensities (compare lanes 5 and 7). The presence of phage episomes in BML10 may explain both the nonstoichiometric ratio of junction fragment hybridization and the increased amount of phage-specific restriction fragment hybridization. To be consistent with this hypothesis, excised cytoplasmic viral genomes would yield HindIII-specific restriction fragments that are shared with purified virus (Fig. 1A, compare lane 5 to lanes 7 and 10). An ~13-kb HindIII fragment contains the *attP* integration target (22).

Figure 2 depicts confirmatory results from the search for episomal phage genomes. Briefly, oligonucleotide primer sets specific to the phage genome, outside the integrating *attP* region, were designed to amplify the entirety of *attP*. If the phage present were integrated into the chromosome, the primers would be pointing away from each other and no PCR product would be observed. PCR amplification products were analyzed after electrophoresis. Positive controls for the ~800-bp amplicon were purified phage genomes (Fig. 2, lane 5) and a recombinant plasmid clone bearing the *attP* region of the ΦE125 genome (lane 6). Both gave the predicted product, as did BML10 (lane 4), indicating that, in the preparations of genomic DNA, unintegrated viral genomes were present. The *cos* site of the phage was found to anneal at 37°C, making genomic circles likely in vivo (22). DNA purified from E125 with the same MasterPure (Epicentre, Madison, Wis.) protocol yielded no *attP*-specific PCR product (lane 2), suggesting that the only copies of the phage present were integrated. These observations, along with the results of the Southern blot analysis shown in Fig. 1, suggest that the PCR product detected in BML10 probably results from extrachromosomal cytoplasmic phage rather than incidentally purified phage from residual culture supernatants. This outcome is probable because uninduced E125 produces spontaneous phage, yet the genomic DNA protocol utilized did not purify enough cytoplasmic phage to foster PCR amplification (Fig. 2, lane 2). The presence of the unintegrated phage genomes (BML10) correlated with the presence of methylated phage genomes (Fig. 1) (22).

A question remaining was whether the m4C present in the ΦE125 genome extended to the host chromosome. Figure 1B suggests that the BML10 host chromosome is not appreciably methylated, since the mode of the HindIII digestion ethidium bromide staining was unaffected by McrBC treatment (compare lanes 7 and 8). Replicate Southern blots, with probes specific to large *B. mallei* chromosomal regions (the rRNA genes), were analyzed in a preliminary follow-up experiment. If the chromosome were methylated, then adding McrBC to any digest would alter the restriction pattern. The restriction analysis and Southern blot analysis both suggested that substantial cytosine modification of the chromosomes did not occur (data not shown).

The observation of methylated extrachromosomal phage DNA from BML10 but not from uninduced E125 suggested that phage replication induction may be connected to the DNA methylation observed. This possibility was explored by using genomic DNA recovered from duplicate E125 cultures grown in parallel that had been either UV induced or uninduced under our previously described induction conditions (22). A control purification of any residual cell-free (or phage) geno-
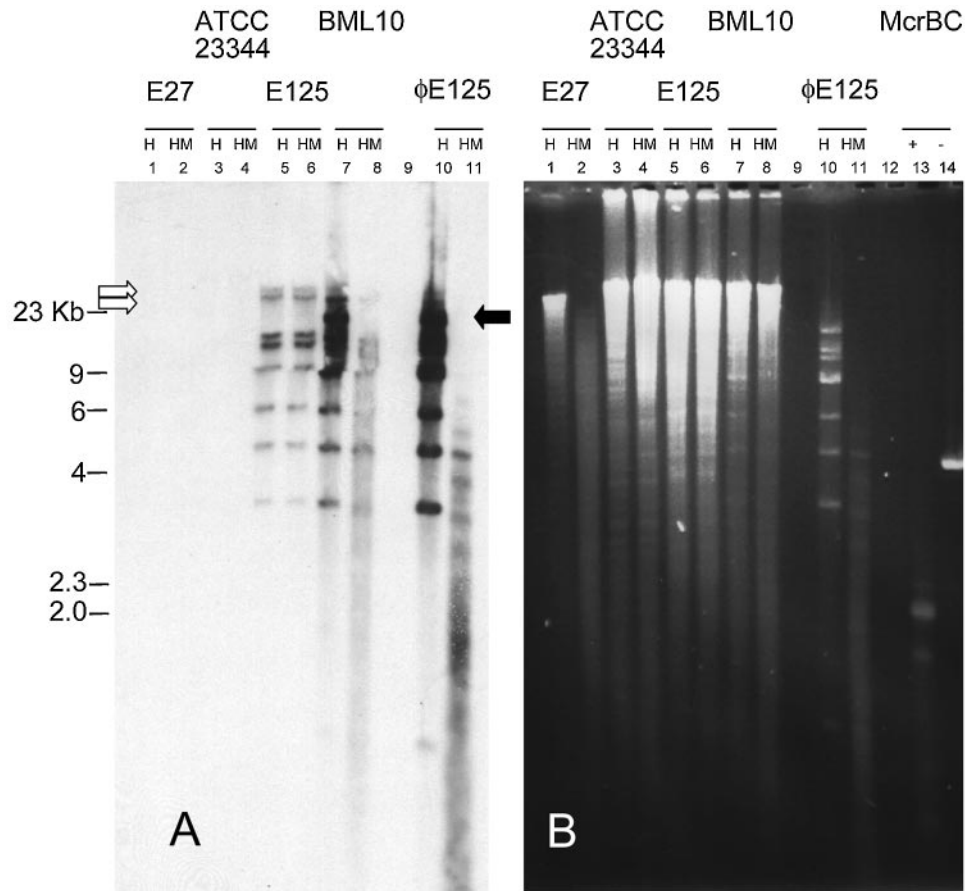
FIG. 1. Southern blot (A) and electrophoretic (B) analysis of the M.BtaII-induced methylation of ΦE125. (A) Approximately 50 μg of the indicated genomic DNA and ~10 μg of ΦE125 purified from plate lysates were subjected to digestion by HindIII (H) according to the NEB-specified conditions. (B) Subsequently, half of each reaction mixture was removed and digested with McrBC (HM) under NEB-specified conditions. The DNAs were blotted by using the alkaline transfer protocol of the TurboBlotter (Schleicher & Schuell, Piscataway, N.J.) and photoimmobilized. The ECL probe (ΦE125::HindIII) was synthesized, hybridized, and detected according to the instructions specified by Amersham. The positive controls for McrBC digestion were E27 genomic DNA (lanes 2) and the NEB-supplied positive control (lane 13). The empty arrows indicate the chromosomal junction fragments and the host chromosome, while the filled arrow indicates the fragment that originates from excised viral genomes.

mic DNA was also attempted by using the culture supernatants as the DNA source (i.e., DNA was purified from cell pellets and supernatants). Following purification, the DNA was subjected to the same *attP* (extrachromosomal phage)-detecting PCR assay, as described for Fig. 2, with one modification. Approximately 1 μg from each UV-induced genomic DNA preparation was either digested with McrBC or mock digested and then subjected to limited rounds of PCR amplification (20 cycles). In this way, any difference in concentration of the amplification product between treated and untreated samples may be interpreted as indicating methylation content. The use of a sensitive detection system like PCR allowed investigation of the medium supernatant in parallel, affording us the ability to eliminate the possibility that contamination of the cell pellet with residual phage could serve as an explanation for any methylated genomes detected. Representative results are depicted in Fig. 3. Not only did the induction of phage replication from E125 by UV treatment foster the cytoplasmic accumulation of extrachromosomal phage, but those accumulated phage were also methylated, since McrBC treatment suppressed the accumulation of the PCR product by more than 50% (Fig. 3, compare lane 1 to lane 2 and lane 3 to lane 4).

Again, as in the experiment in which BML10 was the host, M.BtaII methylation in the E125 chromosome was apparently excluded, since PCR amplification using primer sets designed for the *B. thailandensis* 16S rRNA genes, as well as an ~5-kb segment targeting both the 16S and 23S RNA, was found to not display a difference between McrBC-treated and untreated fractions. Similarly, as predicted by the results shown in Fig. 1B, McrBC treatment had no effect upon genomic restriction fragment distributions obtained from UV-induced E125 (data not shown).

It is currently unclear how the phage selectively modified only its own genome. M.BtaII, either independently or in connection with other phage components, was able to distinguish between the viral genomes (the extrachromosomal phase) and the host chromosome. One simple explanation of the preferential phage episome methylation by M.BtaII would be based on a paucity of chromosomal sites relative to abundant phage sites and a connection of methylase expression with phage induction.
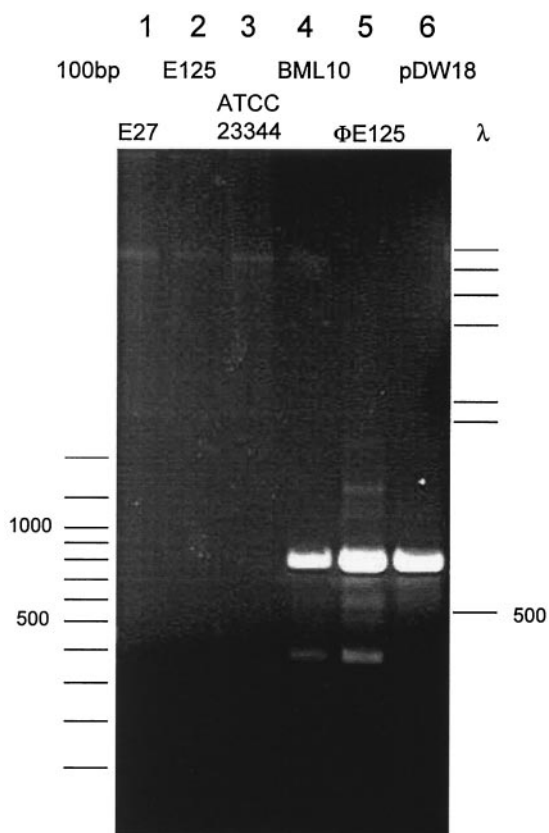
FIG. 2. PCR analysis reveals extrachromosomal phage genomes. Following PCR amplification using 15-s incubations, a 58°C annealing temperature, and 35 cycles, loading dye was added to each 50-µl reaction mixture and 10 µl was loaded and visualized after electrophoresis. Negative controls in this analysis were *B. thailandensis* E27 and *B. mallei* ATCC 23344, as they lack ΦE125. Positive controls were the lysate-recovered ΦE125 DNA and a recombinant ΦE125 plasmid subclone (pDW18) that carries the HindIII fragment containing *attP*. Because these primers lie in the phage genome outside the tRNA gene encoded by *attP*, the amplicon is phage specific and can detect the presence of only unintegrated phage genomes. Both BML10 and E125 were previously demonstrated to contain integrated ΦE125 at the genomic tRNA homologous to *attP* (22). Phage genome methylation correlates with the presence of the unintegrated phage genomes.

A lack of chromosomal M.BtaII sites in the *B. mallei* and *B. thailandensis* regions explored by blotting and PCR assays is not a likely explanation for the observation for several reasons. First, these genomes are enormous (~6 to 7 Mbp) and have a high G+C content. Importantly, the G+C contents of the phage and *Burkholderia* sp. genomes are only modestly different, and both have abundant acceptor cytosines (over 60% G+C) (22). Second, McrBC restriction analysis suggests that the M.BtaII site occurs frequently in the phage genome. There was no 5-kb or larger segment of the phage genome that fully survived cleavage by McrBC (Fig. 1). The mode of restriction fragments obtained from the viral genome following McrBC treatment was approximately 1 kb (Fig. 1). Given that McrBC requires two methylated RC motifs to observe cleavage, this result suggests that M.BtaII has a frequently occurring recognition sequence, perhaps 4 bp or less, because nearly every HindIII fragment was eliminated with McrBC treatment. In order for the HindIII restriction pattern obtained from the

BML10 genomic DNA not to be affected by the McrBC treatment, the cooccurrence of M.BtaII sites with HindIII sites in both chromosomes would have to be less frequent than one event every 23 kb (the limit of gel resolution), such that no chromosomal small HindIII fragments were cooccurring within M.BtaII restriction sites, implying a >8-bp recognition site for M.BtaII. No described 7- to 18-bp C-containing palindromic sites exist in the phage genome at the density necessary to support this hypothesis (~2/kb) (data not shown).

Our group previously reported that induced E125 produced about twice as many phage as BML10. One way to unify this observation with these data, which suggest that BML10 produces more phage episomes, is to posit that BML10 may make as many phage as UV-induced strain E125, but for some reason BML10 is less capable of exporting virions, which leads to their cytoplasmic accumulation.

The necessity of the methylation modification must be governed by the interaction of the virus with its host, as the uninduced *B. thailandensis* E125 provirus was unmethylated yet still produced infectious phage (22). In certain hosts, molecularly differentiating between the proviral and any extra-
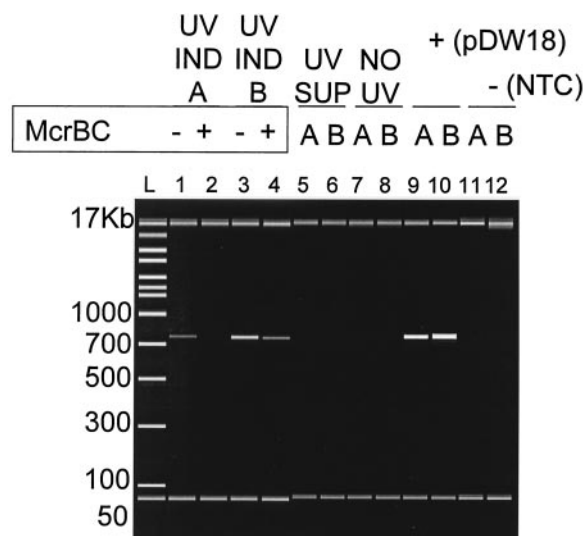

FIG. 3. Induction of replication triggers methylated extrachromosomal phage accumulation in E125. Depicted is a representative digital gel output from an Agilent 2100 bioanalyzer using the DNA 12000 chip system. One microgram of genomic DNA from each of the indicated genomic DNAs was either digested with McrBC or mock digested under the NEB-suggested conditions. A total of ~100 ng of each was then subjected to a limited PCR. Following PCR amplification using 15-s incubations, a 58°C annealing temperature, and 20 cycles, 1 µl of each 25-µl PCR mixture was loaded onto an Agilent 2100 bioanalyzer, which can size and quantify reaction products. Negative controls in this analysis were a water control (NTC) (lanes 11 and 12), any genomic DNA purified from the indicated cell supernatants (UV SUP) (lanes 5 and 6), and uninduced *B. thailandensis* E125 (NO UV) (lanes 7 and 8). The positive amplification control was a plasmid subclone (pDW18) that carries the HindIII fragment containing *attP* (lanes 9 and 10). Because these primers lie in the phage genome outside the tRNA gene carried by *attP*, the amplicon is phage specific and can detect the presence of only unintegrated phage genomes. Both BML10 and E125 were previously demonstrated to contain integrated ΦE125 at the genomic tRNA homologous to *attP* (22). Induced (actively replicating) phage genome methylation correlates with the presence of the unintegrated phage genomes. UV IND, UV induction.

chromosomal phage genomes must be important for some aspect of the phage life cycle and likely connected with the rate of virus replication. Methylation of infectious phage may confer protection against other host restriction systems or modify the expression of viral genes for expression in other hosts. The connections among DNA methylation, extrachromosomal phage replication, and the partitioning of target sequences suggest that this system shares features with not only prokaryotic but also eukaryotic DNA methylation systems. Future studies would likely be aimed at understanding the phage's selective cytosine methylation-targeting mechanism, directly demonstrating the enzymatic activity of M.BtaII, and exploring the potentially epigenetic aspect to extrachromosomal phage replication.

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