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Methylation-dependent DNA Restriction in *Bacillus anthracis*

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Abstract

Bacillus anthracis, the causative agent of anthrax, is poorly transformed with DNA that is methylated on adenine or cytosine. Here we characterize three genetic loci encoding type IV methylation-dependent restriction enzymes that target DNA containing C5-methylcytosine (m5C). Strains in which these genes were inactivated, either singly or collectively, showed increased transformation by methylated DNA. Additionally, a triple mutant with an ~30-kb genomic deletion could be transformed by DNA obtained from Dam⁺Dcm⁺ *E. coli*, although at a low frequency of ~10⁻³ transformants/10⁶ cfu. This strain of *B. anthracis* can potentially serve as a preferred host for shuttle vectors that express recombinant proteins, including proteins to be used in vaccines. The gene(s) responsible for the restriction of m6A-containing DNA in *B. anthracis* remain unidentified, and we suggest that poor transformation by such DNA could in part be a consequence of the inefficient replication of hemimethylated DNA in *B. anthracis*.

Keywords

Restriction; Methylation; *Bacillus anthracis*; Transformation; Plasmids

1. INTRODUCTION

Restriction-modification (R-M) systems constitute a major barrier to the transformation and genetic manipulation of bacteria. These systems are ubiquitous in Eubacteria and Archaea, as is evident from the ever-growing restriction enzyme database, REBASE (Roberts et al., 2010), available online: <http://rebase.neb.com>. Restriction endonuclease enzymes (RE) are classified into four groups, IV, II, III or I, in order of increasing biochemical complexity. All REs and the corresponding DNA methyltransferases (MTases) of the above types have site-specific DNA-binding activities. Restriction endonucleases of types I, II (except for subtype IIM), and III cleave within the specific recognition sequences unless these contain methylated adenine or cytosine residues. Methylation of adenines or cytosines within the recognition sequences by the cognate MTases ensures the protection of host DNA from restriction activity.

The type IV, or methylation-dependent restriction enzymes (MDREs), have a behavior that is opposite that of the other types, in that they cleave only when bases within the recognition sites are methylated. The bacteria encoding these enzymes do not have an MTase associated

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with the RE. The *Escherichia coli* McrBC enzyme, the best studied of the type IV REs and the only one that is commercially available, requires two purine methylcytosine/hydroxymethylcytosine sites separated by 40–3000 base pairs for cleavage (Stewart and Raleigh, 1998; Sutherland et al., 1992). Two other MDREs in *E. coli* are Mrr and McrA. The Mrr enzyme digests DNA containing methylated adenine or cytosine residues while the McrA enzyme is specific for C5-methylcytosine (m5C). The precise sequence requirements of other MDREs remain unknown. The type IV REs are relevant to genetic manipulations in many bacterial species including *E. coli* because transforming DNA prepared from standard *E. coli* strains is methylated. *E. coli* contains three site-specific methylases, of which two are particularly relevant for our work. The Dam methylase (M. EcoKDam, herein termed Dam, encoded by *dam*) acts on A in the sequence GATC, while the Dcm methylase (M. EcoKDcm, herein termed Dcm, encoded by *dcm*) acts on the second C in the sequences CC(A/T)GG. Sites methylated by the third methylase, M. EcoKI, occur rarely and are unlikely to play a significant role in influencing the transformability of DNA. Reviews of bacterial R-M systems are available (Bickle and Kruger, 1993; Tock and Dryden, 2005).

Several *Bacillus* species are known to restrict methylated DNA (Macaluso and Mettus, 1991). A systematic study of the restriction activities of *Bacillus anthracis* was first undertaken by Marrero and Welkos (Marrero and Welkos, 1995). From this study, it became clear that shuttle vectors (specifically, plasmids pLTV1 and pHV33) propagated in Dam methylation-proficient *E. coli* (e.g. HB101) could not transform *B. anthracis*. However, the same vectors prepared from a *dam*[−] *dcm*[−] strain of *E. coli* (e.g., GM2929) were capable of transforming *B. anthracis* at reported efficiencies of 10²–10³ transformants per µg DNA. Additionally, the authors observed that the same plasmids isolated from *B. subtilis* could also be introduced into *B. anthracis* at comparable transformation efficiencies. From the known R-M properties of the strains of *E. coli* and *B. subtilis* used as sources of shuttle vectors, the authors concluded that: (a) *B. anthracis* encodes REs that degrade Dam-methylated DNA (i.e., containing m5A) and (b) it was unlikely that *B. anthracis* encodes REs directed against M. EcoKI- or Dcm-methylated DNA (containing m5C). These findings paralleled those of the earlier study of plasmid transformation of *Bacillus thuringiensis* (Macaluso and Mettus, 1991), except that for this species there was evidence for restriction of both adenine and cytosine-methylated DNA.

Studies in this and other laboratories over a number of years have confirmed that efficient transformation of *B. anthracis* requires that DNA be propagated in DNA methylation-deficient *E. coli*. Typically, DNA is prepared from *E. coli* SCS110, GM2163, or similar strains that are both *dam*[−] and *dcm*[−]. However, certain plasmids propagate poorly in *dam*[−] *E. coli* strains, most likely because the hemimethylated DNA formed in *dam*[−] strains after the first round of replication is not a good substrate for subsequent replication (Aloui et al., 2007; Marinus and Casadesus, 2009; Russell and Zinder, 1987). For the ColE1 plasmid, it is known that this replication block is due to hemimethylation at promoter sequences from which the RNA primer for leading-strand synthesis (RNA II) is transcribed (Patnaik et al., 1990). Additionally, *dam*[−] strains of *E. coli* have an increased rate of mutation owing to the lack of post-replicative mismatch repair (Glickman, 1979; Marinus and Morris, 1974).

For the reasons noted above, we wished to better understand DNA restriction by *B. anthracis*, and to create, if possible, a plasmid-free (avirulent) strain with a significantly lower level of DNA restriction. Such a strain could serve as a host for plasmid constructs intended for later transfer into restriction-competent strains of *B. anthracis* without passing through an intermediate *E. coli* host. During the course of our studies, we determined that *B. anthracis* encodes multiple type IV restriction enzyme activities and restricts DNA containing methylated cytosine residues, a phenomenon that was earlier considered unlikely (Marrero and Welkos, 1995). We also created a strain with disruptions

in three, and possibly four, known MDRE genes that showed reduced restriction activity, such that it can reproducibly be transformed by methylated DNA, albeit at low frequencies, suggesting that additional restriction systems remain to be identified.

2. MATERIALS AND METHODS

2.1 Bacterial strains, plasmids and primers

All bacterial strains and the DNA methylation phenotypes of the *E. coli* strains are listed in Table 1. The plasmid-free *B. anthracis* strain UM44-1C9 (Koehler et al., 1994; Ruhfel et al., 1984) was used for all transformation experiments and mutant construction. Plasmids (and their construction, when required) and oligonucleotide primers used in this study are listed in Tables 2 and 3, respectively. In this paper, gene designations/locus tags with the prefix “BA” refer to the annotation of the Ames strain, Genbank accession AE016879 (Table 4). The corresponding *mrr/mcr* gene designations are those used in the REBASE annotation (<http://rebase.neb.com>). The shuttle plasmid pUTE29 (Koehler et al., 1994) was used for all transformations and was propagated in the listed *E. coli* strains with selection in the presence of 100 µg/ml ampicillin. This yielded plasmids having differing methylation patterns. *B. anthracis* transformants containing pUTE29 were selected on LB medium with 5 µg/ml tetracycline.

2.2 Electroporation of *B. anthracis*

An overnight culture of *B. anthracis* (UM44-1C9 or the mutant strains) in LB broth was diluted 1:200 into fresh LB broth containing 1 g/l of glucose. The culture was shaken (225 rpm) at 37°C until the A_{600} of the culture was 0.15–0.2. The cells were harvested by centrifugation and washed thrice with 1/10 the culture volume of electroporation buffer (EB: 10% sucrose, 15% glycerol, 2 mM potassium phosphate buffer, pH 8.4). This buffer is identical to that used by Quinn and Dancer (Quinn and Dancer, 1990) except that the pH is 8.4 instead of 7.8. EB at 1/100th of original culture volume was used to resuspend the bacteria for electroporation and 200 µl aliquots were used for transformation. Electroporation was carried out in 0.2 cm cuvettes using the Gene Pulser II apparatus (Bio-Rad) at a setting of 1.7 kV, 25 µF, 200 Ω, after the addition of 800–1000 ng of pUTE29 DNA. After electroporation, the bacteria were immediately transferred to 0.8 ml of SOC medium and incubated at 37°C, 225 rpm for 1 h before plating on selective media. Transformation frequencies were calculated as transformants obtained per 10⁶ input colony-forming units (cfu) and for comparison to published values are sometimes also expressed as transformants obtained per µg DNA. To ensure consistency during comparisons of transformation frequencies of the parent and mutant strains, electrocompetent cells of each were prepared simultaneously using the same batch of reagents. Only freshly prepared electrocompetent cells were used for comparison of transformation frequencies, and typical values obtained in simultaneous tests of the mutant and the parent strains are given. (For general use, stocks of electrocompetent cells stored at –70°C can be used for transformation, but with a nearly 10-fold decrease in transformation frequency.) A negative control for transformation (electroporation without pUTE29) was included in each experiment to rule out the spontaneous emergence of tetracycline resistance.

2.3 DNA manipulations

All cloning procedures were carried out in accordance with standard protocols (Sambrook and Russell, 2001). Plasmid DNA for *B. anthracis* transformation was extracted from various *E. coli* hosts using the Hi-speed Plasmid Midiprep kit (Qiagen). Plasmid DNA for routine cloning was extracted from *E. coli* using the Plasmid Miniprep kit (Qiagen) or FastPlasmid (Eppendorf). The GeneClean Spin kit (Qbiogene) was used to gel-purify DNA fragments. PCR products were cloned into the pGEM T-easy vector (Promega) as necessary.

All enzymes required for DNA manipulations were purchased from New England Biolabs and used as per the manufacturer's instructions. *In vitro* methylation of plasmid DNA was carried out using M. SssI or M. HaeIII purchased from New England Biolabs and protocols recommended by the manufacturer. A negative control of an equal amount of plasmid was also subjected to identical handling except that the MTase was not added. At the end of the procedure, both the methylated and the negative control (unmethylated) plasmids were re-purified using the Qiaquick kit (Qiagen), quantitated, and identical amounts (800–1000 ng) were used for the transformation of various strains of *B. anthracis*.

2.4 Database analyses

The online database of restriction enzymes, REBASE (Roberts et al., 2010) (<http://rebase.neb.com>) maintained by New England Biolabs, was used to identify candidate RE of *B. anthracis*. Expression levels were calculated from microarray data (Passalacqua et al., 2009a), obtained as accession E-MEXP-2036 at the ArrayExpress archive (<http://www.ebi.ac.uk/arrayexpress>). Identification of co-transcribed genes was obtained from RNA-Seq data (Passalacqua et al., 2009b), downloaded as accession GSE13543 from the NCBI Gene Expression Omnibus site, (<http://www.ncbi.nlm.nih.gov/geo>).

2.5 Gene knockouts by double cross-over

The vector used for this purpose was pHY304 (Pritzlaff et al., 2001), which contains a mutant pWVO1 origin of replication that is not functional at temperatures >37°C. The vector contains an *ermB* gene from Tn917 that confers resistance to erythromycin in both *E. coli* (400 µg/ml) and *B. anthracis* (5 µg/ml). The procedure used for gene knockouts was described previously (Pomerantsev et al., 2006). *B. anthracis* sequences homologous to those in the chromosomal region to be knocked out were amplified by PCR and cloned into the multiple cloning site of pHY304. These sequences were then disrupted by inserting the *aad9* gene cassette (from *Enterococcus faecalis*) flanked by two *loxP* sites oriented as direct repeats. The *aad9* gene cassette (denoted Ω) confers spectinomycin resistance in *E. coli* and *B. anthracis* (100 µg/ml for each). The site of insertion of *aad9* within the *B. anthracis* sequence was chosen such that the final construct had at least 0.8 kb of homologous *B. anthracis* sequence on each side of *aad9*. The final construct was introduced into *B. anthracis* UM44-1C9 and the transformants were propagated on selective solid medium at the permissive temperature (30°C). A randomly chosen colony was then propagated overnight at 43°C in liquid culture to cure the plasmid, while selecting for spectinomycin resistance. This enriched for bacteria having plasmid integrated into the chromosome via a single cross-over. Subsequent rounds of screening on solid medium for erythromycin sensitivity and spectinomycin resistance allowed detection of clones in which the gene of interest was disrupted by a double cross-over event. All gene disruptions were verified by PCR using one primer to flanking regions of the gene in question (termed the “genomic primer”) but not present in the targeting vector, and another to *aad9* sequences in the targeting vector, viz., Tn5loxPaad9For or Tn5loxPaad9Rev. Depending on the orientation of the *aad9* gene relative to the genomic primer in the mutant, one of the primer pairs would give a product that could not be obtained when using the parent strain (Pomerantsev et al., 2006).

2.6 Generation of multiple unmarked mutations

The Cre-*loxP* method was used as described earlier (Pomerantsev et al., 2006) to successively mutagenize the three loci predicted to encode methylation-dependent restriction enzymes. Briefly, the *aad9* gene within the knockout construct was flanked by two *loxP* sites oriented as direct repeats. After gene inactivation by double crossover and plasmid curing as described above, *aad9* was excised by transforming the mutant with the temperature-sensitive plasmid pCrePA. This plasmid contains the *cre* gene encoding the Cre

recombinase and the *ermB* gene that confers resistance to erythromycin (described above). Cre recombinase expression at the permissive temperature results in the excision of the *aad9* marker, and clones that have undergone this process are identified by spectinomycin sensitivity. Growth of these clones at the restrictive temperature (43°C) results in curing of pCrePA, and cured clones are identified by erythromycin sensitivity. This procedure was repeated to mutate multiple genes without the permanent establishment of spectinomycin resistance.

3 RESULTS

3.1 Methylation-dependent restriction endonucleases (MDRE) in *B. anthracis*

The online database of restriction enzymes, REBASE (Roberts et al., 2010) indicates that *B. anthracis* encodes four potential RE (Table 4). These four RE are all of type IV, methylation-dependent RE (MDRE). Three of these are predicted to be specific for m5C-containing DNA. Database searches showed that these proteins are highly conserved among *B. anthracis* isolates, consistent with the monomorphic nature of this species (Keim et al., 2009). Expression data from microarrays and RNA-seq databases indicate that the McrBP and McrB2P enzymes are more highly expressed than the other two MDRE (Table 4).

3.2 Role of individual MDRE in restriction of DNA

The contributions of each of the MDRE to DNA restriction was evaluated by constructing strains disrupted in one or more of the four RE genes listed in Table 4. Single gene disruptions were done by double cross-over as described previously (Pomerantsev et al., 2006) and briefly outlined in Materials and Methods. The resulting single mutant strains contained a spectinomycin resistance (Ω) cassette flanked by 34-bp *loxP* sites within the targeted gene. For creation of multiple gene knockouts, the Ω cassette was removed by the action of Cre recombinase prior to disruption of an additional RE gene. In one case, the Cre recombinase acted on *loxP* sites separated by 32 genes, leading to excision of a 30-kb region, as reported previously (Pomerantsev et al., 2006).

The mutated strains (Table 1) were tested for their ability to be transformed by plasmid pUTE29 DNA prepared from four *E. coli* strains differing in their methylation ability (Table 5). Each mutant strain was tested in a separate experiment in which it was compared to the parent strain, UM44-1C9. The M0 Ω strain that is deleted for *mrr* showed a significant increase in acceptance of DNA containing m5C, consistent with the expectation that the Mrr enzyme cleaves m5C-containing DNA. However, no transformation of this strain was obtained with m6A-containing DNA, even though the Mrr enzyme was expected to contribute to its restriction based on what is known about the *E. coli* homolog (see <http://rebase.neb.com>). This indicates that other enzymes or factors are dominant in restricting the adenine-methylated plasmid DNA.

The M1 Ω -2 strain has the *mcrBP* gene disrupted by insertion of the Ω cassette, and this is expected to inactivate the downstream *mcrB2P* gene, which is in the same operon. In fact, the *mcrB2P* open reading frame overlaps the 3'-end of the coding sequence of the *mcrBP* gene by 26 base pairs. Thus, in all the work presented here, the phenotypic changes in mutants having the disrupted *mcrBP* gene (containing the Ω cassette) could be due to loss of expression of either or both *mcrBP* and *mcrB2P*. The M1 Ω -2 mutant strain showed a substantial increase in frequency of transformation by plasmids containing m5C (those prepared from *E. coli* GM33). This shows that at least one of these two MDRE play a large part in the restriction of m5C-containing DNA.

The other predicted MDRE that targets m5C sites is designated McrB3P. The *mcrB3P* (BA2283) gene was inactivated by double cross-over to obtain the mutant strain designated

M3Ω. The transformation efficiency of this strain with pUTE29 propagated in different strains of *E. coli* was no greater than that of the *mrr* mutant strain M0Ω (Table 5). However, the data of Table 5 indicate that all three identified *mcr* gene products contribute to the degradation of cytosine-methylated DNA. A comparison of the transformation frequencies obtained with each of the three single mutants (Table 5) shows that the *mrr*[−], *mcrB3P*[−], and *mcrBP*[−] single mutants can be transformed by m5C-containing plasmids. Interestingly, the frequency of transformation of the *mcrBP*[−] mutant by DNA containing only m5C (but not m6A) is comparable to that obtained using unmethylated DNA. This shows, as noted above, that the *mcrBP* locus is the major contributor to the restriction of m5C-containing DNA by *B. anthracis*.

Further evidence of the large role played by the *mcrBP* locus came from studies with DNA methylated *in vitro*. The methylase M. SssI creates m5C modifications in the DNA sequence CG. Plasmid DNA methylated in this way was strongly restricted in the parental UM44-1C9 strain and the two other single mutants tested, but not in the M1Ω-2 mutant strain (Table 6). This result confirmed that obtained in Table 5 for plasmid methylated by growth in the *dam-dcm*⁺ GM33 strain.

The report by Marrero and Welkos (Marrero and Welkos, 1995) notes that methylases capable of modifying the underlined cytosine residues in the sequences CG (M. SssI), GGCC (M. HaeIII), and CCGG (M. HpaII) are encoded in the *B. subtilis* 168 genome. The ability of *B. anthracis* to take up DNA purified from *B. subtilis* 168 was therefore interpreted as an indication of the absence m5C-specific MDREs in *B. anthracis*. However, as noted above, we found that CpG-methylated DNA was incapable of transforming *B. anthracis* UM44-1C9, even though unmethylated DNA transformed the same strain with an efficiency of 2.6×10^2 transformants per μg (0.38 transformants per 10^6 cfu), well within the range of 10^2 – 10^3 transformants per μg observed in the work of Marrero and Welkos) in a parallel experiment (Table 6). When the unmethylated plasmid was modified by M. HaeIII *in vitro*, it was rendered incapable of transforming the parent strain UM44-1C9, even though unmethylated plasmid was able to transform the same strain at a frequency of 2.13 transformants per 10^6 cfu (transformation efficiency of 1.3×10^3 per μg) in a parallel experiment (Table 6). The ability of the various mutant strains (single or multiple) to take up M. HaeIII-methylated plasmid was not tested. Methylation-transformation experiments with M. HpaII were not carried out as its methylation specificity, Cm5CGG, overlaps with the CG methylation specificity of M. SssI.

3.3 Characterization of *B. anthracis* mutant strains lacking multiple MDREs

We also constructed strains that are mutated in more than one restriction enzyme gene (Table 1) by means of the Cre-*lox*-based system developed in our laboratory (Pomerantsev et al., 2006). Thus, we obtained two double mutants, M3-0Ω and M3-0Δ, both defective in *mrr* and *mcrB3*, and a triple mutant, M3-0Δ-1Ω-2, that is additionally defective in *mcrBP* (and probably *mcrB2P* as well, as noted above). Of the two double mutants, M3-0Δ also has a 30-kb deletion in the *mrr-mcrB3P* intergenic region owing to the action of the Cre recombinase on widely separated *loxP* sites in M3-0Ω (Pomerantsev et al., 2006). The triple mutant, by virtue of being derived from this strain by the inactivation of *mcrBP*, also harbors this deletion. The transformation frequencies of these strains relative to the parent strain UM44-1C9 are shown in Table 5. It is notable that M3-0Δ is able to accept m6A-containing DNA at a low frequency, so long as the DNA is not also m5C-methylated. The triple mutant is more transformable when compared to single or double mutants at levels ranging from 10-fold to nearly 1000-fold, depending on the mutant being compared (Table 5, column 5). Additionally, the triple mutant can be transformed with fully methylated DNA at a reproducible, but very low frequency (Table 5, bottom row). Among the genes deleted in the 30-kb stretch in mutants M3-0Δ and its derivative M3-0Δ-1Ω-2 are a peptidase (BA_2297)

and a sporulation control protein, Spo0M (BA_2308). Strains having this large deletion may have added value as hosts for protein expression due to the peptidase deletion and because the sporulation defect (Spo0M) could decrease the possibility of persistent laboratory contamination by spores (Pomerantsev et al., 2006).

4 DISCUSSION

4.1 Type IV restriction enzymes in *B. anthracis*

Few detailed genetic analyses of type IV restriction enzymes of Gram-positive bacteria are available. *B. anthracis* harbors at least three, and possibly four, genes encoding type IV restriction enzymes (or subunits thereof) that specifically degrade DNA containing methylcytosine residues. By making mutations in these genes, we have provided strong genetic evidence that each of these MDRE contribute to restriction of m5C-containing DNA. The *mcrBP-mcrB2P* locus makes the most significant contribution to restriction of the m5C DNA (Table 5), consistent with its higher expression levels compared to the other loci (Table 4).

The apparent contradiction between our results and the earlier inference that *B. anthracis* does not restrict m5C-containing DNA (Marrero and Welkos, 1995) can be resolved when we allow the possibility that the *B. subtilis* 168 strains used in that work were probably cured of the prophages that encode DNA MTases targeting cytosines in CGCG, CCGG or GGCC sequences. *B. subtilis* 168 possesses only one intrinsic DNA MTase, viz., BsuM, which recognizes CTCGAG (XhoI) (Matsuoka et al., 2005). Of the two plasmids used for transformation in the prior study (Marrero and Welkos, 1995), pHV33 does not have any XhoI sites (Guha, 1985; Primrose and Ehrlich, 1981) and pLTV1 has only a single XhoI site (Camilli et al., 1990). Thus, plasmids isolated from *B. subtilis* 168 would probably not contain m5C and not be subject to restriction. This provides a reasonable explanation for the finding that *B. anthracis* is transformable by plasmids that were first propagated in *B. subtilis* 168, but not those propagated in Dam⁺Dcm⁺ *E. coli*. Their additional observation that EcoK modification of transforming DNA probably does not influence transformation remains valid. In this work, an *E. coli* B strain (BL21) has been additionally used as a source of transforming DNA in addition to *E. coli* K-12 derivatives (Table 1). However, it should be noted that M. EcoB methylation of transforming plasmids is not expected to affect the final results of our experiments because the BL21 strain used is mutated in *hsdM* (see Table 1), and therefore deficient in M. EcoB methylation activity.

In this study, the genes encoding putative MDREs in *B. anthracis* were mutated singly and collectively using a Cre-*loxP* system. However, the phenotypes of mutants altered in the *mcrBP* or *mcrB3P* genes cannot be unambiguously attributed to the inactivation of targeted genes, owing to the probability of polar effects. The *mcrB2P* gene, located downstream of the *mcrBP* gene, encodes a protein possessing a PD...(D/E)XK nuclease motif. The inactivation of this gene along with *mcrBP* could also be a likely cause for the enhanced transformability with m5C-containing DNA of the *mcrBP* mutant compared to that of the *mcrB3P* and *mrr* single mutants. The ORF downstream of *mcrB3P*, BA2284, encodes a hypothetical protein with no homology to known proteins and lacks any recognizable motifs. While this gene could conceivably be affected by the inactivation of *mcrB3P*, its role in the restriction of methylated DNA is uncertain.

Even in the multiple mutant having all the putative MDRE inactivated, plasmids containing m6A modifications were still strongly restricted (Table 5). We were unable to identify any candidate genes that might be responsible for this restriction. The genes within the 30-kb genomic region deleted in the triple mutant strain M3-0Δ-1Ω-2 do not include any obvious candidates. Thus, we have considered other mechanisms that might account for the inability

of m6A-containing plasmids to transform *B. anthracis*. One plausible mechanism could be the inefficient replication of hemimethylated DNA, as observed in some bacteria. Dam⁻ *E. coli* and *Salmonella enterica* serovar Typhimurium strains are poorly transformed with fully Dam-methylated DNA, even though they can be transformed at high efficiencies by unmethylated DNA. This occurs because the first round of replication of a methylated plasmid in a Dam⁻ strain produces hemimethylated DNA that, in turn, is a sub-optimal substrate for the replication machinery (Aloui et al., 2007; Russell and Zinder, 1987). Therefore, the inability of m6A-containing DNA to transform *B. anthracis* may not be indicative of restriction by an endonuclease, but a consequence of inefficient replication.

In *E. coli*, the McrBC holoenzyme McrB is involved in DNA recognition and binding, as well as in GTP hydrolysis, while McrC is required to catalyze DNA cleavage (Dryden et al., 2001). Neither McrB nor McrC can individually cause DNA cleavage. In *B. anthracis*, the McrBP and McrB3P proteins contain ATPase/GTPase domains, as determined by a BLAST homology search, and are therefore likely to be McrB-type subunits. McrB2P, on the other hand, harbors a characteristic PD...(D/E)XK nuclease motif (DUF524) (Knizewski et al., 2007). Therefore, it is possible that *B. anthracis* produces two McrBC holoenzymes – McrB3P-McrB2P and McrBP-McrB2P. This could potentially explain the relatively larger gain in transformation efficiency in the mutants with *mcrBP* disruptions, since these are likely to incidentally affect the expression of the *mcrB2P* gene as well. In contrast, the *mcrB3P* mutant would be expected to retain a functional type IV holoenzyme, McrBP-McrB2P. Indeed, inactivation of *mcrBP* seems to essentially abolish m5C restriction whereas disruption of *mcrB3P* does not (Table 5, column 5). The interchangeability of McrB subunits could potentially change the sequence specificity of the holoenzyme or lead to redundancy, and might in this way constitute a unique instance of the generation of alternate McrBC holoenzymes.

Although the work described here has not fully resolved the mechanisms responsible for the restriction of methylated DNA by *B. anthracis*, one practical benefit of the work is evident from the data in column 6 of Table 5. Transformation of *B. anthracis* is often a limiting step in genetic manipulations even when plasmids are prepared from dam⁻dcm⁻ *E. coli* hosts. Notably, the strains mutated in both the *mrr* and *mcrB3P* genes exhibit an increase in transformation frequency of approximately two orders of magnitude relative to parent strain (UM44-1C9) for the unmethylated pUTE29 plasmid. If this effect extends to other commonly used cloning and protein expression shuttle plasmids, then this will facilitate studies that depend on high transformation efficiencies, e.g., those involving preparation of mutant libraries. The formal possibility that wild-type strains of *B. anthracis* harboring both virulence plasmids pXO1 and pXO2 could exhibit R-M properties different from that of the plasmid-free strains described here should also be recognized.

Finally, both this work and that of Marrero and Welkos (Marrero and Welkos, 1995) again highlight the importance of carefully analyzing the R-M properties of both recipient and donor bacteria when planning the transformation experiments that constitute a vital step in genetic manipulation of model systems. Endogenous R-M systems in the recipient organism and the methylation status of transforming DNA are important considerations not only during the inter-species transfer of DNA, but also during inter-strain transfer. Due consideration must also be given to the problem of replication of methylated (or non-methylated) DNA in the bacterial species/strains of interest.

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Research Highlight

- *Bacillus anthracis* is poorly transformed by DNA methylated on adenine or cytosine.
- We inactivated three genes encoding methylation-dependent restriction enzymes.
- The resulting strains showed increased transformation by methylated DNA.
- The genes *mrr*, *mcrBP*, and *mcrB3P* each restricted cytosine-methylated DNA.
- Adenine methylation may cause inefficient replication of hemimethylated plasmid DNA.

Table 1

Bacterial strains used in this study.

Strain	Short descriptor	Relevant characteristics/Genotype/Methylation phenotype	Reference and source
<i>B. anthracis</i> ^a			
UM44-1C9		pXO1 ⁺ pXO2 ⁺ Ind ⁺ Str ^R derivative of the Weybridge (Sterne-type) strain of <i>B. anthracis</i> .	(Koehler et al., 1994), C. Thorne
MrrΩL	M0Ω	UM44-1C9 derivative; <i>mrr</i> knockout, Sp ^R Ω cassette flanked by two <i>loxP</i> sites	This work
McrBPΩL-B2P	M1Ω-2	UM44-1C9 derivative; <i>mcrBP</i> knockout, Sp ^R Ω cassette flanked by two <i>loxP</i> sites. Downstream <i>mcrB2P</i> gene also probably inactive due to a polar effect.	This work
McrB3PΩL	M3Ω	UM44-1C9 derivative; <i>mcrB3P</i> knockout, Sp ^R Ω cassette flanked by two <i>loxP</i> sites	(Pomerantsev et al., 2006)
McrB3P	M3	McrB3PΩL derivative, after removal of the Sp ^R Ω cassette by Cre recombinase action, leaving a single <i>loxP</i> site. (not used in tests of transformation efficiency (Tables 5, 6).	This work
McrB3P-MrrΩL	M3-0Ω	McrB3P derivative; <i>mrr</i> knockout, Sp ^R Ω cassette flanked by two <i>loxP</i> sites. <i>mcrB3P</i> ⁺ <i>mrr</i> ⁻ double mutant. Contains total of 3 <i>loxP</i> sites.	This work
McrB3P-Mrr-LΔ30	M3-0Δ	McrB3P-MrrΩL derivative; <i>mcrB3P</i> ⁺ <i>mrr</i> ⁻ double mutant, with a 30-kb deletion caused by Cre action, and a single residual <i>loxP</i> site.	(Pomerantsev et al., 2006)
McrB3P-Mrr-LΔ30-McrBPΩL-B2P	M3-0Δ-1Ω-2	McrB3P-Mrr-LΔ30 derivative; <i>mcrBP</i> knockout, Sp ^R Ω cassette flanked by two <i>loxP</i> sites. <i>mcrB3P</i> ⁺ <i>mrr</i> ⁻ <i>mcrBP</i> ⁻ triple mutant. Downstream gene <i>mcrB2P</i> also probably inactive due to a polar effect. Contains total of 3 <i>loxP</i> sites.	This work
<i>E. coli</i> ^b			
XL1-Blue		<i>E. coli</i> K-12; Dam ⁺ Dcm ⁺ M. EcoKI ⁺	Stratagene/Agilent
BL21		<i>E. coli</i> B; <i>dcm hsdS</i> (r _B ⁻ m _B ⁻); Dam ⁺ Dcm ⁻ M. EcoB ⁻	Stratagene/Agilent
GM33		<i>E. coli</i> K-12; <i>dam-3</i> ; Dam ⁻ Dcm ⁺ M. EcoKI ⁺	<i>E. coli</i> Genetic Stock Center, (Marinus and Morris, 1973)
SCS110		<i>E. coli</i> K-12; <i>dam dcm</i> ; Dam ⁻ Dcm ⁻ M. EcoKI ⁺	Stratagene/Agilent

^aFor *B. anthracis* strains: Sp^R - spectinomycin resistance; Ω - *aad6* gene with promoter and transcriptional stop signals; L - *loxP* site(s); Δ30 - 30-kb deletion between the *mrr* and *mcrB3P* genes. The genes encoding MDREs have been given the following short numeric descriptors for use in short descriptor names in 2nd column: *mrr* - 0; *mcrBP* - 1; *mcrB2P* - 2; *mcrB3P* - 3.

^bFor *E. coli* strains, only genotypes relevant to this study are listed. For complete genotypes, see <http://cgsc.biology.yale.edu> and <http://www.agilent.com>

Table 2

Plasmids used in this study.

Plasmid	Relevant characteristics ^a	Reference or Source
pUTE29	<i>E. coli-Bacillus</i> shuttle vector, pBC16 origin of rep for <i>bacilli</i> ; Ap ^R in <i>E. coli</i> and Tc ^R in <i>B. anthracis</i> .	(Koehler et al., 1994)
pHY304	Contains Em ^R gene and strongly temperature sensitive pWVO1 replicon for both <i>E. coli</i> and Gram-positive bacteria; Em ^R both in <i>E. coli</i> and <i>B. anthracis</i> .	(Pritzlaff et al., 2001)
pGEM-T Easy	Vector for cloning PCR products by T-A cloning, Ap ^R	Promega
pBS246	Contains two directly repeated <i>loxP</i> sites flanking a multiple cloning site; Ap ^r in <i>E. coli</i>	Invitrogen
pCrePA	pHY304 with the Cre recombinase under control of the promoter for protective antigen (PA).	(Pomerantsev et al., 2006)
pΩL	2.3-kb BamHI fragment with <i>aad9</i> (Ω-sp) inserted between two directly repeated <i>loxP</i> sites of pBS246	(Pomerantsev et al., 2006)
pMrrΩL	Self-ligated pGEM-T Easy with (1) <i>mrr</i> 5' sequences amplified by primers <i>mrr</i> 5' for pr. and <i>mrr</i> 5' rev pr. and cloned into the ApaI-SacII sites; (2) <i>mrr</i> 3' sequences amplified by <i>mrr</i> 3' for pr. and <i>mrr</i> 3' rev pr. and cloned into the PstI-SalI sites; (3) the NotI fragment from pΩL (containing the <i>loxP</i> -Ω-sp- <i>loxP</i> cassette) cloned into the NotI site.	(Pomerantsev et al., 2006)
pHYMrrΩL	pHY304 with the SalI fragment from pMrrΩL consisting of <i>mrr::loxP</i> -Ω-sp- <i>loxP</i> cloned into the SalI site; used for <i>mrr</i> inactivation.	(Pomerantsev et al., 2006)
pMcrB3PΩL	pGEM-T Easy with (1) <i>mcrB3P</i> sequences amplified by McrB3P For 1 and McrB3P Rev, cloned by the T-A method; (2) <i>loxP</i> -Ω-sp- <i>loxP</i> amplified by Tn5loxPaad9For and Tn5loxPaad9Rev, cloned into the BstZ17 I site of <i>mcrB3P</i> giving <i>mcrB3P::loxP</i> -Ω-sp- <i>loxP</i> .	(Pomerantsev et al., 2006)
pHYMcrB3PΩL	pHY304 with the NotI fragment from pMcrB3PΩL consisting of <i>mcrB3P::loxP</i> -Ω-sp- <i>loxP</i> , cloned into the NotI site; used for <i>mcrB3P</i> inactivation.	(Pomerantsev et al., 2006)
pMcrBPΩL	pGEM-T Easy with (1) <i>mcrBP-B2P</i> sequences amplified by McrBP-B2P For 1 and McrBP-B2P Rev, cloned by the T-A method; (2) <i>loxP</i> -Ω-sp- <i>loxP</i> amplified by Tn5loxPaad9For and Tn5loxPaad9Rev, cloned into the EcoRV site of <i>mcrBP</i> giving <i>mcrBP-B2P::loxP</i> -Ω-sp- <i>loxP</i> .	This work
pHYMcrBPΩL	pHY304 with the NotI fragment from pMcrBPΩL consisting of <i>mcrBP-B2P::loxP</i> -Ω-sp- <i>loxP</i> , cloned into the NotI site. Part of the <i>mcrBP</i> sequences were removed by digestion with NcoI and BglII to reduce the size of the plasmid. The NcoI and BglII ends remaining in the plasmid after digestion were rendered blunt using T4 DNA polymerase, and the plasmid self-ligated to give pHYMcrBPΩL; used for <i>mcrBP-B2P</i> inactivation.	This work

^a Abbreviations not defined in Table 1: Ap^R, ampicillin resistance; Tc^R, tetracycline resistance; Em^R, erythromycin resistance.

Table 3

Primers used in this study.

Primer	Sequence (5'-3') ^a	Relevant property	Restriction site(s)
Tn5loxPaad9For	ctgtctcttataacacatctATAA <u>CTTCGTATAATGTATGCTATACGAAGTTATATCGA</u> TTTTTCGTTCCTGTAATACATG	Primer pair to amplify <i>aad9</i> from pUTE29 and add a Tn5-binding sequence (lowercase) and a <i>loxP</i> sequence (bold italics) on either end.	
Tn5loxPaad9Rev	ctgtctcttataacacatctATAA <u>CTTCGTATAATGTATGCTATACGAAGTTATATCGA</u> GGTTTATTGTTTTCTAAAAATCTG		
mrr 5' for pr.	GGGCCCGTCGAGCCCGCTGAATCCCGTACATGTTTTATATC	Primer pair to amplify sequences on the 5' side of <i>mrr</i>	Apal, Sall
mrr 5' rev pr.	CCGCGGCAATGAAAGAAATAGCCCAAAATCATAC		SacII
mrr 3' for pr.	CTGCAGGAAGCGCTCATTCATATGATGAAG	Primer pair to amplify sequences on the 3' side of <i>mrr</i>	Psd
mrr 3' rev pr.	GTCGACCCCGCTGAATCTAATTCGGTACTAATCAATCAAAAG		Sall
MerB3P For	GTTGACGAGAAATTGTTGATTAGC	Primer pair to amplify <i>mcrB3P</i> sequences	
MerB3P Rev	CCGTCCCAATGATTAACTTTAATAC		
MerBP-B2P For 1	GATGAAAATGTTAGAGCAACTCATAATTG	Primer pair to amplify <i>mcrBP-B2P</i> sequences	
MerBP-B2P Rev	GTTCTTTCCCTTTTCTCAATAG		
mrr 5' genomic pr.	GAACTTTTCACCGAAATAATGCCACTG	Primers to <i>B. anthracis</i> genomic sequences not present in the pHY series of vectors used for inactivation of the <i>mrr</i> or <i>mcr</i> genes. Used to verify gene disruptions by PCR.	
MerB3P genomic pr.	GAATATTTAATACATAAGATATTACAGGAGGG		
MerBP-B2P genomic pr.	CAAAAAATCATTTCAGAAAGCTTC		

^a Restriction enzyme sites are underlined.

Table 4

Predicted methylation-dependent restriction enzymes in *B. anthracis*

RE name	Locus Tag and enzyme ID ^a	Putative restriction target(s)	Protein size (aa)	Protein size variations among other <i>B. anthracis</i> strains	Genes that are co-transcribed ^b	Gene expression level (percentile) ^b
Mrr	BA_2317 NP_844702	m5C, m6A	154	N-terminal deletions of 51 and 29 aa in A2012 and Sterne, respectively	BA_2318 to BA_2317 (reverse strand)	10
McrBP	BA_0927 NP_843433	m5C	843	Sterne has 4 aa added to N-terminal	BA_0923 to BA_0928	60
McrB2P	BA_0928 NP_843434	m5C	815	1 aa less at N-termini in A0248, A2012, CDC684, etc.	BA_0923 to BA_0928	85
McrB3P	BA_2283 NP_844669	m5C	606	3 aa added to N-terminal in Sterne	BA_2283 to BA_2286	15

^a Ames strain accession number AE016879 was used as reference. Locus tags for genes in Ames Ancestor (NC_007530) are the same except for the prefix; e.g., BA_2317 corresponds to GBAA_2317. The enzyme ID quoted is with reference to the NCBI database.

^b Identification of co-transcribed genes and calculation of gene expression levels were performed using RNA-Seq (Passalacqua et al., 2009b) and microarray (Passalacqua et al., 2009a) data as described in Materials and Methods.

Table 5

Transformation of *B. anthracis* mutants deficient in one or more restriction enzymes.

<i>B. anthracis</i> recipient		Transformation frequencies ^a			
Strain (short descriptor)	Disrupted RE genes	<i>E. coli</i> strain used as plasmid source Relevant strain methylation activities Bases methylated in plasmid DNA			
		XL-1 Blue Dam+Dcm+m6A, m5C	BL21 Dam+Dcm-m6A	GM33 Dam-Dcm+m5C	SCS110 Dam-Dcm-none
UM44-1C9		0	0	0	5.9
M0Q	<i>mrr</i>	0	0	0.37	12
UM44-1C9		0	0	0.01	14
M1Q-2	<i>mcrBP</i> (<i>mcrB2P</i>) ^b	0	0	36	53
UM44-1C9		0	0	0	82
M3Q	<i>mcrB3P</i>	0	0	0.19	41
UM44-1C9		0	0	0.0015	2
M3-0Q	<i>mrr mcrB3P</i>	0	0	0.72	240
UM44-1C9		0	0	0.014	1.6
M3-0A	<i>mrr mcrB3P^c</i>	0	0.0065	0.52	400
M3-0A-1Q-2	<i>mrr mcrBP mcrB3P^c</i> (<i>mcrB2P</i>) ^b	0.012	0.005	140	200

^aFrequencies of transformation by plasmid pUTE29 prepared from the indicated *E. coli* host strains are reported as transformants per 10⁶ input cfu using a total of 1 μg DNA per transformation. A frequency of 10 transformants per 10⁶ input cfu represents a transformation efficiency of approximately 10⁻²–10⁻⁴ transformants per μg DNA under these conditions. Each box of 2 or 3 rows represents a separate experiment in which the parental strain UM44-1C9 was included as the standard for comparison.

^bThe *mcrB2P* gene is likely to be inactivated due to deletion of the upstream *mcrBP* gene.

^cNo genes predicted to encode restriction enzymes were identified in the 30-kb deletion (see text).

Table 6

Transformation frequency of *B. anthracis* mutant strains with DNA methylated *in vitro*.

<i>B. anthracis</i> recipient		Methylation status of input plasmid DNA	
Strain	Disrupted gene(s)	CpG	none
UM44-1C9		0 ^a	0.38
M0Ω	<i>mrr</i>	0.02	38
M3Ω	<i>mcrB3P</i>	0	18
M1-2Ω	<i>mcrBP</i> (<i>mcrB2P^b</i>)	120	78

^aTransformation frequencies (transformants per 10⁶ input cfu using 1 μg DNA per transformation) of *B. anthracis* mutants with *dam*[−]*dcm*[−]pUTE29 DNA that was cytosine-methylated *in vitro* at CpG sites with M. SssI.

^bThe *mcrB2P* gene is also likely to be inactivated due to disruption of the upstream *mcrBP* gene (see discussion).