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To cite this version:
Mikkel A. Algire, Michael G. Montague, Sanjay Vashee, Carole Lartigue, Chuck Merryman. A Type III restriction-modification system in Mycoplasma mycoides subsp. capri.. Open Biology, 2012, 2 (10), pp.1-8. 10.1098/rsob.120115. hal-02645716

HAL Id: hal-02645716
https://hal.inrae.fr/hal-02645716
Submitted on 29 May 2020

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A Type III restriction–modification system in *Mycoplasma mycoides* subsp. *capri*

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1. Summary

The sequenced genome of *Mycoplasma mycoides* subsp. *capri* revealed the presence of a Type III restriction–modification system (MmyCI). The methyltransferase (modification) subunit of MmyCI (M.MmyCI) was shown to recognize the sequence 5′-TGAG-3′ and methylate the adenine. The coding region of the methyltransferase gene contains 12 consecutive AG dinucleotide repeats that result in a translational termination at a TAA codon immediately beyond the repeat region. This strain does not have MmyCI activity. A clone was found with 10 AG repeats such that the gene is in frame, and this strain has MmyCI activity, suggesting that the expression of the MmyCI methyltransferase may be phase variable.

2. Introduction

Type III restriction enzymes are complexes composed of a methyltransferase subunit (Mod) and a restriction endonuclease subunit (Res) encoded by *mod* and *res* genes, respectively [1]. Using S-adenosyl-L-methionine (SAM) as a methyl donor, the methyltransferase modifies the recognition site independently or complexed with the endonuclease subunit. Restriction activity requires both the Mod and Res subunits and is adenosine-5′-triphosphate (ATP)-dependent. It has been reported that two copies of the recognition site in inverse orientation and on the same DNA molecule are required for cleavage [2].

The genus *Mycoplasma* are wall-less bacteria and are important host-adapted pathogens in a variety of species [3]. Short sequence repeats (SSRs) have been found in the genomes of *Mycoplasmas* and other prokaryotes. These SSRs vary in length and composition, and are often found within the open reading frames of proteins, sometimes altering the reading frame. In such cases, SSRs are indicative of phase variable gene expression [4–10]. Phase variation is the heritable, high-frequency and reversible on/off switching of gene expression. It is common in *Mycoplasma*, and can be mediated by several alternative mechanisms [8,10–12]. During DNA replication, SSRs gain or lose repeat units by slipped-strand mispairing, which changes gene expression by introducing or removing frameshift mutations [8,13]. Host-adapted bacterial pathogens frequently use phase variation to generate diversity in surface structures, such as capsules, lipopolysaccharides and flagella [10,14]. Restriction–modification (R-M) systems are cytoplasmic gene products that can also be modulated by phase variation. The Type I R-M system from *Mycoplasma pulmonis* has been shown to undergo phase variation [15,16]. Numerous Type III R-M systems in host-adapted pathogens, including *Mycoplasma* species, are potentially phase-variable based on...
Mycoplasma putative Type III restriction systems have been identified in DNA helicase [24] and endonuclease [25] (figure 1). Several [23]. The R.MmyCI protein has motifs characteristic of m6A or m4C methyltransferases has motifs characteristic of a reading frame of the gene. The conserved TAA stop codons are coloured green. The alternate shading of grey and white shows the reading frame in which a full-length protein is predicted to be produced. The boxed residues indicate a change in the reading frame of the gene. The conserved TAA stop codons are coloured green. The M. mycoides gene contains one of the conserved stop codons, but has two stop codons upstream that can act to truncate translation. The organism abbreviations are M. syn (M. synoviae 53), M. hy 232 (M. hypneumoniae 232), M. hy J (M. hypneumoniae J), M. hy 7448 (M. hypneumoniae 7448), M. ag PG2 (M. agalactiae PG2), M. pu (M. pulmonis UAB CTP).

3. Results

3.1. Sequence analysis of the MmyCI restriction – modification system

Analysis of the M. mycoides subsp. capri GM12 genome sequence (Genbank accession no. CP001621) revealed an operon of two genes with homology to the mod and res genes of Type III R-M systems [22]. The mmyCImod gene precedes the mmyCires gene. The two genes overlap at the 3’ end of mod. The AG repeats begin at nucleotide 320 in the mod gene. The predicted amino acid sequence of the active site of Res is indicated. (b) A zoomed-in view of the sequence alignments using ClustalX of mod genes from several Mycoplasmas shows a conserved AG repeat of various lengths (red). The alternate shading of grey and white shows the reading frame in which a full-length protein is predicted to be produced. The boxed residues indicate a change in the reading frame of the gene. The conserved TAA stop codons are coloured green. The M. mycoides gene contains one of the conserved stop codons, but has two stop codons upstream that can act to truncate translation. The organism abbreviations are M. syn (M. synoviae 53), M. hy 232 (M. hypneumoniae 232), M. hy J (M. hypneumoniae J), M. hy 7448 (M. hypneumoniae 7448), M. ag PG2 (M. agalactiae PG2), M. pu (M. pulmonis UAB CTP).

Figure 1. The MmyCI restriction – modification system of M. mycoides subsp. capri. (a) The mmyCImod gene is located upstream of mmyCires. The two genes overlap at the 3’ end of mod. The AG repeats begin at nucleotide 320 in the mod gene. The predicted amino acid sequence of the active site of Res is indicated. (b) A zoomed-in view of the sequence alignments using ClustalX of mod genes from several Mycoplasmas shows a conserved AG repeat of various lengths (red). The alternate shading of grey and white shows the reading frame in which a full-length protein is predicted to be produced. The boxed residues indicate a change in the reading frame of the gene. The conserved TAA stop codons are coloured green. The M. mycoides gene contains one of the conserved stop codons, but has two stop codons upstream that can act to truncate translation. The organism abbreviations are M. syn (M. synoviae 53), M. hy 232 (M. hypneumoniae 232), M. hy J (M. hypneumoniae J), M. hy 7448 (M. hypneumoniae 7448), M. ag PG2 (M. agalactiae PG2), M. pu (M. pulmonis UAB CTP).
3.2. Determination of the methylation site of MmyCI

We chose to follow the methyltransferase activity of MmyCI as a means to identify the recognition sequence of the enzyme. Type III Mod subunits can function independently and modify a single recognition site [30], though we found the methyltransferase activity of Mod subunit (R.MmyCl) was greatly enhanced by the presence of the Res subunit (R.MmyCl) to make the entire complex (MmyCI). We examined the ability of the MmyCI complex to methylate DNA with [H\textsuperscript{3}-methyl]-S-adenosyl-l-methionine (H\textsuperscript{3}-SAM) using a plasmid (pRS426-pMyco1) as a potential substrate. In order to avoid DNA cleavage, the methylation reactions were performed without ATP, which is essential for the endonuclease activity of Type III restriction enzymes. The pRS426-pMyco1 plasmid was methylated by MmyCI (data not shown). Further experiments were necessary to clearly identify the recognition site of MmyCI. To this end, pRS426-pMyco1 was subdivided by using PCR to generate nine non-overlapping (approximately 1 kb) fragments that covered the majority of the pRS426-pMyco1 sequence. These subfragments were used as the substrates in separate methylation reactions with MmyCI and H\textsuperscript{3}-SAM. All of the DNA subsections were methylated by the enzyme to various degrees, indicating that the MmyCI recognition site was present in each of the nine subsections. From this result, we reasoned that the recognition site is common, and therefore probably contains four bases. Using computer analysis, 107 four-base DNA candidate sequences were identified by comparing all of the common four-base sequences in the nine DNA subsections. A likely recognition site of 5'-CTCA/TGAG-3' was methylated to a similar extent as the original CTCA containing DNA, demonstrating the recognition site for MmyCI is the four-base sequence CTCA/TGAG (figure 2).

MmyCI has the characteristic motifs of an adenine or cytosine methyltransferase. To determine which strand is methylated by MmyCI, we performed experiment on 5'-AG10 and 5'-AG12 clones in one strand, both or neither. Following methylation with H\textsuperscript{3}-SAM, 60-fold excess unbiotinylated oligo was added to the sample and used to compete with the signal of the unbiotinylated strand, allowing the level of H\textsuperscript{3} incorporation of the biotin labelled strand to be measured after purification with Streptavidin agarose resin (see §5 for details). The double biotin-labelled control showed considerable modification while the unlabelled control sample had a low signal, as expected (figure 2). The bottom strand sample signal is comparable to the double biotin-labelled sample, indicating that the bottom strand (TGAG) was methylated. These results and the presence of characteristic m6A motifs in the protein demonstrate that MmyCI recognizes the sequence 5'-TGAG-3' and methylates the adenine residue.

3.3. Differential expression and activity of mmyClmod-AG10 and -AG12 clones

The sequences of the mmyClmod-AG12 and mmyClmod-AG10 genes predict that there should be differential expression of the methyltransferase gene in the AG10 and AG12 clones. This differential expression suggest that the gene could be phase variable. In order to demonstrate this, we examined the presence of MmyCI in vitro.
The presence of the MmyCI methyltransferase subunit in the M. mycoides clones was determined by Western blot analysis. Lysates were prepared from mmyCImod-AG12 and mmyCImod-AG10 containing clones (clone AG12 and clone AG10, respectively), and probed with polyclonal antibodies raised against the purified M.MmyCI protein (figure 4). The methyltransferase is produced in clone AG10 cells as predicted by the gene sequence. Clone AG12 shows a faint band at the position where M.MmyCI is expected, suggesting a low level of M.MmyCI in the lysate. This may be due to a small minority of clone AG12 cells that have stochastically altered the number of AG repeats and switched the mmyCImod gene ‘on’ or to coincidental background fluorescence.

Purification of MmyCI allowed us to examine the methyltransferase activity of the complex in vivo. Cells with active mmyCImod genes are expected to have DNA that is protected from MmyCI endonuclease activity, whereas the clones with inactive genes will be susceptible to cleavage. Purified MmyCI was incubated with genomic DNA isolated from the two M. mycoides clones (figure 4b). DNA from clone AG12 (frameshifted) was degraded by MmyCI. The DNA from the non-frameshift clone (clone AG10) was not completely digested, indicating that mmyCImod gene encodes a functional methyltransferase.

Genomic DNA from clone AG12 was used as a substrate to further examine the DNA cleavage activity of MmyCI. Removal of the Mod subunit (M.MmyCI) or the Res subunit (R.MmyCI) abolished the DNA cleavage activity, as expected for a Type III restriction enzyme that requires the presence of both subunits for DNA cleavage activity. The reaction was dependent on the presence of ATP. Also, the complete reaction could be inhibited by the addition of 20 μM EDTA (figure 4b).

4. Discussion

We have identified a functional Type III R-M system in M. mycoides subsp. capri, designated MmyCI. MmyCI recognizes and methylates the adenine in the sequence 5'-TGAG-3'. This is a new recognition sequence for a Type III enzyme and this sequence has not been reported for any other R-M system [22].

We have isolated two M. mycoides clones that have AG dinucleotide repeats within the mod gene. The number of repeats in the gene alters the reading frame, indicating that the mod gene could be subject to phase variability by the loss or addition of AG repeat units, although this needs to be demonstrated more thoroughly. It is likely that the putative phase variability of the gene is mediated by

![Figure 3](http://rsob.royalsocietypublishing.org/)

**Figure 3.** Identification of methylated residue by MmyCI. (a) Unlabelled and biotin-labelled oligonucleotides were annealed to generate DNA substrates. (b) Scintillation counts of substrates following methylation, competition with biotin-free oligos and clean-up (representative values shown), indicating the adenine residue of 5'-TGAG-3' is methylated.

![Figure 4](http://rsob.royalsocietypublishing.org/)

**Figure 4.** Differential MmyCI expression between clones. (a) Western blot analysis of M. mycoides lysates. Whole cell lysates of clone AG10 and AG12 were run on a SDS-PAGE gel and transferred to a PVDF membrane for Western blot analysis (50, 25 and 12.5 μg of total protein). Purified M.MmyCI was run as a marker (16, 8 and 2.6 ng). The MmyCI methyltransferase is present in clone AG10 and may be present at a substantially lower level in a population of clone AG12. (b) DNA cleavage by MmyCI. Purified M. mycoides DNA was used as a substrate for the restriction activity of MmyCI. The complete reaction contained 1 μg of DNA, 1 mM ATP, 1.6 μM MmyCI and 1X NEB buffer four supplemented with 1X BSA. Cleavage activity is not observed in the absence of ATP, M.MmyCI or R.MmyCI, or in the presence of 20 mM EDTA.
slipped-strand mispairing of the dinucleotide repeat region during DNA replication events, similar to the *vmc* genes in *M. capricolum* subsp. *capricolum* [7]. While slipped-strand mispairing appears to be a likely mechanism for changing the ‘on/off’ state of the *mod* gene, the signal or inducer of this change is not known.

Several other *mod* genes from Mycoplasmas have been found that also contain various numbers of AG dinucleotide repeats within the coding region. Several of these *Mycoplasma* genes also contain frameshift mutations at the site of their AG repeats, just as the AG12 gene does, suggesting that these genes probably also undergo phase variation. The high degree of homology with M.MmyCI also suggests that these genes encode for functional methyltransferases.

It is thought that bacteria use the phase variability of gene expression to evade the host immune system. The majority of phase-variable gene products from bacteria are located on the surface and are often associated with virulence [10,14]. The biological significance of phase-degree of homology with M.MmyCI also suggests that these genes probably also undergo phase variation. The high repeats, just as the AG12 gene does, suggesting that these genes also contain frameshift mutations at the site of their AG repeats within the coding region. Several of these found that also contain various numbers of AG dinucleotide change is not known.

5. Material and methods

5.1. Expression and purification of MmyCI

5.1.1. M.MmyCI

The coding sequence of the methyltransferase of MmyCI (M.MmyCI) identified in *M. mycoides* subsp. *capri* may be applied to elucidate the biological implications of combinatorial gene changes and differential methylation in *M. mycoides* [28,29].

The restriction endonuclease subunit (R.MmyCI) coding sequence was amplified from genomic DNA in three overlapping pieces using primers that replace TGA codons with TGG codons. Isothermal DNA assembly was used to assemble the gene into pTYB1. Expression and purification of the R.MmyCI protein was performed as above for M.MmyCI.

5.2. Methylation assays

Standard methylation reactions (100 μl) contained 1 μM dsDNA oligo or 1 μg PCR product, 120 nM Hβ-SAM, 500 nM MmyCI and 1X NEB buffer 4. The reactions were incubated at 37°C for 2 h and terminated with 100 μl of phenol. Unincorporated Hβ-SAM was removed by passing 70 μl of the aqueous phase through P-30 Micro Bio spin columns (BioRad). The [Hβ-methyl]-labelled DNA was quantified by scintillation counting.

The sequence of the top strand oligo used to verify the recognition site is 5’-catgttagcttccgatcaggCTCAggcatctattgtggta-3’. CTCA was changed to CACA, GTCA, CTCC or CTAA in the control DNAs. The sequence of the top strand oligos used to examine the effect of the flanking sequence on the recognition site were 5’-catgttagcttccgatgCTCAgtctattgtggta-3’ (underlined nucleotides different from above), 5’-(a)20CTCA(a)21-3’ and 5’-(t)20CTCA(t)21-3’.

Methylation reactions with biotin labelled DNA were performed using 500 nM duplex DNA. The reactions were stopped and the Hβ-SAM was removed as described above. Excess biotin-free oligo was added to a final concentration of 30 μM, the samples were heated at 95°C for 5 min, then placed on the bench top and allowed to cool. Approximately, 50 μl of Streptavidin agarose resin (Thermo Scientific) was used to isolate the biotin-containing DNA. Unbound DNA was removed by washing the resin with 4 ml of H2O. After washing, the resin was resuspended in 100 μl H2O and co-purifying radiolabel was measured.

5.3. Sequence analysis

III R-M systems in *Mycoplasma* species were identified from REBASE [22]. DNA sequences were acquired from Genbank, and initially aligned with CLUSTALX v. 2.0 [35]. The alignment was then refined manually in the vicinity of the AG repeats. Sequences that did not contain the AG repeat region were then removed from the analysis; these genes were also noted as having relatively divergent sequences. The sequences that remained were the *mod* genes corresponding to locus tag MAG1530 from *Mycoplasma agalactiae* PG2 (NC_009497.1); locus tags mph330, mph399 and mph428 from *Mycoplasma hyopneumoniae* 232 (NC_006360.1); MPH7448_0316, MPH7448_0386 and MPH7448_0410 from *M. hyopneumoniae* 7448 (NC_007332.1); MHJ_0308, MHJ_0382, MHJ_0383, MHJ_0399 and MHJ_0423 from
M. mycoides subsp. M. mycoides cells (50, 25 and 12.5 μg of total protein) were electrophoresed on 4 to 12 per cent Bis-Tris polyacrylamide gels (Invitrogen) and transferred to an Immobilon-FL PVDF membrane. The membrane was then incubated with mouse polyclonal antibodies raised against purified M.MmyCI (Precision Antibody, Columbia, MD, USA) for 1 h, washed three times with Tris pH 7.5, 150 mM NaCl, 0.05% Tween-80) containing 5 per cent BSA for 2 h at room temperature. The membrane was then incubated with mouse polyclonal antibodies raised against purified M.MmyCI (Precision Antibody, Columbia, MD, USA) for 1 h, washed three times with TBST and probed with Alexa Fluor 555 donkey antimouse IgG (Invitrogen) for 1 h. After three washes with TBST, the protein was visualized with a Typhoon 9410 imager (GE).

5.4. Western blot analysis

Purified M.MmyCI (16, 8 and 2.6 ng) or a lysate of M. mycoides cells (50, 25 and 12.5 μg of total protein) were electrophoresed on 4 to 12 per cent Bis-Tris polyacrylamide gels (Invitrogen) and transferred to an Immobilon-FL PVDF membrane. The membrane was then incubated in TBST (50 mM Tris pH 7.5, 150 mM NaCl, 0.05% Tween-80) containing 5 per cent BSA for 2 h at room temperature. The membrane was then incubated with mouse polyclonal antibodies raised against purified M.MmyCI (Precision Antibody, Columbia, MD, USA) for 1 h, washed three times with TBST and probed with Alexa Fluor 555 donkey antimouse IgG (Invitrogen) for 1 h. After three washes with TBST, the protein was visualized with a Typhoon 9410 imager (GE).

5.5. DNA cleavage assays

DNA cleavage reactions typically contained 300 ng to 1 μg of substrate DNA, 0.5 mM ATP, 500 mM MmyCI complex (500 nM M.MmyCI and 500 nM R.MmyCI) and 1X NEB buffer 4. The reactions were incubated at 37°C. In order to quench the reactions, 25–50 μl was added to an equal volume of buffer-saturated phenol containing 0.1 M EDTA. After quenching, the aqueous phase was removed and purified with the QIAquick PCR purification kit (Qiagen). The products were separated with a 0.8 or 1.2 per cent e-Gel (Invitrogen).

6. Acknowledgements

We thank Rich Roberts for initial identification of the Type III operon. We also thank the Synthetic Biology team for critical reading of the manuscript. This work was supported by Synthetic Genomics, Inc. M.A.A., M.G.M. and C.M. wrote the main manuscript text. M.A.A. supervised the project. All authors performed experiments and analysed data. All authors reviewed the manuscript.

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