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1 **Methylation of two-component response regulator MtrA in mycobacteria negatively modulates**
2 **its DNA binding and transcriptional activation.**

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17 **Abstract**

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Posttranslational modifications such as phosphorylation, nitrosylation, and pupylation modulate multiple cellular processes in *Mycobacterium tuberculosis*. While protein methylation at lysine and arginine residues is widespread in eukaryotes, to date only two methylated proteins in *Mtb* have been identified. Here we report the identification of methylation at lysine and/or arginine residues in nine mycobacterial proteins. Among the proteins identified, we chose MtrA, an essential response regulator of a two-component signaling system, which gets methylated on multiple lysine and arginine residues to examine the functional consequences of methylation. While methylation of K207 confers a marginal decrease in the DNA binding ability of MtrA, methylation of R122 or K204 significantly reduces the interaction with the DNA. Overexpression of S-adenosyl homocysteine hydrolase (SahH), an enzyme that modulates the levels of S-adenosyl methionine in mycobacteria decreases the extent of MtrA methylation. Most importantly, we show that decreased MtrA methylation results in transcriptional activation of *mtrA* and *sahH* promoters. Collectively, we identify novel methylated proteins, expand the list of modifications in mycobacteria by adding arginine methylation, and show that methylation regulates MtrA activity. We propose that protein methylation could be a more prevalent modification in mycobacterial proteins.

33 **INTRODUCTION**

34 *Mycobacterium tuberculosis* (*Mtb*), the causative agent of tuberculosis, is responsible for nearly one
35 million deaths annually around the globe [1]. It resides dormant in the host for decades without detection
36 and when the immune system wanes, it proliferates and causes active disease. The adeptness of
37 mycobacteria to hijack the host cell can be attributed to the fine-tuning of signaling pathways. Post-
38 translational modifications (PTMs) including serine/threonine phosphorylation, nitrosylation, and
39 pupylation (addition of prokaryotic ubiquitin-like protein) play an important role in regulating
40 mycobacterial physiology and virulence [2-6]. While there are few specific examples of how these
41 modifications affect the function of a protein, more mechanistic insight is required to delineate their
42 regulatory roles. In addition to these modifications, proteins can be post-translationally modified by the
43 addition of methyl groups, catalyzed by S-adenosyl methionine (SAM) dependent methyltransferases [7],
44 at the ϵ -amino group of lysine, guanidino group of arginine, or oxygen in the carboxylate side chain of
45 glutamate [8-10]. Glutamate methylation of methyl-accepting chemotaxis proteins play a biologically
46 conserved role in chemotaxis and provide rotational directionality to bacteria [11].

47 In eukaryotes, methylation of histone proteins at specific lysine residues regulates chromatin
48 architecture and transcription, and aberrant methylation is associated with aging and cancer [12]. Arginine
49 methylation is the most extensively studied protein modification in eukaryotes and its role in DNA repair,
50 RNA metabolism, and transcriptional repair is well established [13]. Guanidino group of arginine is
51 involved in the interaction with DNA; the addition of methyl group directly affects the activity of
52 proteins. Methylation of Sam68 (an adapter protein for Src kinases during mitosis) at arginine residue
53 restrains its binding to Src homology 3 (SH3) domain of phospholipase C γ -1 and methylation at arginine
54 and lysine residues of CHD1 (chromo-helicase/ATPase DNA-binding protein 1) results in a significant
55 decrease in its binding affinity to DNA [8]. Several non-histone proteins, mainly transcription factors and
56 histone- or chromatin-associated proteins are also regulated by methylation [12, 14].

57 In bacteria, however, our understanding of the functional role of lysine or arginine methylation is
58 limited [9]. Lysine methylation is associated with bacterial cell motility of *Synechocystis* sp. and with host
59 colonization and disease initiation by *Pseudomonas aeruginosa* [15]. A recent proteomics study has identified
60 abundant lysine and arginine methylation in *Escherichia coli* [16]. In *Mtb*, lysine residues of Heparin-Binding
61 Hemagglutinin Adhesin (HBHA) and Histone-like Protein (HupB) have been shown to undergo
62 methylation but there are no reports of arginine methylation. HBHA and HupB are both critical for
63 infection by *Mtb* [17] and their methylation imparts protease resistance and thus increased stability,
64 suggesting a role for methylation in disease pathogenesis [18]. Methylation reactions are catalyzed by S-
65 adenosyl methionine (SAM)- dependent methyltransferases where S-adenosyl homocysteine (SAH) and
66 consequently homocysteine (Hcy) are generated as by-products. Methyltransferase reactions are
67 dependent on the presence of balanced amounts of SAM and SAH as they are prone to SAH-mediated
68 inhibition. Under normal conditions, SAH levels are regulated using SahH-mediated reversible hydrolysis

69 of SAH to Hcy. We have previously shown that perturbation of levels of *Mtb* SahH impacts metabolic
70 levels of Hcy and may affect SAH, a potent inhibitor of methyltransferases [19].

71 In this manuscript, we set out to determine the prevalence of methylation in *Mtb* proteins. Nine
72 among the 72 proteins tested were found to be methylated either on lysine or arginine residues. To
73 determine the functional consequences of methylation, we chose MtrA; an essential response regulator of
74 the MtrB-MtrA two-component system (TCS) that regulates cell cycle progression. We show that
75 methylation perturbs MtrA DNA binding activity leading to modulation of its own expression. We also
76 reveal that SahH, an enzyme that is required for SAM synthesis, modulates MtrA methylation. Taken
77 together, we propose that methylation of lysine and arginine residues is an important additional regulatory
78 modification in *Mtb*.

79 **Materials and Methods**

80 *Bacterial strains and growth conditions.*

81 *E. coli* strains DH5 α (Novagen) and BL21-DE3 (Stratagene) were used for cloning and
82 expression of recombinant proteins, respectively. *M. smegmatis* mc²155 (*Msm*) and *Mtb* H37Rv were
83 maintained in Middlebrook 7H9 broth (Difco, BD) containing 10% ADC (Albumin/Dextrose/Catalase)
84 and 0.05% Tween-80 (Merck, USA), supplemented with 25 μ g/ml kanamycin or 50 μ g/ml apramycin
85 when required. For assessing the effect of homocysteine (Hcy) on bacterial growth, *Msm* cells were grown
86 in Sauton's minimal medium supplemented with 0 - 0.8 mM DL-homocysteine (Sigma-Aldrich) at an
87 initial A₆₀₀ of 0.01. Absorbance was measured up to 36 h and colony-forming units (CFUs) were
88 enumerated at 25 h. Reagents and chemicals were purchased from Sigma-Aldrich unless otherwise
89 mentioned.

90 *Generation of plasmid constructs.*

91 We selected 180 protein-coding genes from *Mtb* genome representing a random set across
92 various functional classes (Fig S1). Genes involved in regulation and information processing were over-
93 represented in the list, while conserved hypotheticals and PE/PPE genes (encoding proteins containing
94 Proline-Glutamate or Proline-Proline-Glutamate motifs) were under-represented. We did not select any
95 gene from the categories stable RNAs, insertion sequences and phages, and those with unknown
96 function. The generation of recombinant plasmids using the shuttle vector pVV16 was explained
97 previously [20]. The recombinant clones (2 μ g) were transformed individually in *Msm*. *Mtb* H37Rv
98 genomic DNA was used to amplify *mtrA* (*rv3246c*; 687 bp) using forward and reverse primers containing
99 NdeI and HindIII restriction sites. Digested PCR product was cloned into either pVV16 or the *E. coli*
100 expression vector pET28a and recombinants were selected on kanamycin. *E. coli* K12 genomic DNA was
101 used to amplify *emz* (1353 bp) and cloned into pMAL-c2x at BamHI and HindIII restriction sites,
102 recombinants were selected on ampicillin. Site-specific mutants of pVV16-*mtrA* and pET28a-*mtrA* were
103 generated using QuikChange® XL Site-Directed Mutagenesis Kit (Stratagene) according to the
104 manufacturer's protocol. *Mtb sabH* (*rv3248c*; 1488 bp) was cloned in pVV16 vector at NdeI and HindIII
105 restriction sites. All constructs were confirmed by restriction enzyme digestion and DNA sequencing
106 (Invitrogen). Information about primers and plasmids used in this study is compiled in tables 1 and 2,
107 respectively.

108 For analyzing the effect of SahH on MtrA methylation, the genes encoding these proteins were
109 co-expressed in *Msm*. *Mtb sabH* was cloned in mycobacterial integrative vector pSET152 [21]. For this,
110 pVV-*sabH* was digested with HindIII and the ends were made blunt. A second digestion with XbaI
111 yielded 1.86 kb fragment containing *sabH* under a heat shock gene promoter (*hsp60*). This fragment was
112 ligated to pSET152 pre-digested with XbaI and EcoRV. pSET-*sabH* and pSET152 (2 μ g each) were
113 electroporated in *Msm* competent cells and apramycin resistant transformants were selected. pVV16-*mtrA*
114 plasmid (2 μ g) was then electroporated in competent *Msm* cells harboring either pSET152 or pSET152-

115 *sabH* and the positive clones were selected on apramycin and kanamycin. His₆-MtrA was purified from
116 cells containing both pSET-*sabH* and pVV16-*mtrA* and used for Western blotting.

117 *Expression and purification of recombinant proteins.*

118 For expression and purification of proteins from *Msm*, recombinant clones (2 µg) in pVV16
119 vector were electroporated and recombinants were selected on kanamycin. Expressed proteins were
120 purified as described before [20]. Briefly, *Msm* cells expressing recombinant proteins were cultured
121 individually in 200 ml of 7H9 medium and grown till mid-log phase ($A_{600} \sim 0.8$). The cells were harvested
122 and lysed by sonication in lysis buffer (1X Phosphate-buffered saline (PBS), 1 mM phenylmethylsulfonyl
123 fluoride (PMSF, Sigma-Aldrich), and protease inhibitor cocktail [Roche]). The lysates were centrifuged at
124 14,000 rpm at 4°C for 30 min, and the resulting supernatants containing His₆-tagged proteins were
125 incubated with Ni²⁺-NTA resin (Qiagen). The resin was washed thoroughly with a wash buffer (1X PBS,
126 1 mM PMSF, 20 mM imidazole, and 10% glycerol) and proteins were eluted in the elution buffer (1X
127 PBS, 1 mM PMSF, 200 mM imidazole, and 10% glycerol).

128 For purifying His₆-MtrA from *Mtb*, pVV16-*mtrA* construct (2 µg) was electroporated and
129 recombinants were selected on kanamycin. Recombinant cells were cultured in 200 ml of 7H9 media and
130 grown till mid-log phase ($A_{600} \sim 0.8$). The cells were harvested and lysed by bead beating in lysis buffer
131 (1X Tris-buffered saline (TBS), 1 mM PMSF, 100 µg/ml lysozyme, and protease inhibitor cocktail) using
132 0.1 mm zirconium beads (Biospec). The lysate was centrifuged and the resulting supernatant containing
133 His₆-MtrA was incubated with Co²⁺ superflow resin (Thermo Scientific). The resin was washed
134 thoroughly with wash buffer (1X TBS, 1 mM PMSF, and 10 mM imidazole) and the protein was eluted in
135 the elution buffer (1X PBS, 1 mM PMSF, and 300 mM imidazole).

136 For protein expression in *E. coli*, pET28a- or pMAL-c2x-based plasmids (100 ng) were
137 transformed, and proteins were overexpressed in *E. coli* BL21 (DE3). The recombinant His₆-tagged
138 proteins were purified using Ni²⁺-NTA affinity chromatography (Qiagen) and MBP (Maltose binding
139 protein)-tagged EnvZ was purified using Amylose resin (NEB) as per the manufacturer's instructions.
140 The concentration of purified proteins was estimated by Bradford assay (Bio-Rad). Purified proteins were
141 resolved on SDS-PAGE and analyzed by staining with coomassie brilliant blue R250 (Sigma-Aldrich).

142 To analyze the effect of Hcy on MtrA methylation, *Msm* cells harboring pVV16-*mtrA* were
143 grown in Sauton's medium containing 0.4 mM Hcy. Cells were grown up to $A_{600} \sim 0.8$, harvested, and
144 subjected to Ni²⁺-NTA chromatography for purification of His₆-MtrA, as described above. The proteins
145 were later analyzed by Western blotting. For normalization, His₆-MtrA was purified from cells grown
146 without the addition of Hcy.

147 *Western blot analysis*

148 Purified proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane
149 (Millipore). Membranes were blocked with 3% bovine serum albumin (Sigma-Aldrich) in 1X PBS
150 containing 0.1% Tween-20 (Sigma-Aldrich) (1X PBST₂₀) overnight at 4°C. After blocking, the blots were
151 washed thrice with 1X PBST₂₀ followed by incubation with antibodies for 1 h at room temperature.

152 Methyllysine immunoblots were developed by two separate antibodies from different manufacturers and
153 one representative blot is shown in figure 1a. The antibodies were: anti-methyllysine antibody from
154 Abcam (ab23366; 1:10,000 dilution) and pan anti-mono, dimethyllysine antibody from PTM Biolabs
155 (PTM-602; 1:2000 dilution). Other antibodies and their dilutions used were: anti-MtrA antibody (1:15,000
156 dilution; generated in the lab), anti-acetyllysine antibody (Cell Signaling; 1:3,000 dilution), pan anti-
157 succinyllysine antibody (PTM Biolabs; 1:2,000 dilution), HRP-conjugated anti-His₆ antibody (Abcam;
158 1:20,000 dilution), and HRP-conjugated anti-mouse and anti-rabbit IgG antibodies (Bangalore Genei;
159 1:20,000 dilution). According to the manufacturer, the methyllysine antibodies used here can detect
160 mono- or dimethyllysine with no cross-reactivity to acetyllysine. Antibodies to acetyllysine and
161 succinyllysine have been successfully used in our previous study [20]. Antibodies against recombinant *Mtb*
162 His₆-MtrA were raised in Rabbits with the help of Dr. A. K. Goel (DRDE, Gwalior, India). The
163 specificity of anti-MtrA antibody was validated by using a preparation of purified MtrA protein that had
164 been confirmed by mass spectrometry. Also, these antibodies identified a single protein band
165 corresponding to MtrA when whole cell lysate preparations of *Mtb* were probed. Anti Ef-Tu antibodies
166 were used as previously mentioned [22]. Immunoblots were developed using Immobilon™ western
167 chemiluminescent HRP substrate kit (Millipore) according to the manufacturer's instructions.
168 Quantification of immunoblots was performed using ImageJ software [23].

169 *Mass spectrometry*

170 Recombinant *Mtb* proteins purified from *Msm* were resolved on 12% SDS-PAGE and stained
171 with coomassie brilliant blue R250. The stained bands were sliced from the polyacrylamide gel and
172 subjected to in-gel reduction, carbamidomethylation, and an overnight tryptic digestion at 37°C.
173 Alternatively, protein samples were subjected to chloroform-methanol precipitation and pooled before in-
174 solution digestion and single-shot analysis. Mass spectrometry to identify protein methylation was
175 essentially performed as described [24]. Peptides were separated on a 50 cm reversed-phase column (75
176 mm inner diameter, packed in-house with ReproSil-Pur C18-AQ 1.9 mm resin [Dr. Maisch GmbH]) over
177 a 60- or 120-min gradient using the Proxeon Ultra EASY-nLC system. The LC system was directly
178 coupled online with a Q Exactive HF instrument (Thermo Fisher Scientific) via a nano-electrospray
179 source. Full scans were acquired in the Orbitrap mass analyzer with resolution 60,000 at 200 m/z. For the
180 full scans, 3E6 ions were accumulated within a maximum injection time of 120 ms and detected in the
181 Orbitrap analyzer. The ten most intense ions were sequentially isolated to a target value of 1e5 with a
182 maximum injection time of 120 ms and fragmented by HCD in the collision cell (normalized collision
183 energy of 25%) and detected in the Orbitrap analyzer at 30,000 resolution. Raw mass spectrometric data
184 were analyzed in the MaxQuant environment v.1.5.3.31 and employed Andromeda for database search
185 [25]. The MS/MS spectra were matched against the H37Rv proteome. Enzyme specificity was set to
186 trypsin, and the search included cysteine carbamidomethylation as a fixed modification and methylation
187 of lysine and arginine (+14.015650 Da) as variable modifications. Based on optimized parameters for
188 PTM identification and localization [24], the search engine score was set to a minimum cutoff of 40 for

189 the identification of methylated peptides. Additional annotations on low and high scoring peptides were
190 performed by the 'expert system' for computer-assisted annotation of MS/MS spectra. Up to two missed
191 cleavages were allowed for protease digestion, and peptides had to be fully tryptic. Downstream
192 bioinformatics analysis was done in the Perseus software environment, which is part of MaxQuant. For
193 MtrA mutants, we employed the matching between runs algorithm [26, 27] in MaxQuant to alleviate the
194 stochasticity of shotgun proteomics, which consists of transferring identifications of MS1 features
195 between samples based on accurate mass and retention time values. Identification of lysine acetylation
196 and succinylation was performed as described earlier [20].

197 *In silico analysis*

198 Gene names, protein names, protein subcellular localization, and molecular functions were
199 extracted from Mycobrowser (<https://mycobrowser.epfl.ch/>) and UniProt databases
200 (<http://www.uniprot.org/>). Protein functional categories were obtained as described earlier by Lew *et al.*
201 [28]. Gene essentiality data was procured from Mycobrowser and from previous studies documenting
202 gene essentiality during *in vitro* growth, infection, or growth on cholesterol-containing media [29-32].
203 MtrA crystal structure was obtained from Protein Data Bank (PDB ID: 2GWR) [33] and viewed using
204 UCSF Chimera [34].

205 Putative promoter regions of *Mtb sabH*, or *Msm sabH* were predicted using BPROM
206 (<http://softberry.com>). Probable MtrA-binding sites were searched at these promoter regions using the
207 LASAGNA online tool (<https://biogrid-lasagna.engr.uconn.edu>) [35].

208 *In vitro kinase assay*

209 *In vitro* kinase assay was performed by a protocol described earlier [36]. Briefly, *E. coli* purified
210 His₆-MtrA and mutants (5 µg each) were incubated with MBP-EnvZ kinase (2 µg) in the kinase buffer (50
211 mM Tris-Cl [pH 7.4], 50 mM KCl, 20 mM MgCl₂, 10 mM CaCl₂, and 1 mM DTT) and [γ -³²P]ATP (BRIT,
212 Hyderabad, India) at 37°C for 30 min. The reaction was stopped by adding 2X Laemmli buffer and
213 proteins were resolved on 12% SDS-PAGE followed by autoradiography using Personal Molecular
214 Imager (PMI, Bio-Rad).

215 *Electrophoretic Mobility Shift Assay (EMSA)*

216 DNA region encompassing *Mtb oriC* (205 bp) [37], putative *Mtb sabH* promoter (*sabH*_{Mt}-Pr, 199
217 bp), or putative *Msm sabH* promoter (*sabH*_{Ms}-Pr, 201 bp) were PCR amplified, and purified products were
218 end-labeled with [γ -³²P]ATP using T4 Polynucleotide Kinase (Roche) as per manufacturer's instructions.
219 Varying amounts of His₆-MtrA and its site-specific mutants were phosphorylated using 2 µg MBP-tagged
220 *E. coli* EnvZ in the kinase buffer and 1 mM ATP at 37°C for 30 min. Phosphorylated MtrA and MtrA
221 mutants (10-100 µM) were incubated with the labeled DNA probes at 4°C for 30 min in a buffer
222 containing 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM MgCl₂, 1 mM ATP, and 5% glycerol in a
223 total volume of 20 µl. Reaction samples were resolved using 5% non-denaturing polyacrylamide gel in

224 0.5X Tris/Borate/EDTA buffer. Gels were dried and subjected to autoradiography in Personal Molecular
225 Imager (BioRad).

226 *RNA isolation and real-time PCR.*

227 RNA isolation and quantitative real-time PCR (qRT-PCR) were performed using the protocols
228 described previously [38] with few modifications. Briefly, log phase *Msm* cells were lysed in TRIzol®
229 (Invitrogen) by bead beating using 0.1 mm zirconium beads. RNA was precipitated using isopropanol,
230 washed with 70% ethanol, and dissolved in nuclease-free water. Before performing cDNA synthesis,
231 RNA was treated with DNase (Ambion) according to the manufacturer's protocol to remove traces of
232 genomic DNA. cDNA was synthesized from total RNA using random primers according to the protocol
233 provided by the supplier (Thermo Scientific), and then used for measuring the expression of *mtrA* or
234 *sabH* with gene-specific primers. qRT-PCR was performed using SYBR Green master mix (Roche) as per
235 previously described protocols [39]. The data obtained were analyzed using the $\Delta\Delta C_T$ method and the
236 relative fold change in expression was calculated. *Msm* housekeeping gene *sigA* (encoding Sigma factor A)
237 or 16S rRNA, was used as a control. The primers were sequence-specific for each gene analyzed, with
238 PCR products between 100 and 200 bp.

239 For studying the effect of Hcy on *mtrA* or *sabH* expression, *Msm* cells were grown in an
240 increasing concentration of Hcy and gene expression was measured using qRT-PCR. For assessing the
241 effect of SahH on *mtrA* expression, *Msm* cells containing pVV16 or pVV16-*sabH* were used.

242

243 RESULTS

244 *Multiple Mtb proteins are methylated on lysine and arginine residues.*

245 In *Mtb*, the proteins involved in metabolism, respiration, and cell wall-related processes form the
246 majority of functional proteome compared with regulatory and signaling proteins (Fig S1a) [28].
247 Moreover, in *E. coli* the proteins involved in regulation and signaling represent a low copy number group
248 as compared with the proteins involved in translation, protein folding, and other constitutive functions
249 [40]. Therefore, for our study, we chose 180 candidate protein-coding genes belonging to different
250 functional classes (Fig S1a & b and Table S1) with a focus on less prevalent regulatory and signaling
251 proteins. We selected very few genes from “PE and PPE proteins” and “conserved hypotheticals” and
252 did not select any genes from the categories “Stable RNAs”, “Insertion sequences and phages”, and
253 “Unknown”. These genes were cloned into mycobacterial expression vector pVV16 that contains a
254 carboxy-terminal His₆-tag and the constructs were electroporated into *Msm*, a non-pathogenic model
255 organism that provides appropriate cellular milieu close to *Mtb*. We successfully purified 72 out of the 180
256 proteins under non-denaturing conditions, while others failed to express or purify. Purified proteins were
257 probed with anti-methyllysine to determine their methylation status where Histone protein served as a
258 positive control. The identity of each purified protein was confirmed by re-probing the immunoblot
259 membranes with an anti-His₆ antibody. Apart from identifying the purified target proteins, the anti-His₆
260 antibody also detected the presence of a consistent protein band corresponding to *Msm* chaperon protein
261 GroEL, which is highly abundant and contains a C-terminal histidine-rich tail (MSMEG_1583) [41], and a
262 few other contaminating proteins (Fig 1a). Among the 72 recombinant proteins, ten proteins were
263 recognized by the anti-methyllysine antibody, suggesting the presence of methylation on lysine residues
264 (Fig 1a). However, we could not detect a distinct methylated band for PykA. To validate the protein
265 identity and the methylation of the ten western blot-positive candidate proteins, we performed high-
266 resolution mass spectrometry. We were successful in detecting 20 methyllysine sites belonging to 7
267 candidate proteins except LldD1, LldD2, and Tpi (Fig 1b, Fig S2, Table S2). Interestingly, we also
268 detected 18 methylarginine sites in 7 candidate proteins, which included LldD2 and Tpi (Fig 1b, Fig S2,
269 Table S2). Together, we identified 20 methyllysine and 18 methylarginine sites in 9 out of 10 western blot-
270 positive proteins.

271 *MtrA is methylated in Mtb*

272 Subsequently, we set out to investigate the biological significance of methylation on lysine and
273 arginine residues. Towards this, we chose MtrA as the candidate protein, which was methylated on both
274 lysine and arginine residues. MtrB-MtrA is one among the 11 TCS systems present in *Mtb* where MtrB is
275 the sensor histidine kinase and MtrA is the cognate response regulator. High throughput transposon
276 mutagenesis experiments suggested *mtrA* to be an essential gene for *in vitro* growth of *Mtb* [29]. To
277 determine if MtrA is methylated in *Mtb*, pVV16-*mtrA* expression construct was expressed in *Mtb*, and
278 purified His₆-tagged protein was probed with anti-MtrA and anti-methyllysine antibodies. Consistent with
279 the results obtained in *Msm* (Fig 1a), MtrA was found to be methylated in *Mtb* (Fig 2b).

280 MtrA is a 228 amino acid (aa) long protein with a 102 aa long N-terminal response regulator
281 domain and a 93 aa long C-terminal winged helix-turn-helix DNA binding domain homologous to *E. coli*
282 OmpR (Fig 2a & Fig S3) [42, 43]. Mass spectrometry data showed that MtrA was methylated on six
283 arginine residues and seven lysine residues (Fig 1b). Analysis of these 13 methylated residues showed that
284 R122 is a conserved residue present in the linker region, and K204 and K207 are adjacent to the DNA
285 recognition helix (Fig 2c & Fig S3). Therefore, we examined the roles of R122, K204, and K207 by
286 mutating them individually to methionine residues, the closest structural mimic to dimethyllysine [44].
287 Wild type and mutant MtrA proteins were expressed in *Msm* and the purified proteins were probed with
288 anti-methyllysine antibody to compare their relative methylation (Fig 3a). Densitometric analysis of blots
289 suggested that mutating R122, K204, or K207 individually resulted in decreased overall methylation
290 levels, albeit the extent of reduction varied. While MtrA_{K204M} and MtrA_{K207M} had considerable decrease in
291 the extent of methylation (95% and 80%, respectively; *p* value < 0.0001), MtrA_{R122M} mutant only lost
292 marginal methylation (30%) (Fig 3b). The contribution of these three amino acid residues towards total
293 MtrA methylation was further analyzed by a quantitative proteomics-based method to determine the
294 extent of methylation at ten sites in MtrA. A heat-map was generated representing the methylation
295 intensities of identified peptides in MtrA, MtrA_{R122M}, MtrA_{K204M}, and MtrA_{K207M}. The fold change of
296 signal intensities at specific sites in MtrA mutants relative to that in MtrA are shown in Table S3. The
297 comparison shows that mutating any of these residues negatively affects the methylation at other sites;
298 K204M or K207M completely abolish the methylation at four other sites (Fig. 3c). We observed a
299 background signal for amino acid position 207 in the MtrA_{K207M} mutant, which was due to the ‘match
300 between run’ event rather than a bonafide fide MS/MS signal. Moreover, the signal was only 0.4%
301 compared with that in MtrA-WT signal, suggesting that it most likely represents the noise (Table S3).
302 Similarly, background signal was also observed for amino acid position 122 in MtrA_{R122M} mutant, which
303 may be due to noise but could not be attributed to ‘match between run’ event. Collectively, the data
304 indicate that multiple methylated residues of MtrA act co-operatively and K204 and K207 are crucial for
305 MtrA methylation.

306 *Methylation of MtrA is critical for DNA binding.*

307 Binding of MtrA to DNA is contingent upon its phosphorylation on D56 residue by the sensor
308 kinase MtrB [42]. Once phosphorylated, MtrA is known to regulate DNA replication by binding to the
309 repeat nucleotide motifs at the origin of replication (*oriC*) [37]. To evaluate the role of methylation of
310 MtrA on its DNA binding ability, we chose the 205 bp long region of *oriC* as a probe to perform EMSA
311 with purified MtrA and its methylation site mutants MtrA_{R122M}, MtrA_{K204M}, and MtrA_{K207M}. To
312 phosphorylate and activate MtrA, we utilized EnvZ, a homolog of MtrB in *E. coli* that has been used in
313 several previous studies [36, 37, 45]. MtrA, MtrA_{R122M}, MtrA_{K204M}, and MtrA_{K207M} proteins were
314 incubated with EnvZ in the presence of [γ -³²P] ATP and their phosphorylation status was analyzed by
315 autoradiography. As anticipated, EnvZ was found to be autophosphorylated likely on the histidine
316 residue (Fig 4a; upper band). In addition to the autophosphorylated EnvZ, we detected efficient

317 phosphorylation of MtrA and its site-specific mutants (Fig 4a; lower band). Moreover, phosphorylation of
318 MtrA was found to be similar for wild type and mutant proteins suggesting that EnvZ does not
319 differentiate between these substrates (Fig 4a).

320 Next, we evaluated the DNA binding activity of wild type MtrA with or without EnvZ
321 incubation by EMSA using radiolabeled *oriC* fragment as the DNA probe. It is apparent from the data
322 that there is no DNA: protein complex formation if either MtrA or EnvZ is absent (Fig 4b). We observed
323 DNA binding only upon incubation of phosphorylated MtrA with radiolabeled *oriC* DNA fragment and
324 the binding efficiency was dependent on the concentration of MtrA (Fig 4b). These results show that,
325 EnvZ efficiently phosphorylates MtrA *in vitro*, and phosphorylated MtrA proficiently interacts with the
326 DNA (Fig 4a & 4b).

327 Finally, we compared the DNA binding activity of MtrA and MtrA mutants that were
328 phosphorylated by EnvZ (Fig 4c). Equal amounts of phosphorylated MtrA, MtrA_{R122M}, MtrA_{K204M}, or
329 MtrA_{K207M} were incubated with *oriC* DNA probe and EMSA was performed. While we could detect
330 DNA: protein complex with MtrA, and MtrA_{K207M} proteins; mutants MtrA_{R122M} and MtrA_{K204M} showed
331 marginal or no binding, respectively. Since lysine residues can be modified by other PTMs such as
332 acetylation, we analyze whether MtrA was a target of any of these other modifications. We performed
333 additional mass spectrometric analysis to identify lysine modifications on His₆-MtrA_{Mtb} expressed and
334 purified from *Msm*. Mass spectrometric analysis showed the presence of acetylation and succinylation on
335 MtrA and both modifications were found to be on K207 residue, but not on K204 residue, suggesting
336 that the only modification detected on K204 is methylation (Fig S4). Taken together this data suggest that
337 the methylation of R122 and K204 plays an important role in modulating the interaction of MtrA with
338 DNA.

339 *Perturbation of metabolic intermediate levels influences MtrA methylation.*

340 Next, we tried to identify mechanisms that regulate protein methylation. Methylation reactions
341 are catalyzed by SAM-dependent methyltransferases where SAH and consequently Hcy are generated as
342 by-products (Fig 5a). We have previously shown that perturbation of levels of *Mtb* SahH impacts
343 metabolic levels of Hcy and may affect SAH, a potent inhibitor of methyltransferases [19]. Interestingly,
344 *Mtb sabH* (encoding SahH), an essential gene, is present in the genomic vicinity of *mtrA* [29]. This led us
345 to hypothesize that SahH-mediated perturbation in the levels of SAH or Hcy may impact methylation of
346 proteins like MtrA.

347 To test our hypothesis, we first evaluated the effect of increasing Hcy on the growth of *Msm*.
348 Bacteria were grown in minimal growth medium containing varying concentrations of Hcy and their
349 growth was measured. We found that increasing concentration of Hcy negatively affects bacterial growth
350 in a concentration-dependent manner (Fig 5b & 5c). Results suggested that higher than 0.4 mM Hcy
351 resulted in more than a log-fold decrease in *Msm* CFUs during the exponential growth phase. Therefore,
352 we decided to use a sub-lethal concentration of 0.4 mM for further experiments. We analyzed methylation
353 of MtrA purified from *Msm* grown in the presence or absence of 0.4 mM Hcy using immunoblotting. In

354 line with our hypothesis, the addition of Hcy resulted in a ~70% decrease in methylation of MtrA (Fig 5d
355 and 5e).

356 Next, we addressed the influence of overexpressing SahH on MtrA methylation. We analyzed
357 methylation of MtrA purified from *Msm* containing an integrated copy of *Mtb* SahH. We observed that
358 overexpression of SahH also resulted in a ~70% decrease in MtrA methylation levels, presumably because
359 of perturbed SAH levels as SAH is a potent inhibitor of methyltransferases (Fig 5f and 5g). Collectively,
360 the data suggest that perturbation of metabolic intermediates negatively modulates MtrA methylation.

361 *MtrA* methylation negatively regulates transcriptional activation.

362 In Fig 4, we showed that the methylation mimetic mutant of K204 (K204M) does not bind with
363 the DNA. As a corollary, methylation of MtrA should negatively modulate MtrA-mediated transcriptional
364 activation whereas a decrease in the methylation should positively modulate transcriptional activation.
365 Results in Fig 5 showed that the addition of Hcy or overexpression of SahH results in decreased
366 methylation of MtrA. Taken together, we theorized that the addition of Hcy or overexpression of SahH
367 would increase the transcriptional activation by MtrA. MtrA is known to bind to its own promoter and
368 regulates its expression [37]. Thus, we monitored the expression level of *mtrA* in the presence of an
369 increasing concentration of Hcy. *Msm* cells were grown in minimal medium supplemented with increasing
370 concentration of Hcy and *mtrA* expression was measured using quantitative real-time PCR (qRT-PCR). In
371 line with our hypothesis, we observed increased transcription of *mtrA* with an increasing concentration of
372 Hcy (Fig 6a). In these qRT-PCR reactions, the expression was normalized with respect to the expression
373 of *sigA*. To reconfirm these results, we performed a new set of qRT-PCR reactions in the presence of 0.4
374 mM Hcy, except that the expression of *mtrA* was normalized with respect to the expression of 16S rRNA
375 (Fig 6b). The results were in agreement with the data presented in Fig 6a, confirming that the addition of
376 Hcy increases the expression of *mtrA*. Next, we examined the expression levels of *mtrA* upon expression
377 of SahH by utilizing *Msm* harboring pVV16-*sabH* plasmid (Fig 6c). We observed a ~6-fold increase in the
378 transcript levels of *mtrA* in the presence of overexpressed SahH.

379 Subsequently, we asked if the addition of Hcy impacts the expression of *sabH* and if so, does
380 MtrA binds to the promoter region of *sabH*. To address this question, we evaluated the expression of
381 *Msm sabH* in the presence of an increasing concentration of Hcy (Fig 6d). The results showed a direct
382 correlation between Hcy concentration and expression of *sabH_{Mt}*. Besides *mtrA* promoter regions, we
383 identified MtrA-binding sites in the putative *sabH* promoter region. Thus to examine if MtrA binds to
384 putative *sabH_{Mt}* (*sabH_{Mt}-Pr*) and *sabH_{Mt}* (*sabH_{Mt}-Pr*) promoter regions, we performed EMSA with
385 radiolabeled *sabH* promoter regions from *Msm* and *Mtb*, respectively. While only EnvZ or
386 unphosphorylated MtrA does not bind with the DNA, we observed robust binding of phosphorylated
387 MtrA with both *sabH_{Mt}-Pr* and *sabH_{Mt}-Pr* DNA fragments (Fig 6e). Most importantly, MtrA_{K204M} mutant
388 that showed abrogated binding with *oriC* fragment in the previous EMSA experiments (Fig 4c) failed to
389 bind with both *sabH_{Mt}-Pr* and *sabH_{Mt}-Pr* DNA fragments, confirming that methylation of MtrA negatively
390 modulates DNA binding and hence its activity both *in vitro* and *in vivo*.

391 Discussion

392 Covalent modification of side chains of multiple amino acids in proteins regulates their activity
393 and function thus controlling cellular processes [46]. In addition to phosphorylation, which has been
394 extensively investigated, multiple additional modifications have been identified with the help of high
395 throughput mass spectrometry or by candidate-specific approaches. In this report, we used a candidate
396 approach to identify proteins that are methylated on lysine residue, and the methylation of the positive
397 candidates was validated by mass spectrometry. Most of the candidate proteins that were chosen for the
398 study belonged to regulatory protein class (Supplementary Fig. 1a & b), followed by intermediary
399 metabolism, and cell wall and cell processes. While a high throughput mass spectrometry approach may
400 have provided a more comprehensive list of methylated proteins, with our approach we detected
401 methylation of proteins in the functional categories that are relatively less prevalent. We have used a
402 similar candidate approach previously to identify novel acylated proteins in *Mtb* [20]. In an independent
403 study, western blot analysis of MtHU (HupB) expressed and purified from *Msm* revealed the presence of
404 acetylation, and the target sites were subsequently identified by mass spectrometry [47]. We identified a
405 total of 10 proteins by western blot and the mass spectrometry analysis showed nine of them to be
406 methylated on lysine residues. Identification of ten western blot positive methylated proteins among the
407 72 candidates suggest that methylation could be a more frequent modification in mycobacterial proteins
408 and warrant future large-scale analyses of the whole proteome. We propose that the present study be used
409 in parallel with global proteomics-based approaches in order to have an unbiased analysis of both over-
410 and under-represented protein functional categories in the whole proteome.

411 Analysis of *Mtb* genome suggests the presence of 57 probable methyltransferases- 29 of them
412 may be involved in intermediary metabolism and respiration, 12 of them are probable lipid
413 methyltransferases, 8 could be involved in RNA methylation, and 7 in DNA methylation
414 (<https://mycobrowser.epfl.ch/>). To date, only three methyltransferases-Rv1988, Rv2966c, and MamA-
415 have been functionally characterized. Rv1988 is a secretory methyltransferase that enters the host nucleus
416 and methylates histone H3 at arginine residues and regulates the expression of genes involved in
417 combating reactive oxygen species [48]. Rv2966c is also a secretory methyltransferase that localizes to the
418 host nucleus and methylates host DNA at cytosine residues [49]. MamA is a DNA N6-adenine
419 methyltransferase that regulates the expression of multiple genes that provides fitness during hypoxia
420 [50]. Methylation of *Mtb* HBHA and HupB by unknown methyltransferase(s) renders them proteolytic
421 resistant [18]. Recently, a host methyltransferase is shown to methylate *Mtb* HupB to confer protection
422 against invading bacilli [51]. Thus far, lysine/arginine methyltransferases that can act on the bacterial
423 protein targets have not been characterized in mycobacteria. Identification of lysine/arginine methylation
424 of many essential *Mtb* proteins indicate mycobacterial methyltransferases might play important role in
425 pathogenesis and physiology of mycobacteria. Elucidating the specificities and mode of substrate
426 recognition of methyltransferase would help in understanding the biological significance of protein
427 methylation.

428 To elucidate the functional relevance of methylation in mycobacteria, we chose MtrA, an
429 essential response regulator of TCS MtrB-MtrA in *Mtb*. MtrB is a non-essential membrane-bound sensor
430 kinase that transfers a phosphate group to a conserved aspartate residue (D56) in MtrA. MtrA binds to
431 the promoters of *ripA* (encoding peptidoglycan hydrolase), *fbpA* (encoding secreted antigen 85B), *fbpB*
432 (encoding cell wall mycolyl hydrolase), *dnaA* (encoding replication initiator protein) and *oriC* (origin of
433 replication) and regulates cell cycle progression [37, 52]. Although phosphorylation at D56 is the primary
434 regulatory mechanism for MtrA, the protein has also been reported to be pupylated at K207 [53] and
435 acetylated at K110 [54]. We now show that MtrA is modified by lysine/arginine methylation, lysine
436 acetylation, and lysine succinylation. Different lysine modifications occurring on MtrA might play a role
437 in regulating different aspects of MtrA, such as methylation-mediated regulation of DNA-binding activity
438 and pupylation-mediated regulation of protein turnover rate. Methylation of MtrA on arginine and lysine
439 residues was found to negatively regulate its DNA binding function (Fig 4). Arginine methylation
440 regulates several mammalian processes associated with gene expression but is largely unrecognized in
441 bacteria [8]. Proteomics analysis has revealed >25 arginine methylated proteins in *Leptospira interrogans*, but
442 no functional role has been assigned to them [55]. In most of these proteins, lysine methylation occurs in
443 conjunction with arginine methylation on the same protein as is the case with mycobacterial MtrA. On
444 the contrary, all the arginine methylated proteins of *Desulfovibrio vulgaris* do not contain methyllysine [56].
445 Although a dimethylarginine was spotted on a *D. vulgaris* transcriptional response regulator DVUA0086,
446 its functional role remained obscure. Further revelation of the role of arginine methylation in bacteria is
447 therefore essential.

448 Methyltransferase reactions are dependent on the presence of balanced amounts of SAM and
449 SAH as they are prone to SAH-mediated inhibition. Under normal conditions, SAH levels are regulated
450 using SahH-mediated reversible hydrolysis of SAH to Hcy. Hcy supplementation may allow the net flux
451 of this reversible reaction towards SAH synthesis, which can negatively regulate methyltransferases
452 activity. In a similar vein, overexpression of SahH may lead to depletion of SAH, which in turn leads to
453 lower levels of SAM, a substrate for methyltransferases, thus influencing the activity. In agreement with
454 this hypothesis, we observed that the addition of Hcy or overexpression of SahH led to decreased
455 methylation of MtrA (Fig 5). DNA binding experiment suggests that methylation negatively regulates
456 MtrA interaction with DNA (Fig 4) and overexpression of SahH or addition of Hcy decreases MtrA
457 methylation. In accordance, SahH or Hcy were found to increase *mtrA* transcription which may lead to
458 altered expression of genes targeted by MtrA such as *ripA*, *fbpB*, and *dnaA* and regulate cell cycle
459 progression. It is to be noted that SahH has previously been found to be associated with differential
460 DNA and RNA methylation in eukaryotes [57-59], thus pointing towards a more general implication of
461 SahH in regulating one-carbon metabolism. In mycobacteria, one-carbon metabolism pathway involving
462 SAM and methionine biosynthesis has been proposed as a powerful target for anti-mycobacterial agents
463 [60]. Mycobacterial strains deficient in SAM and methionine biosynthesis were found to be remarkably
464 vulnerable in host tissues. Interestingly, such metabolic perturbation was shown to be associated with

465 altered methylation at DNA and other important metabolites like biotin. In another study, disruption of
466 one-carbon metabolism by antifolate molecules led to efficient killing of *Mtb* [61]. These studies suggest
467 the significance of studying regulators of one-carbon metabolism and highlight SahH as a promising drug
468 target.

469 In summary, the present study provides a framework for elucidation of protein methylation in
470 mycobacteria. We report the addition of protein arginine methylation to the growing list of regulatory
471 PTMs in mycobacteria and suggest that methylation of MtrA at lysine and arginine residues regulates its
472 activity. This study provides an orchestration of methylation and TCS signaling and therefore illuminates
473 the critical role of methylation in bacterial physiology.

474

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484 **Data availability:** Original mass spectrometry spectra are submitted in Sup Figure 2. All the reagents
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486

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664

665 **Figure legends**

666 **Figure 1. Multiple *Mtb* proteins are methylated on lysine and arginine residues.** (a) Ten
667 recombinant proteins containing His₆-tag were purified using Ni²⁺-NTA beads from *Msm*. Purified
668 proteins were loaded on SDS-PAGE, transferred on nitrocellulose, and probed with anti-methyllysine or
669 anti-His₆ antibody. (b) Table shows the number and location of methylation sites in the recombinant
670 proteins by mass spectrometry. The corresponding supplementary image number of mass spectra is also
671 mentioned.

672 **Figure 2. *MtrA* is methylated in *Mtb*.** (a) Pictorial representation of MtrA domain organization
673 showing the location of different methylated sites. (b) His₆-MtrA was overexpressed and purified from
674 *Mtb*. Purified protein was probed with anti-methyllysine antibody (upper panel) and anti-MtrA (lower
675 panel) antibody. Histone was used as a positive control. (c) Structural representation of MtrA (PDB ID:
676 2GWR). Response regulator domain (blue), linker region (green), and DNA binding domain (pink) are
677 visible with the three crucial methylated sites marked red.

678 **Figure 3. *MtrA* is methylated at lysine and arginine residues.** (a) MtrA and its mutants were over-
679 expressed and purified from *Msm*. Purified proteins were loaded on SDS-PAGE, transferred on
680 nitrocellulose, and probed with anti-methyllysine antibody. Ponceau-stained membrane image is shown in
681 the lower image. (b) Densitometric analysis of the western blot shown in (a). The bar graph depicts
682 intensities obtained after normalization with protein amounts detected by ponceau staining. The intensity
683 of methylated MtrA was considered as 100% and relative methylation intensities of mutants are plotted.
684 Data (mean \pm s.d.) are from three individual replicates. **** $P \leq 0.0001$, as determined by two-tailed
685 unpaired Student's *t*-test. (c) Heat map showing the effect of mutation of R122, K204, and K207 residues
686 on methylation intensities at other sites. Each row depicts the residue at which quantitative analysis was
687 performed and each column represents the protein analyzed. Mass spectrometric intensities are color-
688 coded according to the key given below the heat map (log₂ scale).

689 **Figure 4. Role of methylated residues in DNA-binding activity.** (a) MtrA WT and mutants were
690 expressed and purified from *E. coli* and equal amounts were phosphorylated by EnvZ in the presence of
691 γ [³²P]ATP. The reactions were resolved on SDS-PAGE, coomassie stained (lower panel), and
692 autoradiographed (upper panel). (b) Radiolabelled *Mtb oriC* DNA probe was synthesized using γ [³²P]ATP
693 by PCR. DNA binding assay was performed in the presence of unphosphorylated (6 μ g, lane 2) or
694 phosphorylated MtrA (6 μ g and 2 μ g, lanes 4 and 5). As a control, reactions were performed in the
695 absence of MtrA (lane 3) or without any protein (lane 1). The reactions were resolved on native PAGE
696 and gels were autoradiographed. DNA: protein complex and the unbound DNA are shown. (c)
697 Radiolabelled *oriC* probe was incubated in the presence of 0-100 μ M phosphorylated MtrA, MtrA_{K204},
698 MtrA_{K207}, or MtrA_{R122} proteins. The reactions were resolved on native PAGE and gels were
699 autoradiographed. DNA: protein complex and the unbound DNA are shown.

700 **Figure 5. The perturbation of metabolic intermediate levels influences *MtrA* methylation.** (a)
701 Reaction showing the synthesis of Hcy from SAH catalyzed by SahH. (b & c) *Msm* cells were grown in

702 the presence of 0-0.8 mM Hcy and growth was measured. Data (mean \pm s.d.) are from four individual
703 replicates. (b) A_{600} was plotted as a function of time. (c) Graph shows $\text{Log}_{10}(\text{CFU/ml})$ calculated during
704 the exponential growth phase as a function of Hcy concentration. (d & e) MtrA was expressed and
705 purified from *Msm* using Ni^{2+} -NTA chromatography in the absence or presence of 0.4 mM Hcy. (d)
706 Immunoblotting was performed using anti-methyllysine antibody followed by an anti-His₆ antibody. (e)
707 Graph showing the relative methylation of MtrA in the presence of Hcy with respect to methylation of
708 MtrA in the absence of Hcy. Methyllysine intensities were normalized to MtrA protein levels as measured
709 by anti-His₆ immunoblot. Data (mean \pm s.d.) are from three individual replicates. (f & g) MtrA was
710 expressed and purified from *Msm* strain that overexpressed SahH using Ni^{2+} -NTA chromatography. (f)
711 Immunoblotting was performed using anti-methyllysine antibody followed by anti-His₆ and anti-MtrA
712 antibodies. (g) Graph showing the methylation of MtrA in the presence of overexpressed SahH relative to
713 the methylation of MtrA in the presence of vector control. Methyllysine intensities were normalized to
714 MtrA protein levels as measured by anti-His₆ immunoblot. Data (mean \pm s.d.) are from three individual
715 replicates. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, as determined by two-tailed unpaired Student's *t*-test.

716 **Figure 6. MtrA methylation negatively regulates transcriptional activation.** (a-c) *mtrA* expression
717 was analyzed using qRT-PCR in *Msm* cultures grown in the presence of Hcy (a & b) or SahH
718 overexpression (c). Expression level of *mtrA* was analyzed with respect to *sigA* (a & c) or 16S rRNA (b).
719 (d) *sabH* expression was analyzed using qRT-PCR in *Msm* cultures grown in the presence of Hcy with
720 respect to *sigA*. Data (mean \pm s.d.) are from six (a) or three (b & c) biological triplicates. * $P \leq 0.05$,
721 ** $P \leq 0.01$, **** $P \leq 0.0001$ as determined by two-tailed unpaired Student's *t*-test (compared to control
722 values). (e) DNA binding assay was performed using putative *sabH* promoter fragments from *Mtb*
723 (*sabH_{Mt}*-Pr) or *Msm* (*sabH_{Mt}*-Pr). MtrA and MtrA_{K204M} were used in unphosphorylated or phosphorylated
724 forms. The reactions were resolved on native PAGE and gels were autoradiographed. DNA:protein
725 complex and the unbound DNA are shown.

726

727 **Table 1.** List of primers used in this study.

Primer Name	Primer sequence (5'→3') ^b
MtrA F	GTCCCGATGTGGTGACATATGGACACCATGAGGC (NdeI)
MtrA R	GCATCGTCGCCGGCGAAGCTTCGGAGGTCCGGCCTTG (HindIII)
EnvZ F	ACGGCTCGGATCCATGAGGGCGATTGCGCTTC (BamHI)
EnvZ R	CCTTCGCCTCAAGCTTATTTACCCTTCTTTTG (HindIII)
MtrAK204M F	GTCCAGCGTCTGCGGGCCATGGTTCGAAAAGGATCCCGAG
MtrAK204M R	CTCGGGATCCTTTTCGACCATGGCCCGCAGACGCTGGAC
MtrAK207M F	CTGCGGGCCAAGGTCGAAATGGATCCCGAGAACCCGACTG
MtrAK207M R	CAGTCGGGTTCCTCGGGATCCAATTCGACCTTGGCCCGCAG
MtrAR122M F	GGTGCGGGCGCGGCTGCGCATGAACGACGACGAACCCGCCG
MtrAR122M R	CGGCGGGTTCGTCGTCGTTTCATGCGCAGCCGCGCCCGCACC
SahH F	GGATGAAAGCCCATATGACCGGAAATTTGG (NdeI)
SahH R	TGGGCGATTTTGCGTAAGCTTTCGGGGTGGGA (HindIII)
OriC F	CACGGCGTGTCTTCCGAC
OriC R	GTCGGAGTTGTGGATGACGG
SahH _{M5} -pr F	GCGCTGGCGATGAGCTACG
SahH _{M5} -pr R	GCACACTCATGCCGACAACC
SahH _{Mt} -pr F	GCGGCTGTGCTTGAGCTACG
SahH _{Mt} -pr R	GCTCACAGGGATCCGAGCG
MtrART F	CCATCGTTCTGCGTGGTGAG
MtrART R	GGTCAGCATGACGATCGGC
SahH-RT F	GCGCCAAGAAGATCAACATC
SahH-RT R	CTCGGACAGCACGATGATC
SigA-RT F	CGTTCCCTCGACCTCATCCA
SigA-RT R	GCCCTTGGTGTAGTCGAACTTC
16S-RT F	AATTCGATGCAACGCGAAGA
16S-RT R	GCGGGACTTAACCCAACATC

728 ^aF' denotes forward primer and 'R' denotes reverse primer.

729 ^bRestriction sites/mutations are underlined and Restriction enzymes are mentioned in parenthesis.

730

731 **Table 2:** List of plasmids used in this study.

Plasmid construct	Description	Reference or source
pVV16	Mycobacterial expression vector with kanamycin resistance	[22]
pET28a	<i>E. coli</i> expression vector with His ₆ -tag and kanamycin resistance	Novagen
pMAL-c2x	<i>E. coli</i> expression vector with MBP-tag and ampicillin resistance	New England BioLabs
pSET152	Mycobacterial integrative vector with apramycin resistance	[21]

732

Figure 1

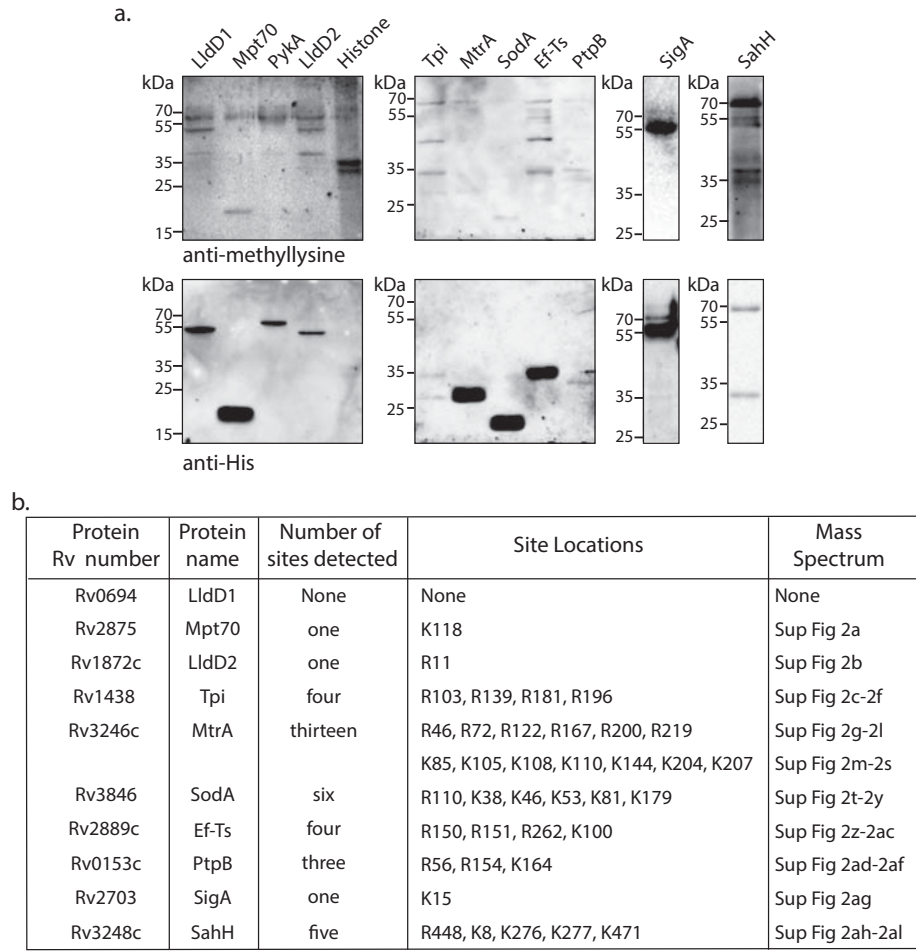


Figure 2

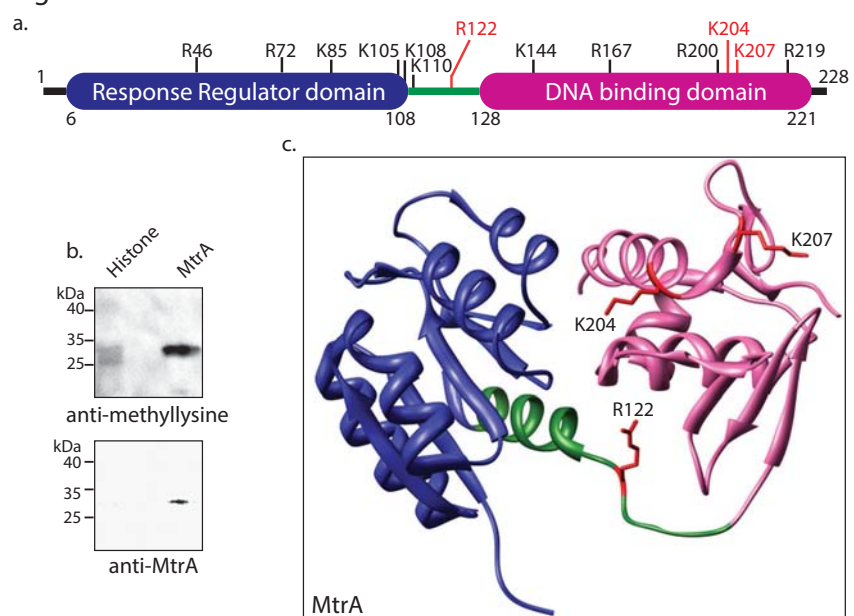


Figure 3

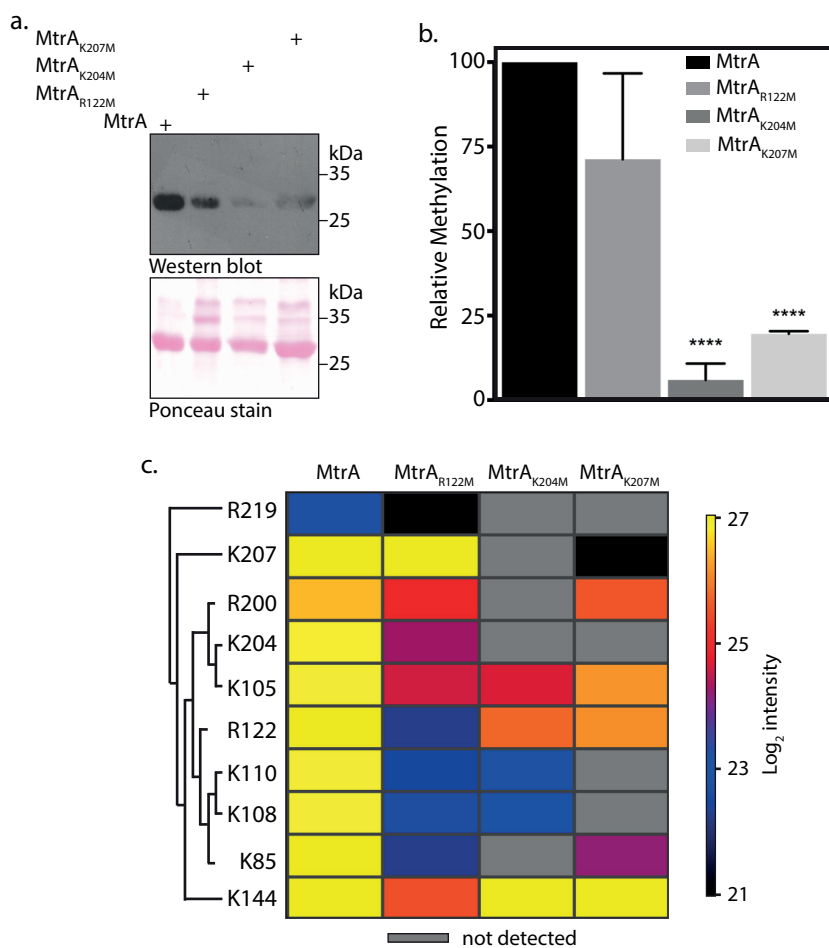


Figure 4

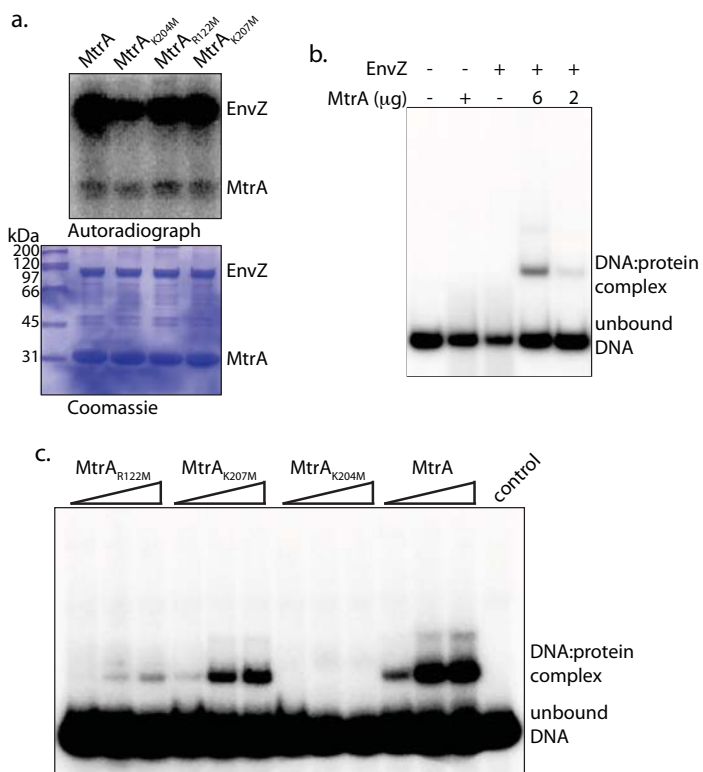


Figure 5

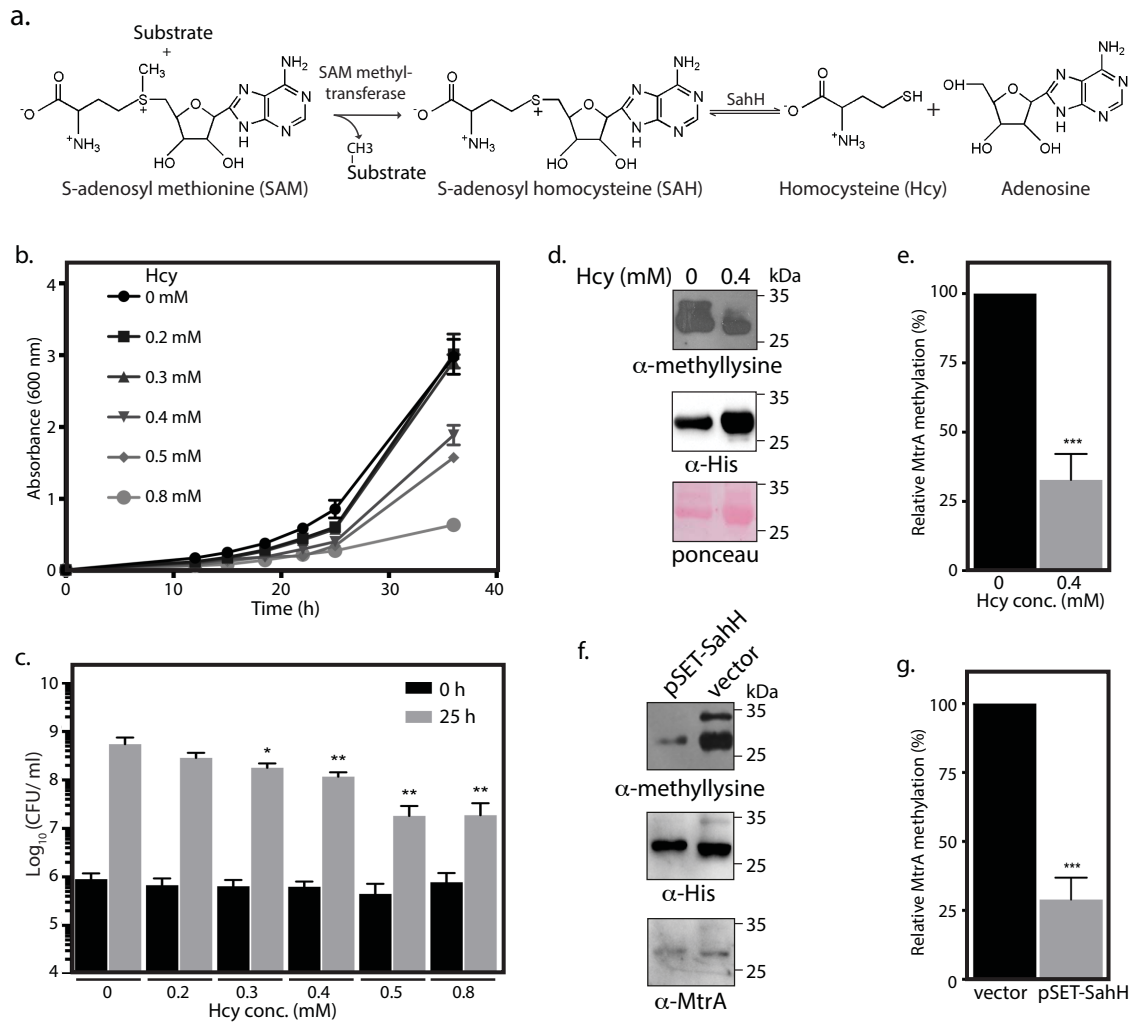


Figure 6

