Precision methylome characterization of *Mycobacterium tuberculosis* complex (MTBC) using PacBio single-molecule real-time (SMRT) technology

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**ABSTRACT**

Tuberculosis (TB) remains one of the most common infectious diseases caused by *Mycobacterium tuberculosis* complex (MTBC). To panoramically analyze MTBC’s genomic methylation, we completed the genomes of 12 MTBC strains (*Mycobacterium bovis*; *M. bovis* BCG; *M. microti*; *M. africanum*; *M. tuberculosis* H37Rv; H37Ra; and 6 *M. tuberculosis* clinical isolates) belonging to different lineages and characterized their methylomes using single-molecule real-time (SMRT) technology. We identified three \(^{\text{m6}}\text{A}\) sequence motifs and their corresponding methyltransferase (MTase) genes, including the reported *mamA*, *hsdM* and a newly discovered *mamB*. We also experimentally verified the methylated motifs and functions of *HsdM* and *MamB*. Our analysis indicated the MTase activities varied between 12 strains due to mutations/deletions. Furthermore, through measuring ‘the methylated-motif-site ratio’ and ‘the methylated-read ratio’, we explored the methylation status of each modified site and sequence-read to obtain the ‘precision methylome’ of the MTBC strains, which enabled intricate analysis of MTase activity at whole-genome scale. Most unmodified sites overlapped with transcription-factor binding-regions, which might protect these sites from methylation. Overall, our findings show enormous potential for the SMRT platform to investigate the precise character of methylome, and significantly enhance our understanding of the function of DNA MTase.

**INTRODUCTION**

Tuberculosis (TB) has been the subject of a global health emergency, with more than 9 million new cases of active disease and nearly 1.5 million deaths annually (1). As the primary pathogen causing tuberculosis, *Mycobacterium tuberculosis* (MTB) belongs to the *Mycobacterium tuberculosis* complex (MTBC), all of whose members can cause tuberculosis in humans or other organisms. Besides *M. tuberculosis*, the MTBC group mainly consists of some genetically related *Mycobacterium* species (sharing more than 99% identity at the nucleotide level) including: *Mycobacterium bovis*; *M. bovis* BCG; *Mycobacterium microti*; *M. africanum*; and *Mycobacterium canettii* (2). The MTBC members have different methods of host adaptation and pathogenicity: *M. tuberculosis* and *M. africanum* mainly infect humans, whereas *M. bovis* now rarely causes disease in humans and mainly infects animals such as cattle. *M. microti* has been reported to mainly infect animals such as voles and also rarely infects humans, while the at-
tenuated \textit{M. bovis} BCG strain is derived from a virulent \textit{M. bovis} strain, and is well known as a vaccine against tuberculosis.

Among MTBC clinical isolates, the main human-adapted strain lineages are classified into seven groups (Lineage 1–7, L1–7) according to their geographic distribution: Indo-Oceanic (L1); East Asian (L2); East African-Indian (L3); Euro-American (L4); West African 1 (L5); West African 2 (L6); and Ethiopian (L7). L1–L4 comprise the majority of human-adapted strains which are responsible for global human TB cases, while L5 and L6 are restricted to West Africa, and L7 is only associated with those cases surfacing in the Horn of Africa (3). The animal-adapted strains are regarded as lineage 8 (L8) (4). Alternatively, based on the presence or absence of a \textit{M. tuberculosis} specific deletion (TbD1), MTBC strains can also be classified as ancient strains (L1; L5–L7) and modern strains (L2–L4) (4).

There are some genomic studies concerning MTBC strains including DNA methylation, which have been reported in recent years (5–8). However, due to their high GC content (∼65%), and the PE/PPE multigene families (accounting for ∼10% of the genome) having repetitive sequences of PE/PPE regions (7), only 48 MTBC genomes have been completed so far, while most of the others (more than 1500) are only draft assemblies (http://www.ncbi.nlm.nih.gov/genome/genomes). Next-generation sequencing (NGS) platforms, such as the Illumina HiSeq platform, have had technical difficulties in finishing whole genomes with a high GC content and large numbers of repetitive sequences (9). Meanwhile, DNA methylation in MTBC genomes was also studied using various methods and technologies, including liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS), restriction digest susceptibility and Sanger sequencing (8,10). This line of research revealed the existence of N6-methyladenine (\textsuperscript{m6}A) and 5-methylcytosine (\textsuperscript{m5}C) within MTBC genomes. Two DNA methyltransferases (MTases), MamA and HsdM, which are responsible for \textsuperscript{m6}A modification, were discovered, of which MamA MTase has been well characterized. Its targeted methylated sequence motif (CTCCAG) was also identified. Interestingly, the function of MamA appears to be different from that of most MTases in prokaryotes, whose primary function of DNA methylation is DNA self-recognition via restriction-modification (R-M) systems that protect the organism against invading DNA (11). Instead, MamA appears to operate more like an ‘orphan’ MTase, such as Dam in \textit{Escherichia coli}, because so far the cognate restriction enzyme has not been discovered in MTB genomes (12). It has been reported that the ‘orphan’ MTases may play an important role in chromosome stability, mismatch repair, replication, etc. (12,13). MamA has also been proved to be capable of influencing gene expression and fitness during hypoxia (13).

Single-molecule real-time (SMRT) sequencing, recently developed by Pacific Biosciences, can achieve unbiased GC coverage with extraordinarily long reads (up to 20 kb), therefore it is suitable for use in sequencing the genomes with high GC content and large numbers of repetitive regions like MTBC strains. Furthermore, SMRT sequencing allows direct genome-wide detection of diverse modified bases (\textsuperscript{m6}A, \textsuperscript{m4}C, \textsuperscript{m5}C, phosphorothioate modification, etc.) by monitoring the kinetic variations (KV) of the single base (14), in which \textsuperscript{m6}A provides the strongest signals. Remarkably, SMRT sequencing provides a novel strategy for characterizing the ‘precision methylome’ by detecting the modification status of each site and even each sequence-read (15,16). This method enables the MTase activity to be intricately characterized at a whole-genome scale. In previous years, many bacterial methylosomes (\textit{E.coli}, Mycoplasma genitalium, etc.) have been determined using SMRT sequencing technology (17–20). However, until now, the panoramic analysis of MTBC’s genomic methylation has not been reported. We have reason to believe that methylome characterization of MTBC strains will help us to completely understand the genomic function.

In this study, we completed the genomes of 12 MTBC strains and analyzed their respective methylomes using SMRT sequencing technology. The results revealed that there were three \textsuperscript{m6}A motifs and three corresponding \textsuperscript{m6}A DNA MTase genes encoded within the MTBC genomes, including the reported \textit{mamA} (Rv3263), the \textit{hsdM} (Rv2756c) (8) and a newly discovered MTase gene (Rv2024c), which we termed \textit{mamB} (Mycobacterial adenine methyltransferase B). We report, to our knowledge for the first time, verification of the methylated motifs and functions for both the HsdM and MamB MTases. Interestingly, we found that the activities of the three MTases varied within different lineages, probably due to either mutations or deletions inactivating the corresponding MTases. Furthermore, based on the information obtained from SMRT sequencing, we investigated the methylation status of each modified site and even each modified sequence-read. By determining the ‘methylated-motif-site ratio’ and ‘the methylated-read ratio’, we were able to discover the ‘precision methylome’ of the MTBC strains, which enabled an intricate analysis of MTase activity on the scale of the whole-genome.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions**

All MTBC strains used in this study are listed in Supplementary Table S1. The MTBC strains were grown in Lowenstein–Jenden media or Middlebrook 7H10 supplemented with 10% OADC (Oleic Albumin Dextrose Catalase, Becton Dickinson), glycerol and 0.05% Tween 80.

**MIRU-VNTR genotyping**

We used the VNTR-15 scheme as described in MIRU-VNTRplus (http://www.miru-vntrplus.org/) for genotyping, comprising the following markers: Mtbub04; ETRC; MIRU04; MIRU40; MIRU10; MIRU16; Mtbub21; QUB11b; ETRA; Mtbub30; MIRU26; MIRU31; Mtbub39; QUB26; and QUB4156. Each MIRU-VNTR locus was amplified individually, and electrophoresis of products on agarose gels was carried out as described previously (21). The copy number at each locus was calculated with BioNumerics software.
Large sequence polymorphism (LSP) determination of deletions in pks15/1

A large sequence polymorphism (LSP) (pks15/1) was used to differentiate the clinical TB strains by using the primers in Supplementary Table S2. Deletion of a 7-bp region in the polyketide synthase gene pks15/1 is present in the Euro-American lineage of M. tuberculosis (22).

SMRT sequencing

Genomic DNA from the MTBC strains was extracted using TIANamp Bacteria Genomic DNA Kit (Tiangen Biotech Co. Ltd., Beijing, China). Pacific Biosciences RSII DNA sequencing system (Pacific Biosciences, Menlo Park, CA, USA) was used as the sequencing platform. A 10-kb SMRTbell library was prepared from sheared genomic DNA (>5 μg) using a 10-kb template library preparation workflow according to the manufacturer’s recommendation, with an additional bead clean-up before primer annealing. The library was bound with P4 polymerase and complexes were loaded on to version V3 SMRT cells. These were sequenced using 165 min movies. Two SMRT cells were used for each strain, yielding output data with average genome coverage of ~100X.

Bioinformatic analyses of SMRT sequencing data

De novo assembly of the insert reads was performed with the Hierarchical Genome Assembly Process (HGAP.3) algorithm in SMRT Portal (version 2.2.0). Gap closing was finished by PBJelly (23). Circularization was achieved by manual comparison and removal of a region of overlap, and the final genome was confirmed by remapping of sequence data. rRNA and tRNA predictions were performed using RNAmer (24) and tRNAscan-SE (25), respectively. Genes were predicted using Prodigal version 2.60 (26). We first assigned annotation by comparison with the well annotated H37Rv (GenBank accession NC_000962) and the NCBI non-redundant (NR) database was used to further assign these annotations for all the sequences with no hits. The average nucleotide identity with H37Rv (GenBank accession NC_000962) was calculated through ANI on EzGenome (http://www.ezbiocloud.net/ezgenome/ani). Promoter regions were analyzed using Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html) and PePPER (http://pepper.molgenrug.nl/index.php/promkaryote-promoters).

Genome-wide detection of base modification and the sequence motifs were identified by selecting the top 1000 kinetic hits and subjecting a ±20 base window around the detected base to MEME-ChIP (27), and then compared with the predictions in REBASE (28).

Through measuring ‘the methylated-motif-site ratio’ and ‘the methylated-read ratio’, we were able to obtain the ‘precision methylome’ of the MTBC strains. ‘The methylated-motif-site ratio’ represented the percentage of modified motif sites within all the motif sites and was obtained automatically by SMRT Portal (RS_Methylation_and_motif_Analysis protocol, Version 2.2.0). ‘The methylated-motif-site ratio’ = ‘number of methylated-motif-sites’ / ‘number of motif-sites’ × 100%.

‘The methylated-read ratio’ represented the percentage of the modified reads within the total number of reads (modified reads + unmodified reads) which were mapped to the site. It was also calculated automatically by ‘RS_Methylation_and_motif_Analysis’ protocol (Version 2.2.0). ‘The methylated-read ratio’ = ‘number of reads mapped to the sites which have a modified base’ / ‘number of all reads mapped to the sites’ × 100%

Single nucleotide polymorphism (SNP) identification and Phylogenetic analysis

Single nucleotide polymorphisms (SNPs) were analyzed by MAUVE 2.3.1 (29) using the 12 genomes in the study and 22 published genomes (Supplementary Table S3). Paired-end reads from an M. tuberculosis Ethiopia strain were mapped to the H37Rv reference genome (GenBank accession NC_000962) using BWA 0.5.9 (30) and SNPs were called using SAMtools 0.1.19 (31). SNPs called in repetitive regions of the H37Rv genome, defined as exact repetitive sequences of ≥25 bp in length, identified using RepeatMasker (32), were excluded. The SNPs present in all strains were used to construct a phylogenetic tree on the basis of single nucleotide polymorphism (SNP) present in the maximum likelihood using MEGA 6.06 (33). A strain of Mycobacterium canettii was included as an outlier.

m5C bisulfite sequencing and data analysis

m5C methylation was detected by bisulfite sequencing (34). Genomic DNA (100 ng) was used to construct the DNA library. They were sonicated into 100–500 bp fragments and then subjected to end repair, dA tailing, ligation and gel purification. Bisulfite conversion was performed using the EZ DNA methylation-Gold kit (Zymo Research). The library was sequenced by HiSeq 2000. Adapter and low-quality base trimming was performed by Trimmomatic version 0.32 (35) with default parameters. Filtered paired-end reads were mapped against the reference genomes by Bismark (version 0.12.2) (36).

MTase cloning

MamB and HsdM MTase genes (Rv2024c and Rv2756c) were amplified from the genomic DNA of M. bovis 30 and
cloned into the plasmid pRRS as described previously (37). The gene-specific oligonucleotide primers used for PCR are described in Supplementary Table S2. Restriction sites diagnostic for the predicted methylation pattern were incorporated into the 3’-end oligonucleotides. The presence or absence of specific methylation was determined by digesting the constructs with appropriate restriction enzymes and sequencing the plasmid with the MTase gene by PacBio RSII. The host strain used for cloning was E. coli ER2796 (38). E. coli strains were cultured in LB broth or on LB plates supplemented with ampicillin (final concentration 100 g/ml) as required. Various truncated Type I MTases were established to validate the function of both the modification subunit (HsdM) and the specificity subunit (HsdS).

Restriction digests

Plasmid DNA was prepared with a Qiagen Miniprep kit and then cleaved with the appropriate diagnostic restriction endonucleases (BclI and Eco47III, respectively (New England Biolabs)) for assaying the methylation state for HsdM endonucleases (BclI and Eco47III, respectively (New England Biolabs)). Plasmid DNA was prepared with a Qiagen Miniprep kit and then cleaved with the appropriate restriction enzymes and sequenced by SMRT sequencing technology (14). All strains were de novo sequenced, including M. microti which had not been sequenced before. The average of the sequencing coverage was ∼100X. These new genomes were all completed with the use of SMRT Portal and PBJelly software (Supplementary Figure S1). All the genome data have been deposited in NCBI with the GenBank accession numbers CP010329–CP010340 for M. tuberculosis (Mtb) F1, Mtb F28, M. bovis BCG 26, M. bovis 30, M. microti 12, M. africanum 25, Mtb 2242, Mtb 2279, Mtb22115, Mtb37004, Mtb 22103 and Mtb26105, respectively. The bioinformatic analysis provided the general genome information (Table 1 and Supplementary Table S4), including GC% content (∼65.6%); the size of the respective genomes (∼4.34–4.43 Mb); and the number of predicted protein-coding genes (∼4000–5000). As shown in Supplementary Figure S1 and Table S4, these predicted genes were generally distributed evenly across the plus and minus strands, which accounted for the vast majority of the whole genome as consistent with other prokaryotes (42).

When compared with the M. tuberculosis H37Rv reference genome (NC_000962) (7), our 12 MTBC genomes showed more than 99.70% identity at the nucleotide level when using ANI on EzGenome (http://www.ezbiocloud.net/ezgenome/ani). The genome structure of all the MTBC strains was then explored by multiple genome alignment using progressive MAUVE (29). No extensive translocations, duplications or inversions were found in the genomes except for strain Mtb 2242, which contained a 1.8 Mb inverted fragment with the same transposase gene on both ends (Supplementary Figure S2). We investigated further by designing different primer sets in order to amplify the junction regions by PCR. These PCR results confirmed the presence of the 1.8 Mb inversion (data not shown).

To implement the phylogenetic analysis, a total of 30 598 SNPs were discovered in the non-repetitive regions of the MTBC genomes and then used to construct a genome-wide maximum-likelihood phylogeny (Figure 1, Supplementary Table S5). Our tree topology comprised seven main lineages (L1–L7) and one animal-adapted lineage (L8), which matched satisfactorily with the published trees (6,43). Of the six clinical isolates, two strains (Mtb 2242 and Mtb 2279) belonged to L2 (Beijing type); three strains (Mtb 22115, Mtb 37004 and Mtb 22103) belonged to L4 (Euro-American lineage); and the remaining strain (Mtb 26105)...

### RESULTS

#### General bioinformatic analysis for 12 MTBC strains

Twelve MTBC strains, including six reference strains and six clinical isolates, were sequenced by SMRT sequencing technology (14). All strains were de novo sequenced, including M. microti which had not been sequenced before. The average of the sequencing coverage was ∼100X. These new genomes were all completed with the use of SMRT Portal and PBJelly software (Supplementary Figure S1). All the genome data have been deposited in NCBI with the GenBank accession numbers CP010329–CP010340 for M. tuberculosis (Mtb) F1, Mtb F28, M. bovis BCG 26, M. bovis 30, M. microti 12, M. africanum 25, Mtb 2242, Mtb 2279, Mtb22115, Mtb37004, Mtb 22103 and Mtb26105, respectively. The bioinformatic analysis provided the general genome information (Table 1 and Supplementary Table S4), including GC% content (∼65.6%); the size of the respective genomes (∼4.34–4.43 Mb); and the number of predicted protein-coding genes (∼4000–5000). As shown in Supplementary Figure S1 and Table S4, these predicted genes were generally distributed evenly across the plus and minus strands, which accounted for the vast majority of the whole genome as consistent with other prokaryotes (42).

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### Table 1. The general genome information of 12 MTBC strains

| Strain No. | Species          | ATCC No./Lineage | Average read size (kb) | Sequencing coverage (x) | Completed genome size (Mb) | GC content (%) | Gene number | Average gene size (bp) |
|------------|------------------|------------------|------------------------|-------------------------|---------------------------|----------------|-------------|-----------------------|
| F1         | M. tuberculosis H37Rv | 27294/L4         | 4.39                   | 120                     | 4.43                      | 65.61          | 4320        | 927                   |
| F28        | M. tuberculosis H37Ra | 25177/L4         | 4.03                   | 94                      | 4.42                      | 65.60          | 4179        | 959                   |
| 30         | M. bovis         | 19210/L8         | 4.39                   | 94                      | 4.34                      | 65.60          | 4198        | 932                   |
| 26         | M. bovis BCG     | 35735/L8         | 5.37                   | 99                      | 4.35                      | 65.61          | 4780        | 809                   |
| 12         | M. microti       | 19422/L8         | 6.81                   | 128                     | 4.37                      | 65.63          | 4323        | 910                   |
| 25         | M. africanum     | 35711/L6         | 5.30                   | 95                      | 4.39                      | 65.58          | 4801        | 813                   |
| 2242       | M. tuberculosis   | L2               | 4.90                   | 89                      | 4.42                      | 65.60          | 4467        | 888                   |
| 2279       | M. tuberculosis   | L2               | 5.13                   | 97                      | 4.41                      | 65.59          | 4601        | 858                   |
| 22115      | M. tuberculosis   | L4               | 5.74                   | 123                     | 4.40                      | 65.57          | 4213        | 946                   |
| 37004      | M. tuberculosis   | L4               | 6.66                   | 125                     | 4.42                      | 65.60          | 4231        | 943                   |
| 22103      | M. tuberculosis   | L4               | 4.37                   | 101                     | 4.40                      | 65.61          | 4186        | 952                   |
| 26105      | M. tuberculosis   | L3               | 4.97                   | 101                     | 4.43                      | 65.63          | 4200        | 952                   |
belonged to L3 (East African-Indian lineage). Of the reference strains, *M. africanum* 25 belonged to L6 (West African 2) while, as expected, the other reference strains belonged to the corresponding lineages: Mtb F1 and Mtb F28 to L4 (Euro-American lineage); and *M. bovis* 30, *M. bovis* BCG 26, and *M. microti* 12 to the animal-adapted lineage (L8).

**DNA methylome analysis of 12 MTBC strains using SMRT sequencing**

The genome-wide distribution of methylated bases in 12 MTBC strains was determined using SMRT sequencing technology. Through bioinformatic analysis of the SMRT sequenced data, N6-methyladenine (\(m^6A\)) could be detected with a high level of accuracy. In addition to \(m^6A\), a significant number of bases were reported as ‘modified bases’. Among these suspected sites, almost all of the reported bases failed to show any obvious methylated patterns after analysis with the SMRT Portal analysis platform. The reported modifications were subsequently verified by whole genome amplification (WGA) technology as previously described, this being based on the multiple displacement amplification (MDA) method (44). This WGA testing revealed that almost all of the modifications which did not have specific patterns were in fact false positives. We also observed that the IPD values (average 7.28) of \(m^6A\) were usually higher than those of the other modifications (average 2.13). Incidentally, no \(m^5C\) modification was found in the genomes of the twelve MTBC strains when using bisulfite sequencing, this being consistent with some previous reports (8).

Three \(m^6A\) sequence motifs were detected in the 12 MTBC strains by SMRT Portal analysis (Table 2). In addition to the previously identified methylated sequence motif CTCCAG (5’ to 3’ direction; the detected methylated base was indicated A, and T represented the thymine pairing with the methylated adenine on the complementary strand) (8), 2 adenine methylation motifs were also identified in the 12 MTBC strains: an asymmetric motif CACGC\(A\) and a characteristic Type I motif G\(A\)\(TN4R\)\(TAC\) (Supplementary Figure S1 and Table 2). This concurred with the predictions presented on the REBASE website (http://tools.neb.com/~vincze/genomes/index.php?page=M). The methylation of the motifs varied in the 12 MTBC strains, with all of the 3 methylated motifs being discovered in 4 of the ancient strains (*M. africanum*; *M. microti*; *M. bovis* BCG; and *M.
bovis). However, this was not the case with the modern strains. We found two methylated motifs (GATN4RTAC and CACGCAG) in the L2 clinical isolates (Mtb 2242 and Mtb 2279), two methylated motifs (CTCCAG and CACGCAG) in the L3 clinical isolate Mtb 26105, while for the L4 lineage strains, it became more complicated. The L4 reference strains, *M. tuberculosis* H37Rv and *M. tuberculosis* H37Ra, contained only one methylated motif CTCCAG; two L4 clinical isolates (Mtb 22115 and Mtb 37004) contained two methylated motifs (CTCCAG and CACGCAG); and another L4 clinical isolate, Mtb 22103, contained all of the three modified sequence motifs which were found in the ancient strains (Table 2). The unmethylated motifs in these genomes could be ascribed to the functional inactivation of the three related MTase genes which contained some missense mutations and partial deletions (discussed in detail in the next section).

The three motifs were then plotted as different colored tracks on the Circos plots to exhibit their distribution across the whole genomes of the 12 MTBC strains. This showed that they were distributed randomly across these genomes (Supplementary Figures S1 and S3). We then analyzed the distribution of the motifs separately within gene regions (GRs) and intergenic regions (IGRs). The motif GATN4RTAC was identified as being preferentially located within IGRs when compared with the other two motifs: about 12–13% of the GATN4RTAC motifs were located in IGRs, whereas less than 10% of the other motifs CTCCAG and CACGCAG were within IGRs (Supplementary Table S6 and Figure S4). We subsequently calculated the GC content of IGRs and GRs, respectively. The average GC content of IGRs and GRs is 63.4% and 65.8%, which may account for the difference in distribution of the motif GATN4RTAC (12–13%) and other two motifs (10%). Further analysis of the motifs occurring in IGRs revealed a relative enrichment of the motif GATN4RTAC at −70 to −80 bp from the start codon (Supplementary Figure S5A). A promoter was also predicted to be located in this region by using Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html) and PepPER (http://pepper.molgenrug.nl/index.php/prokaryote-promoters). This proximity to the promoters suggested that the methylated sequence motif might be involved in regulating promoter activity. Incidentally, relative enrichments of motifs CTCCAG and CACGCAG were found at −10 bp to −20 bp and −30 bp to −40 bp from the start codon, respectively (Supplementary Figure S5B and S5C).

We then proceeded to investigate the COG functional category of genes containing three sequence motifs (Supplementary Figure S6). Among the COG categories, only motif GATN4RTAC displayed a significant enrichment in COG category L (replication, recombination and repair) in two L2 clinical isolates (Mtb 2242 and Mtb 2279). This is because these two L2 clinical isolates contained higher copies of IS6110 with the sequence of motif GATN4RTAC.

### Analysis of the unmethylated motif

SMRT sequencing revealed that not all modified motif sites were detectable as being modified (17). The results in Table 3 show that there were some GATN4RTAC and CTCCAG sites which were detected as always being unmethylated within the MTBC genomes. Amongst these sites, most were detected as being unmethylated on both strands, but some sites were shown as being hemi-methylated (methylated on only one strand). The sequence coverage (∼100X) was sufficient to support these findings (17). From a total of about 4000 CTCCAG sites within the MTBC genomes, fewer than 20 (5–20 loci, ≤0.5%) were identified as always being unmethylated. The only exception was the Mtb 37004 genome which contained 122 unmethylated sites (about 3%). With regard to the GATN4RTAC motif (∼700 loci), the genomes of three ancient strains (*M. africanum* 25; *M. bovis* BCG 26; and *M. bovis* 30) also had more unmethylated sites (66–151 loci, 9.4–22.3%).

To investigate the distribution of unmethylated sites within the 12 MTBC strains, we analyzed their positions in both GRs and IGRs. As shown in Table 3, a total of

| Strain No. | CTCCAG | CACGCAG | GATN4RTAC |
|-----------|--------|---------|-----------|
| Mtb F1    | 3902   | 825     | 724       |
| Mtb F28   | 3904   | 803     | 730       |
| M. bovis 30 | 3828  | 806     | 686       |
| M. bovis BCG 26 | 3842  | 817     | 698       |
| M. microti 12 | 3860  | 813     | 706       |
| M. bovis BCG 26 | 3872  | 829     | 730       |
| M. microti 12 | 3872  | 824     | 722       |
| M. microti 12 | 3872  | 824     | 716       |
| M. microti 12 | 3872  | 824     | 716       |
| M. microti 12 | 3872  | 824     | 716       |
| M. microti 12 | 3872  | 824     | 716       |

*The number of motifs includes ones on the plus and minus strands.

*The methylated nucleotide in the motif is shown as bold and underlined letter. The underlined letter represents the thymine pairing with the methylated adenine on the complementary strand.
Table 3. The unmethylated sites among 12 MTBC strains

| Strain No. | Total GR IGR % in IGR | Total GR IGR % in IGR |
|------------|------------------------|------------------------|
| Mtb F1     |                        |                        |
| Mtb F28    |                        |                        |
| M. bovis 30| 89(11)†               | 73(9)                  |
| M. bovis BCG 26 | 151(21)       | 124(20)               |
| M. microti 25 | 66(14)        | 54(10)                 |
| Mtb 2224   | 23(3)                 | 14(2)                  |
| Mtb 2279   | 20(4)                 | 12(4)                  |
| Mtb 22115  |                        |                        |
| Mtb 37004  | 122(62)               | 113(59)                |
| Mtb 22103  | 28(6)                 | 22(6)                  |
| Mtb 26105  |                        |                        |
| Average    | 22.99                 | 9.09                   |

GR: Gene Region; IGR: Intergenic Region.
†The number in the parentheses indicates the number of hemi-methylated sites.

∼23% of unmethylated GATN₄RTAC sites were located within the IGRs in seven MTBC strains, which was ∼10% above the proportion of the other sites in IGRs (∼12%, Supplementary Table S6). In M. microti, Mtb 2242 and Mtb 2279, the proportions were even as high as 40–50%. To systematically investigate this location bias of the unmethylated GATN₄RTAC sites within IGRs, the distances between the unmethylated sites and the start codon were determined (Supplementary Figure S7). In comparison with most of the MTBC strains, the unmethylated GATN₄RTAC sites were located within 50 bp upstream from the start codon. Since most of the promoters in the 12 MTBC strains were predicted to be located 70–80 bp upstream from the start codon, these sites might always remain unmethylated so as to ensure that the related gene is transcribed smoothly. With regard to the unmethylated CTCCAG sites, they had approximately the same distribution proportion within IGRs (∼9.1%, Table 3) as the motif did (6.1–8.2%, Supplementary Table S6).

Exploration of the precision methylome by determining ‘the methylated-motif-site ratio’ and ‘the methylated-read ratio’

In order to investigate the precision methylome across all of the 12 MTBC genomes, we determined ‘the methylated-motif-site ratio’ by calculating the percentage of modified motif sites within all the motif sites (modified motif sites + unmodified motif sites) (Table 2). For the CTCCAG motifs, except Mtb 37004 (about 97%), the methylated-motif-site ratios for the MTBC strains were greater than 99.5%. However, this was not the case for the GATN₄RTAC motifs: the methylated motif site ratios (about 77.7–90.7%) for the three ancient strains (M. africanum 25; M. bovis BCG 26; and M. bovis 30) were noticeably lower than those for the other strains (about 97%). In addition, after calculating the methylated-motif-site ratio for GATN₄RTAC with the permutations of the four Ns, it could be seen that no nucleotide selection preference of the N sites was found to be responsible for the differences between these strains (data not shown). With regard to the CACGCAG motifs, all the sites (100%) were determined to be fully methylated for the tested strains with active MTases.

Looking at each methylated motif site, not all of the sequence-reads covering the site were methylated (15,17). To investigate the DNA methylation heterogeneity (or partial methylation), we analyzed ‘the methylated-read ratio’ of three motifs in the MTBC strains by calculating the percentage of the modified reads within the total number of reads (modified reads + unmodified reads) which were mapped to the site. This provided us with a more precise way of characterizing the methylome. Figure 2A showed that

Table 4. The average methylated read ratio of 12 MTBC strains

| Stain No. | CTCAG | CACGCAG | GATN₄RTAC |
|-----------|-------|---------|-----------|
| Mtb F1    | 96.2  | /       | /         |
| Mtb F28   | 96.17 | /       | /         |
| M. bovis 30 | 94.5  | 97.2    | 88.81     |
| M. bovis BCG 26 | 95.7  | 97.29   | 82.43     |
| M. microti 25 | 95.86 | 97.6    | 95.51     |
| M. africanum 25 | 94.28 | 97.92   | 90.41     |
| Mtb 2242   | /     | 97.99   | 95.73     |
| Mtb 2279   | /     | 97.1    | 94.05     |
| Mtb 22115  | 95.69 | 98.52   | /         |
| Mtb 37004  | 82.06 | 98.38   | /         |
| Mtb 22103  | 94.47 | 97.7    | 93.82     |
| Mtb 26105  | 95.88 | 98.49   | /         |

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Figure 2. Distribution of the methylated read ratio of three motifs in the MTBC stains. The horizontal axis shows the strain name. The vertical axis shows the number of motifs with diverse methylated read ratio (30–60%, 60–90%, 90–100%). The methylated read ratio indicates the percentage between the reads containing the methylated base and the total reads mapped to the site. For example, as for one methylated site, if its methylated read ratio is 60% and there are 100 reads covering (mapped to) this methylated site, this means that 60 reads contain the methylated base and the other 40 reads have no methylation.

(A) Distribution of the methylated read ratio of CTCCAG motif in the MTBC stains. (B) Distribution of the methylated read ratio of GATN4RTAC motif in the MTBC stains. (C) Distribution of the methylated read ratio of CACGCAG motif in the MTBC stains.

most of the CTCCAG sites (78.25–87.11%) were almost fully methylated (methylated-read ratio: 90–100%) in the MTBC strains with the exception of Mtb 37004, with the average methylated-read ratio (82.06%) of Mtb 37004 being significantly lower than that for the other strains (average 95.40%) (Table 4). Here, only 34.58% of the CTCCAG sites in Mtb 37004 showed a high-level methylated-read ratio (90–100%); 56.42% displayed a mid-level methylated-read ratio (60–90%); and 9% exhibited a low-level methylated-read ratio (30–60%) (Figure 2A). In contrast, the average methylated-read ratios of the GATN4RTAC sites in the three ancient strains (M. africanum 25, 90.41%; M. bovis BCG 26, 82.43%; and M. bovis 30, 88.81%) were lower than the other four MTBC strains (93.82–95.73%) (Table 4 and Figure 2B), while, as shown in Figure 2C, most of the CACGCAG sites (88.89–95.48%) showed a high level methylated-read ratio (90–100%) in the MTBC strains. Interestingly, we observed that there were very few motifs with a 100% methylated-read ratio, which was probably because the methylated motifs underwent passive demethylation (45) during replication before they had time to be methylated again.

DNA methyltransferases in MTBC strains

In the search for genes which were homologous to known DNA MTases in the REBASE database (28), we identified three homologous genes which were responsible for the three methylated motifs in the MTBC genomes (Figure 3). Apart from the reported mamA (8), the other two genes were predicted to separately encode a Type I MTase and a Type IIG MTase. Type I MTase (M) gene is generally located between its cognate restriction enzyme gene (R) and a specificity subunit encoding gene (S). The three parts (S–M–R) together function as a Type I system which recognizes a bipartite motif comprising two short sequences (3–5 nt) separated by 5–8 non-specific nucleotides (46). The predicted Type IIG MTase was encoded by the Rv2024c gene which we termed mamB. In general, Type IIG MTases only methylate one strand of the asymmetric recognition sequences (47).

In order to determine whether the two predicted MTases were responsible for the two newly detected methylated motifs (GATN4RTAC and CACGCAG), we implemented the restriction digestion and SMRT sequencing of plasmids containing cloned MTase genes in methyltransferase-free E. coli ER2796 (38). It has been shown previously that the Type I MTase encoded by Rv2756c gene, which was named as HsdM, is responsible for N6A modification (8), but its methylated motif has not been characterized. The genome sequence analysis showed that the core of the HsdM Type I MTase consisted of three subunits: specificity subunit 1 (designated as S1); MTase subunit (designated as M); and specificity subunit 2 (designated as S2). Four genes encoding two pairs of antitoxin and RNase (designated as A1, R1, A2 and R2, respectively) were present between M and S2 (Supplementary Figure S8A1). However, the cognate restriction enzyme was not found in the MTBC strains, suggesting that it might be an orphan MTase. An MTase was defined as an orphan MTase if we were unable to detect a restriction endonuclease with the same target site as the MTase in the proximity of the MTase gene (48). To determine the function of the four parts (S2-R2A2R1A1-M-S1) of HsdM Type I MTase, different subunit-combinations were cloned and tested for their resistance to BclI cleavage (the methylation-sensitive BclI restriction site (5′-TGATCA-3′) overlaps the HsdM methylated motif site (GATN4RTAC)) (Supplementary Figure S8A1). Regarding the full length of the sequence (S2-R2A2R1A1-M-S1), S2-M-S1 and S2-M, the electrophoresis results showed an almost com-
Figure 3. Three MTase genes and corresponding methylated sequence motifs in 12 MTBC strains. *M. bovis* 30 strain is located in the central position and the 11 other MTBC strains are located around it. Only active MTase genes were shown in the figure. The red circles mark the order change of the two MTase genes due to a large-scale inversion (about 1.8 Mbp) in Mtb 2242.

Complete resistance to BclI cleavage (Supplementary Figure S8A2: lanes 5, 8 and 11). However, no resistance to BclI cleavage was observed at the GATN\_RTAC motif site if only the M-S1 genes were present (Supplementary Figure S8A2: lane 14). As expected, the cleavage results were confirmed by SMRT sequencing of the plasmids with different subunit-combinatorial hsdM-expressing clones (Supplementary Figure S8A3). Therefore, we concluded that HsdM was able to act on the motif GATN\_RTAC, and that during this process the S2 subunit was essential for MTase activity while the S1 subunit and the four gene products (between the S2 and M subunits) appeared to be dispensable by comparison. The reason why S1 subunit is dispensable may be because the target recognition domain (TRD) for S1 subunit was predicted not exist using the Interpro database (http://www.ebi.ac.uk/Tools/pfa/iprscan5/). Using the same method, we also confirmed that the CACGCAG motif was recognized and methylated by the MamB MTase (Supplementary Figure S8B1), while, similar to the situation with most organisms with Type IIG MTases, only hemi-methylation was detected at the asymmetric recognition site (Supplementary Figure S8B2) (47).

Based on the methylated motifs detected by SMRT sequencing, all the active MTases of the 12 MTBC strains were determined (Figure 3). The relative location of the *mamB* and *hsdM* in the Mtb 2242 genome was different from the others due to the large genomic inversion region (1.8 Mb) (Supplementary Figure S2). Subsequently, through multiple sequence alignment for all the MTBC MTase sequences, some mutations and deletions were dis-
Table 5. Distribution of SNPs/Indels in three MTBC MTase genes among 12 MTBC strains

| Strain No. | HsdS2 | HsdM | Activity |
|------------|-------|------|----------|
| Mbh F1     | /     | C917T(P306L) | Inactive |
| Mbh F28    | /     | C917T(P306L) | Inactive |
| M. bovis 30| C279G | /    | Active   |
| M. bovis BCG 26 | C279G | /     | Active   |
| M. microti 12 | C279G | /    | Active   |
| M. africanum 25 | C279G | /     | Active   |
| Mbh 2242   | /     | /    | Active   |
| Mbh 2279   | /     | /    | Active   |
| Mbh 22115  | /     | C917T(P306L) | Inactive |
| Mbh 37004  | /     | /    | Inactive |
| Mbh 22103  | /     | /    | Inactive |
| Mbh 26105  | T356G(L119R) | / | Inactive |

| Strain No. | MamA | Activity |
|------------|------|----------|
| Mbh F1     | /    | Active   |
| Mbh F28    | /    | Active   |
| M. bovis 30| A809C(E270A) | Inactive |
| M. bovis BCG 26 | G61A(D21N) | /   | Inactive |
| M. microti 12 | /    | /     | Active   |
| M. africanum 25 | G527A(G176D) | / | Active   |
| Mbh 22115  | /    | /     | Active   |
| Mbh 37004  | G527A(G176D) | / | Active   |
| Mbh 22103  | /    | /     | Active   |
| Mbh 26105  | /    | /     | Active   |

| Strain No. | MamM | Activity |
|------------|------|----------|
| Mbh F1     | C139T(R47W) | /     | Truncated (1520-4821) |
| Mbh F28    | C139T(R47W) | /     | Truncated (1520-4821) |
| M. bovis 30| /     | C865T(R289C) | /     | Active   |
| M. bovis BCG 26 | /     | C865T(R289C) | /     | Active   |
| M. microti 12 | /     | A661C(D21L) | /     | Active   |
| M. africanum 25 | /     | C865T(R289C) | /     | Active   |
| Mbh 2242   | /     | /     | Truncated (1520-4821) |
| Mbh 2279   | /     | C865T | /     | Active   |
| Mbh 22115  | G141A | /     | C448G    |
| Mbh 37004  | /     | /     | C2448G   |
| Mbh 22103  | /     | /     | C448G    |
| Mbh 26105  | /     | /     | C448G    |

Synonymous mutations are marked as blue; Non-synonymous mutations are marked as red.

**”** indicates no mutations in corresponding strains.

Underlined mutations indicate the reported mutations inactivating the MTases.
covered (Table 5). Combining this with the analysis of the MTase activities, it is therefore reasonable to infer the impact of these mutations and deletions on the related MTase activities. On the one hand, we found that some missense mutations did not influence the MTase activity, as all the three MTases were active within the four ancient strains (M. africanum 25; M. microti 12; M. bovis BCG 26; and M. bovis 30) and one L4 clinical isolate (Mt 22103). From this we could deduce that the missense mutations G1374T and A1442C in M. africanum 25 had no significant effect on the HsdM activity. Additionally, the missense mutations: A661C in M. microti 12; C865T in the four ancient strains (M. africanum 25; M. microti 12; M. bovis BCG 26; and M. bovis 30); and A1972G in M. africanum 25, did not affect the activity of MamB. Among these, G1374T was also reported in L6 (43). On the other hand, we could infer from Figure 3 and Table 5 that some missense mutations and deletions could disrupt the activities of MTases within MTBC strains. Here, the activity of HsdM was lost within two reference L4 strains (Mt F1 and Mt F28) and two L4 strains (Mt 22115 and Mt 37004) due to the missense mutation G518A (Gly173Asp) and A809C in the four ancient strains (M. africuam 25; M. microti 12; M. bovis BCG 26; and M. bovis 30). We could also infer from Figure 3 that the missense mutation C917T (Pro306Leu) as detailed previously (8). From Table 5A, we could reasonably infer that the missense mutation G518A (Gly173Asp) and/or T356G (Leu19Arg) within the L3 clinical isolate (Mt 26105) might lead to the inactivation of the HsdM. This missense mutation (G518A) was also reported for typing L3 (43). As detailed in the literature, we also found that MamA became inactive as a result of the reported missense mutation A809C (Glu270Ala) (Table 5B) (8). With regard to MamB, it was the deletion in RvD1 (H37Rv related deletion) that caused MTase activity to be defective within two reference L4 strains (Mt F1 and Mt F28) (Table 5C). This is the first time that all of the above missense mutations have been reported to be associated with MTase activity within MTBC strains, with the exception of C917T in the hsdM and A809C in mamA (8).

These two important mutations that inactivate the MTases were investigated further by using the 161 published MTBC genomes including: 122 L2 strains (among these, 112 strains belonging to the Beijing sub-lineage); 37 L4 strains; and 2 L3 strains (6). We found that the missense mutation A809C (Glu270Ala) in mamA was present in all of the L2 strains, so it could be used in L2 lineage genotyping. This SNP has only been previously reported for typing within the L2.2 Beijing sub-lineage (43). Bioinformatic analysis also revealed that the missense mutation C917T (Pro306Leu) in hsdM was discovered in 35 out of the 37 L4 strains while, incidentally, the deletion within mamB, due to various forms of RvD1 in different strains, was present in the modern TB strains of L2, L3 and L4.

The evolution of the three MTBC DNA MTases

In order to explore the evolution of the three MTBC DNA MTases, we chose 1493 microbial species with complete genomes and built a phylogenetic tree for each MTase based on the protein sequences of each MTase. From Supplementary Figure S9A, we found that only 36 species contained MamA (Type II MTase), including all of the MTBC strains (four MTBC species), more than half of the non-tuberculosis mycobacteria (NTM) species (12 of 22 species), and Mycobacterium leprae, indicating that MamA might be indispensable to MTBC. Previous research reported that MamA could influence gene expression in M. tuberculosis (8). With regard to MamB MTase, there was a total of 60 species (including 4 MTBC species) which contained this (Supplementary Figure S9B). However, it was a surprise that MamB was not discovered in all of the 22 NTM species as these are considered to be the closest ancestors to MTBC (49). Since the MamB was discovered in many phyla in addition to Actinobacteria, such as Proteobacteria, Firmicutes and Bacteroidetes, we ascertained that the ancestor of NTM might have lost the MamB at an ancient evolutionary node (49). In comparison with MamA and MamB, HsdM MTase showed a much wider distribution across 302 species including 4 MTBC species, 6 NTM species and 292 other species (Supplementary Figure S9C). This indicates that HsdM MTase may be even more conserved than Type II MTases, because it may be evolutionarily constrained by the necessity of interacting with R and S subunits. Furthermore, we built a phylogenetic tree of the HsdS2 (S for specificity) recognition subunit as described previously (Supplementary Figure S10). Incidentally, in addition to the 12 MTBC strains that we sequenced, we also found the 3 MTase genes in all of the 36 published complete MTBC genomes, thereby suggesting that all the MTBC strains should contain the three MTase genes. It is interesting that the three DNA MTases are strongly conserved over MTBC strains, which needs further research.

DISCUSSION

Within this research, we have characterized the methylomes of 12 MTBC strains belonging to different MTBC lineages at single-base resolution using SMRT sequencing technology. Three m6A sequence motifs and their corresponding MTase genes were identified within MTBC strains. The evolution of the three MTases was also analyzed and discussed. Furthermore, through analyzing the ‘the methylated-motif site ratio’ and ‘the methylated-read ratio’, we determined the precise methyleome of the MTBC strains.

The precision methylome revealed the existence of unmethylated motif sites among the MTBC strains which had active MTases. To explore the formation mechanism of unmethylated motif sites, we studied the unmethylated sites that were found most frequently within the tested MTBC strains (Table 3). For both the GATN4RTAC and CTCCAG sites, the 10 most frequent unmethylated sites within gene regions appeared in at least 2 strains. Of particular interest, we found that three of the unmethylated sites occurred in all of the tested strains (Table 6), including two GATN4RTAC sites in pyrroline-5-carboxylate dehydrogenase gene (rocA) and cobalt-precorrin-6x reductase gene, respectively, and one CTCCAG site in the gene encoding for transmembrane transporter MmpL4. Supplementary Table S7 also revealed one unmethylated GATN4RTAC site in an intergenic region occurring in all of the tested strains. The appearance of these unmethylated sites across the tested strains increased the reliability of unmethylated site identification.

In order to investigate further why some sites always remained unmethylated, we analyzed the bind-
ing regions of 119 transcription factors (TF) from the TB Database (http://genome.tdb.org/annotation/genome/tdb/RegulatoryNetwork.html). We found that most of the unmethylated sites overlapped with TF binding regions (Supplementary Tables S7 and S8), and indeed some of them were overlapped with multiple TF binding regions (Supplementary Figure S11). From this finding we presumed that these sites were probably bound with some TFs, and thereby protected themselves from methylation (Supplementary Figure S12). Several publications concerning SMRT sequencing have also stated that some proteins, such as the Fur regulon in *Caulobacter crescentus* (50), might bind to certain motif sites in order to prevent methylation. This phenomenon is also commonly found in *E. coli*, in which some regulators (such as OxyR, SeqA) compete with Dam for occupation of a certain motif site (12). Alternatively, it is also possible that these unmethylated sites are protected from methylation by a higher-order chromatin structure (17).

The precision methylome of the MTBC strains also enables us to deeply investigate the activity of MTase at a whole-genome scale. Here, we discuss that relationship in more detail (Figures 2 and 3, Tables 2 and 4). For the CACGCAG motifs, the MTBC strains with active MamB displayed a 100% methylated-motif-site ratio, with most of them also displaying a high-level methylated-read ratio (90–100%), indicating that MamB was capable of high methylation activity within the MTBC strains. Of the CTCAG motifs, all of the MTBC strains, except Mtb 37004, also displayed high methylation activities, shown by a high methylated-motif-site ratio (more than 99.5%) and a high-level methylated-read ratio (90–100%) in most motif sites. With respect to Mtb 37004, its methylated-motif-site ratio (~97%) was a little lower than that of the other strains, but its average methylated-read ratio (82.06%) was noticeably lower than that of the other strains (95.40%) (Table 4). We presumed that a point mutation (G527A) in the catalytic domain of MamA (51) in Mtb 37004 might be the cause of this low methylation activity (Table 5B). In this instance, Mtb 37004 was revealed to be a good example of how to precisely analyze the activity of MTase by measuring the ‘methylated-motif-site ratio’ and the ‘methylated-read ratio’. For the GATnRTAC motifs, three ancient strains (*M. africanaum* 25; *M. bovis* BCG 26; and *M. bovis* 30) exhibited lower methylated-motif-site ratios (82.43–90.41%) and an average methylated-read ratio (87.22%) by comparison with those of the other modern strains (94.78%). This might be due to the variety of HsdM MTase methylation activity within the different MTBC lineages.

In summary, this study showed the enormous potential of the PacBio SMRT platform for characterizing the ‘precision methylome’. Not only could it detect the modified bases at single-base resolution, but it also could predict the modified sequence motif throughout the whole genome. Moreover, SMRT sequencing revealed that not all modified motif sites were detected as modified, and that there were a few unmodified motif sites. We determined the ‘methylated-motif-site ratio’ by calculating the percentage of modified motif sites within a total number of motif sites. SMRT sequencing was capable of even more. It could detect the modification status of each sequence read. Our findings demonstrated that, as seen for one modified motif site, not all reads covering the site were detected as modified. We obtained the ‘methylated-read ratio’ by calculating the percentage of modified reads within the total number of reads that had been mapped to the site. Overall, the characterization of the precision methylome significantly enhances our understanding of the function of DNA MTase.

To date, although the PacBio SMRT Sequencing technology has been used for the detection of many modified nucleotides throughout the whole genome, such as "m^5^A, "m^4^C, "m^5^C, 5hmC and some damaged bases, its precision analysis for modification is still limited to "m^5^A (17). This situation warrants further study in order to perfect the precise characterization of more modifications in the future.

### Table 6. The summary of top 10 frequent genes with unmethylated sites shared in 12 MTBC strains

| Motif | Synonym | Gene annotation |
|-------|---------|-----------------|
| GATnRTAC | Rs1187  | Essential pyrroline-5-carboxylate dehydrogenase RocA |
|         | Rs2070c | Non-essential preoccin-6A reductase |
|         | Rs1753c | Essential PPE family protein PPE24 |
|         | Rs0112  | Essential GDP-mannose 4,6-dehydratase |
|         | Rs2963  | Non-essential integral membrane protein |
|         | Rs3341  | Essential homoserine O-acetyltransferase |
|         | Rs0409  | Non-essential membrane bound polyketide synthase |
|         | Rs1461  | Essential Fe-S cluster assembly protein SurB |
|         | Rs2130c | Essential D-glucopyranoside ligase |
|         | Rs2984  | No-data polyphosphate kinase |

| CTCAG | Rs0450c | Essential transmembrane transport protein MmpL4 |
|       | Rs1562c | No-data malto-oligosaccharase trehalohydrolase |
|       | Rs1461  | Essential Fe-S cluster assembly protein SurB |
|       | Rs2501c | No-data acetyl-CoA carboxylase subunit alpha |
|       | Rs1917c | Non-essential PPE family protein, PPE34 |
|       | Rs3282  | Essential Maf-like protein |
|       | Rs0400c | Essential acetyl-CoA dehydrogenase FadE7 |
|       | Rs1664c | Non-essential polyketide synthase |
|       | Rs2174c | Essential alpha-(1-6)-mannopyranosyltransferase A |
|       | Rs1552c | No-data fumarate reductase flavoprotein subunit |

| MTBC Strain | F1 | F28 | 26 | 30 | 12 | 25 | 2242 | 2279 | 22115 | 37004 | 22103 | 26105 |
|-------------|----|-----|----|----|----|----|------|------|-------|-------|-------|-------|
| M. tuberculosis | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| M. bovis | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| M. smegmatis | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| M. leprae | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| M. avium | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| M. africanum | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| M. caprae | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| M. fortuitum | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| M. smegmatis | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| M. leprae | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| M. avium | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| M. caprae | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |
| M. fortuitum | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA | NA |

E/N: essential / nonessential genes. NA: Not applicable. ‘-‘ indicates gene with hemimethylated motifs. ‘++’ represents the gene with methylated motifs on both strands.
ACCESSION NUMBERS
The SRA accession number for the sequencing data reported in this paper is SRR064893. The GenBank accession numbers are CP010329–CP010340.

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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