Precision methylome and *in vivo* methylation kinetics characterization of *Klebsiella pneumoniae*

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Abstract

Klebsiella pneumoniae (*K. pneumonia*) is an important pathogen that can cause severe hospital-/community-acquired infections. To panoramically analyze *K. pneumoniae*'s methylation features, we completed the whole genome sequences of 14 *K. pneumoniae* strains covering various serotypes, multilocus-sequence typings (MLSTs), clonal groups (CG), viscosity/virulence and drug-resistances, and further characterized their methylomes using Pacbio-SMRT and bisulfite technologies. We identified 15 methylation motifs (13 6mA and two 5mC motifs), among which eight were novel ones. Their corresponding MTases were further validated. Additionally, we analyzed the genomic distribution of G**A**TC and C**C**WGG methylation motifs shared by all strains, and identified differential distributive patterns of some hemi/un-methylated G**A**TC motifs tending to locate in the intergenic regions (IGRs). Specifically, we characterized the *in vivo* methylation kinetics at single-base resolution on a genome-wide scale by simulating the dynamic processes of replication-mediated passive demethylation and MTase-catalyzed re-methylation. The slower methylation-rates of the G**A**TC motifs in the replication origins (*oriC*) and IGRs suggest an epigenetic mechanism implicated in the regulation of replication-initiation and transcription. Our findings illustrate the first comprehensive dynamic methylome map of *K. pneumonia* at single base resolution, and provide an efficient means and important reference for a better understanding of epigenetic regulation in bacteria.

KEYWORDS: *Klebsiella pneumonia*; DNA methylation; epigenomics; 6mA; 5mC; Dam

Introduction

Klebsiella pneumonia (K. pneumoniae), the important member of the *Enterobacteriaceae*, can cause severe hospital- and community-acquired infections (*e. g.*, pneumonia, genitourinary tract infection, and septicemia). There are some typing methods for *K. pneumoniae* strains, such as serotype, multilocus-sequence typings (MLSTs), and clonal group (CG) [1,2]. The related studies indicated that the hypervirulence phenotype usually corresponded to K1/K2/K57 serotypes and CG23-ST23 [1-3], while multidrug-resistance (MDR) phenotype often corresponded to CG258- ST11/ST258 [4].

Several studies on DNA methylation of *K. pneumoniae* strains using molecular biological techniques have identified three DNA methylases (MTases) and corresponding motifs, including two restriction-modification (R-M) systems (M.KpnI: GG<u>T</u>**A**CC; M.KpnBI: CAA**A**N_6R<u>T</u>CA) and one orphan MTase (Dam: G**A**<u>T</u>C) [5−7]. Further research on Dam indicated the epigenetic mechanism in regulating mismatch repair, virulence and pathogenicity of *K. pneumoniae* strains [8].

Recent rapid progress on high-throughput sequencing techniques, such as Pacific single-molecule real-time (SMRT) sequencing for accurate detection of modified bases (mainly 6mA) on a genome-wide scale and bisulfite sequencing for efficient analysis of genome-wide 5mC [9,10], has greatly facilitated investigations of DNA methylome in bacteria. It is well known that 6mA and 5mC are the two most important types of DNA methylation in prokaryote [9]. To date, many bacterial methylomes have been precisely determined using the above two techniques, including *E. coli*, *Mycoplasma genitalium*, *Bifidobacterium breve*, *Clostridium difficile*, *Campylobacter jejuni*, *Helicobacter pylori* [9−13] and *Mycobacterium tuberculosis* complexes (MTBC) reported by our group [14].

Through precisely and comprehensively analyzing the bacterial methylome, a lot of valuable information has been revealed, including methylation motifs and their corresponding methyltransferases, motif distributions on genomes, and the related epigenetic regulation mechanisms in bacteria [9,15,16]. Most identified MTases and the corresponding motifs belong to R-M system, which primary function is to prevent (cleave) the invading DNA and protect genomic DNAs through methylation-mediated mechanism [9−14]. Distinctively, some orphan MTases (no cognate restriction enzymes) and the corresponding motifs exert multiple epigenetic regulation functions

in bacteria [15−18]. Among them, Dam/G**A**TC motif is the most well-known because of its prevalent existence in almost all the *Enterobacteriaceae* bacteria and involvement in the epigenetic regulation of replication, transcription and mismatch repair [15−17,19−21]. In particular, its regulatory role in replication initiation has been studied in *E. coli*. Its *oriC* region contains five DnaA boxes and 11 G**A**TC motif sites. The replication-mediated passive demethylation causes the hemi-methylated G**A**TC motifs adjacent to the DnaA boxes that are specifically recognized and bound by SeqA, further leading to the competitive occupation of the motif sites between Dam and SeqA [17]. As a result, the re-methylation of the motifs is delayed, which in turn prevents the initiation cascade for chromosome replication induced by *DnaA* protein [17,19]. The re-methylation of the upstream G**A**TC motifs of the third and fifth *DnaA* boxes are the rate-limiting steps for DNA replication initiation in *E.coli* strains [17]. In addition, Dam also participates in the transcriptional regulation of the downstream genes (*e. g*., *opvAB* in *Salmonella enterica*) [15].

Although several MTases and corresponding motifs have been revealed in *K. pneumoniae* strains, the whole methylome has not been reported so far. Here, we obtained whole-genome sequences of *K. pneumoniae* strains of 14 various types, and then characterized their methylomes using SMRT combined bisulfite sequencing techniques. Fifteen methylation motifs were identified (13 6mA and two 5mC methylation motifs), including eight novel ones corresponding to eight novel MTases termed as *kamA* ~ *G* (*K. pneumoniae* adenine methyltransferase A ~ *G*) and *kcmA* (*K. pneumoniae* cytosine methyltransferase A). We further analyzed the distribution pattern of the G**A**TC and C**C**WGG methylation motifs shared by all *K. pneumoniae* strains. Importantly, by establishing a mathematical model to simulate the dynamic process of passive demethylation and re-methylation of each motif in the exponential phase, we characterized the genome-wide *in vivo* methylation kinetics at single-base resolution. The motifs at different genomic locations showed different re-methylation rates, and the G**A**TC motifs in *oriC* region and intergenic regions (IGRs) had slower re-methylation rates. Our findings indicate potential roles of epigenetic regulation in the replication initiation and transcription of *K. pneumonia* genome, and provide important reference and insights into our better understanding of *K. pneumonia* epigenomics.

Results

General bioinformatic analysis of 14 *K. pneumoniae* strains

We first obtained the whole genome sequences of 14 *K. pneumoniae* strains with various serotypes, MLSTs, CGs, viscosity/virulence and drug-resistances (Table S1, S5) by SMRT sequencing followed by correction using Illumina sequencing (**Figure 1**). The genome data have been deposited in National Genomics Data Center with the GSA accession number CRA003482 for strains of NTUH-K2044, 11492, 11420, 11454, 12208, 11311, 23, 11305, N201205880, 309074, 13190, 283747, 721005, 11021 and 11305. We then constructed a phylogenetic tree using 76 complete genomes of *K. pneumoniae* strains (14 from ours and 62 from online) (Figure S1, Table S5). Our 14 *K. pneumoniae* strains covered many common CGs and MLSTs of *K. pneumoniae* strains in China (*e. g*., CG23-ST23 and CG258-ST11) [22], indicating the well representativeness yet diversity of *K. pneumoniae* strains selected in our study.

The bioinformatic analyses provided the general genome information (**Table 1**, Figure 1), including genome size (5.20-5.54 Mb), GC content (57.4-57.9%), predicted protein-coding gene number (4990-5485), gene length (886-922 bp), and the percentage of coding region (88.26-90.23%) [23]. In addition, each *K. pneumoniae* strain contained 1-8 plasmids, with lower GC content (47.27-54.85%), lower percentage of coding region (75.98-88.63%), and shorter average gene length (596-831 bp) (Table 1).

Additionally, the relatively conserved genomic sequences and structures among the 14 *K. pneumoniae* strains were also indicated: ANI analysis revealed more than 99% identity among the 14 *K. pneumoniae* strains; no extensive translocations, duplications or inversions were found in the *K. pneumoniae* genomes except for strains of 11492 and 11454 each containing a large inverted fragment on the genomes (Figure S2).

Known and novel methylation motifs and corresponding methyltransferases in *K. pneumoniae* strains

A total of 13 6mA and two 5mC methylation motifs were identified in all *K. pneumoniae* strains (**Table 2-4**) by SMRT and bisulfite sequencing techniques (Table S2, S6), including seven known and eight novel methylation motifs (**Table 5**, Table

S10). It is worth noting that G**A**TC and C**C**WGG motifs were shared by all strains. The other motifs were shared by at most two *K. pneumoniae* strains (Table 2-4). Further analyses indicated the relationship between the motif and stain types (serotype, MLST and CG). The MT**C**GAK motif existed in NTUH-K2044 and 11492 belonging to K1 serotype and ST23-CG23, the common types of hypervirulent *K. pneumoniae* strains. The CC**A**YN$_7$TTYG motif was shared by two strains (11311 and 23) of hypervirulent serotype K57 and ST412. The CC**A**GN$_7$RTTC motif was present in strains 11305 and 13190 belonging to MDR CG147.

Modification analysis indicated that not all motif sites were fully-methylated (methylated on both strands, Table 2-4). A minority of motif sites (< 30%) were detected as being hemi-methylated (methylated on one strand only) or un-methylated within the *K. pneumoniae* genomes. The only exception was the MT**C**GAK motif in the NTUH-K2044 and 11492 genomes showing over half of hemi/un-methylated sites (56.03-56.77%). Further analysis indicated that the un-methylated MT**C**GAK motif prefered to have a guanine (G) in front of the motif (Figure S3, Table S11).

To search for the responsible MTases, we first predicted 22 MTases that might be responsible for the 15 methylated motifs from *K. pneumoniae* genomes in the REBASE database (http://rebase.neb.com/rebase/rebase.html) [24]. Among them, seven MTases and their corresponding motifs had been verified in previous studies (Table 5) [24]. To determine the MTases responsible for the eight newly detected methylated motifs, we performed the restriction digestion and SMRT/bisulfite sequencing using the plasmids containing the predicted MTase genes in methyltransferase-free *E. coli* ER2796. Crossover validations identified the corresponding eight MTases that could specifically recognize and methylate the respective eight novel motifs (Table 5, Table S10, Figure S4-S5).

We further analyzed the distribution of 15 MTase genes on the genomes of the *K. pneumoniae* strains. Thirteen genes were located on the chromosomes and two others were located on the plasmids (**Figure 2**). In addition, within the 15 identified MTases, there were 10 Type I MTases, three Type II MTases and two classical orphan MTases (Dam and Dcm). Here *dam* and *dcm* genes were carried by all *K. pneumoniae* strains, and responsible for the methylation of G**A**TC and C**C**WGG motif, respectively. Among the three newly identified Type II MTases, KamC and KamD were predicted to be Type IIG enzymes, which function of endonuclease and methyltransferase were

executed by a single gene (Table S10).

Differential distributions of GA<u>T</u>C and CCW<u>G</u>G motifs on *K. pneumoniae* genomes

Among the 15 methylation motifs, the G**A**<u>T</u>C and C**C**W<u>G</u>G had the most extensive distributions in all of the 14 *K. pneumoniae* strains, each containing ~30,000 G**A**<u>T</u>C and ~20,000 C**C**W<u>G</u>G modified sites (Table 2, 4). The distribution features of the above two motifs on the genomes of *K. pneumoniae* strains were further analyzed, and differential/uneven distribution on the genomes were observed (Figure 1). Both motifs exhibited some high-density and low-density regions on the genomes, where the genes were clustered into different COG functional categories (Figure S7). Importantly, the G**A**<u>T</u>C motif showed the highest distribution density (~34 sites/kb) in the *oriC* region (average density: 5-6 sites/kb) of the 14 *K. pneumoniae* genomes (Figure S8). In contrast, the C**C**W<u>G</u>G motifs didn't display such enrichment in the *oriC* region.

We then compared the density distributions of G**A**<u>T</u>C and C**C**W<u>G</u>G motifs on the 14 *K. pneumoniae* genomes and the simulated genome with the same base composition (**Figure 3**, Figure S9-10). The result revealed that their distribution densities on the *K. pneumoniae* genomes (1kb consecutive window) were higher than those on the simulated genome, indicating the high-density/nonrandom distribution of these two motifs on the *K. pneumoniae* genome. To explore the underlying causes for their high-density distributions, we investigated the impact of selection pressure on these two motifs by calculating the Ka/Ks ratios of the corresponding fragments in GRs (more than 90% of the two motifs were located in GRs) (Figure S11). We observed that the amino acid (AA) codons with two motifs (two AA codons for G**A**<u>T</u>C motif, two or three AA codons for C**C**W<u>G</u>G motif) were under strong negative/purifying selection with Ka/Ks ≈ 0.09/0.09 in relative to the ratio of 0.39/0.54 for the scramble sequences in GRs.

Distribution patterns of the methylated, hemi-methylated and un-methylated GA<u>T</u>C and CCW<u>G</u>G motifs on *K. pneumoniae* genomes

We identified three methylation patterns (methylated, hemi-methylated and un-methylated) for the G**A**<u>T</u>C and C**C**W<u>G</u>G motifs. Most G**A**<u>T</u>C (84.26-98.13%) and

C**C**WGG (77.58-98.84%) sites were detected as methylated state (Table 2, 4), whereas a small percentage of motif sites were hemi-methylated (< 15%) or un-methylated (< 10%) on the *K. pneumoniae* genomes. Further analysis demonstrated that the ratio of hemi-methylated G**A**TC motifs were (~6.48%) much higher than that of un-methylated G**A**TC motifs (~0.38%), while the hemi-methylated C**C**WGG motifs (~3.60%) showed a similar proportion as the un-methylated ones (~2.94%).

We then investigated the distribution ratio of methylated, hemi-methylated and un-methylated G**A**TC and C**C**WGG motifs in gene regions (GRs) and IGRs. The hemi/un-methylated G**A**TC motifs preferred to locate in IGRs, since its ratio (7.14%/34.11%) in IGRs was significantly higher than that of methylated G**A**TC motifs (5.23%) ($P < 0.01$, **Figure 4**A, Table S12). The analysis of the "fraction of methylated reads" (FRAC value) for the motifs in GRs/IGRs also supported the above findings that the hemi/un-methylated G**A**TC motifs were inclined to locate in the IGRs (Figure 4B). In contrast, the hemi/un-methylated C**C**WGG motifs (~7.49%) showed a little lower distribution ratio in IGRs compared with the methylated C**C**WGG motifs (~7.68%, Table S13).

The analysis of the G**A**TC motifs' density in 5' upstream region (USR), coding sequence (CDS) and 3' downstream region (DSR) also supported the above conclusion: the methylated motifs displayed a higher density in GRs, while the hemi-/un-methylated motifs presented a higher density in IGRs (both 5' USR and 3' DSR) (Figure 4C). We further explored the hemi/un-methylated G**A**TC sites shared in the 5' USR regions of 14 *K. pneumoniae* strains: 13 hemi/un-methylated G**A**TC sites corresponding to 11 genes were observed (Table S14). We also detected 12 high-density hemi/un-methylated clusters (no less than three consecutive hemi/un-methylated motifs in at least two strains) in the 5' USR regions (Table S15).

We then analyzed the sequence conservation of the DNA fragments (20 nt on both sides) containing hemi/un-methylated or methylated motifs to determine the causes leading to their differential distributions in IGRs. The results showed that the fragments with hemi/un-methylated G**A**TC motifs (44 nt) exhibit higher conservation than those with methylated G**A**TC motifs in IGRs (Figure S12).

Methylation kinetic characteristics of GATC and CCWGG motifs during growth cycle of *K. pneumoniae* strains

Since there is no demethylase in bacteria, the *in vivo* methylation kinetics characterization is based on the dynamic equilibrium between the replication-mediated passive demethylation and MTases-catalyzed re-methylation [18]. To explore the features of methylation kinetics of G**A**TC and C**C**WGG motifs during the growth cycle, we first characterized the methylomes of the NTUH-K2044 and 11492 strains at the exponential phase (1 h), transition-to-stationary phase (4 h) and stationary phase (24 h) (**Figure 5**A). By comprehensively analyzing genome-wide sequencing coverages and the fractions of methylated reads (methylated-read ratio/FRAC value), G**A**TC and C**C**WGG motifs were found to exhibit distinct kinetic features during the growth cycle (Figure 5B, Figure S13). As for G**A**TC motifs, the methylated-read ratios were more than 90% throughout the genomes in the three phases (Figure 5B), although the sequencing coverages varied on the genomes during the growth cycles (Figure S13), indicating that the G**A**TC motifs could be re-methylated in a very short time after the passive demethylation caused by replication: *i.e.*, the re-methylated rate was almost identical to the passive demethylation rate.

In contrast, the C**C**WGG motif showed a much slower re-methylation rate. First, in the exponential phase (1 h), the methylated-read ratio of C**C**WGG motifs in the *oriC* regions (55.66 ± 18.54%) was much lower than that in the replication termination (*Ter*) regions (82.63 ± 12.71%) (Figure S13B). Secondly, the average methylated-read ratio in the transition-to-stationary phase (54.50 ± 27.14%, 4 h) was close to that of the exponential phase (60.50 ± 17.71%, 1 h), but not the stationary phase (80.28 ± 19.05 %, 24 h) (Figure S13B). This finding suggested a much slower re-methylation rate compared to the replication-mediated passive demethylation rate for C**C**WGG motifs, which resulted in the above differences in the methylated-read ratios.

To quantify the re-methylation time per motif in the exponential phase, we first investigated the replication kinetics of the aforementioned two *K. pneumoniae* strains. Figure S13 showed that the genome sequencing coverage in the *oriC* regions was more than twice as much as that of the *Ter* regions (2.93 and 3.02 folds for the 11492 and NTUH-K2044 strains, respectively), indicating that the next round of replication was re-initiated before the replication termination in the exponential phase. We then constructed a replication model (Figure 5C) by fitting genome coverage data, and obtained the ratio (t_D/t_R) of doubling time (t_D) to replication termination time (t_R) (see "Materials and methods" for detail). The doubling time (t_D) in the exponential phase

also reflects the re-initiation time. Interestingly, we obtained the same t_D/t_R values (0.59) for the two *K. pneumoniae* strains (Figure 5D), suggesting a similar regulatory mechanism for replication cycle, which should be intrinsically carried by *K. pneumoniae* strains. Two t_D values were then calculated through fitting the growth curves (~34.64 min for strain 11492; ~27.95 min for NTUH-K2044, Figure 5A), therefore we could infer the t_R from the t_D/t_R values (~58.71 and ~47.36 min for strain 11492 and NTUH-K2044, respectively).

We further obtained the re-methylation time per motif (**Table 6**) through simulating the dynamic processes of passive demethylation and re-methylation in the exponential phase based on the five parameters, such as methylation-read fraction of each motif ($M_{(x)}$), initial methylation-read fraction of each motif ($M^0_{(x)}$), t_D, t_R and distribution density of the first replication forks ($P_{(x_1)}$, see "Materials and methods" for detail).

In general, the mean re-methylation time of 6mA was shorter (3.52 and 3.46 min for G**A**TC and GR**A**CRAC motifs, respectively) than that of 5mC (9.23 and 4.55 min for C**C**WGG and MT**C**GAK motifs, respectively) in the exponential phase (Table 6). As for a certain motif, the re-methylation time in the NTUH-K2044 strain was shorter than that in the 11492 strain (Figure S14). In addition, the flanking bases could influence the re-methylation rates of the G**A**TC and C**C**WGG motifs. When the flanking bases were C/G rather than T/A, G**A**TC motif showed a faster re-methylation rate, which was totally reversed for the C**C**WGG motif (Table 6, Figure S15).

Slower re-methylation rate of the GATC motifs in intergenic and *oriC* regions at the exponential phase

To investigate the role of G**A**TC motifs in transcriptional regulation of *K. pneumoniae* strains, we analyzed the re-methylation rates of the motifs in IGRs (including 5' USRs and 3' DSR) at the exponential phase (**Figure 6**A). The results indicated the slower re-methylation motifs in IGR regions (3.94 ± 5.82 min) than that in GR regions (3.50 ± 5.10 min). Since most 5' USRs in bacteria are overlapped with the promoter regions and involved in the transcriptional regulation [25], we further explored the COG functional category of genes with top 5% slow re-methylation sites (> 7.04 min/motif) in 5' USRs (Table S16). Four enriched functional categories ("Cell cycle control, cell division, chromosome partitioning", "Carbohydrate transport and metabolism", "Intracellular trafficking, secretion, and vesicular transport" and "Translation,

ribosomal structure and biogenesis") were observed (Table S16), of which at least 5% of genes contained a slow re-methylation sites (> 7.04 min/motif) in 5' USRs. Among them, the "carbohydrate transport and metabolism" functional category owned the most genes (22 genes) with slow re-methylation rates in 5' USRs, including three hemi/un-methylated motifs shared in 14 *K. pneumoniae* strains and even one hemi/un-methylated motif-clusters (Table S16).

To further explore the role of G**A**TC motifs in replication regulation of *K. pneumoniae* strains, we analyzed the re-methylation rates of the motifs in *oriC* regions (Figure 6B). The G**A**TC motifs in *oriC* region had the highest distribution density (~34 sites/kb) (Figure S8) and slower re-methylation rates (10.35 ± 8.69 min). Among the 14 G**A**TC motifs in *oriC* region, eight and seven motifs with slow re-methylation rates (> 7.04 min/motif) were enriched in the upstream sequence of the fourth DnaA binding site (DnaA box) and the adjacent AT-rich region in strain 11492 and NTUH-K2044, respectively (Figure S16).

Discussion

In this study, we characterized the precision methylome of 14 *K. pneumoniae* strains with different serotypes, MLSTs, CGs, viscosity/virulence and drug-resistances, using SMRT and the bisulfite sequencing techniques, and identified 15 DNA methylation motifs (eight novel ones) including 13 6mA motifs and two 5mC motifs corresponding to 13 R-M system MTases and two orphan MTases, respectively. Two motifs (G**A**TC and C**C**WGG) and their respective orphan MTases (Dam and Dcm) appeared to be the most important since they were present in all *K. pneumoniae* strains with the most extensive distributions on genomes in relative to other modified motifs (Table 2, 4). This feature was also reported in almost all members of the *Enterobacteriaceae* family [21,24]. Functional analysis in previous reports indicated that these two motifs, especially G**A**TC, could exert multiple functions including transcriptional regulation, cell-cycle controlling and mismatch repair in *E.coli* and *S. enterica* [15−17,19−21]. We also demonstrated their high-density distributions on *K. pneumoniae* genome comparing with that on simulated genome (Figure 3, Figure S9-10), which was probably caused by purifying selection (Figure S11) leading to their evolutionary conservation (Figure S12) as reported in other bacterial genome [26]. This well-conserved feature also implies an essential role of these two motifs in *K.*

pneumoniae.

The hemi/un-methylated G**A**TC motifs were found to possess the tendency of IGRs (including 5' USR and 3' DSR) localization (Figure 4A, C). Since promoters in *K. pneumoniae* were also predicted to be located across the 5' USR regions (Figure S17), thus, these hemi-/un-methylated G**A**TC motifs in 5' USR might be protected from the methylation by competitively binding of some regulators to the promoter regions [17, 19]. In *E.coli* strains, this feature facilitates the epigenetic regulation of the downstream gene expression [27]. The status of these motifs suggested similar epigenetic mechanisms in *K. pneumoniae* strains, and might be the consequence of selection during long-term evolution (Figure S11), which was supported by our findings that the genomic fragments with hemi/un-methylated G**A**TC motifs in IGRs had higher sequence conservation (Figure S12).

Importantly, by establishing a mathematical model to simulate the dynamic process of passive demethylation and re-methylation of each motif in the exponential phase, we obtained the re-methylation time of each motif throughout the whole genome (Table 6, Figure S14). Our studies revealed that the motifs at different genomic locations showed different re-methylation rates. We could reasonably infer that the slower re-methylation rates in some sites/regions might also be due to the competitive binding of certain proteins in order to prevent methylation [17, 19]. Thus, the slow re-methylation rates could precisely reflect the methylation-mediated epigenetic regulations at these sites/regions *in vivo*.

There are two methylation-mediated epigenetic regulations: transcription and replication, which were examined by methylation kinetic analysis in our study (Figure 6, Figure S16). Firstly, transcriptional regulation analysis indicated that the G**A**TC motifs in IGRs presented the slower re-methylation rates than those in GRs (Figure 6). This is in agreement with the distribution characteristics of the G**A**TC motifs in IGRs: lower methylated-read ratios and more hemi/un-methylated motifs (Figure 4A, 4B). As described above, most IGRs in bacteria overlap with the promoter regions and participate in the transcriptional regulation [25]. Thus, the promoter regions with slow re-methylation motifs should be the locations where the G**A**TC motifs with hemi/un-methylated status function as transcription regulators in *K. pneumoniae* strains. Similarly, this should also be the consequence of the competitive binding between Dam and some transcription regulatory proteins (such as OxyR) to these sites/regions

[15,27,28], which is analogous to the epigenetic transcriptional regulation mediated by the competition between DNA methyltransferases and CTCF in CpG islands of eukaryotic cells [29]. Secondly, replication regulation analysis identified 7 - 8 slow G**A**TC motifs enriched in the upstream sequence of the fourth DnaA binding site (DnaA box) and the adjacent AT-rich region (Figure S16). SeqA has been shown to prefer to bind to these motifs for the purpose of lowering re-methylation rates [30] and preventing the initiation cascade for chromosome replication [17,30]. Therefore, re-methylation of these motifs in the fourth DnaA box and AT-rich regions should represent the main rate-limiting steps for triggering initiation of DNA replication in *K. pneumoniae* strains. Our findings also uncovered many promoter regions with slower re-methylation motifs in *K. pneumoniae* strains (Figure 6), therefore, it is reasonably speculated that epigenetic regulation in bacteria is very complex rather than simple as we believed.

Previous studies on MTase kinetics mainly focused on *in vitro* analysis [31−33]. Several reported analyses about the *in vivo* methylation kinetics, including one publication about bacteria, only studied the overall, but not single-motif methylation kinetics [34−36]. Our study is the first to characterize the *in vivo* methylation kinetics at single motif resolution in whole genome, thus, offer an efficient means and valuable resources for a better understanding of epigenetic regulation in bacteria.

Materials and methods

Strain information, growth curve, and phenotypic characterization

The information of 14 *K. pneumoniae* strains used in this study was shown in Table S1. The strains were cultured overnight in Luria-Bertani (LB) medium at 37 °C. 1 ml of overnight culture suspensions was then transferred to a flask containing 200 ml of LB medium and cultured in a shaker at 200 rpm. The growth curve of bacteria was determined by recording OD_{600} values at different time-points. This experiment was performed in triplicate.

Drug susceptibility test was performed by VITEK 2, and the drug-resistance phenotypes were determined by Clinical and Laboratory Standards Institute (CLSI) standards (https://clsi.org/standards/products/microbiology/documents/m100/). The string test of *K. pneumoniae* strains was conducted, and hypermucoviscosity was defined by viscous strings with a length of more than 5 mm [37].

Genomic DNA Extraction, sequencing, assembly, correction and annotation

The genomic DNA was extracted using TIANamp Bacteria Genomic DNA Kit (Tiangen Biotech Co. Ltd., Beijing, China). The whole-genome sequencing was implemented by PacBio RSII platform using P6/C4 chemistry. Each strain was sequenced with 1-2 SMART cells with genome coverage of more than 50× (Table S2−S3).

De novo assembly of the genome was performed using Hierarchical Genome Assembly Process 3 (HGAP3) in SMRT Portal (version 2.2.0). Gap closing was completed by PBJelly [38]. On the basis of Blast results, genome circularization was finished by manually removing the contigs overlapped regions.

To correct the polymer-errors, we re-sequenced the strains using Illumina sequencing (Table S4). Paired-end libraries were prepared as previous description, and clean reads were obtained after eliminating redundant and low-quality raw reads. Paired reads were extracted and then mapped onto the assembled genome sequences to obtain unique mapped reads using BWA. Pilon v.1.13 was subsequently used to polish genome sequences using unique map reads.

Genome sequences were annotated in the Subsystem Technology (RAST) pipeline with Rapid Annotation [39]. Unannotated genes were then predicted by alignment in the NCBI non-redundant (NR) database using BLAST. The protein functions were annotated by Clusters of Orthologous Groups (COG). tRNAs and rRNAs were predicted by tRNAscan-SE and “search_for_rnas” tools, respectively.

Genome structure and phylogenetic analysis

The average nucleotide identity (ANI) and coverage were calculated by ANI on EzGenome (http://www.ezbiocloud.net/tools/ani) and online BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple alignments of genomic sequences were performed using the Mauve multiple alignment software [23].

Single nucleotide polymorphisms (SNPs) were detected by MUMmer based on the 14 genomes in this study and 62 published genomes (Table S5) using HS11286 as reference. Prank [40] was used to annotate the protein coding genes of the 76 *K. pneumonia* genomes, and then used Roary to predict 3173 core genes[41]. The SNPs (117,142 SNPs) in core genes were used to construct a phylogenetic tree on the basis

of maximum likelihood using FastTree [42], followed by decoration using software evolview v2 (http://www.evolgenius.info/evolview/) [43].

Genome-wide detection of 6mA and the related motifs using the SMRT sequencing data

The SMRT Portal (version 2.2.0) was applied to detect genome-wide 6mA modification and related motifs using the standard settings in the RS_Modification_and_Motif_Analysis.1 protocol as previously described (https://smrt-analysis.readthedocs.io/en/latest/SMRT-Pipe-Reference-Guide-v2.2.0/). We then identified the motifs through selecting the top 1000 kinetic hits and subjected a window of ± 20 base around the detected modified base to MEME-ChIP [44], followed by comparing them with the predicted MTase targeting motif sequences in REBASE [24].

Three methylation patterns (methylated, hemi-methylated and un-methylated) were identified for DNA motifs within the *K. pneumoniae* genome. A methylated motif is the sites were fully-methylated on both strands, whereas the hemi-methylated motif is the sites were methylated on one strand only. The un-methylated motif is the sites were not modified on both strands. A methylated site was defined as methylated-read ratio > 0.8. The “methylated-read ratio” is the percentage of methylated reads in the total number of reads mapped to the sites, which was obtained from “frac = decimal” in “modification.gff” file.

Genome-wide detection of 5mC and the related motifs using the bisulfite sequencing data

The 5mC methylation was detected by bisulfite sequencing as previously reported (Table S6, S7). Trimmomatic (v0.32) [45] was used to trim adapters and low-quality bases using default parameters. Clean reads were mapped on the reference genomes by Bismark (version 0.12.2) [46]. We then identified the motifs through subjecting a window of ± 20 base around the detected modified base to MEME-ChIP [44], followed by comparing with the predicted MTase targeting motif sequences in REBASE [24].

MTases cloning and verification

The predicted MTase genes were amplified from bacterial genomic DNAs using the gene-specific primers (Table S9), and cloned into plasmid pRRS as previously described [14]. The corresponding methylation sensitive restriction-site sequences (used to detect the activity of MTases) were included in the 3'-end oligonucleotides. The recombinant plasmids were then transformed into bacterial host ER2796 (not containing known MTase genes), followed by bacterial culture overnight. The plasmid DNAs were prepared using Qiagen Miniprep kit. The appropriate restriction enzymes were then used to determine the presence or absence of methylated motifs in recombinant plasmid. The digestion reaction were conducted for 4 h at 37 ºC and run on 1% agarose gels. The methylation motifs were also double detected by sequencing the recombinant plasmid by SMRT/bisulfite sequencing.

Density distributions of GA<u>T</u>C and CCW<u>G</u>G motifs on the *K. pneumoniae* and simulated genomes

The simulated genome was generated based on the same length and GC content as those in the *K. pneumoniae* genomes. We then calculated the number of motifs in 1kb consecutive segments, so as to determine the density of GA<u>T</u>C and CCW<u>G</u>G motifs across the *K. pneumoniae* and simulated genomes.

We counted the number of motifs in each 2kb-size non-overlap sliding window along the genome sequence of *K. pneumoniae* strains. By fitting the in normal distribution in R using the "pnorm" function, top 5% regions with higher motif counts were selected as high-density regions, and the tail 5% regions with fewer motif counts were selected as low-density regions.

Ka/Ks analysis of the GA<u>T</u>C and CCW<u>G</u>G "motif sequences" and corresponding "scramble sequences"

We firstly extracted the minimum DNA sequences (2~3 codons) containing the motifs from the open reading frames of genes, in order to obtain "motif sequences". The corresponding "scramble sequences" were obtained by random shuffling (eliminating the "motif sequences"). The top-10 frequent "scramble sequences" were used in subsequent analysis. We then identified the reference sequences of "motif sequences"/ "scramble sequences" on reference genome (HS11286) by multiple sequence alignment (mafft) [47]. The "motif sequences", "scramble sequences" and

corresponding reference sequences of each strain were respectively concatenated, and their Ka/ks ratios were calculated by paraAT [48].

Sequence conservation analysis of the GA<u>T</u>C and CCW<u>G</u>G motifs

We extracted the methylated motifs and their flanking sequences (20 nt) in IGRs from one genome, and then obtained the corresponding sequences of 13 other genomes by multiple sequence alignment. Using the above 14 sequences, the conservation scores were calculated by phylop [49]. The conservation scores of the hemi-/un-methylated motif with 20-nt flanking sequences in IGRs were calculated by the same method.

Calculation of the genomic replication time (t_R) through simulating genomic coverage plots

The genomic coverage plots reflect the accumulated copy numbers across genomes. Since the replication forks always proceed from origin of replication (*oriC*) to doubling point (s_{DP}), only a few ones can get close to the doubling point in the exponential phase. As a result, the sequencing coverage in *oriC* region is much higher than that in doubling point. In each cell, the copy number of each site (s) depends on its relative position to the first replication fork (s_1) and the minimal time of successive initiations (re-initiation). The re-initiation time is consistent with the doubling time (t_D) in the exponential phase, reflecting the growth rate of *K. pneumoniae* strains.

After initiation, the replication forks should go forward bi-directly at similar speeds in the exponential phase. Therefore, the genome coverage plots are symmetrical near the doubling point (s_{DP}). We divided the coverage plots into the left ($s \leq s_{DP}$) and right ($s \geq s_{DP}$) parts $s \leq s_{DP} s \geq s_{DP}$by doubling point, and simulated the curves independently. We then obtained the relative position ($x \in (0,1]$) of each genomic site shown as equation (1). S_G represents the half length of genome.

$$\begin{cases} x = s/S_G & (s \leq s_{DP}) \\ x = 2 - s/S_G & (s \geq s_{DP}) \end{cases} \quad (1)$$

We subsequently calculated the relative distance of the first replication fork when another one at *oriC* is re-initiated (Δx_R) as equation (2). t_R represents the genomic replication time.

$$\Delta x_R = t_D/t_R \qquad (0 \leq t_D \leq t_R) \quad (2)$$

By constructing the replication frequency matrix of genomic sites (x) with different

distances from the first replication fork (x_1), the copy number of the genomic site ($f_{(x,x_1)}$) can be determined as equation (3). When the genomic site is in front of the first replication fork ($x \geq x_1$), its copy number is one; when the site is between the first and second replication forks ($x_1 - \Delta x_R \leq x \leq x_1$), its copy number is two. The rest was deduced by analogy.

$$f_{(x,x_1)} = 2^{(x_1-x)/\Delta x_R} \quad (3)$$

Next, we used the B distribution to assess cell population density with different genomic positions of the first replication forks ($x_1 \in [0,1]$). Since x is not continuous data (with the step size of $1/S_G$), the cell population density of the first replication forks ($P_{(x_1)}$) at each genomic site was determined by the difference of accumulated densities ($I_x(\alpha, \beta)$) of the adjacent sites.

$$P_{(x_1)} = I_{(x_1)}(\alpha,\beta) - I_{\left(x_1-\frac{1}{S_G}\right)}(\alpha,\beta) \quad (4)$$

The genome coverage plots could be determined as the integral of the copy numbers of each genomic site in the cellular populations ($H(x)$) as shown in the equation (5). The integral was achieved based on $P_{(x_1)}$ and the relative distance between x and x_1 ($x_1 - x$). By substituting equation (2)-(4) into equation (5), we established the mathematical model to fit the genome coverage plots as equation (6).

$$H(x) = \int_0^1 f_{(x,x_1)} * P_{(x_1)} d_{x_1} \quad (5)$$

$$H(x) = \int_0^1 2^{(x_1-x)*t_R/t_D} * \left(I_{(x_1)}(\alpha,\beta) - I_{\left(x_1-\frac{1}{S_G}\right)}(\alpha,\beta) \right) d_{x_1} \quad (6)$$

We then substituted equation (1) into equation (6), and repeatedly fit the genome coverage plots by selecting continuous parameters with step sizes of 0.1. We finally obtained the optimal solutions of Δx_R and B distribution parameters (α,β) through Goodness of Fit Test. Since t_D could be calculated from growth curve of each *K. Pneumoniae* strain, we could obtain the replication time (t_R) by the equation (2).

Calculation of the re-modification time of motifs

In the exponential phase, methylation-read fraction ($M_{(x)}$) of each motif was determined by its initial methylation-read fraction ($M^0_{(x)}$), replication-induced passive de-methylation and methyltransferase-catalyzed re-methylation. Based on $M_{(x)}$ in the exponential phase and B distribution parameters (α,β), we calculated the proceeding

distance of the corresponding replication fork when the hemi-methylated motif was re-methylated (Δx_M). As shown in the equation (7), Δx_M corresponds to the ratio of mean re-methylation time (t_M) to the genomic replication time (t_R).

$$\Delta x_M = t_M / t_R \tag{7}$$

When the motif x is located at downstream of replication fork ($x_j \leq x$), its methylation-read fraction ($M_{(x)}$) remain unchanged because of no replication-induced passive de-methylation. When the motif x is located at upstream of replication fork with a longer distance ($x_j \geq x + \Delta x_M$), the methylation-read fraction is also unchanged because of the completion of re-methylation of motif x. Thus, we only considered the de-methylation effect of the replication forks in a certain range ($x \leq x_j \leq (x + \Delta x_M)$) on $M_{(x)}$ in the mathematical derivation. j represents the serial number of the replication fork affecting $M_{(x)}$.

If the hemi-methylated motifs can be fast re-modified before next replication fork ($t_M \leq t_D$), we only need to evaluate the de-methylation effect of one replication for all replication forks in the range of $x \leq x_j \leq x + \Delta x_M$. Here we include the first replication forks (x_1) and other effective replication forks (x_j) in the range. We then substituted the accumulated B distribution density of first replication forks ($x + (j-1)\Delta x_R \leq x_1 \leq x + \Delta x_M + (j-1)\Delta x_R$) for that of the corresponding effective replication forks ($x \leq x_j \leq x + \Delta x_M$). Due to semi-conservative replication, we further obtain Δx_M from equation (8). N represents the upper limit of j, which is determined by Δx_R and the relative genomic position (x) of methylated motifs.

$$\begin{cases} M(x) = M^0_{(x)} * \left(1 - 0.5 * \sum_{j=1}^{N} \begin{pmatrix} I_{(x+\Delta x_M+(j-1)*\Delta x_R)}(\alpha,\beta) - \\ I_{(x+(j-1)*\Delta x_R)}(\alpha,\beta) \end{pmatrix}\right) \\ N = \lceil (1-x)/\Delta x_R \rceil \end{cases} \tag{8}$$

If the methylation rate is slow, the hemi-methylated motifs may not be re-modified when the next replication fork crosses them ($t_M > t_D$). In this case, the effect of multiple passive de-methylation on $M_{(x)}$ should be considered. n represents upper limit of multiple passive de-methylation times (i) as equation (9), which is determined by the ratio of mean re-methylation time (t_M) to re-initiation time(t_D).

$$n = \lceil t_M / t_D \rceil = \lceil \Delta x_M / \Delta x_R \rceil \tag{9}$$

We then converted genomic position of effective replication forks (x_j) into that of the corresponding first replication forks (x_1), and substituted accumulated B

distribution density of x_1 ($x + (j-1) * \Delta x_R \leq x_1 \leq x + j * \Delta x_R$) for the x_j population density ($x \leq x_j \leq x + \Delta x_M$). Considering the differential influences on $M_{(x)}$ with the case of $j < n$ and $n \leq j \leq N$, we calculated Δx_M as the equation (10) by comprehensive calculations.

$$\begin{cases} M(x) = M_{(x)}^{0} * \left(\begin{array}{l} 1 - \sum_{j=1}^{n-1} \sum_{i=1}^{j} 0.5^{i} * \left(\begin{array}{l} I_{(x+j*\Delta x_R)}(\alpha,\beta) - \\ I_{(x+(j-1)*\Delta x_R)}(\alpha,\beta) \end{array} \right) \\ -\sum_{j=n}^{N} \sum_{i=1}^{n-1} 0.5^{i} * \left(\begin{array}{l} I_{(x+j*\Delta x_R)}(\alpha,\beta) - \\ I_{(x+(j-1)*\Delta x_R)}(\alpha,\beta) \end{array} \right) \\ -0.5^{n} * \sum_{j=n}^{N} \left(\begin{array}{l} I_{(x+\Delta x_M+(j-n)*\Delta x_R)}(\alpha,\beta) - \\ I_{(x+(j-1)*\Delta x_R)}(\alpha,\beta) \end{array} \right) \end{array} \right) \\ n = \lceil t_M / t_D \rceil = \lceil \Delta x_M / \Delta x_R \rceil \\ N = \lceil (1-x) / \Delta x_R \rceil \end{cases} \tag{10}$$

Based on the growth curve and genome coverage plots of the sequencing data, we deduce some parameters in different *K. pneumoniae* strains, such as t_D, t_R and $I_x(\alpha, \beta)$. The mean re-methylation time (t_M) of each motif could be further calculated by substituting the above parameters, the equation (2) and (7), as well as the methylation-read fraction in exponential phase ($M_{(x)}$) and stationary phase ($M_{(x)}^{0}$) into the equation (10).

Data availability. The genome data have been submitted to GSA at the National Genomics Data Center (CRA003482).

CRediT author statement

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Competing interests

The authors have declared no competing interests.

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Figure legends

Figure 1 Circos plots displaying the general genomic information of 14 *K. pneumoniae* strains (NTUH-K2044; 11492; 11420; 11454; 11311; 12208; 11311; 23; 11021; N201205880; 309074; 11305; 13190; 283747 and 721005)

The circles are as follows (from outside to inside): (1) A physical map scaled in megabases (Mb) from the base 1, which is the start of putative replication origin; (2) Coding sequences transcribed in a clockwise direction; (3) Coding sequences transcribed in a counterclockwise direction; (4) G+C content in a window of 2 kb sliding (red and green indicates G+C content that is higher and lower than average, respectively); (5) GC (G-C/G+C) skew in a window of 2 kb sliding (orange and green indicates GC skew above and below zero, respectively); (6) G**A**TC motifs; (7) C**C**WGG motifs. The other circles inside indicate the other 13 methylation motifs.

Figure 2 The 15 MTase genes and corresponding methylated sequence motifs for the 14 *K. pneumoniae* strains

15 MTase genes and corresponding methylated sequence motifs in 14 *K. pneumoniae* strains. Hyper-virulence strains and low-virulence strains were separately labeled in red and green letters. The large circles represents chromosome genome , and small circles around represents plasmid genome. Only the MTases with enzymatic activity are shown in this figure.

Figure 3 Density distribution of the GATC/CCWGG motifs on the NTUH-K2044 genome and random generated genome

The orange histograms show the density distribution of G**A**TC/C**C**WGG motifs on the random generated genome, which follow Poisson distribution with λ = 3.73/2.89 (total number of G**A**TC/C**C**WGG motifs×1000 per genome size). The green histograms show the density distribution of G**A**TC/C**C**WGG on the NTUH-K2044 genome, which also follow Poisson distributions with λ = 5.64/3.62.

Figure 4 Distribution of GATC/CCWGG motifs with different methylation patterns in GR and IGR regions

A. Bar plots showing the ratio of G**A**TC/C**C**WGG motifs with different methylation patterns in GRs and IGRs of 14 *K. pneumonia* strains. The blue, yellow and green bars indicate the ratios of methylated, hemi-methylated and un-methylated motifs in GRs and IGRss. **B**. Box plots showing the methylated levels of G**A**TC/C**C**WGG motifs in GRs and IGRs (red and blue boxes). **C**. Frequency distribution of the

methylated (top) and hemi/un-methylated (bottom) G**A**T̲C/C**C**WG̲G motifs in GRs and IGRs of 14 *K. pneumonia* strains. The dotted grey lines represent the position of the start and stop codons.

Figure 5 Dynamic methylation analysis of GAT̲C and CCWG̲G motifs of NTUH-K2044 and 11492

A. Growth curves of *K. pneumonia* NTUH-K2044 (red) and 11492 (blue). X-axis represents the growth time, including lag phase, exponential phase, transition-to-stationary phase and stationary phase. Y-axis represents the logarithms of normalized O.D. value. At 1, 4 and 24 h, aliquots of bacterial cultures were collected and sequenced using Pacbio and Illumina platforms. The doubling time (tD) of the two strains are also labelled in the plot. **B**. Genome-wide methylation level versus genome position for the two motifs (G**A**T̲C and C**C**WG̲G) at exponential phase. The bold fitting lines approximate the average methylation levels across the genomes (5 kb window size). **C**. Schematic diagram showing the process of DNA replication in exponential phase. (1) The origin replication complex binds to oriC region. (2) The first round of replication will be initiated when original replication complex binds to oriC region. (3) The second round of replication begins before completion of the first round replication. (4) Six replication forks are generated in one bacterium. (5-6) The first round of replication completes, followed by a new cell replication cycle. **D**. Fitting of the genomic coverages with the mathematical model of replication. The genomic coverage plots of 11492 (upper) and NTUH-K2044 (lower) strains are shown in grey; the mathematically fitting curves are shown in red. The equation for fitting plot is also shown in the figure.

Figure 6 Comparison of the methylation time of GACT motifs in different genome regions

A. Box plot shows the methylation time of G**A**T̲C motifs in *oriC* and other regions. **B**. Box plot shows the methylation time of G**A**T̲C and C**C**WG̲G motifs in GR and IGR regions. ** $P < 0.01$, *** $P < 0.001$.

Tables

Table 1 General genomic information of the 14 *K. pneumoniae* strains

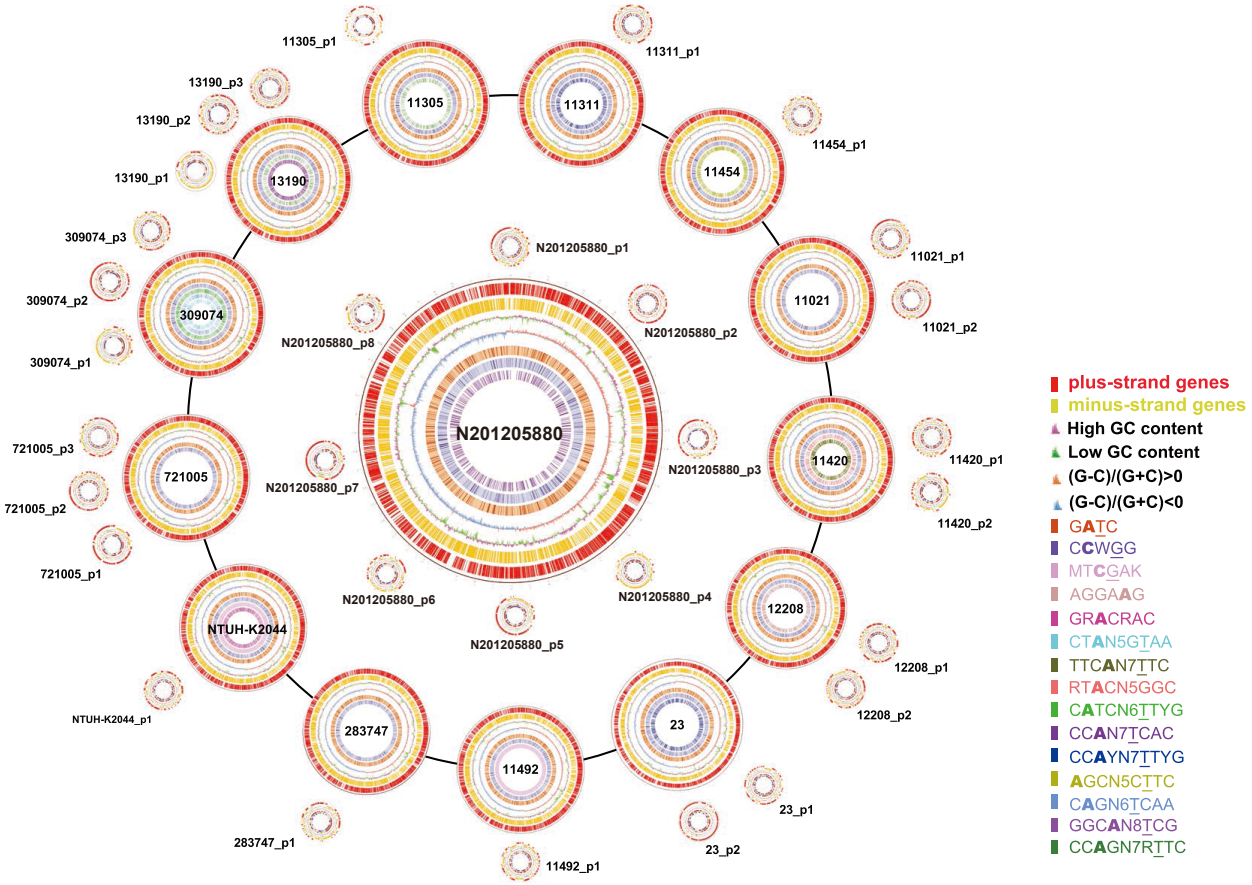
Table 2 Three types of modification patterns of GATC motif from the 14 *K. pneumoniae* strains

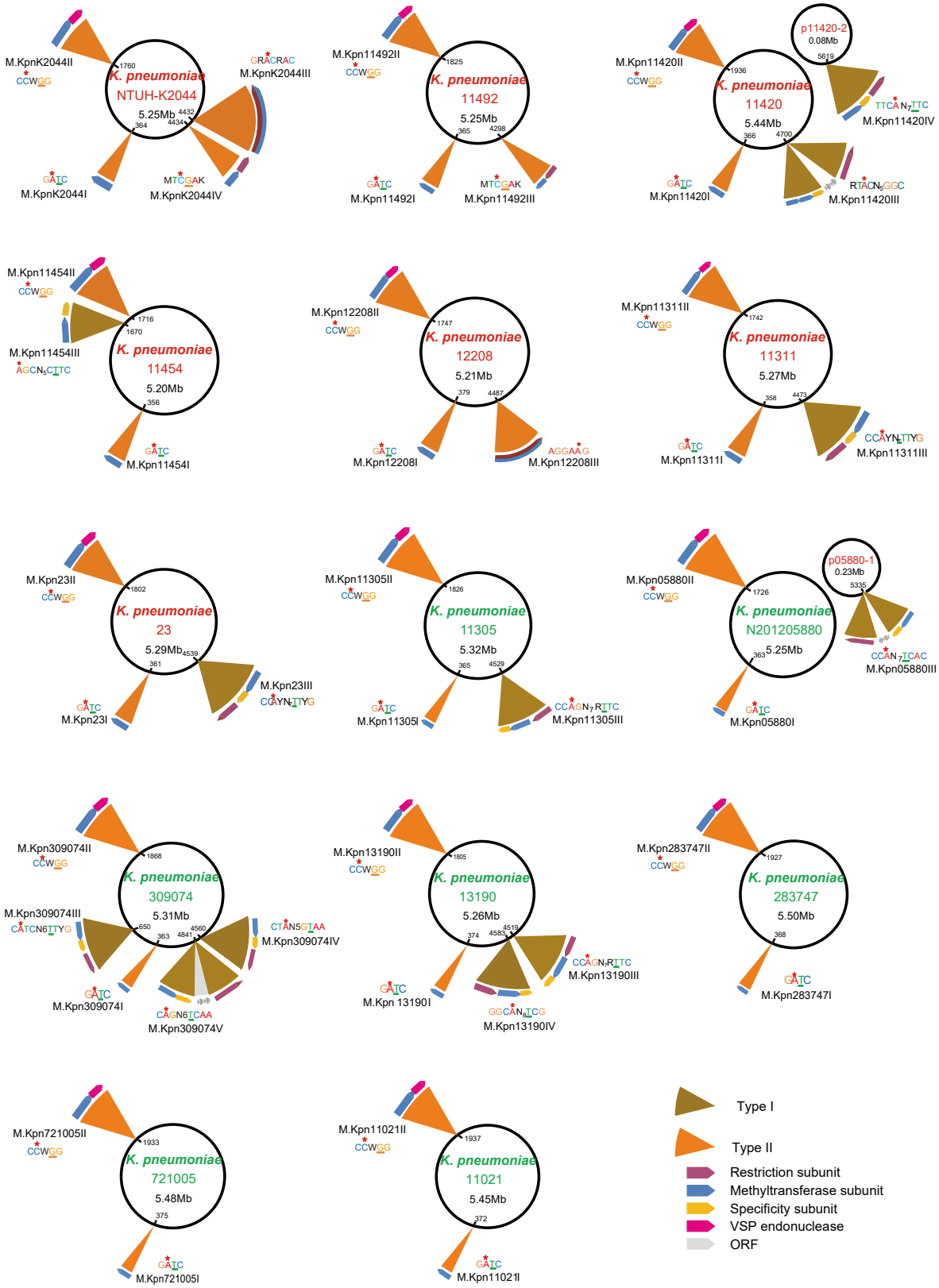
Table 3 Modification patterns of the 12 motifs with 6mA among the 14 *K. pneumoniae* strains

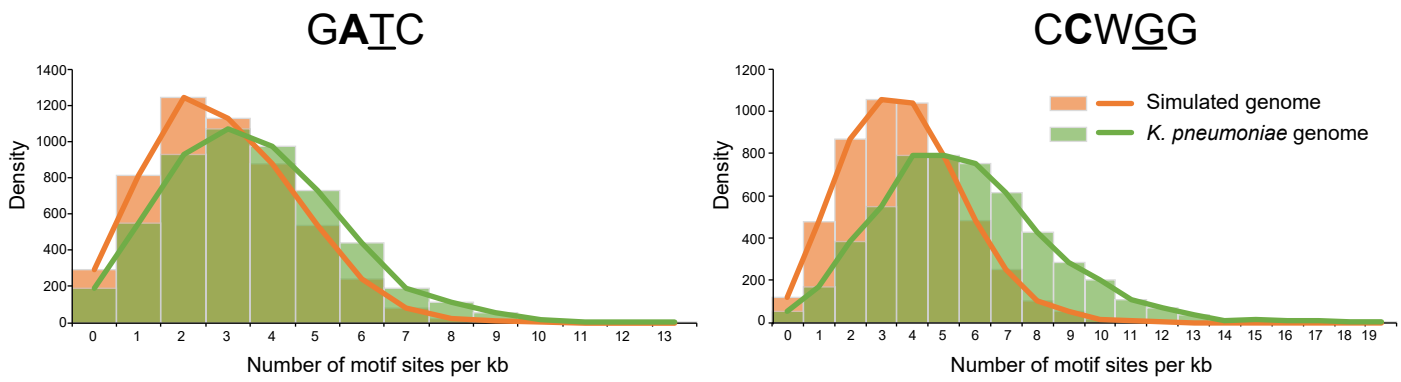
Table 4 Modification patterns of the two motifs with 5mC among the 14 *K. pneumoniae* strains

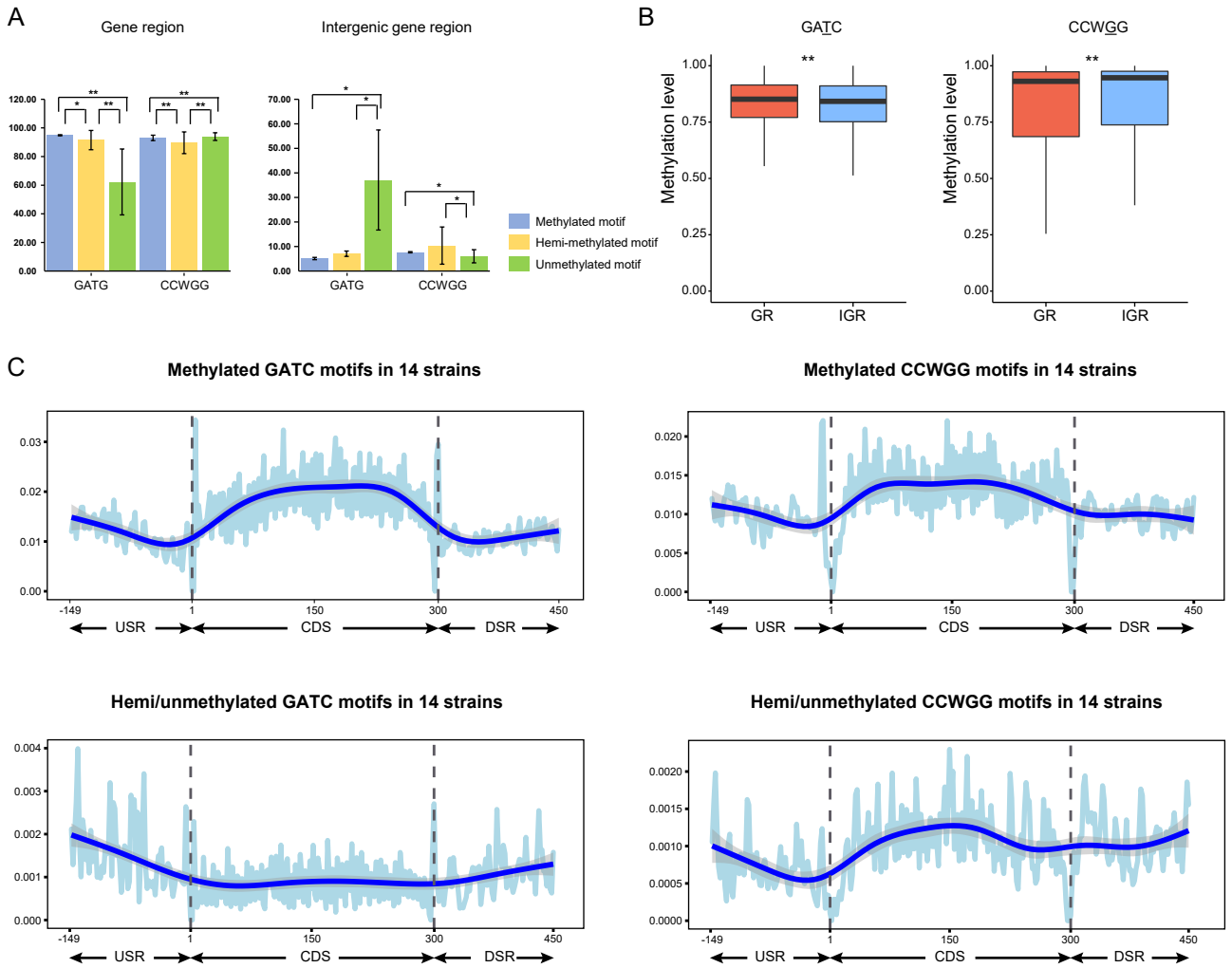
Table 5 15 methylated motifs and corresponding DNA MTases from the 14 *K. pneumoniae* strains

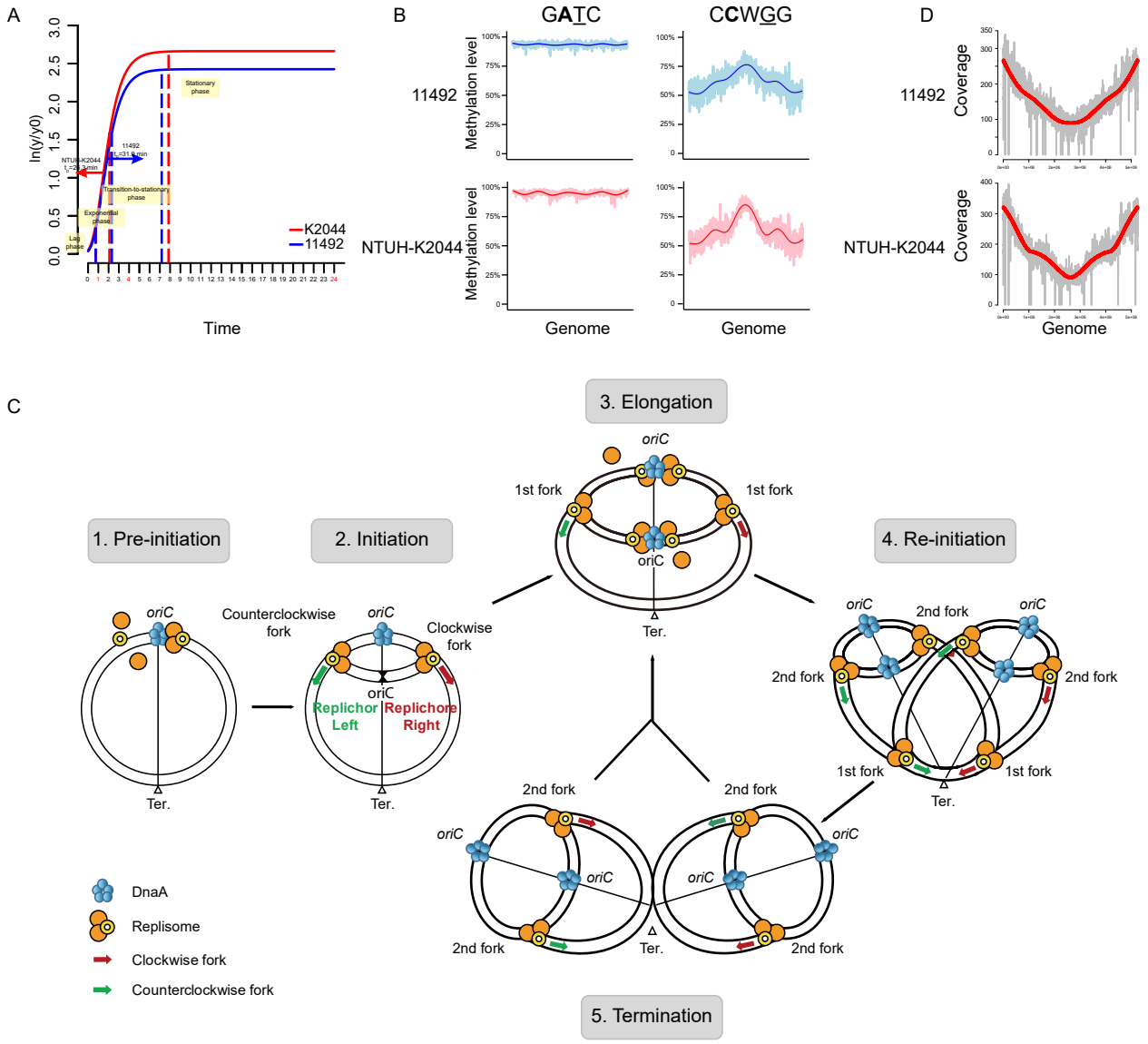
Table 6 Average methylation time per motif in the two *K. pneumoniae* strains (11492 and NTUH-K2044)


N201205880
11305
11311
11454
11021
11420
12208
23
11492
283747
NTUH-K2044
721005
309074
13190
11305_p1
11311_p1
11454_p1
11021_p1
11021_p2
11420_p1
11420_p2
12208_p1
12208_p2
23_p1
23_p2
11492_p1
283747_p1
NTUH-K2044_p1
721005_p1
721005_p2
721005_p3
309074_p1
309074_p2
309074_p3
13190_p1
13190_p2
13190_p3
N201205880_p1
N201205880_p2
N201205880_p3
N201205880_p4
N201205880_p5
N201205880_p6
N201205880_p7
N201205880_p8
plus-strand genes
minus-strand genes
High GC content
Low GC content
(G-C)/(G+C)>0
(G-C)/(G+C)<0
GATC
CCWGG
MTCGAK
AGGAAG
GRACRAC
CTAN5GTAA
TTCAN7TTC
RTACN5GGC
CATCN6TTYG
CCAN7TCAC
CCAYN7TTYG
AGCN5CTTC
CAGN6TCAA
GGCAN8TCG
CCAGN7RTTC


K. pneumoniae
NTUH-K2044
5.25Mb
M.KpnK2044II
CCWGG
GRACRAC
M.KpnK2044III
GATC
M.KpnK2044I
MTCGAK
M.KpnK2044IV
K. pneumoniae
11492
5.25Mb
M.Kpn11492II
CCWGG
GATC
M.Kpn11492I
MTCGAK
M.Kpn11492III
p11420-2
0.08Mb
K. pneumoniae
11420
5.44Mb
M.Kpn11420II
CCWGG
TTCAN7TTC
M.Kpn11420IV
GATC
M.Kpn11420I
RTACN5GGC
M.Kpn11420III
M.Kpn11454II
CCWGG
M.Kpn11454III
AGCN5CTTC
K. pneumoniae
11454
5.20Mb
GATC
M.Kpn11454I
M.Kpn12208II
CCWGG
K. pneumoniae
12208
5.21Mb
GATC
M.Kpn12208I
AGGAAG
M.Kpn12208III
M.Kpn11311II
CCWGG
K. pneumoniae
11311
5.27Mb
GATC
M.Kpn11311I
CCAYN7TTYG
M.Kpn11311III
M.Kpn23II
CCWGG
K. pneumoniae
23
5.29Mb
GATC
M.Kpn23I
M.Kpn23III
CCAYN7TTYG
M.Kpn11305II
CCWGG
K. pneumoniae
11305
5.32Mb
GATC
M.Kpn11305I
CCAGN7RTTC
M.Kpn11305III
M.Kpn05880II
CCWGG
p05880-1
0.23Mb
K. pneumoniae
N201205880
5.25Mb
CCAN7TCAC
M.Kpn05880III
GATC
M.Kpn05880I
M.Kpn309074II
CCWGG
K. pneumoniae
309074
5.31Mb
M.Kpn309074III
CATCN6TTYG
CTAN5GTAA
M.Kpn309074IV
GATC
M.Kpn309074I
CAGN6TCAA
M.Kpn309074V
M.Kpn13190II
CCWGG
K. pneumoniae
13190
5.26Mb
GATC
M.Kpn 13190I
CCAGN7RTTC
M.Kpn13190III
GGCAN5TCG
M.Kpn13190IV
M.Kpn283747II
CCWGG
K. pneumoniae
283747
5.50Mb
GATC
M.Kpn283747I
M.Kpn721005II
CCWGG
K. pneumoniae
721005
5.48Mb
GATC
M.Kpn721005I
M.Kpn11021II
CCWGG
K. pneumoniae
11021
5.45Mb
GATC
M.Kpn11021I
Type I
Type II
Restriction subunit
Methyltransferase subunit
Specificity subunit
VSP endonuclease
ORF


GATC
CCWGG
Density
Number of motif sites per kb
Simulated genome
K. pneumoniae genome


A
Gene region
Intergenic gene region
GATG
CCWGG
Methylated motif
Hemi-methylated motif
Unmethylated motif
B
GATC
CCWGG
Methylation level
GR
IGR
C
Methylated GATC motifs in 14 strains
Methylated CCWGG motifs in 14 strains
Hemi/unmethylated GATC motifs in 14 strains
Hemi/unmethylated CCWGG motifs in 14 strains
USR
CDS
DSR


A
ln(y/y0)
Time
Lag phase
Exponential phase
Transition-to-stationary phase
Stationary phase
NTUH-K2044
11492
K2044
B
GATC
CCWGG
11492
NTUH-K2044
Methylation level
Genome
D
Coverage
Genome
C
1. Pre-initiation
2. Initiation
3. Elongation
4. Re-initiation
5. Termination
oriC
Ter.
Counterclockwise fork
Clockwise fork
Replichore Left
Replichore Right
1st fork
2nd fork
DnaA
Replisome
Clockwise fork
Counterclockwise fork

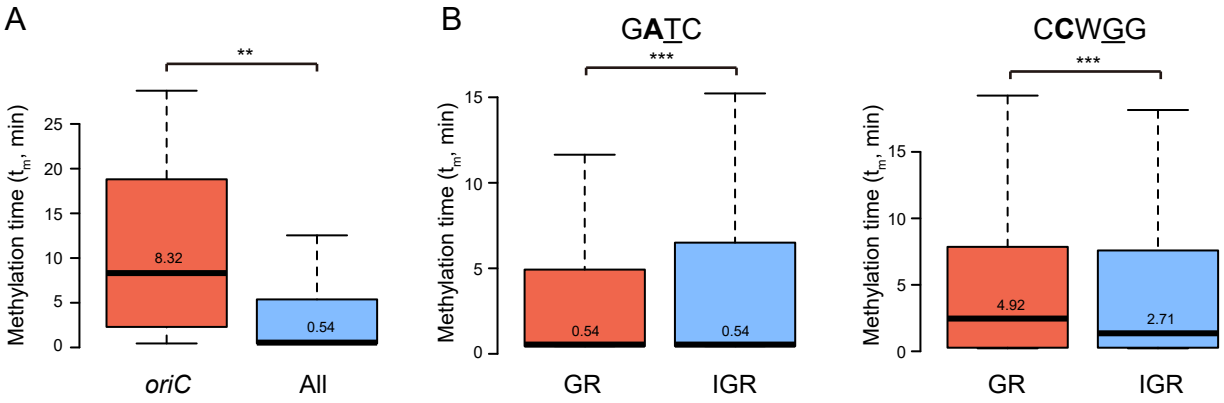

A
**
Methylation time (t_m, min)
25
20
15
10
5
0
8.32
0.54
oriC
All
B
GATC
***
Methylation time (t_m, min)
15
10
5
0
0.54
0.54
GR
IGR
CCWGG
***
Methylation time (t_m, min)
15
10
5
0
4.92
2.71
GR
IGR

Table 1 General genomic information of the 14 *K. pneumoniae* strains

Strains No.	Chromosome/ plasmid	Genomic size (Mb)	GC content (%)	Gene number	Average gene size (bp)	Coding region (%)	tRNAs	rRNAs
NTUH-K2044	NUTH-K2044_chr	5.25	57.68	5050	917	90.09	86	25
	pNUTH-K2044-1	0.22	50.17	243	701	83.28	0	0
11492	11492_chr	5.25	57.45	5017	922	88.43	87	25
	p11492-1	0.19	50.37	202	724	82.72	0	0
11420	11420_chr	5.44	57.46	5264	912	88.35	88	25
	p11420-1	0.23	50.10	257	688	83.43	0	0
	p11420-2	0.08	50.56	101	605	84.45	0	0
11454	11454_chr	5.20	57.60	4990	917	88.35	86	25
	p11454-1	0.21	50.67	225	717	83.74	0	0
12208	12208_chr	5.21	57.59	5067	910	89.42	82	25
	p12208-1	0.33	47.27	353	709	83.79	0	0
	p12208-2	0.21	49.69	232	698	82.90	0	0
11311	11311_chr	5.27	57.56	5048	918	88.26	86	25
	p11311-1	0.20	49.93	209	733	82.74	0	0
23	23_chr	5.29	57.54	5120	910	88.31	85	25
	p23-1	0.20	49.93	212	730	82.76	0	0
	p23-2	0.11	49.46	106	831	87.78	2	0

Strains No.	Chromosome/ plasmid	Genomic size (Mb)	GC content (%)	Gene number	Average gene size (bp)	Coding region (%)	tRNAs	rRNAs
11305	11305_chr	5.32	57.46	5186	901	88.37	88	25
	p11305-1	0.06	53.29	60	690	75.98	0	0
N201205880	N201205880_chr	5.21	57.50	5106	901	88.85	87	25
	pN201205880-1	0.23	51.08	240	758	82.03	0	0
	pN201205880-2	0.15	52.33	146	755	88.63	0	0
	pN201205880-3	0.11	54.85	134	661	83.30	0	0
	pN201205880-4	0.11	49.31	108	815	88.07	1	0
	pN201205880-5	0.08	50.29	88	733	84.69	0	0
	pN201205880-6	0.08	54.00	96	596	78.08	0	0
	pN201205880-7	0.07	52.85	83	657	86.33	0	0
	pN201205880-8	0.06	52.45	69	609	82.19	0	0
309074	309074_chr	5.31	57.46	5181	907	90.23	89	25
	p309074-1	0.19	53.44	197	735	83.60	0	0
	p309074-2	0.07	51.83	85	668	83.78	0	0
	p309074-3	0.05	49.03	65	667	85.67	0	0
13190	13190_chr	5.26	57.50	5172	897	89.34	88	25
	p13190-1	0.31	47.26	316	703	84.09	0	0
	p13190-2	0.08	53.86	85	649	79.42	0	0

Strains No.	Chromosome/ plasmid	Genomic size (Mb)	GC content (%)	Gene number	Average gene size (bp)	Coding region (%)	tRNAs	rRNAs
	p13190-3	0.04	45.72	50	674	86.66	0	0
283747	283747_chr	5.50	57.37	5441	891	90.07	85	25
	p283747-1	0.15	54.40	192	574	78.82	0	0
721005	721005_chr	5.48	57.38	5468	886	89.57	85	25
	p721005-1	0.27	46.48	283	716	82.85	0	0
	p721005-2	0.16	53.92	199	627	82.14	0	0
	p721005-3	0.06	51.76	77	631	88.59	0	0
11021	11021_chr	5.45	57.46	5386	893	89.67	85	25
	p11021-1	0.24	51.69	271	682	82.43	0	0
	p11021-2	0.17	53.48	217	605	81.85	0	0

Table 2 Three types of modification patterns of GATC motif from the 14 *K. pneumoniae* strains

Strain No.	Motif [a]	Total motif number [b]	Methylated motif (%)	Hemi-methylated motif (%)	Un-methylated motif (%)
NTUH-K2044	G**A**TC	30,727	30,151 (98.13%)	551 (1.79%)	25 (0.08%)
11492	G**A**TC	30,316	30,271 (99.85%)	31 (0.10%)	14 (0.05%)
11420	G**A**TC	31,847	26,835 (84.26%)	4669 (14.66%)	343 (1.08%)
11454	G**A**TC	30,278	28,046 (92.63%)	2142 (7.07%)	90 (0.30%)
12208	G**A**TC	31,512	30,695 (97.41%)	790 (2.51%)	27 (0.09%)
11311	G**A**TC	30,623	29,520 (96.4%)	1076 (3.51%)	27 (0.09%)
23	G**A**TC	31,275	26,523 (84.81%)	4423 (14.14%)	329 (1.05%)
11305	G**A**TC	30,369	29,222 (96.22%)	1122 (3.69%)	25 (0.08%)
N201205880	G**A**TC	33,171	28,007 (84.43%)	4852 (14.63%)	312 (0.94%)
309074	G**A**TC	31,208	30,384 (97.36%)	782 (2.51%)	42 (0.13%)
13190	G**A**TC	31,419	28,766 (91.56%)	2515 (8.00%)	138 (0.44%)
283747	G**A**TC	31,335	30,156 (96.24%)	1126 (3.59%)	53 (0.17%)
721005	G**A**TC	32,242	31,521 (97.76%)	687 (2.13%)	34 (0.11%)
11021	G**A**TC	32,080	27,907 (86.99%)	3968 (12.37%)	205 (0.64%)

Note: [a] The methylated nucleotide in the motif is shown as bold letter. The underlined letter represents the thymine pairing with the methylated adenine on the complementary strand. [b] The motif number includes the ones on the plus and minus strands of chromosomes and plasmids.

Table 3 Modification patterns of the 12 motifs with 6mA among the 14 *K. pneumoniae* strains

Strain No.	Motif [a]	Total motif number [b]	Methylated motif (%)	Hemi-methylated motif (%)	Un-methylated motif (%)
NTUH-K2044	GR**A**CRAC*	2104	2060 (97.91%)	/	44 (2.09%)
11420	RT**A**CN$_5$GGC*	1275	1156 (90.67%)	/	119 (9.33%)
	TTC**A**N$_7$TTC*	853	598 (70.11%)	231 (27.08%)	24 (2.81%)
11454	**A**GCN$_5$CTTC	991	930 (93.84%)	61 (6.16%)	/
12208	AGGA**A**G*	2864	2848 (99.30%)	/	16 (0.7%)
11311#	CC**A**YN$_7$TTYG*	667	608 (91.15%)	58 (8.70%)	1 (0.15%)
23#	CC**A**YN$_7$TTYG*	687	534 (77.73%)	145 (21.11%)	8 (1.16%)
11305^	CC**A**GN$_7$RTTC	342	325 (95.03%)	17 (4.97%)	/
N201205880	CC**A**N$_7$TCAC*	543	433 (79.74%)	102 (18.78%)	8 (1.47%)
309074	CT**A**N$_5$GTAA	165	164 (99.39%)	1 (0.61%)	/
	C**A**GN$_6$TCAA*	439	413 (94.08%)	26 (5.92%)	/
	C**A**TCN$_6$TTYG	634	589 (92.90%)	45 (7.10%)	/
13190^	CC**A**GN$_7$RTTC	379	338 (89.18%)	40 (10.55%)	1 (0.26%)
	GGC**A**N$_8$TCG	1065	874 (82.07%)	184 (17.28%)	7 (0.66%)

Note: [a] The methylated nucleotide in the motif is shown as bold letter. The underlined letter represents the thymine pairing with the methylated adenine on the complementary strand. Degenerate bases used in our recognition sequences are listed in the following: R = G or A, Y = C or T, M = A or C, K = G or T, S = G or C, W = A or T, B = not A (C or G or T), D = not C (A or G or T), H = not G (A or C or T), V = not T (A or C or G), N = A or C or G or T. [b] The motif number includes the ones on the plus and minus strands of

chromosomes and plasimds. [*] Newly reported motifs. [#] Both strain 11311 and 23 possess motif CC**A**YN7TTYG. ^ Both strain 11305 and 13190 possess motif CC**A**GN7RTTC.

Table 4 Modification patterns of the two motifs with 5mC among the 14 *K. pneumoniae* strains

Strain No.	Motif [a]	Total motif number [b]	Methylated motif (%)	Hemi-methylated motif (%)	Un-methylated motif (%)
NTUH-K2044	C**C**WGG	19,530	16,117 (82.52%)	1756 (8.99%)	1657 (8.49%)
	MT**C**GAK*	5410	2339 (43.23%)	270 (4.99%)	2801 (51.78%)
11492	C**C**WGG	19,284	19,015 (98.61%)	86 (0.45%)	183 (0.94%)
	MT**C**GAK*	5386	2368 (43.97%)	258 (4.79%)	2760 (51.24%)
11420	C**C**WGG	20,175	18,828 (93.32%)	839 (4.16%)	508 (2.52%)
11454	C**C**WGG	19,314	14,983 (77.58%)	2367 (12.26%)	1964 (10.17%)
12208	C**C**WGG	20108	19,874 (98.84%)	35(0.17%)	199 (0.99%)
11311	C**C**WGG	19,668	19,126 (97.24%)	299 (1.52%)	243 (1.24%)
23	C**C**WGG	19,941	18,668 (93.62%)	963 (0.48%)	310 (0.16%)
11305	C**C**WGG	19,460	18,964 (97.45%)	261 (1.34%)	235 (1.21%)
N201205880	C**C**WGG	21,115	20,867 (98.83%)	38 (0.18%)	210 (0.99%)
309074	C**C**WGG	19,966	17,464 (87.47%)	1460 (7.31%)	1042 (5.22%)
13190	C**C**WGG	20,060	19,149 (95.46%)	694 (3.46%)	217 (1.08%)
283747	C**C**WGG	20,156	19,141 (94.96%)	574 (2.85%)	441 (2.19%)
721005	C**C**WGG	20,832	20,470 (98.26%)	145 (0.70%)	217 (1.04%)
11021	C**C**WGG	20,736	18,352 (88.50%)	1358 (6.55%)	1026 (4.95%)

Note: [a] The methylated nucleotide in the motif is shown as bold letter. The underlined letter represents the guanine pairing with the methylated cytosine on the complementary strand. Degenerate bases used in our recognition sequences are listed in the following: R =

G or A, Y = C or T, M = A or C, K = G or T, S = G or C, W = A or T, B = not A (C or G or T), D = not C (A or G or T), H = not G (A or C or T), V = not T (A or C or G), N = A or C or G or T. [b] The motif number includes the ones on the plus and minus strands of chromosomes and plasimds. [*] Newly reported motifs.

Table 5 15 methylated motifs and corresponding DNA MTases from the 14 *K. pneumoniae* strains

Motif [a]	Modification	R-M/orphan[b]	MTase	Comments
G**A**TC	6mA	orphan	Dam	Known
RT**A**CN$_5$GGC	6mA	RM-I	KamA	New
TTC**A**N$_7$TTC	6mA	RM-I	KamB	New
GR**A**CRAC	6mA	RM-II	KamC	New
AGCN$_5$CTTC	6mA	RM-I	M.KpnGH01II	Known
AGGA**A**G	6mA	RM-II	KamD	New
CC**A**YN$_7$TTYG	6mA	RM-I	KamE	New
CC**A**N$_7$TCAC	6mA	RM-I	KamF	New
CC**A**GN$_7$RTTC	6mA	RM-I	M.KpnAATI	Known
CT**A**N$_5$GTAA	6mA	RM-I	M.Kpn35657I	Known
C**A**GN$_6$TCAA	6mA	RM-I	KamG	New
C**A**TCN$_6$TTYG	6mA	RM-I	M.Kpn39795II	Known
GGC**A**N$_8$TCG	6mA	RM-I	M.KpnAATIV	Known
C**C**WGG	5mC	orphan	Dcm	Known
MT**C**GAK	5mC	RM-II	KcmA	New

Note: [a] The methylated nucleotide in the motif is shown as bold letter. The underlined letter represents the guanine pairing with the methylated cytosine on the complementary strand. Degenerate bases used in the recognition sequences are listed in the following: R = G or A, Y = C or T, M = A or C, K = G or T, S = G or C, W = A or T, B = not A (C or G or T), D = not C (A or G or T), H = not G (A or C or T), V = not T (A or C or G), N = A or C or G or T. [b] The MTase prediction was based on the sequence alignment with REBASE database (http://rebase.neb.com/rebase/rebase.html). The predicted MTases were further classified as Type I, Type II or orphan MTases according to the annotation information.

Table 6 Average methylation time per motif in the two *K. pneumoniae* strains (11492 and NTUH-K2044)

Motif sequence	No. of motif (%)	Methylation time (mean ± sd, min)
G**A**TC	59424 (100%)	3.5 ± 0.5
SG**A**TCS (fast)	17041 (28.7%)	3.4 ± 0.5**
WG**A**TCW (slow)	9803 (16.5%)	3.8 ± 0.5***
C**C**WGG	35516 (100%)	9.2 ± 4.9
SC**C**WGGS (slow)	13573 (38.2%)	9.6 ± 4.9***
WC**C**WGGW (fast)	5800 (16.3%)	8.2 ± 4.5***
MT**C**GAK	10467 (100%)	4.5 ± 0.5
GR**A**CRAC	2002 (100%)	3.5 ± 0.4

Note: Degenerate bases used in our recognition sequences are listed in the following: R = G or A, Y = C or T, M = A or C, K = G or T, S = G or C, W = A or T, B = not A (C or G or T), D = not C (A or G or T), H = not G (A or C or T), V = not T (A or C or G), N = A or C or G or T. *** means that the methylation times of the motifs with certain flanking sequences show the significant difference ($P < 0.001$) comparing with the average methylation time of total motifs.