Methylation analysis of *Klebsiella pneumoniae* from Portuguese hospitals

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*Klebsiella pneumoniae* is an important nosocomial infectious agent with a high antimicrobial resistance (AMR) burden. The application of long read sequencing technologies is providing insights into bacterial chromosomal and putative extra-chromosomal genetic elements (PEGEs) associated with AMR, but also epigenetic DNA methylation, which is thought to play a role in cleavage of foreign DNA and expression regulation. Here, we apply the PacBio sequencing platform to eight Portuguese hospital isolates, including one carbapenemase producing isolate, to identify methylation motifs. The resulting assembled chromosomes were between 5.2 and 5.5Mbp in length, and twenty-six PEGEs were found. Four of our eight samples carry *bla* _CTX-M-15_, a dominant Extended Spectrum Beta Lactamase in Europe. We identified methylation motifs that control Restriction–Modification systems, including GATC of the DNA adenine methylase (Dam), which methylates N6-methyladenine (m6A) across all our *K. pneumoniae* assemblies. There was a consistent lack of methylation by Dam of the GATC motif downstream of two genes: *fosA*, a locus associated with low level fosfomycin resistance, and *tnpB* transposase on IncFIB(K) plasmids. Overall, we have constructed eight high quality reference genomes of *K. pneumoniae*, with insights into horizontal gene transfer and methylation m6A motifs.

*Klebsiella pneumoniae* (Kp) are Gram-negative bacteria that are found in the normal flora of the mouth, intestines, skin and faeces, but in other parts of the body, such as the lungs, can cause severe morbidity with a diverse disease spectrum that can culminate in complicated invasive infections. This pathogen is increasingly recognized as an important etiologic agent of healthcare associated infections. Kp has also been identified as a key route of introduction and dissemination of antimicrobial resistance (AMR) genes into other clinically significant pathogens1,2. In Europe, Kp with resistance to fluoroquinolones and carbapenems, and third-generation cephalosporins has been increasing, leading to reduced treatment options.3,4

The genome sequencing of Kp clinical isolates can provide insights into AMR, but also epigenetic information superimposed over nucleotide sequences.4 The formation of epigenetic lineages enables the adaptation of bacterial populations to harsh or changing environments and modulates the interaction of pathogens with their eukaryotic hosts.4–7. Epigenetic signals control DNA–protein interactions and can cause phenotypic change in the absence of mutation.8 A common mechanism of epigenetic signalling is DNA methylation by orphan methyltransferases (MTases) such as Dam, which have roles in chromosome replication and segregation, nucleoid organization, cell cycle control, and DNA repair.3,9. DNA methylation is also the key element of Restriction–Modification (R–M) systems that not only provide defence against foreign DNA, but also encourage bacterial evolution by driving the persistence of plasmids and other mobile genetic elements.10,11

Single Molecule, Real-Time (SMRT) platforms detect DNA modifications by measuring variation in the polymerase kinetics of DNA base incorporation during sequencing. The approach has the ability to detect genome-wide MTase N6-methyladenine (m6A) and N4-methylcytosine (m4C) target motifs at coverage levels recommended for assembly, and reveal phase variation of related genes.4,5. DNA adenine methylase (Dam),
which methylates m6A in the GATC sequence, plays a key role in DNA mismatch repair, as well as in bacterial virulence and gene expression, including in some strains of Kp10,13–15. R–M systems have also been observed on bacterial plasmids where they may contribute to their maintenance16. Kp is known to have type I and type II R–M systems16. The two systems have different mechanics and their distinct motif types have been reviewed elsewhere4,8. Briefly, type I R–M systems consist of specificity, modification and restriction subunits. The first two subunits are usually located together on a chromosome and are under the control of the same promoter17. The restriction subunit in the type I R–M system recognises long bipartite motifs, such as GGCAN8TCG. While different between chromosomes and plasmids, especially the GATC motifs methylated by orphan MTase Dam.

**Table 1. Klebsiella pneumoniae assemblies generated for the analysis. All isolates sourced from blood, except * from urine; HSM Hospital de Santa Maria; HSAC Hospital St Antonio dos Capuchos; MLST sequence type; ** Busco score is based on genes set enterobacteriales_odb10; *** Kp1363 matches six out of seven ST76 alleles; N50 is defined as the sequence length of the shortest contig at 50% of the total genome length.**

| ID      | Hospital  | Isolation Year | Fold coverage | N50 Mbps | Busco** complete / fragmented (%) | Non-chromosomal Contigs >1kbps | MLST-type | K locus type | O locus type |
|---------|-----------|----------------|---------------|----------|-----------------------------------|-------------------------------|------------|--------------|--------------|
| Kp1363  | HSAC      | 2005           | 197           | 5.34     | 89.4/7.3                          | 1                             | 76**       | KL10         | O3/O3a       |
| Kp1208  | HSM*      | 2006           | 130           | 5.37     | 98.5/0.2                          | 2                             | 35         | KL22         | O1v1         |
| Kp1264  | HSM       | 2007           | 131           | 5.23     | 97.5/0.9                          | 4                             | 147        | KL64         | O2v1         |
| Kp1675  | HSAC      | 2008           | 428           | 4.02     | 98.9/0.2                          | 4                             | 48         | KL62         | O1v1         |
| Kp2209  | HSM*      | 2008           | 504           | 5.37     | 98.5/0.5                          | 4                             | 133        | KL116        | O1v1         |
| Kp2564  | HSM       | 2009           | 36            | 5.39     | 98.7/0.2                          | 6                             | 11         | KL111        | O3b          |
| Kp2958  | HSM       | 2010           | 187           | 5.48     | 97.5/1.1                          | 3                             | 14         | KL2          | O1v1         |
| Kp3860  | HSM       | 2013           | 241           | 5.39     | 97.3/1.1                          | 2                             | 307        | KL102        | O2v1         |

Results

Genome assemblies and phylogeny. Eight Kp isolates with different multi-locus sequence types (MLSTs)20 were sourced from two hospitals in Lisbon, Portugal, between 2005 and 2013, and sequenced on the PacBio RSII technology (Table 1). The assembled chromosomes were between 5.2 and 5.5 Mbp in length and had GC content values between 57.2 and 57.7%. By contrast, 26 putative extra-chromosomal genetic elements (PEGEs) ranged in length between 3.6 and 284.2 Kbp and did not segregate into clusters based on sequence length. The PEGEs have a GC content between 41.4 and 54.1%, except for an outlying 9.9 Kbp plasmid with 60.1%. This outlying plasmid had 100% coverage and 93% identity to several plasmids of Gram-negative bacteria (e.g. CP027616.1, CP023430.1) (Table 1). To put our samples in a broader context we constructed a maximum likelihood phylogenetic tree based on the alignment of seven MLST informative and nineteen core genome loci, which contained 83 representative Kp MLST groups sourced from the NCBI database (Fig. 1)21. All our isolates were the nearest neighbour of isolates with the same MLST, but there was varying heterogeneity in genetic distance between the samples from same MLST, resulting from using 19 additional loci that could differentiate geographical and temporal differences. For example, the nearest neighbour to our Kp3860 isolate (ST307) was one collected in Malta in the same year with an identical MLST and no sequence differences across the genes analysed. On the other hand, our Kp2209 isolate had some divergence from another ST133 sample isolated in Thailand eight years earlier.

Antimicrobial resistance loci. Consistent with their Portuguese origin, four of our eight samples carry blaCTX-M-15, a dominant extra-spectrum beta-lactamase in Europe and a source of resistance to third-generation cephalosporins22. Two isolates, Kp1675 and Kp1264, carry the gene on a chromosomal region flanked by Tn2 (Tn3 family) and IS26 (family IS6) mobile genetic elements. Two further isolates, Kp3860 and Kp2209, carry blaCTX-M-15 gene on IncFIB(K) plasmids. Finally, in addition to chromosomal blaCTX-M-15, Kp1264 also carries it on IncFIA(HI1) plasmid which belongs to the same incompatibility group as IncFIB(K)23. All five blaCTX-M-15 fragments (4 isolates) are embedded in the 9500 nt sequence that is near identical between samples (>99.98% similarity), though Kp2209 has only 50% coverage compared to 93% coverage for the other sequences. This 9500 nt sequence also carries quinolone and aminoglycoside resistance genes. In three isolates (Kp1675, Kp1264 and Kp3860), the region containing blaCTX-M-15 also contains blagmcr, which is a penicillinase and a major correlate of resistance to piperacillin/tazobactam and co-amoxiclav in Kp and E. coli, and is commonly associated with co-carriage of acr(6′)-Ib-cr, which restricts aminoglycoside and fluoroquinolone treatment options24,25.
Only the Kp2564 isolate bore a KPC-3 carbapenemase coding gene (blaKPC-3), which is located on an IncFIA plasmid. This carbapenemase is thought to have originated in clonal group 258 (CG258), to which this sample belongs. KPC-3 has now spread across Kp MLSTs globally, including in Portugal, where its prevalence has increased dramatically over the last decade. All samples were tested for susceptibility to major anti-microbials (Table S1). Sample Kp2564 was the only one resistant to imipenem, which is consistent with presence of blaKPC-3.

Of all samples only Kp1264 appeared resistant to fosfomycin.

**Methylation analysis.** Seven of the eight samples had sufficient quality and coverage to perform DNA methylation analysis using the SMRT portal. For the other sample (Kp2564), we successfully identified methylation motifs, but low sequencing coverage meant that for non-GATC motifs the detection of motifs methylation state was unreliable. After comparing the generality of motifs versus the frequency of motif methylation (Figure S1) for each isolate, we removed the motifs for which less than 60% of motif occurrences were methylated. For Kp2564 we focused only on GATC, AACN5RTGC and GCAYN5GTT motifs, the latter two being part of same type I bipartite recognition sequence. While our raw results did contain four m4C modifications, the highest modification rate was 17% and therefore omitted from further analysis.

All seven high quality assemblies contained a GATC recognition motif of the Dam based type R–M system. A total of ten type I R–M system motifs were found. For each of these ten we identified specific MTases (Table 2) by comparing their specificity subunits to those in the REBASE database. REBASE has 100% matches of the DNA target recognition domain. The recognition sequence motif on plasmid_5 of Kp2564 was the exception with 82% highest amino acid identity in the target domain. For Kp1363, SMRT Analysis returned four motifs (CGACCN4TGG, CGAYDN4TGG, CCAN4GATCA and CCAN5RTCG) without partner motif strings. The first and second motifs are imperfect reverse complements of the fourth. The third motif is likely to be recognised by the same specificity subunit, but may show low methylation (60%) due to the
against foreign DNA. To assess if this is reflected in the number of motifs on the upstream sequences capture gene promoters, whereas downstream sequences provide a control. Relatively evenly distributed. Therefore, we focused on unmethylated motifs 50bps upstream and downstream of the gene. Upstream sequences capture gene promoters, whereas downstream sequences provide a control. 

TnpB fosA

Most isolates (> 3/7) (see Data S1 for the full list of identified genes). All such cases had a GATC motif, with only one isolate (Kp1264) not having the unmethylated motif downstream of the gene, so the unmethylated GATC should not be directly affecting promoter region; however, the lack of methylation may indicate that MTase cannot access the site.

| Assembly | Main motif/partner motif | Modified fraction | Number of motifs | Specificity subunit |
|----------|--------------------------|------------------|-----------------|--------------------|
| Kp1208   | GATC                     | 98%              | 63,002          |                    |
| Kp1208   | GAYN6TTTG/CAAN6RTCA      | 96%/88%          | 463             | chr: 557,963–561,058 |
| Kp1264   | CCAGN7RTTC/ GAAYN7CTGG   | 94%/94%          | 347             | chr: 1,565,436–1,567,199 |
| Kp1264   | GATC                     | 98%              | 61,076          |                    |
| Kp1264   | GGCAN8TCCG/ CGAN8TGCc    | 98%/91%          | 1042            | chr: 1,638,656–1,639,996 |
| Kp1363   | CCAN4GATCA               | 60%              | 447             | plasmid_1: 100,843–102,264 |
| Kp1363   | CCAN5RTCAG               | 99%              | 2313            | plasmid_1: 100,843–102,264 |
| Kp1363   | CGACCN4TGG               | 98%              | 276             | plasmid_1: 100,843–102,264 |
| Kp1363   | CGAydN4TGG               | 98%              | 1613            | plasmid_1: 100,843–102,264 |
| Kp1363   | GAAAYN8TCCG/ CGAN8R TTC  | 97%/96%          | 459             | chr: 1,946,700–1,947,668 |
| Kp1363   | GATC                     | 99%              | 61,266          |                    |
| Kp1675   | GATC                     | 99%              | 64,248          |                    |
| Kp2209   | GATC                     | 99%              | 64,264          |                    |
| Kp209    | GATGn6TTTG/ CAA6CATC     | 99%/99%          | 1029            | plasmid_1: 2570–3844 |
| Kp2564   | AAcN5RTGC/ GCAYN5GTT     | 26%/26%          | 998             | plasmid_3: 94,898–96,112 |
| Kp2564   | GATC                     | 36%              | 63,282          |                    |
| Kp2958   | AcaN5TGC/ GTCA9N5TTG     | 98%/96%          | 319             | chr: 4,378,992–4,379,303 |
| Kp2958   | AGCN5CTTC/ GCA9N5GCT     | 100%/98%         | 1024            | chr: 1,411,097–1,412,662 |
| Kp2958   | GATC                     | 98%              | 64,966          |                    |
| Kp3860   | GAA6N6GGG                | 97%              | 586             | chr: 1,856,401–1,857,603 |
| Kp3860   | GATC                     | 99%              | 63,388          |                    |

Table 2. List of high-quality m6A motifs. Only the GATC motif was present in multiple isolates, consistent with widespread presence of DNA adenine methylase among Gammaproteobacteria. Kp2564 has low methylation rates due to low sequencing coverage.

Due to a lack of a corresponding restriction enzyme in Kp, the GATC motif is not involved in the defence against foreign DNA. To assess if this is reflected in the number of motifs on the Kp chromosome and PEGEs, we examined the relative abundance of GATC motifs, as measured by the ratio of total length of observed motifs to those of genomic sequence. For the GATC motif, there was much greater abundance on the chromosomes compared to PEGEs (Wilcoxon P < 3 × 10⁻⁶) (Table S2, Fig. 2a). We found the same result in 673 high quality assemblies from the NCBI RefSeq database (all N50s > 4.5Mbp; Fig. 2b), where the GATC abundance on the chromosome (mean 2.25%; standard deviation 0.017%) was greater than on PEGEs (mean 1.52%; std. dev. 0.088%) (Wilcoxon P < 3 × 10⁻⁶) (Table S2, Fig. 2a). We found the same result in 673 high quality assemblies from the NCBI RefSeq database (all N50s > 4.5Mbp; Fig. 2b), where the GATC abundance on the chromosome (mean 2.25%; standard deviation 0.017%) was greater than on PEGEs (mean 1.52%; std. dev. 0.088%) (Wilcoxon P < 0.001). We also investigated the abundance of type I R–M system motifs (i.e. non-GATC) by comparing the share of chromosomes occupied by the motifs that are recognised by assembly's MTases versus the share occupied by recognition sequences from other assemblies. There was no strong difference in the abundance of type I recognition motifs on chromosomes or between chromosomes and PEGEs (Wilcoxon P > 0.230). Similarly, there was no strong difference between PEGEs that were recognised by their own assembly's R–M system compared to those that were not recognised (Wilcoxon P = 0.187).

Unmethylated motifs. Methylation motifs (see Table 2) covered a large portion of the genome and were relatively evenly distributed. Therefore, we focused on unmethylated motifs 50bps upstream and downstream of the genes. Upstream sequences capture gene promoters, whereas downstream sequences provide a control. We focused on the genes and motifs that have higher than expected number of unmethylated motifs (Table 3) in most isolates (> 3/7) (see Data S1 for the full list of identified genes). All such cases had a GATC motif, with only fosA and tnpB family (IS2 group) transposase genes having a consistently unmethylated motif downstream. TnpB is a key component of mobile genetic elements' transposition mechanism, and in four samples that had the gene, it was located on a large (197–284kbp) IncFIB plasmid containing various heavy metal and AMR loci. The fosA gene provides Kp with an inherent low-level resistance to the fosfomycin antibiotic. Only one isolate (Kp1264) was resistant to fosfomycin (Table S1), but it was one of two isolates (Kp1264 and Kp1363; Fig. 3) that had the R–M system inserted after fosA. Both fosA and tnpB genes lacked methylation at a GATC motif downstream of the gene, so the unmethylated GATC should not be directly affecting promoter region; however, the lack of methylation may indicate that Mase cannot access the site.

Of the seven isolates with high quality site methylation data, only Kp1264 (ST147) and Kp1363 (ST76) did not have the unmethylated motif downstream of fosA. In both isolates, the required GATC motif was missing.
Figure 2. (a) The share of genomic elements occupied by a GATC motif in the assemblies in Table 1. The largest non-chromosome contig, 284kbp, has abundance ratio of 1.8%. (b) GATC abundance in 673 high quality assemblies of Kp taken from NCBI database limiting sequences to those that have N50 in excess of 5Mbp. The mean chromosomal GATC abundance in (a) and (b) is the same.

Table 3. Genes which have an unmethylated GATC motif 50 bps upstream/downstream in at least four samples. Every sample has either one copy of the gene or no copies. The p-value null hypothesis is probability of gene's GATC motif being unmethylated is the same as probability of any GATC motif being unmethylated. None of the non-GATC motifs had a p-value below 0.05. NM = no motif. *Missing start codon.
Figure 3. The fosA gene and surrounding region. (Black bars) methylated GATC motifs; (red bars with red dot above) unmethylated GATC motifs downstream of fosA on the same strand; (green bars with green dot above) unmethylated GATC on the opposite strand of GntR family transcription regulator coding sequence. Kp1264 and Kp1363 lack a GATC motif downstream of fosA due to an insertion of endonuclease. The other samples all have a single unmethylated GATC motif downstream of fosA on the same strand as fosA coding sequence. The only other unmethylated GATC in these regions are the motifs on GntR family transcription regulator.

Table 4. Genes and proteins encoded by coding sequences immediately downstream on opposite strand of chromosomal fosA. Together these account for 99.6% of downstream elements in 3584 K. pneumoniae samples. For a given MLST, all samples normally have the same gene downstream of fosA. Genes yjiR_1 and yjiR_2 are truncated versions of the GntR family transcription regulator.
due to insertion of an endonuclease gene (> 3 kbp) (Fig. 3). To assess the frequency of this insertion we analysed available *Kp* assemblies (n = 3584) from the NCBI database. We found that 99.4% of assemblies had the *syrM1* gene upstream of chromosomal *fosA*. The exceptions were 23 samples from diverse geographical regions and sequence types (Table S3). Further, we observed a limited variety of genes downstream on the opposite strand of *fosA* (Table 4). The top five genes accounted for 96.2% of the assemblies, with the predominant one being the GntR family transcription regulator (68.3%). For the ST147 sequence type (NCBI, n = 131), we found most assemblies (130/131) had toxic protein SymE in the same position. The outlying assembly (GCA_004145685.1_ASM414568v1_genomic) had a truncated version of SymE due to the scaffold ending in the coding sequence. For the ST76 sequence type, all NCBI isolates (n = 7) and Kp1363 (one locus mismatch) had coding sequence of endoribonuclease SymE downstream of *fosA* (Table 4).

For a random subset of unmethylated motifs (from Table 3), we examined the IPDs of individual reads to evaluate the existence of distinct subpopulations with regard to the methylation status of genes, thereby investigating potential intra-populational epigenetic diversification (Figure S4). Standard SMRT Analysis output files report the methylated fraction of reads in the sample, but only for motifs that were called as methylated. We modified the SMRT Analysis algorithms to produce per base IPD values for all nucleotides. After analysing the underlying reads, we found that IPD levels in some genes, including *fosA* and *dksA* (DnaK suppressor protein), differentiated two subpopulations corresponding to methylated and unmethylated cells. By contrast, we observed no methylated subpopulation in *mglB* (D-ribose transporter subunit RbsB).

### Discussion

Across seven *Kp* isolates with high quality PacBio data, we identified the common Dam based methylation mechanism involving GATC motifs, as well as several type I R–M systems. With a type I R–M system, we observed that the abundance of recognition motifs did not vary between chromosomes of different assemblies, but that some deletion of recognition motifs has occurred in PEGEs. This observation supports the role of type I R–M system as a defence mechanism against invasion by foreign DNA, but not its regulatory function, as chromosomal abundance was not correlated to specificity subunit. In contrast, there was a clear difference in abundance of the GATC motif on chromosomes and PEGEs, which suggests that the motif has a function as identified by previous research.

Because GATC motifs are known to have a regulatory function, we expected to find that some genes had consistently unmethylated GATC in gene promoter regions. However, the finding of consistently unmethylated GATC downstream of genes (e.g. *fosA* and *tnpB*) is intriguing. This outcome may be an indication of a secondary structure that is inaccessible to MTase, or that the GATC motif downstream of *fosA* and *tnpB* is a distant promoter or regulatory region. The phenotypic impact of this GATC motif requires follow-up experiments ideally via single nucleotide site-directed mutagenesis as to prevent methylation with minimum downstream effects. Analysis of this region has also led us to identify the limited variety of recombination events around *fosA* and their potential MLST specificity. Toxic protein SymE is part of the plasmid toxin-antitoxin system. To assess its prevalence in *Kp* we evaluated 131 randomly chosen ST147 samples from the NCBI database. We found the same *fosA*-SymE sequence in all but one of them, but in no other major ST. The potential impact of the SymE insertion on fosfomycin resistance should be evaluated in follow-up experiments. The other coding sequences in that location do not appear to be part of the toxin-antitoxin system, and therefore this locus has the potential to evaluate the robustness of a *Kp* phylogeny based on sequence types. We would expect that sequence types with the same insertion would cluster together, and the opposite observation would indicate possible recombination of MLST genes.

The general lack of resistance to fosfomycin in *Kp* species, despite decades of active use of fosfomycin for infection control, is in contrast to the emergence of resistance to other antimicrobials. However, *Kp* has been reported to acquire resistance in vitro within 24 hours of exposure to fosfomycin; though this resistance does not seem to persist as clinical studies report the limited spread of fosfomycin resistance. The identified unmethylated GATC motif downstream of *fosA* may be correlated with the rapid acquisition of fosfomycin resistance in individual isolates. Our dataset is too small to draw any robust statistical inference, but the only sample in our study resistant to fosfomycin, Kp1264, is one of the two samples which lacks a GATC motif downstream of *fosA* (Fig. 3). The plasmid toxin-antitoxin system downstream of *fosA* in both Kp1363 and Kp1264 requires constant transcription, which means the genomic region is accessible to transcription machinery; whereas unmethylated GATC may be the result of DNA regional conformation inaccessible to Dam. It is unlikely that a lack of methylation downstream of the *fosA* gene is itself the fosfomycin resistance mechanism. If correlation exists, the more likely explanation is that conformation of the genomic region that prevents methylation, also prevents fosA transcription. This hypothesis is particularly relevant for the treatment of complicated infections by carbapenemase-producing strains, for which fosfomycin is often used in combination with another drug (e.g. colistin) as a last resort therapeutic option.

Overall, our work has provided new insights into methylation and potential MLST specificity. We have generated eight new reference genomes for *Kp* and analysed their methylomes. The findings reinforce the role of type I motifs as a defence mechanism against foreign DNA. We also identified a higher than expected rates of the GATC motif on *Kp* chromosomes which, together with absence of respective endonuclease, support existence of Dam function such as DNA mismatch repair and gene expression regulation. We identified two genes, *fosA* and *tnpB*, which have an unmethylated GATC motif downstream of each locus. Methylation analysis may be useful for identification of distant regulatory regions or frequent secondary DNA structures. In particular, a lack of methylation downstream of *fosA* genes could explain not only rapid emergence of resistance in individual samples, but also lack of widespread resistance in *Kp*. Further, using a bioinformatics approach we detected the presence of both methylated and unmethylated sequencing reads existing within individual samples, potentially...
Methods
Sample collection, culture and sequencing. All eight samples were collected from Hospital de Santa Maria (n = 6) and Hospital St Antonio dos Capuchos (n = 2) in Lisbon, Portugal. The samples were cultured and tested for AMR as described previously. DNA was extracted from strain cultures, grown overnight at 37°C on Mueller–Hinton Agar. DNA extraction was carried out using the Cetyl trimethylammonium bromide (CTAB) method previously described using Tris–Acetate buffer (10 mM, pH 8). Our samples were generated without TET1 oxidation; thus we could not examine 5mC methylation. All DNA samples were sequenced at the Genome Institute Singapore on the PacBio RSII platform.

PCR and Sanger sequencing-based MLST analysis was based on fragments of seven housekeeping genes: rpoB (beta-subunit of RNA polymerase), gapA (glyceraldehyde 3-phosphate dehydrogenase), mdh (malate dehydrogenase), pgi (phosphoglucoisomerase), phoE (phosphorine E), infB (translation initiation factor 2), and tonB (periplasmic energy transducer). Details of the MLST scheme including amplification and sequencing primers, allele sequences and MLSTs are available on the Institute Pasteur’s MLST Web site (https://biggsdb.pasteur.fr/klebsiella/klebsiella.html).

Bioinformatic analysis. A summary of the bioinformatic and analysis pipeline is provided (Figure S3). Data assembly was performed using three different software tools (HGAP3, Canu and Flye). HGAP3 is part of the SMRT Analysis toolkit (version 2.3), while Canu and Flye software are standalone. Short read polishing was not performed due to > 100-fold long read coverage for each sample. We have aligned raw reads to the assemblies and examined region ± 2000 nt around fosA gene in each assembly. We did not observe any SNPs, InDels or alignment abnormalities. Between samples, the coverage of the region ranged from 77- to 209-fold. Generated assemblies were compared using the Busco enterobacteriales (odb10) dataset. For each sample the assembly with highest Busco complete gene count was selected. The sum of complete and fragmented genes yielded the same “best” assemblies. While the HGAP3 assembly had marginally higher N50 score, in most cases its Busco scores were substantially lower. All but one of the selected assemblies had a scaffold longer than 5Mbp.

The outlying assembly, Kp1675, had a chromosome split into two scaffolds which was considered an acceptable trade-off for a 5.3% higher share of complete genes. The in silico resistance profile, MLST, O-locus and K-locus types were determined using the kleborate tool. Plasmid and Insertion Sequence (IS) identities were established using PlasmidFinder and ISFinder.

Methylation analysis. We used the generated assemblies to perform methylation detection by applying the PacBio SMRT Analysis toolkit (v2.3). To discriminate between natural variation in IPD and true methylation the toolkit relies on expected distribution of unmethylated IPDs given the nucleotide sequence context. When IPD value exceeds the probability threshold, we used default p-value 0.01, the relevant base is called as modified. The calculations are implemented in KineticWorker.py, which is part of SMRT Analysis toolkit. After comparing the generality of motifs versus the frequency of motif methylation for each isolate, we removed the motifs for which less than 60% of motif occurrences were methylated. We retained two motifs for the Kp2564 isolate which had methylation fractions below 60% (Table 2), where the low level of methylation was result of insufficient sequencing coverage.

NCBI datasets. To assess the abundance of GATC motif on Kp chromosomes, we downloaded from the NCBI RefSeq database all Kp assemblies where their longest chromosomal contig was at least 4.5Mbp (n = 673). For an analysis of the prevalence of GATC motif downstream of fosA gene, we downloaded all Kp assemblies in the NCBI pathogen database (as of November 15, 2019; n = 3584). For reconstruction of a Kp phylogenetic tree we determined the MLST of each of the 3584 assemblies using kleborate software. For each MLST which had more than nine samples from different geographical locations (city, region, or country) and year of collection data, we included a single randomly selected assembly for phylogenetic reconstruction. A total of 83 isolates were used in the construction of the phylogenetic tree. The tree was constructed using IQTREE software with the GTR + F + G4 model.

Ethical statement. The study was approved by the Hospital de Santa Maria and Hospital St Antonio dos Capuchos Ethics Committees, and all methods were performed in accordance with the relevant guidelines and regulations, including informed consent from all patients.

Data availability
The assembled sequences are deposited in the European Nucleotide Archive (project PRJEB38289). Accession numbers and metadata are presented in Table S1. The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary files.

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Author contributions
J.Pe., S.C., A.D., and T.G.C. conceived and directed the project. A.D. coordinated sample collection. J.P., R.E. and A.M. undertook sample processing and DNA extraction. P.Fd.S., M.L.H. and S.C. coordinated sequencing. J.P., R.E. and A.M. interpreted results. A.S., J.Ph. and JC performed bioinformatic and statistical analysis. We gratefully acknowledge the Scientific Computing Group for data management and compute infrastructure at Genome Institute of Singapore for their assistance. The MRC eMedLab computing resource was used for bioinformatics and statistical analysis.

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Competing interests
The authors declare no competing interests.

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