Recurrent emergence of *Klebsiella pneumoniae* carbapenem resistance
mediated by an inhibitory *ompK36* mRNA secondary structure

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Materials and Methods

General cloning, molecular biology and strain generation

All in vitro assays and animal infections with KP were carried out using ICC8001, a strain derived by animal passage from ATCC43816(1).

A homologous recombination technique resulting in scarless markerless mutants was used to generate all strains based on the Standard European Vector Architecture platform pSEVA612S (JX560380.2) mutagenesis plasmid. Briefly, mutants were generated in two sequential recombination steps. The first step integrates the mutagenesis plasmid into the genome generating a merodiploid. The mutagenic plasmid was introduced by three part conjugation and homologous recombination promoted by lambda-recombinase expressed from a helper plasmid. The second step induces double strand DNA breaks by induction (L-arabinose) of I-SceI expression from the helper plasmid. Double stranded breaks are induced at I-SceI sites located on the chromosomally integrated mutagenesis vector, flanking the final integration region. This technique has been used to efficiently recombineer in KP (1) and other Enterobacteriaceae (2) as described in respective manuscripts.

Mutagenesis vectors were generated by routine molecular biological techniques. These included plasmid preparation (Monarch Plasmid Miniprep Kit, NEB), genomic DNA extraction from bacterial cells (DNEasy, QIAGEN), polymerase chain reaction (Q5 High-Fidelity 2x Master Mix and OneTaq, Quick-Load 2X Master Mix, both NEB), PCR clean-up (Monarch PCR and DNA Cleanup Kit, NEB) and gel extraction (Monarch Gel Extraction Kit, NEB), site directed mutagenesis (KLD enzyme blend, NEB) and Gibson assembly (Gibson Assembly Master Mix, NEB). Sanger sequencing was carried out to check vector construction and seamless integration into the genome (Eurofins Genomics). Custom primers in this study were made by Sigma-Aldrich.
**ompK35 mutagenesis**

*ompK35* wild-type ORF (+500bp flanking regions) was cloned from ICC8001 genomic DNA with primers (P) 1/P2 and ligated by Gibson Assembly into pSEVA612S, linearised with P3/P4. The ORF was removed by inverse PCR to generate the genomic deletion vector (P5/P6) and plasmid recircularized from PCR product (KLD enzyme blend, NEB).

**ompK36 mutagenesis**

ICC8001Δ*ompK36* was generated in previous work (Vector 5 in Wong et al., 2019). *ompK36* wild-type ORF (+500bp flanking regions) was cloned from ICC8001 genomic DNA with primers P7/P8 and ligated by Gibson assembly into pSEVA612S, linearised with P3/P4. In screening colonies by Sanger Sequencing we found the 25c>t mutation and this was not generated by site directed mutagenesis.

**sfGFP vectors and -14a>t vectors**

*Leucine 9 CTG-sfGFP (wild-type) and Leucine 9 TTG-sfGFP (25c>t transition).* The wild-type and 25c>t Sec signal sequences were cloned from the *ompK36* vectors generated above with P9/P10. sfGFP was amplified from our previously published glmS site insertion vector (Vector 13 in Wong et al., 2019) with P11/P12 and chimeric fusion generated in the mutagenesis vector ligated by Gibson Assembly.

Leucine 8 CTG-sfGFP and Leucine 8 TTG-sfGFP were generated by site-directed mutagenesis using P13/P14 and P15/P16 respectively using the Leucine 9 CTG-sfGFP (wild-type) as the template.

**5’ untranslated region mutagenesis of Leucine 9 TTG-sfGFP (25c>t transition).** Mutants were generated by site-directed mutagenesis as follows: -32(a>t) (P17/P18), -31(a>t) (P17/P19), -30(a>t) (P17/P20), -29(a>t) (P17/P21), -25(a>t) (P22/P23), -23(a>t) (P22/P24), -21(a>t) (P22/P25), -20(a>t) (P22/P26), -18(a>t) (P22/P27), -17(a>t) (P22/P28), -16(a>t) (P22/P29), -14 a>t(a>t) (P22/P30) using leucine 9 TTG sfGFP as the template. The same primer pair
(P22/P30) was used to introduce the -14a>t mutation into Leucine 8 CTG-sfGFP, ompK36WT and ompK36WT(25c>t) vectors.

The 24c>t mutation was introduced by SDM using primers P31 and P32.

**Introduction of pKpQIL KPC-2 plasmid**

We introduced pKpQIL KPC-2 by conjugation using an *E. coli* donor following our previously published protocol(1).

**Outer membrane preparations and gel electrophoresis**

Outer membrane proteins were purified according to a previously described protocol with several modifications(3). Saturated overnight cultures of bacteria were grown in LB (10g/L NaCl) and sonification was performed at 25% amplitude for 10 bursts of 15 seconds each. 10 μg of protein was separated by SDS-PAGE using 12% acrylamide Mini-protean TGX precast gels (Bio-Rad, USA). Gels were stained with Coomassie (Sigma-Aldrich) and imaged on a ChemiDoc XRS+ (Biorad, USA).

**RNA isolation and reverse transcription quantitative PCR (RT-qPCR)**

300 μl of an LB overnight culture of KP were treated with RNAprotect Bacteria reagent (Qiagen) and centrifuged at 5000 xg for 10 min. The bacterial pellet was digested in 100 μl TE buffer with 15 mg/ml Lysozyme (Sigma-Aldrich) and 20 μl Proteinase K (Qiagen) for 10 min according to manufacturer’s guidelines. RNA was isolated using the RNeasy minikit (Qiagen) following the manufacturer’s instructions, and RNA concentration was determined using a Nanodrop 2000 spectrophotometer (ThermoFisher Scientific). 1 μg of RNA was treated with RNase-free DNase (Sigma-Aldrich) for 1 h at 37 ºC and cDNA was then synthesized using a Moloney murine leukemia virus (MMLV) reverse transcription kit with random primers following the manufacturer’s protocol (Promega). To check for the presence of remaining DNA, a reaction mixture without the MMLV reverse transcriptase was also included (NRT). qPCR was performed using the Power Up SYBR Green master mix (ThermoFisher Scientific) and the
following primers 33/34 (ompK36) and 35/36 (rpoB). The assay was run on a StepOnePlus System (Applied Biosystems) and results were analysed using the StepOne software (Applied Biosystems). Relative gene expression levels were analyzed by using the $2^{-\Delta CT}$ (where $C_T$ is threshold cycle) method.

**IVIS sfGFP fluorescence measurement**

Saturated overnight cultures of strains were diluted 1:2000 in PBS. 20ul of suspension was plated onto an agar plate and allowed to dry. Following overnight incubation (16 hours exactly) plates were removed and imaged on an IVIS Spectrum CT imaging platform (1 second exposure, Ex 465nm/Em520nm). Fluorescence over the area of the spot was quantified using Living Image Software v5.4.3 (Perkin Elmer).

**Meropenem Minimum Inhibitory Concentrations (MIC)**

The meropenem MIC of strains was assessed by broth microdilution by broth microdilution in biological triplicate according to the ISO 20776-1:2019 standard and the median value taken as the final MIC.

**Animal work**

8-10 week old, female, 18-20g BALB/c mice were purchased from Charles River, UK. All animal work took place in the institution animal facility (Association for Assessment and Accreditation of Laboratory Animal Care accredited) under the auspices of the Animals (Scientific Procedures) Act 1986 (PP7392693). Work was approved locally by the institutional ethics committee.

**Housing and Randomisation**

Upon arrival animals were independently randomized into cages of 5 animals and housed for an acclimatisation period of 1 week. Mice received food and water *ad libitum* and were housed in a 12hour/12hour light dark cycle. Identification of animals within groups was achieved by ear notching without anaesthesia at least 24 hours in advance of procedures.
Intratracheal administration of inoculum and infection

Anaesthesia was induced by intraperitoneal injection (BD Microlance 27G 13mm needle) of 80mg/kg ketamine and 0.8mg/kg medetomidine. Pre-intubation body temperature was maintained by contact with a heat mat (Harvard Apparatus, U.K) and eye lubricant (2.0mg/g carbomer, Alcon UK) was applied.

Intubation was achieved as previously described (1) by placement of a 21G catheter (21G IV peripheral catheter (Insyte) BD Medical) using a fibreoptic intubation set (Kent Scientific) following the enclosed instructions.

Inoculum (dose defined in graphs) was prepared by dilution of saturated overnight cultures into phosphate buffered saline (PBS) (total inoculum volume 50ul) and following inspiration it was dispersed with 2 x 200ul air flushes. An aliquot of inoculum was enumerated for each administration and all inoculums were ±10% of the stated figure.

Mice recovered at 32°C (Warm Air System, Safety Cabinet Version red, VetTech, UK) until spontaneous movement and received 0.8mg/kg subcutaneous (BD Microlance 27G 13mm needle) atipamezole to reverse the α-agonist into the neck scruff.

At the experimental end-point animals were anesthetized with ketamine 100mg/kg and 1mg/kg medetomidine by intraperitoneal injection (BD Microlance 27G 13mm needles). Following the induction of anaesthesia, blood was collected by a transdiaphragmatic inferior approach cardiac puncture (BD Microlance 25G 25mm needles) and animals were then humanely killed, under anaesthesia, by cervical dislocation.

An aliquot of blood (20ul) was taken and diluted into 180ul of hypotonic lysis solution (1mM EDTA in water) for enumeration of bacterial counts. The rest was placed into a microtainer (BD Microtainer for serum collection, BD Medical) and allowed to clot for 1 hour. This was then spun according to the manufacturer’s guidance and aliquoted into cryobanking tubes (Greiner Bio-One) and frozen at -80 °C until analysis.
Lungs were dissected out, weighed and then homogenised in genemacs C-tubes (Miltenyi Biotec) containing 3 ml complete RPMI media (RPMI supplemented with 10% heat inactivated FBS, 10 mM HEPES and 1 mM Sodium Pyruvate) using a gentleMac Octo dissociator (Miltenyi Biotec) using the program m_lung_1 twice.

Bacteria were enumerated from the lung homogenate and blood by dilution in PBS and plating onto solidified LB agar containing 50ug/ml rifampicin (Merck, UK).

**Antibiotic delivery**

We combined the meropenem (100mg/kg) with cilastatin (100mg/kg) (Merck, UK), a renal dihydropyridinase inhibitor, to reduce the in vivo metabolism of meropenem as the murine enzyme isoform has increased activity against this carbapenem compared to human enzyme. Drugs were diluted in dH₂O. dH₂O alone was administered to vehicle control animals. Doses were delivered at 6 hourly intervals as previously employed in a murine carbapenemase producing KP model(4). The drug mixture was delivered by intraperitoneal infection (BD Microlance 27G 13mm needles) to animals at 6 hourly intervals and the iliac fossa used was alternated between doses.

**Cytokine bead assay**

Serum IFN-γ was assessed using a custom-made mouse panel 13-plex kit (LEGENDplex, BioLegend) following the manufacturer's instructions. Cytokine levels were acquired using a FACSCalibur flow cytometer (BD Biosciences), and analyses were performed using LEGENDplex data analysis software (BioLegend). A biological repeat was considered valid only if at least 80% of all the samples run were above the limit of detection; any values below the detection limit within a repeat such as this were assumed to be the lowest value detectable by the assay for statistical analysis. Values below the detection limit in graphs were displayed as 1 for visualisation.
Determination of RNA structures using DMS-MaPseq

**In vitro DMS modification**

Plasmids encoding the wildtype and mutant *ompk36* were isolated using miniprep (Zymo) and amplified by PCR using a forward primer containing the T7 promoter sequence (P37) and a reverse primer (P38). The PCR product was used for T7 Megascript *in vitro* transcription (ThermoFisher Scientific) according to manufacturer's instructions. Next, the RNA was purified using RNA Clean and Concentrator™-5 (Zymo). 10µg RNA was denatured at 95°C for 1 min. Denatured RNA was refolded by incubating in 340mM sodium cacodylate buffer (Electron Microscopy Sciences) and 5mM MgCl$_2^+$, such that the volume was 97.5µl, for 20 min at 37°C. Then, 0.5% DMS (Millipore-Sigma) was added and incubated for 4 mins at 37°C while shaking at 800 r.p.m. on a thermomixer. Subsequently, DMS was neutralized by adding 60µl β-mercaptoethanol (BME) (Millipore-Sigma). The DMS-modified RNA was purified using RNA Clean and Concentrator™-5 (Zymo) and eluted in 10µl water.

**DMS-MaPseq library generation of whole-length *in vitro* DMS-modified RNA**

500ng of RNA purified from previous steps was used as input. DMS-MaPseq libraries were prepared using IDT’s xGen™ Broad-Range RNA Library Prep Kit with slight modifications. Briefly, RNA was fragmented using manufacturer's instructions for 2 minutes without adding reagent F2 (dNTPs). After 2 minutes, fragmentation mix was placed on ice immediately. The mixture was then added with the TGIRT reverse transcriptase mix, which consists of 1µl TGIRT, 1µl water, 1µl enzyme R1 (RNAse inhibitor) and 1µl DTT, and incubated at room temperature for 30 minutes. Then, F2 (dNTPs) was added and the fragmented RNA mixture was reverse transcribed under the conditions: 20°C for 10 mins, 42°C for 10 mins, 55°C for 60 mins, denaturation by adding 1µl 4M NaOH at 95°C for 3 mins. The mixture was neutralized by adding 2µl of 4M HCl and the volume of neutralized mixture brought up to 50µl. Then, the cDNA was cleaned up using 1x volume ratio of SPRI beads (Beckman Coulter) and eluted in 10µl EDTA TE. Samples were then adapted, extended, ligated and amplified for 10 cycles.
following IDT’s instructions. The libraries (~300-400 bp) were gel-purified on an 8% TBE polyacrylamide gel (ThermoFisher Scientific) and precipitated using isopropanol. The libraries were then loaded on iSeq-100 sequencing flow cell with iSeq-100 High-throughput sequencing kit and library was run on iSeq-100 (paired-end run, 151 x 151 cycles).

In vivo DMS modification, total RNA extraction and rRNA subtraction

For in vivo DMS modification, 500µl of exponentially growing *E. coli* were incubated with 10µl DMS for 3 mins at 37°C while shaking at 800 r.p.m. on a thermomixer. DMS was quenched by adding 500µl 30% BME, followed by 3 min 30% BME wash and 1x PBS wash. Then, bacterial pellets were resuspended and incubated at room temperature for 5 mins in 500µl RNAprotect® bacteria reagent (QIAGEN). Samples were centrifuged at 16000g for 5 mins and supernatant removed. Pellets were resuspended in 100µl of 15mg/ml lysozyme solution plus 20µl proteinase K and mixed by vortex for 10 seconds. Samples were incubated at room temperature for 10 mins, vortexing for 10 seconds every 2 mins. Next, samples were added with 350µl of buffer RLT plus BME (10µl BME for every 1ml of RLT), vortex to mix. Then, 250µl of 96-100% ethanol was added to samples. RNA was extracted using the RNeasy® Mini kit (QIAGEN) following manufacturer’s instructions. DNA was digested from 5-10µg of total RNA per sample using the TURBO DNA-free kit (ThermoFisher Scientific) and purified using RNA Clean and Concentrator™-5 kit (Zymo). Following that, ribosomal RNAs were depleted using the Ribominus™ Transcriptome Isolation kit for bacteria (ThermoFisher Scientific) following manufacturer’s instructions. RNA was purified using RNA Clean and Concentrator™-5 (Zymo) and eluted in 10µl water.

DMS-MaPseq library generation of target sequence-specific in vivo DMS-modified RNA

To reverse transcribe, rRNA-depleted total RNA purified from the previous steps was added to 4µl 5x FS buffer, 1µl dNTP, 1µl 0.1M DTT, 1µl RNase Out, 1µl 10uM reverse primer (P39) and 1µl TGIRT-III (Ingex). The reaction was incubated for 1.5h at 60°C. Then, to degrade the RNA, 1ul 4M NaOH was added and incubated for 3min at 95°C. The cDNA was purified in
10μl water using the Oligo Clean and Concentrator™ kit (Zymo). Next, 1μl of cDNA was amplified using Advantage HF 2 DNA polymerase (Takara) for 30 cycles according to the manufacturer’s instructions (P40/P41). The PCR product was purified using E-Gel™ SizeSelect™ II 2% agarose gel (Invitrogen). RNA-seq library for 300bp insert size was constructed following the manufacturer’s instructions (NEBNext Ultra™ II DNA Library Prep Kit). The library was loaded on iSeq-100 sequencing flow cell with iSeq-100 High-throughput sequencing kit and library was run on iSeq-100 (paired-end run, 151 x 151 cycles).

DMS-MaPseq analysis

To determine the DMS signal, FASTQ files were processed and analyzed using the DREEM (Detection of RNA folding Ensembles using Expectation-Maximization clustering) pipeline (5). Briefly, reads were trimmed using TrimGalore (github.com/FelixKrueger/TrimGalore) to remove Illumina adapters. Trimmed paired reads were then mapped to ompk36WT (accession number CP009208.1, annotated as ompC4), 25c>t and double mutant using Bowtie2 with the parameters: --local --no-unal --no-discordant --no-mixed -L 12 -X 1000. A bit vector was generated for each pair of aligned reads and their mutational signatures were analyzed using the DREEM algorithm(5). The ratio of mismatches and deletions to total coverage at each nucleotide position was calculated to quantify the population average DMS reactivity at each position. Then, DMS reactivities were normalized to the median of the top 5% of DMS reactivities to a scale of 0 to 1. The normalized DMS signals were used as folding constraints for predicting RNA secondary structures with the program RNAstructure v.6.0.1(6). RNA secondary structures were visualized using VARNA v.3.93 (7).
Primers used

P1 GCCAGTATAGGGATAACAGGGTAATCGCGTACCACGCCGGTGTCTG
P2 CGCCGGATTACCCCTGTATCCCTAGGAAATCACAATTATGTTACG
P3 ATTACCCCTGTATCCCTATAC
P4 TAGGGATAACAGGGTAATCCG
P5 TCTGCAGTACACCCCTTC
P6 CATTATTTATTACCTC
P7 GATTACGCCGGATTACCCCTGTATCCCTAGGCTAATTGATTGATTAATAG
P8 GGCCAGTATAGGGATAACAGGGTAATAGCCCCACAGGTTGACCCAGC
P9 GTTGCAAGCTGCATAAC
P10 CGCATTTTGCTGCCTGCTAC
P11 GTACGAGGCCGCAAAATGCGCGTAAAGGCGAAGAGCTG
P12 GATTATGCAGCTTGCAACCGCGAAGTAATCTTTTCG
P13 TTAACCCCTTTCATGTTATTTAACCCCTCTGGTTGTTATATG
P14 AGTACTGTCCCTGCTGGTTACCCG
P15 AGTACTGTCCCTGCTGGTTACCCG
P16 TTAACCCCTTTCATGTTATTTAACCCCTCTG
P17 GCATATAACAAACAGAGGGTTAATAAC
P18 CTTTAATTATGCCACTGC
P19 CTTTAATTATGCCACTGC
P20 TTTATATTATGCCACTGC
P21 CATTATATTATGCCACTGC
P22 CTTTTATTATGCCACTGC
P23 GCTTATAACAAACAGAGGGTTAATAAC
P24 GCATTTAAACAAACAGAGGGTTAATAAC
P25 GCATATTACAAACAGAGGGTTAATAAC
P26 GCATATATCAAACAGAGGGTTAATAAC
P27 GCATATAACTAACAGAGGGTTAATAAC
P28 GCATATAACATACAGAGGGTTAATAAC
P29 GCATATAACAATCAGAGGGTTAATAAC
P30 GCATATAACAAACTGAGGGTTAATAAC
P31 TACTGTCCCTTCTGGTACCGG
P32 CTTCAACTTTTCATGTATTAACCCTCTG
P33 CCAGACCTACAACGCAACT
P34 CGCTCCAGATCCTTTACCTTTTAG
P35 AAGGCGAATCCAGCTTGTTCAGC
P36 TGACGTTGCATGTGCACCCATCA
P37 TAATACGACTACTTATAGGAAAAAGGCATATAACAAACAGAGGG
P38 AAGAGTATACCGAGGTTAACACCGGA
P39 CCTGAACGTTGTATTCCC
P40 GGAAAAGGCATATAAC
P41 CCTGAACGTTGTATTCCC
Supplementary Figures

Figure S1.

Representative Coomassie gel image of outer membrane (OM) preparations demonstrating no change in OmpK36 expression conferred by the introduction of a 24c>t mutation in in both WT and WT+GD *ompK36* backgrounds.
Figure S2.

A. DMS reactivity in the Shine-Dalgarno Sequence

B. $ompK36_{WT}$

C. $ompK36_{WT(25C4)}$
A. Side-by-side normalized dimethyl sulfate (DMS) signal per nucleotide in the Shine-Dalgarno sequence from full length in vivo-transcribed and refolded \( \text{ompK36}_{\text{WT}} \), \( \text{ompK36}_{\text{WT}(25c\rightarrow t)} \) and DMS-untreated \( \text{ompK36}_{\text{WT}} \). Higher values correspond to increased base accessibility. DMS signal (± SD) of 2 biological repeats for nt -14a through -10g are shown.

B&C. DMS-constrained structure models of the 5' end of \( \text{ompK36}_{\text{WT}} \) (B) and \( \text{ompK36}_{\text{WT}(25c\rightarrow t)} \) (C). Nucleotides are coloured by normalised DMS signal. The SDS in RNA structures are highlighted in grey. Arrows indicate the -14 position and the 25c>tt positions.

Representative Coomassie gel image of outer membrane (OM) preparations demonstrating no change in OmpK36 expression conferred by the introduction of a 24c>t mutation in in both WT and WT+GD \( \text{ompK36} \) backgrounds.
Supplementary Table Legends

**Dataset S1.** Metadata and genotyping data for a curated collection of 1450 KP ST258/512 genomes. See corresponding excel file.

**Dataset S2.** Metadata and genotyping data for 16,086 KP genomes available in Pathogenwatch. See corresponding excel file.

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