Supporting information

A cell-free strategy for profiling intracellular antibiotic sensitivity and resistance

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Supporting Text

Molecular biology and bacterial strains

E. coli MG1655 and DH10β (NEB) strains and all molecular biology was performed as previously described[38]. *K. pneumoniae* strain ATCC 13882 was purchased from Deutsche Sammlung Von Mikroorganismen and Zellkulturen (DSMZ). *K. pneumoniae* clinical strains ST258-T1b and NJST258-1 were previously described[1,2]. The strains were maintained and grown in standard Luria-Bertani, or specific media described within the supporting information. Clinical strains used for minimum inhibitory concentration assays (*K. pneumoniae* M6 and NCTC 13368, *A. baumannii* ATCC 17978 and AYE (BAA-1710), *E. coli* NCTC 12923) were acquired from ATCC or NCTC and maintained on Tryptic Soy Broth. All mutant strains of *K. pneumoniae* ATCC 13882 were generated at UKHSA (**Table S3**) using laboratory adapted evolution and sequenced.

Growth measurements

A single colony of *K. pneumoniae* ATCC 13882 was inoculated into 5 mL of desired media and incubated at 30°C, 200 rpm for 16-18 h. The pre-culture was diluted 1:100 in 50 mL of fresh sterile media in a 250 mL conical flask. Optical density at 600 nm (OD_{600}) was recorded in a UV-Vis spectrophotometer (Agilent, Cary 60), using sterile media as blank. Subsequently, OD_{600} measurements were taken every h (the culture was diluted 1 in 10 when $OD_{600} \geq 1$) and a growth curve was plotted and visualised using GraphPad Prism version 9.0.

Whole cell assays with minimum inhibitory evaluation (MIC assay)

In this study, the MIC_{50} and MIC_{90} values are defined as the concentration of compound needed to cause 50 and 90% inhibition respectively. A single colony was taken from a freshly grown agar plate of each strain and inoculated into 3 mL of sterile tryptic soy broth. The pre-cultures were incubated at 37°C for 16-18 h with shaking at 200 rpm and then diluted to an OD_{600} of 0.01, equivalent to 1 x 10^6 colony-forming units (CFU) per mL. This was further sub-cultured into a sterile clear flat-bottom 96-well plate to 5 x 10^5 CFU per mL and dosed with one compound per plate. Each compound was serially diluted in 100 µL of TSB broth to which 100 µL of culture was added. Wells containing media and compound dilution were included as negative control while wells with bacterial strain only were used as positive control. The plates were incubated at 37°C. During this incubation, bacterial growth was determined by reading absorbance at 600 nm, on a CLARIOstar microplate reader (BMG, LabTech) with an attached plate-stacker enabling measurements of optimal density at 600 nm (OD_{600}) every 30 min for a total of 20 h. Background signal was eliminated by subtracting absorbance in blank wells. Data was normalised to the OD_{600} of positive control wells or wells with lowest concentration of compound (whichever was higher) and expressed as percentage growth. MIC cut-off values were determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Data is fitted to either a symmetric (4-parameter) or asymmetric (5-parameter) logistic regression model and visualised using GraphPad Prism version 9.0.

K. pneumoniae cell extract preparation

K. pneumoniae ATCC 13882 was grown overnight on LB agar at 30°C. A single colony was then inoculated into 5 mL of LB and grown at 30°C, 200 rpm for 6-8 h (unless specified otherwise). 50 mL of fresh sterile media was then inoculated with 250 µL of pre-culture and incubated at 30°C, 200 rpm for 14-16 h. The following day, the overnight culture was diluted 1:100 in 500 µL growth media in Ultra-Yield™ (Thomson, USA) baffled flasks and incubated at 30°C, 220 rpm until the OD_{600} reached 2.5-3.0. Cells were transferred into pre-chilled 250 mL centrifuge bottles and centrifuged (10,000 x *g*, 4°C, 12 min). The supernatant was discarded. Then, cells were washed with 20 mL S30A buffer, recombined into a 30 mL centrifuge bottle and centrifuged (15,000 x *g*, 4°C, 12 min). The supernatant was carefully discarded, and wash step was repeated. The cell pellet was resuspended, aliquoted into 2 mL polypropylene (PP) tubes and centrifuged (18,000 x *g*, 4°C, 12 min). The residual supernatant was discarded using a pipette tip. Using a pipette with the tip cut to increase bore size, the cell pellets were combined into a 50 mL PP tube for cell lysis by sonication. Cells were sonicated in an ice-water bath, in either a 50 mL PP tube or aliquoted into 1.5 mL PP tubes. Settings used were: 20 kHz frequency, 50% amplitude, 10 sec pulse on time, 10 sec pulses off time, 500 J energy input/mL of wet-cell pellet. The lysate was centrifuged (18,000 x *g*, 4°C, 10 min), the supernatants were pooled and then aliquoted into 1.5 mL PP tubes. The lysates were incubated at 37°C for 60 min (unless otherwise stated) and then clarified by centrifugation (18,000 x *g*, 4°C, 10 min). When required, clarified lysate was pooled and dialysed for 3 hr at 4°C in S30B buffer. The extract was subsequently clarified by centrifugation (18,000 x *g*, 4°C, 10 min), aliquoted into 200-500 µL fractions and stored at -80°C. Total protein concentration of the crude extract was estimated using Bradford assay with a BSA standard, as previously described[39]. Using this method, the typical protein concentration of crude extracts from *K. pneumoniae* was 20-24 mg/mL.

WGS of *K. pneumoniae* strains

Genomic DNA was purified using a Wizard genomic DNA purification kit (Promega). DNA was tagged and multiplexed with the Nextera XT DNA kit (Illumina). Whole-genome sequencing of *K. pneumoniae* isolates was performed by UKHSA-GSDU (UK health Security Agency Genomic Services and Development Unit) on an Illumina (HiSeq 2500) with paired-end read lengths of 150 bp. A minimum 150 Mb of Q30 quality data were obtained for each isolate. FastQ files were quality trimmed using Trimmomatic. SPAdes 3.15.3 was used to produce draft chromosomal assemblies, and contigs of less than 1 kb were filtered out. FastQ reads from antibiotic-exposed isolates were subsequently mapped to the genome sequence of pre-exposed strain ATCC 13882 using BWA-MEM (version 0.7.17). Bam format files were generated using Samtools (version 1.6), VCF files were constructed using SNIPPY (Version 4.6.0) GATK2 Unified Genotyper (version 0.0.7) (50). They were further filtered using the following filtering criteria to identify high-confidence SNPs: mapping quality, _60; genotype quality, 40; variant ratio, _0.9; read depth, _10. All the above-described sequencing analyses were performed using Galaxy (www.useqlaxy.org). BAM files were visualized in Integrative Genomics Viewer (IGV) version 2.12.2 (Broad Institute).

G. mellonella killing assays

Wax moth larvae (*G. mellonella*) were purchased from Livefood UK and were maintained on wood chips in the dark at room temperature. The larvae were stored for

no longer than 2 weeks. Bacterial infection of *G. mellonella* was carried out essentially as described[3]. K. pneumoniae were diluted in PBS to an OD_{600} of 0.002 or 0.01, representing low or high doses (in reference to **Figure 7**). Determination of intracellular bacterial numbers was performed as previously described[4], except that appropriate dilutions were plated out onto LB agar plates supplemented with the selection antibiotic, which were incubated overnight at 37 °C to allow the bacteria to grow. Data analysis and visualisation was performed using GraphPad Prism version 9.0.

Supporting Results

Growth Optimisation of the *K. pneumoniae* ATCC 13822 CFE system

To establish a *K. pneumoniae* ATCC 13882 cell-free system, we followed the previous *E. coli* cell-free protocol with some modifications[39]. Cells were grown at 37°C, and harvested at an OD_{600} of 2.5, followed by cell-lysis by sonication, and then finally, extract processing and clarification. Data for ATCC 13882 growth and CFE reaction optimisation is shown in **Figure S1 to S10**. Approximate protein yields for cell extracts were between 20-22 mg/mL, as determined by Bradford assay. Cell-free reactions were prepared with 10 nM plasmid DNA, or without, and incubated for 6-12 hr at 30°C to monitor for either eGFP or mScarlet-I fluorescence.

Optimisation of the CFE reaction for Drug Screening

First, we scaled-up extract preparation and optimised the magnesium and potassium glutamate concentration. We discarded extract batches with less than 50% of the maximum recorded activity (11.0 ± 0.73 μM) to standardise the robustness of the assay. Measured from two biological repeats, this data provides a strong S:B ratio of 134:1 between the error margins (**Figure 1b**). To validate the specificity and sensitivity, we next tested a selection of control antibiotics. This included kanamycin and erythromycin, both ribosomal inhibitors. Ampicillin was also included as a non-specific inhibitor, since its target is cell wall biosynthesis, which should not affect CFE activity. For the clinical antibiotics with intracellular targets, estimated IC_{50} values were within the expected range – i.e., low micromolar (**Figure S15**). The CFE assay was insensitive to ampicillin up to 100 μM, the highest concentration tested within the model. To validate the experimental approach, a robust Z' score was also determined between 0.72 to 0.88 for eight 384-well microtiter plates (**Figure 2a**) – a minimum robust Z' score of 0.5 is recommended for a new assay[5].

A typical industrial high-throughput screening (HTS) campaign requires enhanced assay stability at room temperature for several hours. The *K. pneumoniae* CFE reaction requires all three components (cell extract, energy solution and DNA) to initiate protein synthesis. We incubated cell extracts with plasmid DNA or energy solution for several hours before addition of the remaining component. Interestingly, the cell extract and DNA mixture remained stable for up to 4 hours at room temperature without significant loss of mScarlet-I yield (**Figure S16)**. In contrast, the cell extract and energy solution showed a time-dependent loss of activity, with a >50% decrease after 2 hours. This suggests the energy solution is the major source of instability due to metabolic activity within the cell extracts, whereas the DNA is stable. Finally, we tested the *K. pneumoniae* CFE system with increasing amounts of dimethylsulphoxide (DMSO), which is required for drug solubility in bioassays. The CFE reaction tolerated up to 2.5% DMSO (v/v) without a significant change in activity (**Figure S17**).

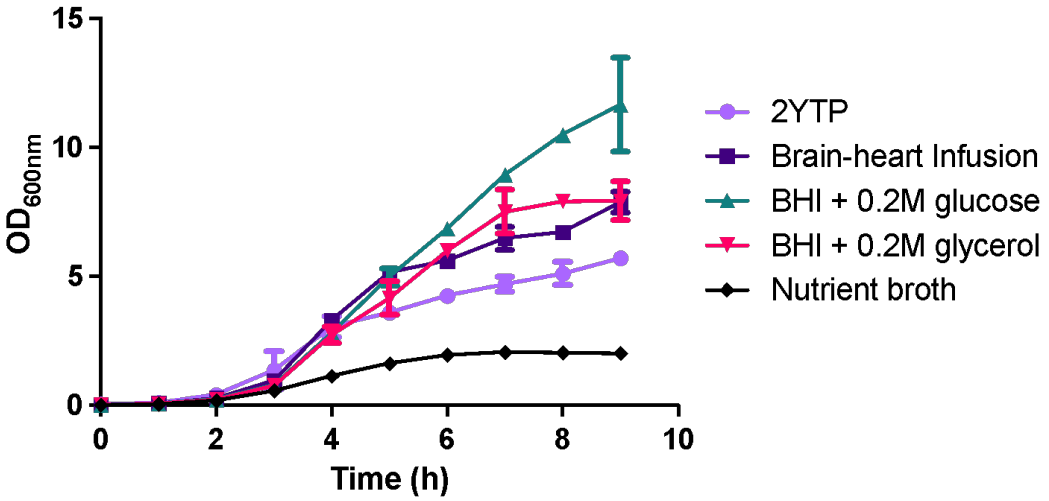

Figure S1. Growth optimisation I. Growth curve of *K. pneumoniae* ATCC 13882 in various rich media. Cells were cultivated in 50 mL liquid cultures grown in 250 mL baffled flasks at 30°C, 200 rpm. Data is shown as mean ± standard error of mean (*n* = 2 biological repeats).

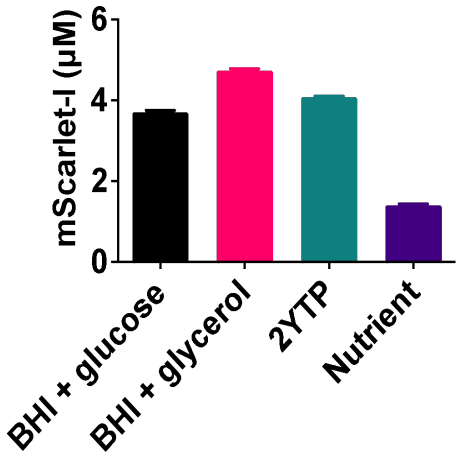

Figure S2. Growth optimisation II. *K. pneumoniae* ATCC 13882 CFE activity from extracts generated from different growth media at an OD_{600} of 2.0. Cell-free reaction conditions: 8 mg/mL cell extract, 10 nM pTU1-A-SP44-mScarlet, standard energy solution, incubated at 30°C for 15 hours. Data is shown as mean ± standard deviation of three technical measurements.

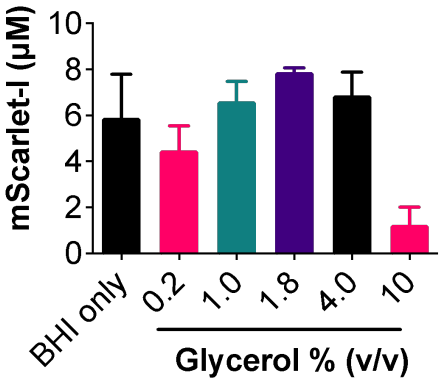

Figure S3. Growth optimisation III. *K. pneumoniae* ATCC 13882 CFE activity from extracts grown in BHI supplemented with and without glycerol. Cells were cultivated in 50 mL BHI media with varying concentrations of glycerol at 30°C, 200 rpm, and harvested at an OD_{600} of 2.0. Cell-free reaction conditions: 8 mg/mL cell extract, 10 nM pTU1-A-SP44-mScarlet, standard energy solution and 30°C for 15 hours. Data was shown as mean ± standard error of mean (*n* = 2 biological repeats).

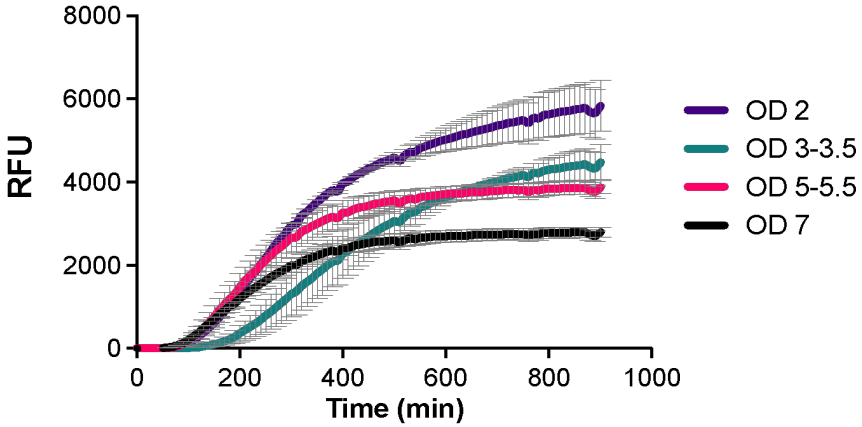

Figure S4. Time course showing synthesis of the mScarlet-I protein in *K. pneumoniae* ATCC 13882 CFE from extracts harvested at different growth stages. Cells were grown in 50 mL BHI media with 1.8% glycerol (v/v/) at 30°C, 200 rpm and harvested at the mentioned OD600. Cell-free reaction conditions: 8 mg/mL cell extract, 10 nM pTU1-A-SP44-mScarlet, standard energy solution and 30°C for 15 hours, with fluorescence measurement every 10 min. Data is shown as mean ± standard error of mean (*n* = 2 biological repeats).

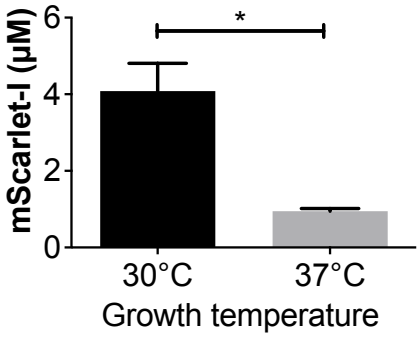

Figure S5. Effect of growth temperature on *K. pneumoniae* ATCC 13882 CFE activity. Cells were cultivated from 50 mL cultures in BHI supplemented with 1.8% (v/v) glycerol, at 30 °C or 37 °C until an OD_{600} of 2.0-2.5 was reached. Data is shown as mean ± standard error (*n* = 2 biological repeats). *p<0.05 following a one-tailed unpaired *t*-test.

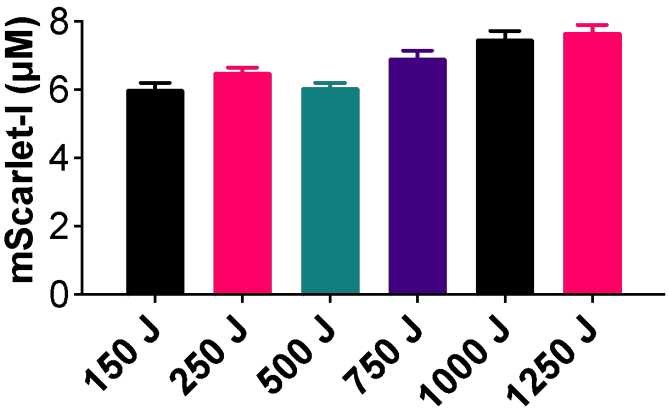

Figure S6. Optimisation of sonication energy input for *K. pneumoniae* ATCC 13882 CFE activity. Cell-free reaction conditions: 8 mg/mL cell extract, 10 nM pTU1-A-SP44-mScarlet, standard energy solution and incubated at 30°C for 15 hours. Data is shown as mean ± standard error of mean (*n* = 2 biological repeats).

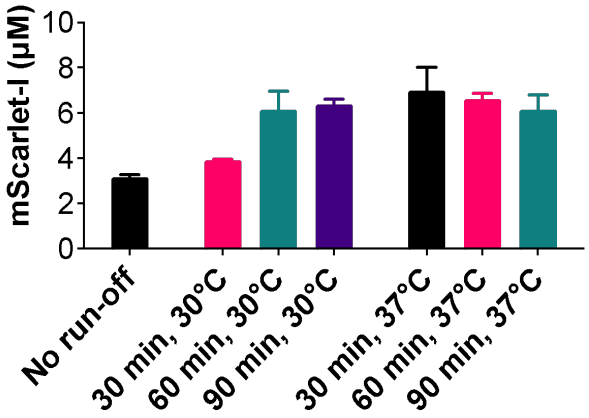

Figure S7. Optimisation of incubation ("run-off reaction") post cell-lysis for *K. pneumoniae* ATCC 13882 CFE activity. Cell-free reaction conditions: 8 mg/mL cell extract, 10 nM pTU1-A-SP44-mScarlet, standard energy solution and 30°C for 15 hours. Data is shown as mean ± standard error of mean (*n* = 2 biological repeats).

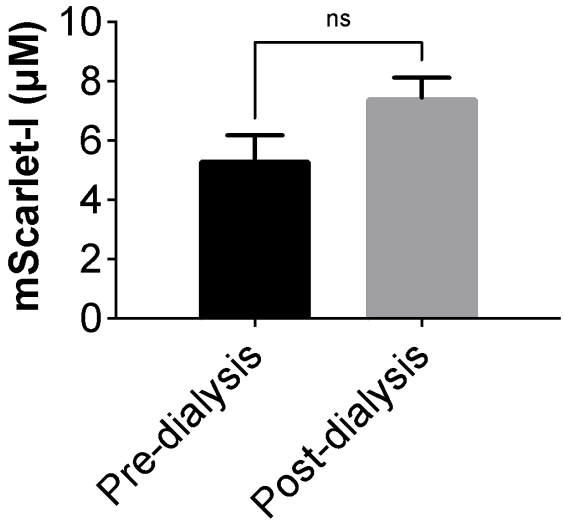

Figure S8. Effect of dialysis on *K. pneumoniae* ATCC 13882 CFE activity. Cell-free reaction conditions: 8 mg/mL cell extract, 10 nM pTU1-A-SP44-mScarlet, standard

energy solution and 30 °C for 15 hours. Extracts were dialysed against 1 L of S30B buffer for 3 h at 4 °C. Data is shown as mean ± standard error of mean for three biological repeats. P value > 0.05 following a two-tailed paired *t*-test (*n* = 3 biological repeats).

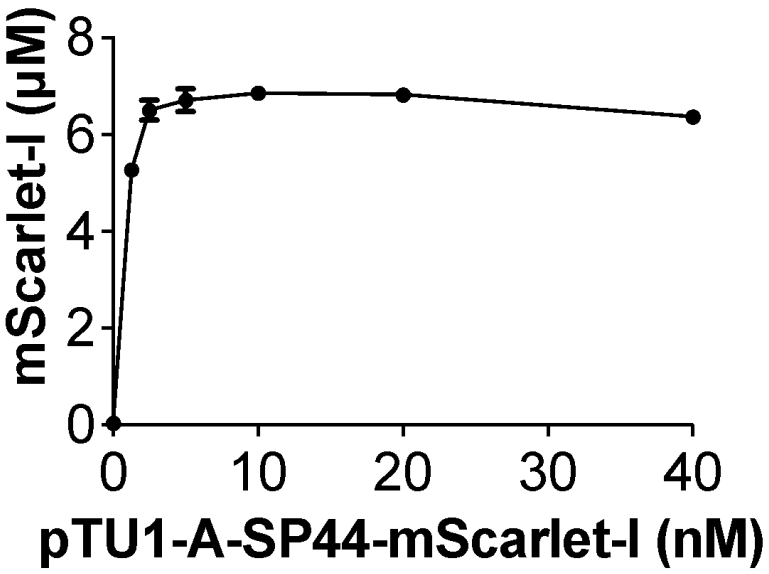

Figure S9. Optimisation of DNA concentration for optimal *K. pneumoniae* ATCC 13882 CFE activity. Cell-free reaction conditions: 8 mg/mL cell extract, 0-40 nM pTU1-A-SP44-mScarlet, standard energy solution and 30°C for 15 hours. Data is shown as mean ± standard deviation of one representative dataset with three technical repeats (*n* = 2 biological repeats).

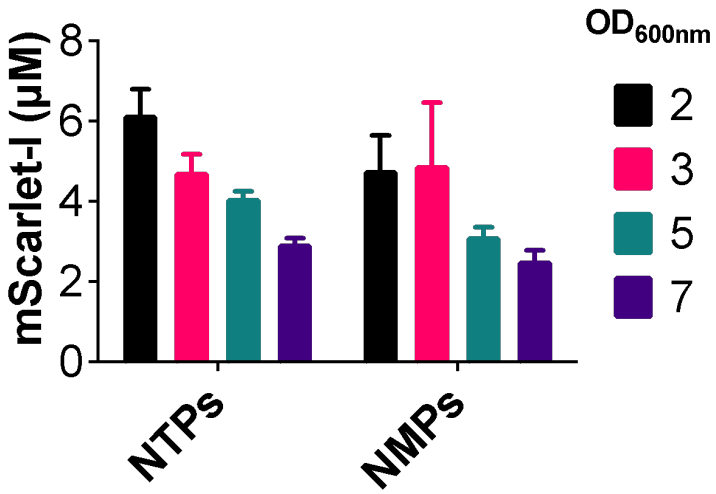

Figure S10. Comparing the effect of nucleotide triphosphates (NTPs) or monophosphates (NMPs) as primary energy source in *K. pneumoniae* ATCC 13882 CFE activity of extracts harvested at different growth stages. Cell-free reaction conditions: 8 mg/mL cell extract, 10 nM pTU1-A-SP44-mScarlet, standard energy solution with either NTPs or NMPs and incubated at 30°C for 15 hours. Data is shown as mean ± standard error of mean (*n* = 2 biological repeats).

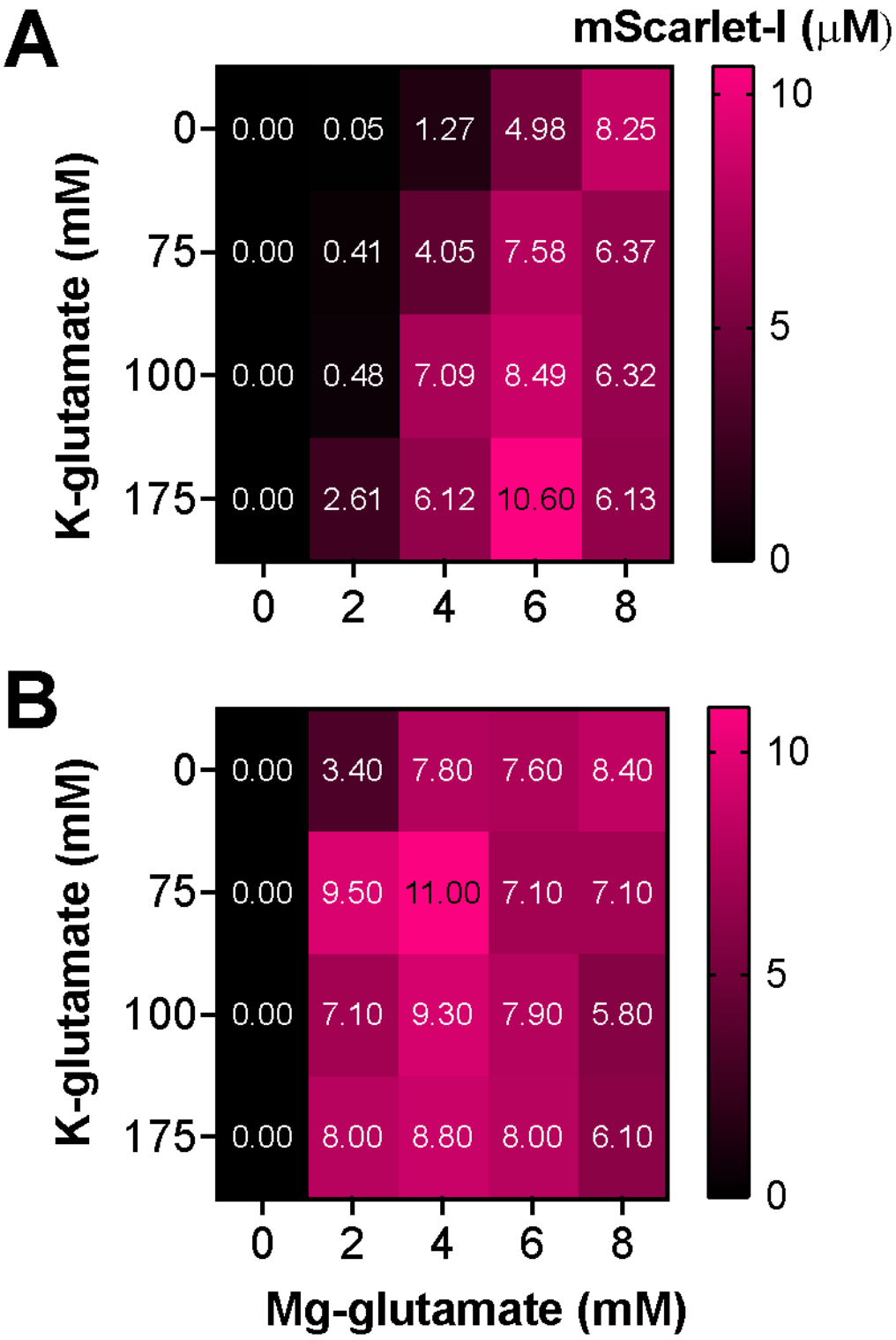

Figure S11. Optimisation of Mg-glutamate and K-glutamate concentrations for the *K. pneumoniae* ATCC 13882 cell extract used for library screening experiments. Top (A) and bottom (B) panels show two independent batches of cell extract. Each time a new batch was prepared, this optimisation step was performed to ensure maximal activity and consistency between the experiments. Cell-free reaction conditions: 8 mg/mL cell extract, 10 nM pTU1-A-SP44-mScarlet, standard energy solution and 30°C for 15 hours. Data is shown as mean of three technical repeats.

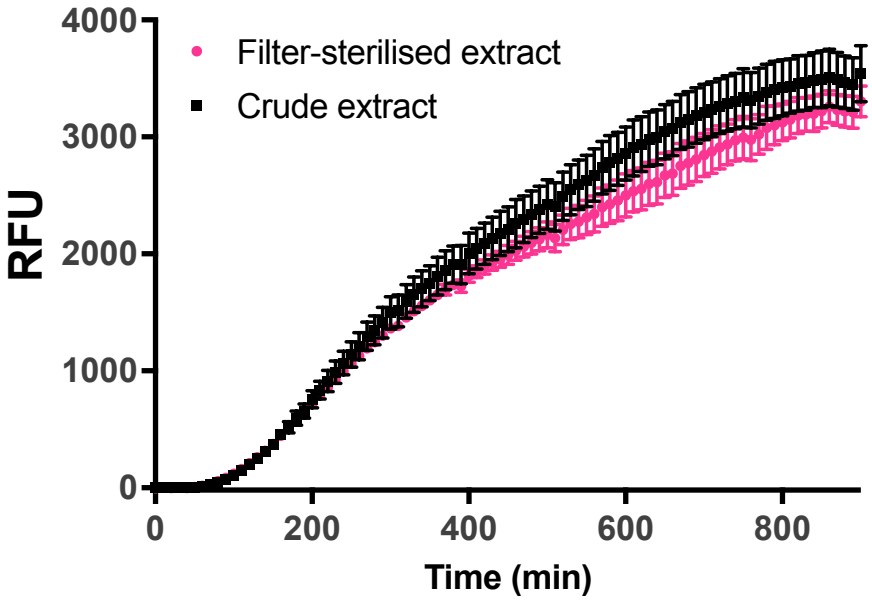

Figure S12. Relative activity (mScarlet-I fluorescence) of extracts before and after filter-sterilisation. Reactions were prepared and measured using the standard methods outlined.

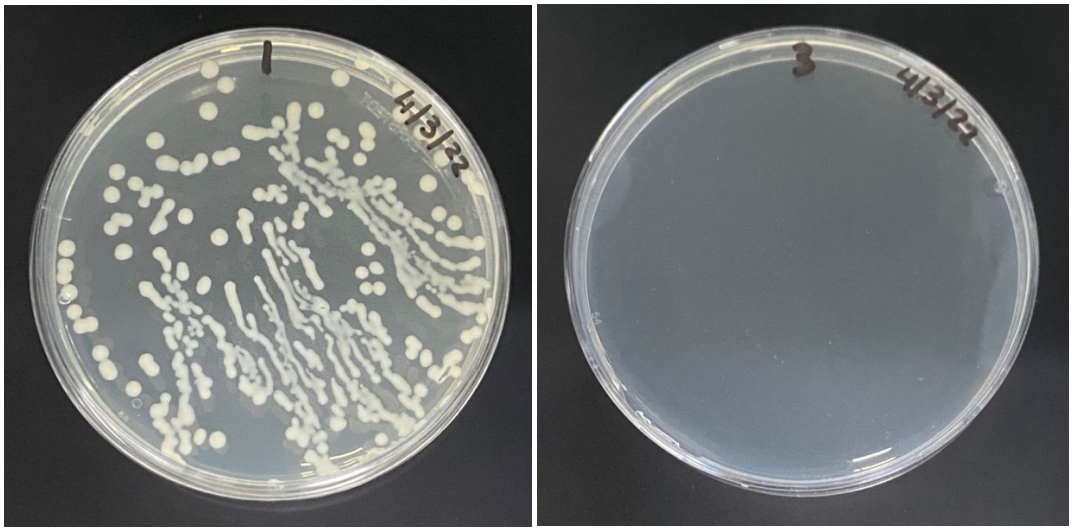

Figure S13. Recovery of viable colony forming units from *K. pneumoniae* ATCC 13882 cell-extracts. 100 μL of 20 mg/mL cell-extract was spread onto a nutrient agar plate before (left panel) and after filtering (right panel) and incubated at 37ºC for 16 hours.

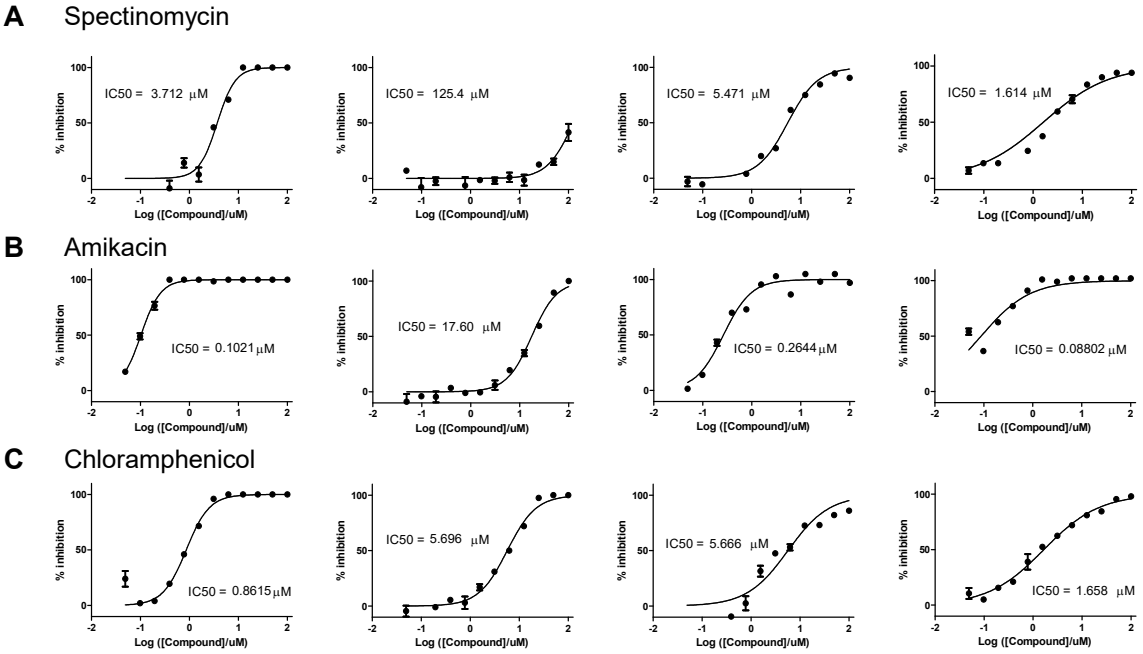

Figure S14. Antibiotic-resistance profiling for *K. pneumoniae* NJST258-1. Predicted (based on RESfinder) resistance was tested for spectinomycin (**A**), amikacin (**B**) and chloramphenicol (**C**). Panels (left to right) represent four biological repeats of extracts grown under standard conditions in BHI medium. Note the extracts exhibit variable resistance, likely due to absence of positive selection during cell extract processing. Data is shown as mean and standard deviation of three technical replicates.

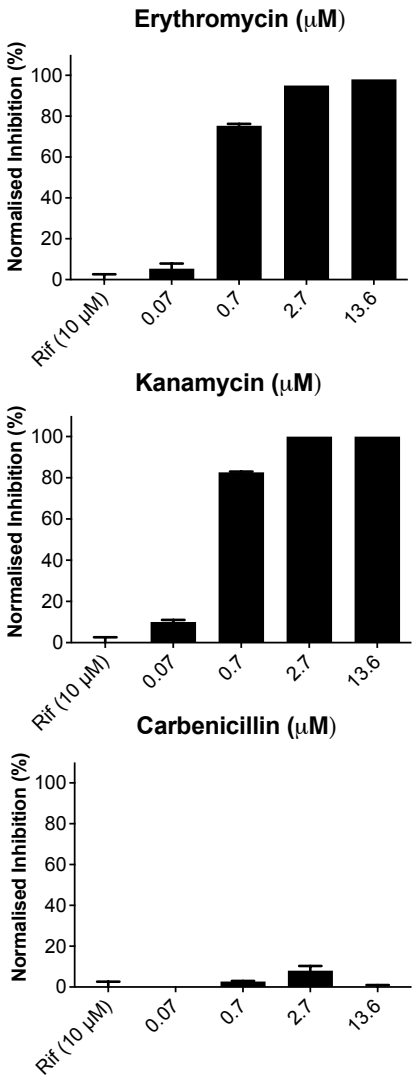

Figure S15. Preliminary antimicrobial inhibition of *K. pneumoniae* ATCC 13882 CFE. Data presented as normalised activity (%) for three technical repeats.

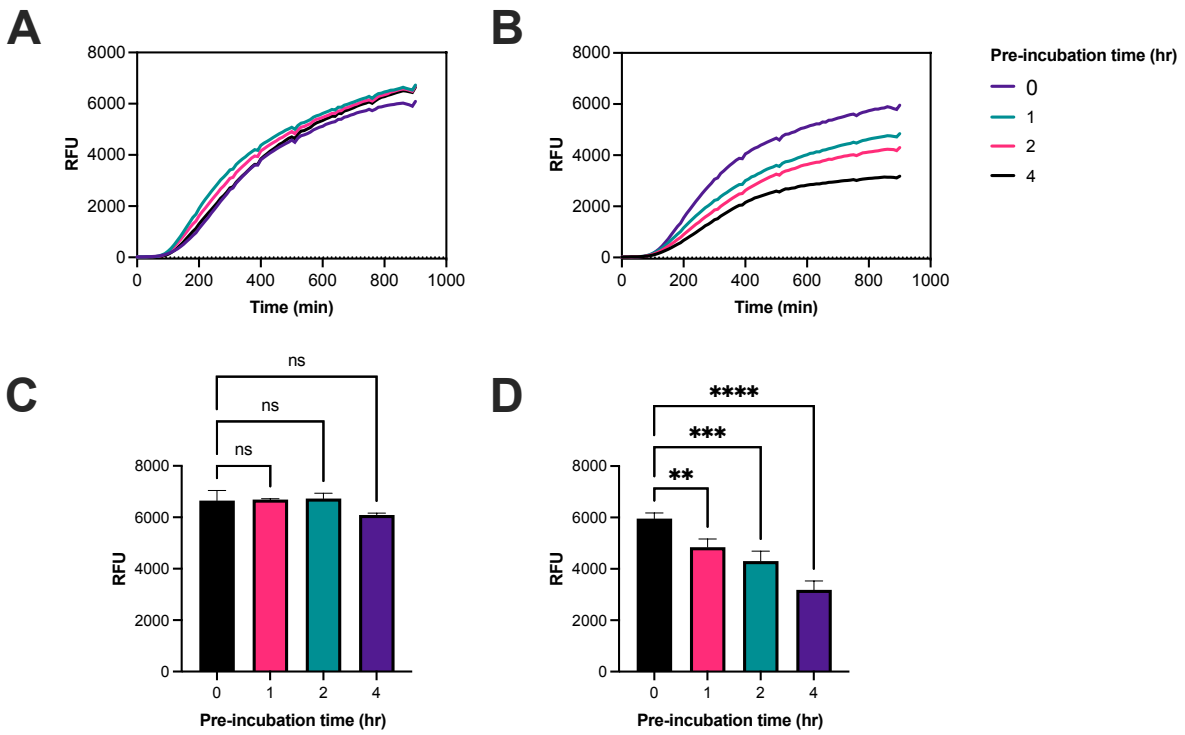

Figure S16. Effect of incubation time during reaction setup on assay stability. Real-time measurement of mScarlet-I fluorescence with (**A**) extracts pre-incubated at room temperature with plasmid DNA (**B**) extracts pre-incubated at room temperature with with energy mix. (**C**) End-point fluorescence data corresponding to panel A. (**D**) End-point fluorescence data corresponding to panel B. Details of experiment: After pre-incubation, the missing component of the reaction (energy mix or DNA), also incubated at room temperature, was added to complete reaction composition. The remaining protocol was followed as outlined in the methods. Standard deviation is representative of three technical measurements.

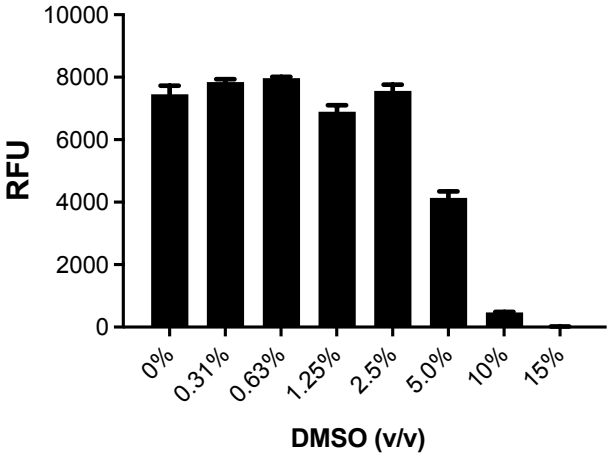

Figure S17. Effect of DMSO (v/v) concentration on *K. pneumoniae* ATCC 13882 CFE activity. Data is shown as mean and standard deviation of three technical measurements and is representative of two independent experiments.

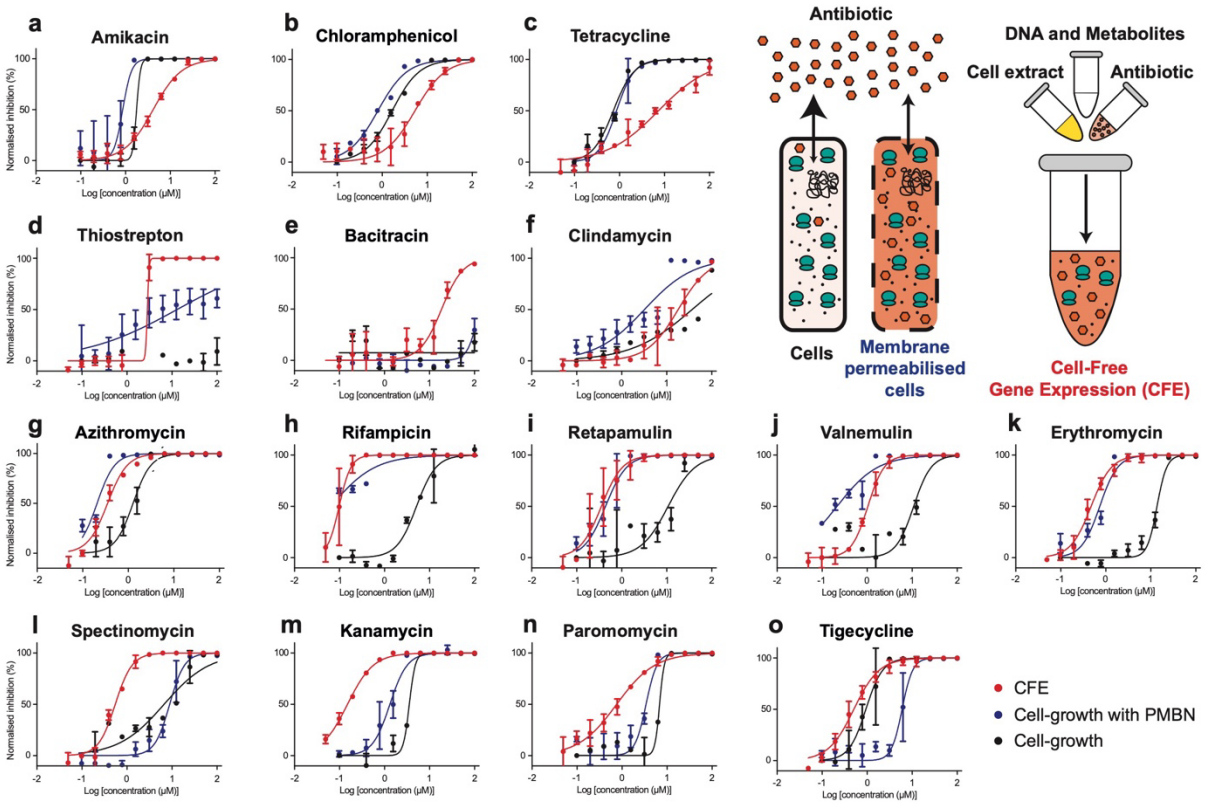

Figure S18. Antibiotic inhibition of *K. pneumoniae* cell-free and whole cells. Antibiotics displaying a similar activity profile between the assays were grouped as follows: (**a-c**) Compounds that were more sensitive in whole cells; (**d-f**) Compounds with weak activity in cells; (**g-k**) Compounds with similar activity in cell-free and cells treated with PMBN; (**i-o**) Compounds with at least an order of magnitude higher sensitivity in cell-free. The data is normalised to provide a relative comparison of Log(concentration) versus normalised inhibition (%) between whole cell and cell-free assays. Data is presented as an average and error bars represent standard deviation. CFE data is the mean and standard deviation of two independent experiments, each with three technical repeats. Each whole cell assay data is mean and standard deviation from two independent experiments).

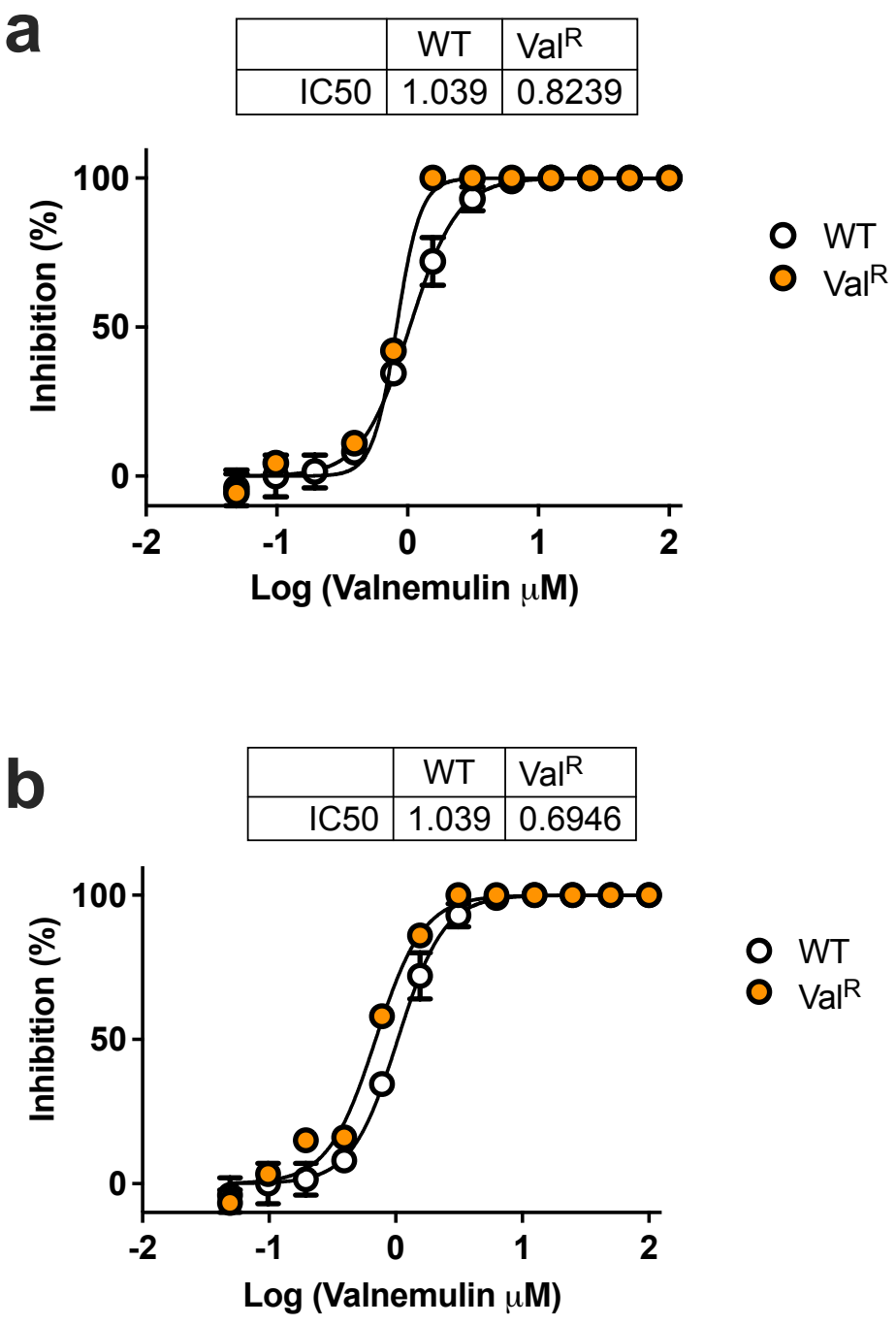

Figure S19. Dose-response curve of valnemulin inhibition of the ATCC 13882 (WT) and Val^R CFE systems. Panels (a) and (b) represent two biological repeats, plotted separately, in comparison to a representative dataset for WT. Data for WT is shown as mean and standard error of mean of two independent experiments. See main methods for reaction setup and fluorescence settings.

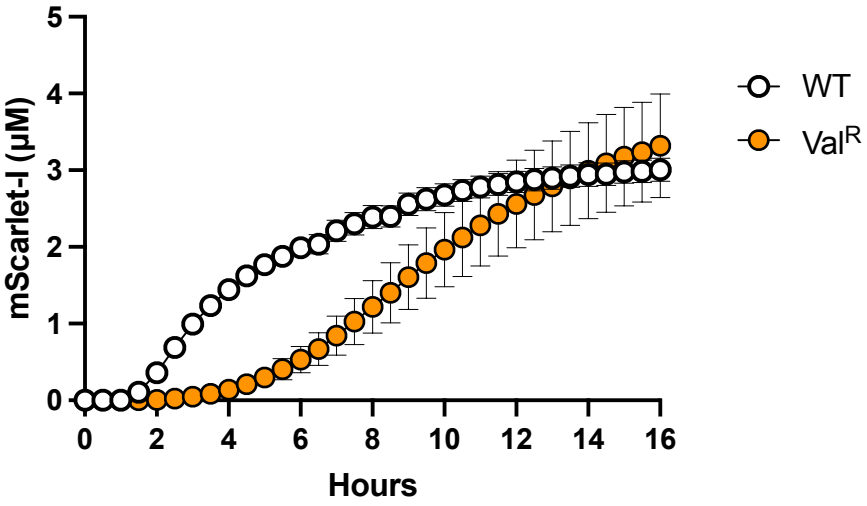

Figure S20. Real-time protein synthesis of the ATCC 13882 (WT) and Val^R CFE systems. See main methods for reaction setup and fluorescence settings.

Table S1 – Plasmids

Plasmid name	Promoter	RBS	Gene	Terminator	AddGene No.
Pr-deGFP-MGapt	OR2-OR1-Pr	TTTGTTTAACTTTAAGAAGGAGA	deGFP	T7	#67734
pTU1-A-SP44-mScarlet-I	SP44	GTACTTTAACTTTAAGAAGGAGA	mScarlet-I	Bba_B0015	#163756

Table S2

	K. pneumoniae DSM30102		*K. pneumoniae* M6		*K. pneumoniae* 13368		*E. coli* MG1655		*E. coli* 12923		*A. baumannii* 17978		*A. baumannii* AYE	
PMBN added	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**	**No**	**Yes**
Tetracycline	2.0	2.2	3.4	7.8	54.6	38.0	5.4	3.8	4.5	3.6	2.7	8.4	>200.0	>200.0
Tigecycline	2.4	8.7	2.6	11.4	15.2	23.1	0.9	6.9	1.4	11.2	1.6	9.4	2.4	12.0
Amikacin sulphate	2.8	1.5	5.9	1.7	5.8	1.7	11.2	1.6	23.2	4.1	2.9	1.6	60.7	77.1
Azithromycin	3.0	0.4	6.0	0.5	27.6	2.0	3.1	0.1	5.8	0.2	0.1	0.1	15.2	4.0
Kanamycin	4.3	3.3	12.0	5.6	154.7	87.3	11.8	6.6	29.0	13.1	3.8	2.7	>200.0	>200.0
Choramphenicol	7.9	3.9	18.0	6.1	103.7	11.8	28.9	6.8	21.2	7.1	>200.0	>200.0	>200.0	>200.0
Paramomycin	11.4	5.8	11.5	11.0	>200.0	>200.0	24.6	11.3	74.6	17.9	10.5	4.6	>200.0	>200.0
Rifampicin	16.2	0.7	30.4	0.7	16.3	1.5	10.1	0.1	9.1	0.3	2.8	0.1	7.2	0.4
Valnemulin	21.2	2.0	80.9	8.3	138.0	16.5	28.9	0.7	108.0	0.9	13.5	0.1	24.8	0.8
Erythromycin	25.0	1.5	90.4	6.1	48.9	25.6	45.1	1.9	62.2	2.6	3.9	0.4	12.7	1.0
Retapamulin	31.0	1.2	113.1	6.2	>200.0	15.4	28.0	0.7	45.5	0.4	107.2	7.9	148.3	13.8
Spectinomycin	44.5	21.0	23.2	21.9	>200.0	47.4	41.4	59.7	155.6	101.0	48.9	41.8	>200.0	>200.0
Bacitracin zinc	>200.0	83.0	>200.0	>200.0	>200.0	32.0	>200.0	35.2	>200.0	39.1	40.6	40.6	38.1	52.6
Clindamycin	>200.0	12.4	76.8	18.1	112.2	56.7	>200.0	10.3	>200.0	7.0	55.9	49.1	55.6	8.0
Thiostrepton	>200.0	5.3	>200.0	6.9	>200.0	11.9	>200.0	34.3	>200.0	20.3	>200.0	0.4	>200.0	4.9

Antibiotic inhibition data was calculated using a Gompertz equation for MIC determination[6]. MIC (μM) values in excess of the concentration range tested, are resistant to the antibiotic (or >200 μM) tested.

Table S3. Whole genome sequence summary of ATCC 13882 antibiotic resistant strains.

Adaptation	Gene	Mutation	Locus	Function	MIC (μM)	MIC after passage (μM)
Untreated control	*wcaJ*	Y69C	WM93_23710	undecaprenyl-phosphate glucose phosphotransferase	-	-
Rifampicin	*rpoB*	H526L	WM93_07445	DNA-directed RNA polymerase subunit beta	12.5	>200
Kanamycin	*cyoA*	W138STOP	WM93_12140	cytochrome ubiquinol oxidase subunit II	6.25	50
	fusA	A592V	WM93_04045	elongation factor G		
Tetracycline	*spoT*	10 bp del starting n2030	WM93_05500	(p)ppGpp synthetase	3.13	12.5
	oqxR	24 bp insertion after n201 (duplication of n177-201)	WM93_00285	transcriptional regulator		
Chloramphenicol	-	I264L	WM93_12915	acetoin utilization protein	12.5	>200
	oqxR	24 bp insertion after n201 (duplication of n177-201)	WM93_00285	transcriptional regulator		
Valnemulin	*rplC*	N149Y	WM93_04015	50S ribosomal protein L3	25	>200
Paromomycin	*sbmA*	Del n109	WM93_11755	microcin B17 transporter	12.5	>200
	fusA	D585G	WM93_04045	elongation factor G		
	ubiB	9 bp del starting n39	WM93_07275	ubiquinone biosynthesis regulatory protein kinase		

K. pneumoniae ATCC 13882 adaptation. All Locus tags based off *K. pneumoniae* strain ATCC 35657 (CP015134.1). An untreated control was also included in experiment and sequence analysis to verify for random genomic mutations.

Supporting information reference

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