Supplemental Data File

Materials and Methods

Bacterial strains and culture conditions

Isolate genus identity was initially determined using conventional biochemical methods and ID-GN cards (Vitek 2, bioMérieux, France) and confirmed by a PCR-based method (1). Fresh isolates were sub-cultured twice on 5% blood agar plates (Thermo Scientific, Malaysia) for 24 h at 35°C prior to each experiment.

Susceptibility studies

Minimum inhibitory concentrations (MICs) of ampicillin/sulbactam, ciprofloxacin, gentamicin, imipenem, meropenem, aztreonam, piperacillin/tazobactam, polymyxin B, tigecycline, ceftazidime, amikacin and cefepime were obtained by microbroth dilution using commercial but custom-made microbroth dilution panels (Trek Diagnostics, East Grinstead, UK) with a higher antibiotic concentration range, performed according to the manufacturer's recommendations.

In vitro time-growth studies and modelling of bacteria growth kinetics

An overnight culture of the original/post antibiotic-exposure isolate was diluted into pre-warmed cation-adjusted Mueller Hinton broth (Ca-MHB) and incubated further at 35°C until reaching log-phase growth. The bacterial suspension was diluted with Ca-MHB according to absorbance (at 630 nm), and 24 ml of the suspension was then transferred to 50-ml sterile conical flasks. The final concentration of the bacterial suspension in each flask was approximately 5 log_{10} CFU/ml (ranging from 1×10^5 CFU/ml to 5×10^5 CFU/ml). The exponential

growth of the bacterial population over 24 h was analysed using an adapted mathematical model (2).

Whole-genome DNA sequencing and data analysis

The paired polymyxin-susceptible and -resistant isolates were grown overnight, and genomic DNA was extracted from the cultures to construct sequencing libraries using the Illumina TruSeq sample prep kit according to the standard protocol for whole-genome shotgun sequencing on the Illumina HiSeq2000 platform in order to produce paired-end fragment reads of approximately 2x100 base pairs.

The Illumina-sequenced reads for each of the twenty isolates were *de-novo* assembled using the VelvetOptimiser software (https://github.com/tseemann/VelvetOptimiser) to obtain a draft genome assembly consisting of contigs which were ordered and oriented relative to a finished reference AB genome (NC_017162.1) with the software Mauve (3). *In silico* multilocus sequence typing (MLST) was performed for each isolate using their respective assembled contigs with the online software tool MLST 1.7 (https://cge.cbs.dtu.dk/services/MLST/), selecting the Institut Pasteur MLST scheme (4).

The draft genome assemblies obtained from the initial polymyxin-susceptible isolates were then used as the reference for each XDR-AB lineage, with the genome annotation performed on each genome assembly using the software PROKKA version 1.8 (5) to identify genes and their locations. For each XDR-AB lineage, the sequenced reads for both polymyxin-susceptible and -resistant isolates were mapped using BWA-MEM version 0.7.4 (6) to the reference genome assembly, keeping only reads that were identified to be a

proper pair by the software PICARD version 1.99. Genetic variants of single-nucleotide polymorphisms (SNPs) and small insertions and deletions (INDELs) were subsequently identified from the processed reads with the software SAMtools version 0.1.19 (7) using the combination of mpileup and bcftools assuming a diploid organism model. The genetic variants were extracted and annotated with the software tool SnpEff version 3.5a (8).

To identify insertion sequences (IS) that were different between each isolate pairs, we first screened all the draft genome assemblies to identify all insertion sequences present using the ISFinder database (9). We then determined the presence or absence of these IS sequences in the isolates with ISMapper (10) on the sequencing reads, where the IS 'hits' were compared against a reference genome. GC2 isolate pairs 2 and 3 were compared against the reference strain 1646-2 (CP001921), while the other GC1 isolate pairs were compared against the reference strain A1 (CP010781). We manually confirmed the insertion of ISAba1 carrying a AbaR4-like genomic island disrupting the *lpxC* gene by inspection of the SPAdes de-novo assembly graph (11) using Bandage (12) for a low confidence IS insertion identified using ISMapper in isolate 8B.

Electron microscopy studies

To visualise and relate the polymyxin B resistance mechanisms that developed in XDR-AB post-polymyxin B exposure, scanning electron microscopy (SEM) and transmission electron microscopy (TEM) techniques were employed. For SEM viewing, the isolates were post-fixed in 1% osmium tetroxide in phosphate-buffered saline (PBS) (pH 7.4) for 1h at room temperature. Subsequently, the specimen was dehydrated at the time of removal through a series of increasing concentrations of ethanol. Critical-point drying was done

with a critical-point dryer for 1.5 to 2.0 hours. The specimens were mounted on aluminium stubs with adhesive carbon tape and sputter-coated with a layer of gold. The coated specimens were examined under a scanning electron microscope. Digital images were taken at various magnifications.

For TEM viewing, the isolates were post-fixed in a similar procedure as for SEM viewing. Next, the specimen was dehydrated at the time of removal through a series of increasing concentrations of ethanol before being embedded in acetone resin. The resin was incubated for one day at 4°C before being incubated in an oven at 40°C for an hour. The specimen was replenished with fresh resin and incubated in an oven at 45°C for an hour and the process was repeated with each increment of 5°C till 55°C. Finally, the specimen was embedded in fresh resin at 60°C for 24 hours. Digital images were taken at various magnifications.

References for Methods

1. **Chen TL, Siu LK, Wu RC, Shaio MF, Huang LY, Fung CP, Lee CM, Cho WL.** 2007. Comparison of one-tube multiplex PCR, automated ribotyping and intergenic spacer (ITS) sequencing for rapid identification of Acinetobacter baumannii. Clin Microbiol Infect **13:**801-806.
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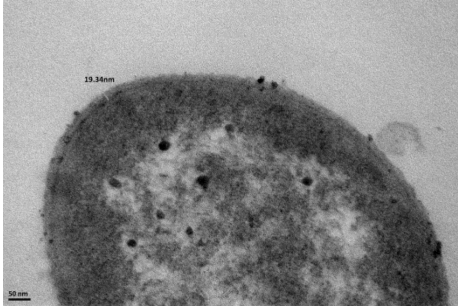
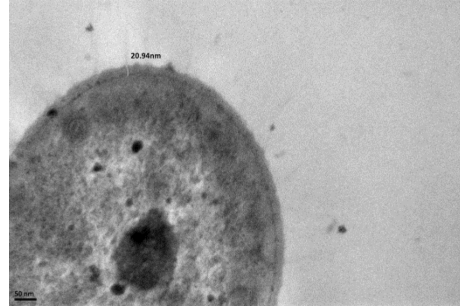
12. **Wick RR, Schultz MB, Zobel J, Holt KE.** 2015. Bandage: interactive visualization of de novo genome assemblies. Bioinformatics doi:10.1093/bioinformatics/btv383.

Figure 1. TEM & SEM images of *A. baumannii* isolate 3 (parent: A-B and polymyxin B-resistant: C-D)

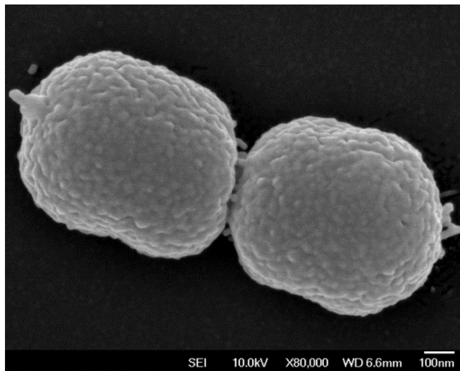
A (200,000x magnification)



C (200,000x magnification)



B (80,000x magnification)



D (80,000x magnification)

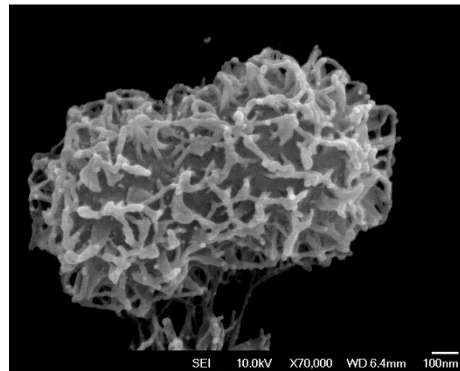
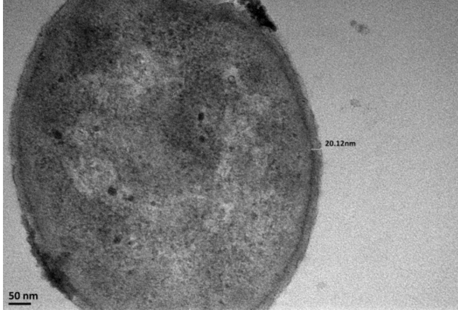
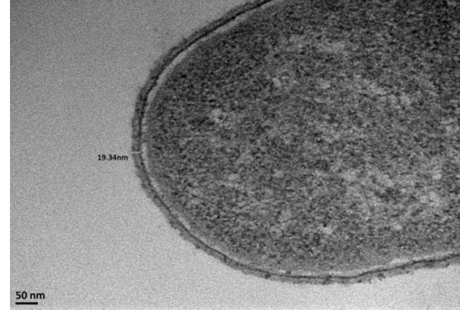


Figure 2. TEM & SEM images of *A. baumannii* isolate 6 (parent: A-B and polymyxin-B resistant: C-D)

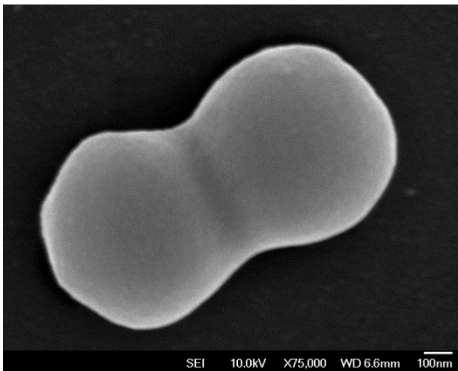
A (200,000x magnification)



C (200,000x magnification)



B (75,000x magnification)



D (45,000x magnification)

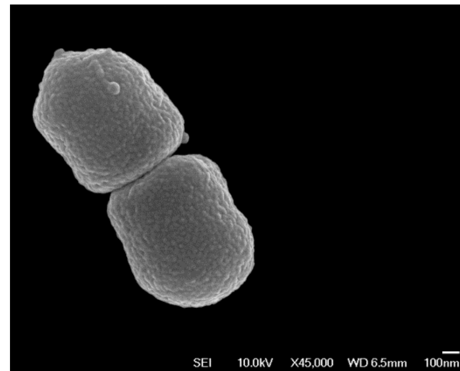
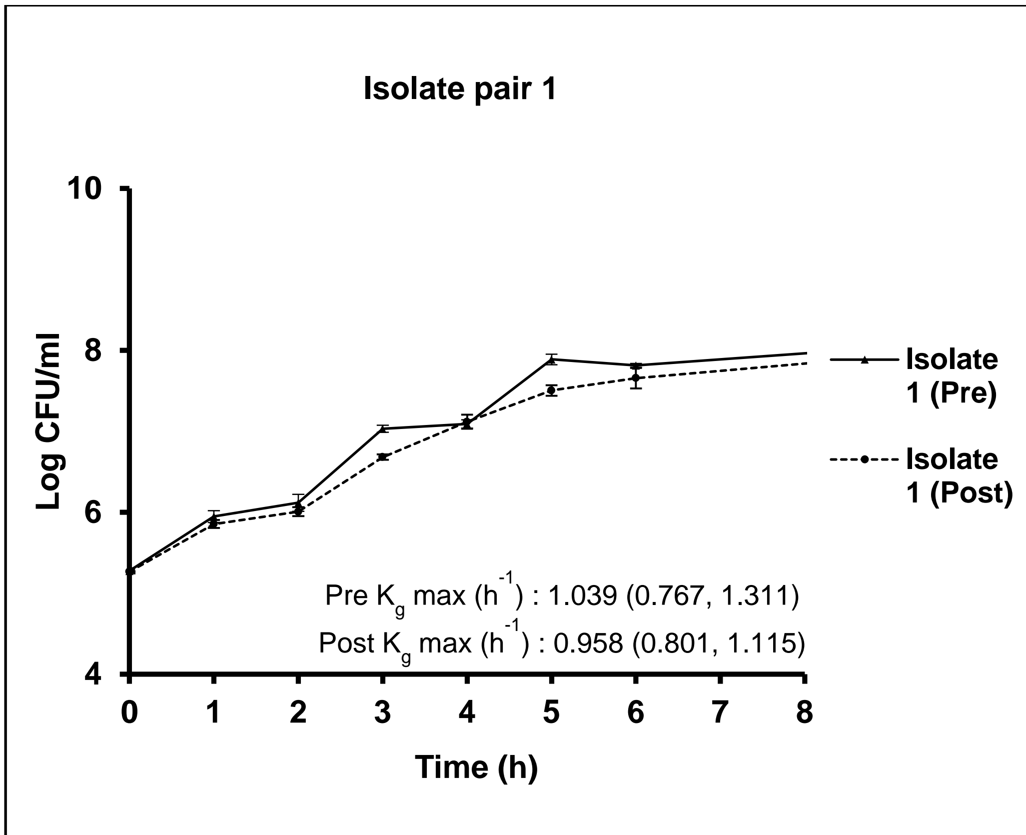
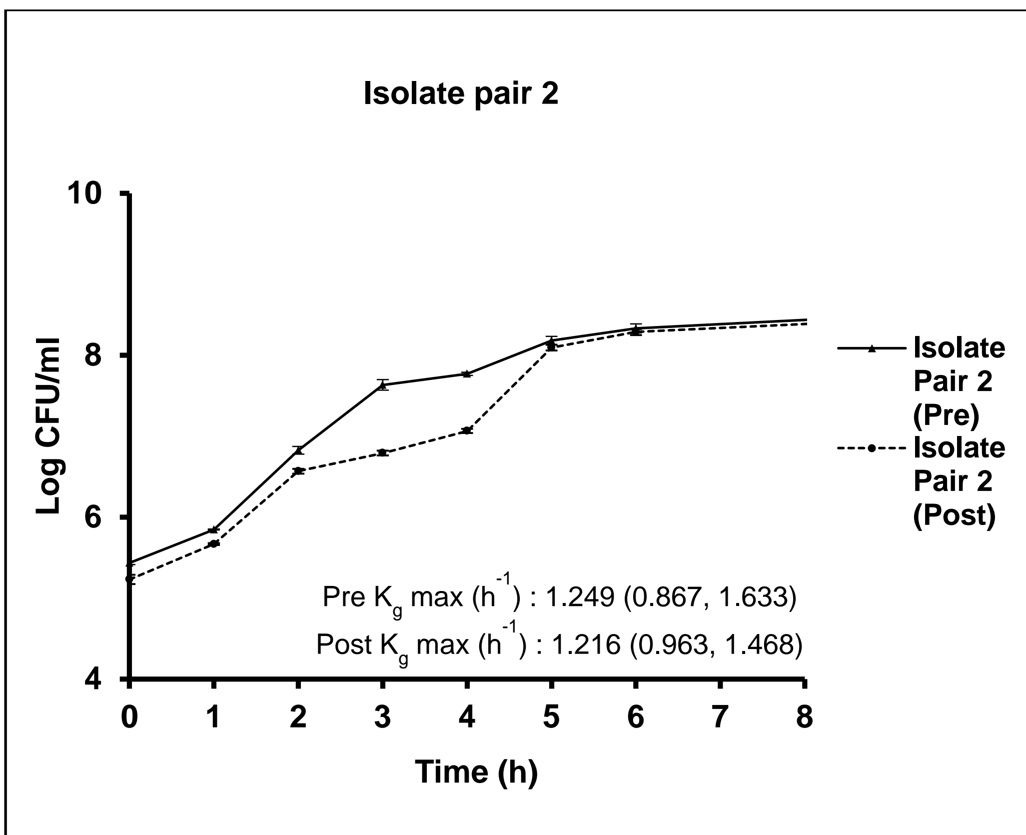
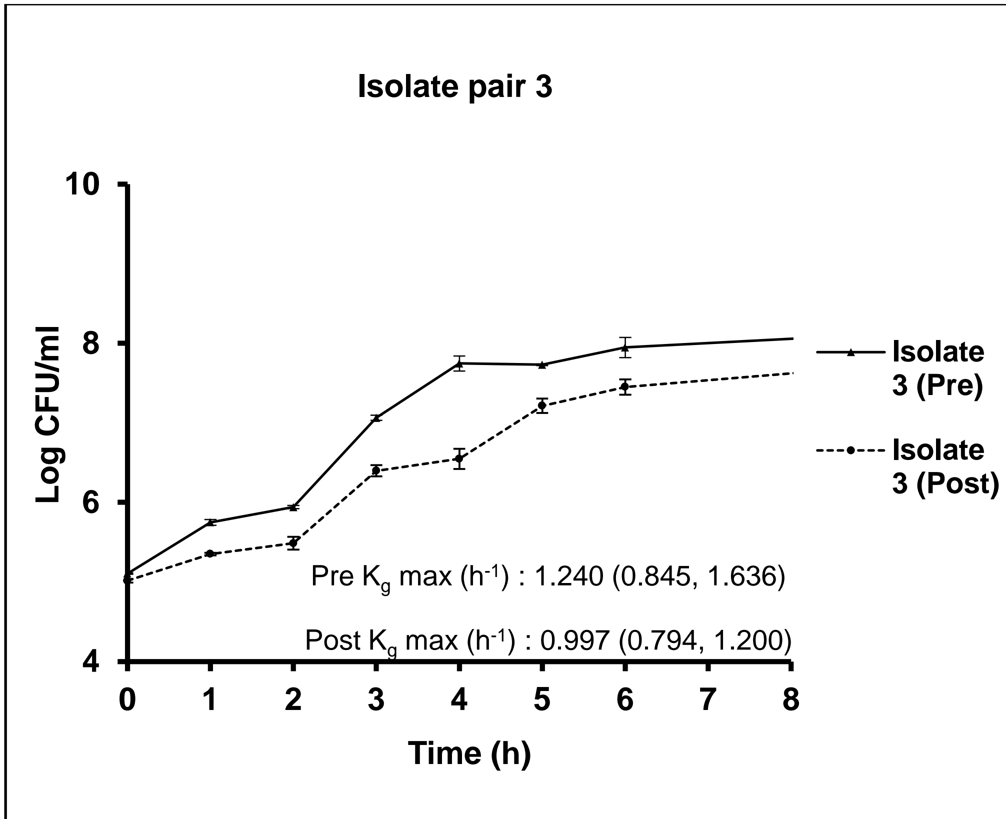
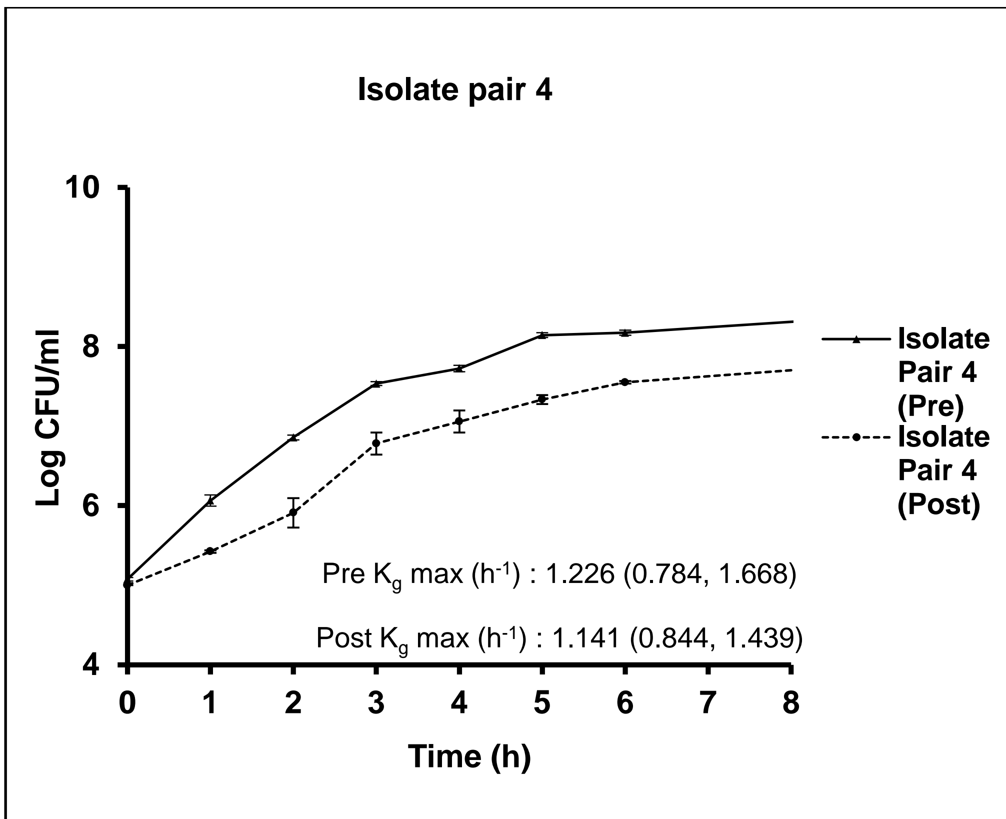


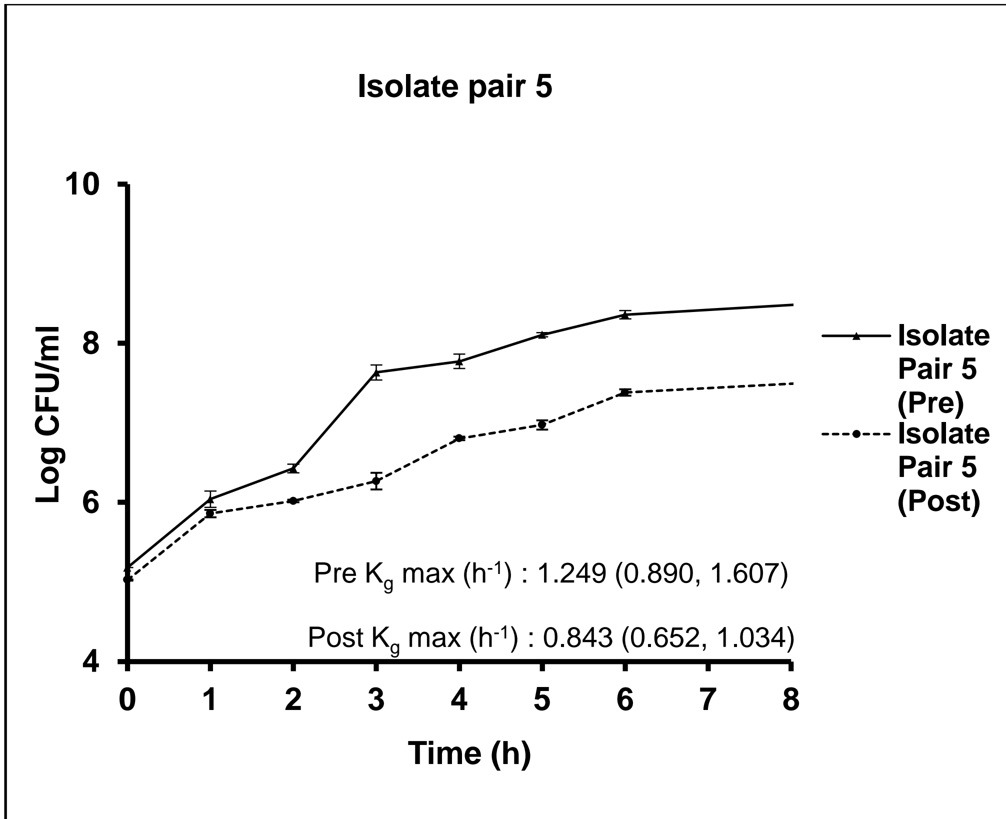
Figure 3. *In vitro* growth curves for the 10 pairs of XDR-AB isolates; pre: pre-polymyxin B exposure & post: post-polymyxin B exposure



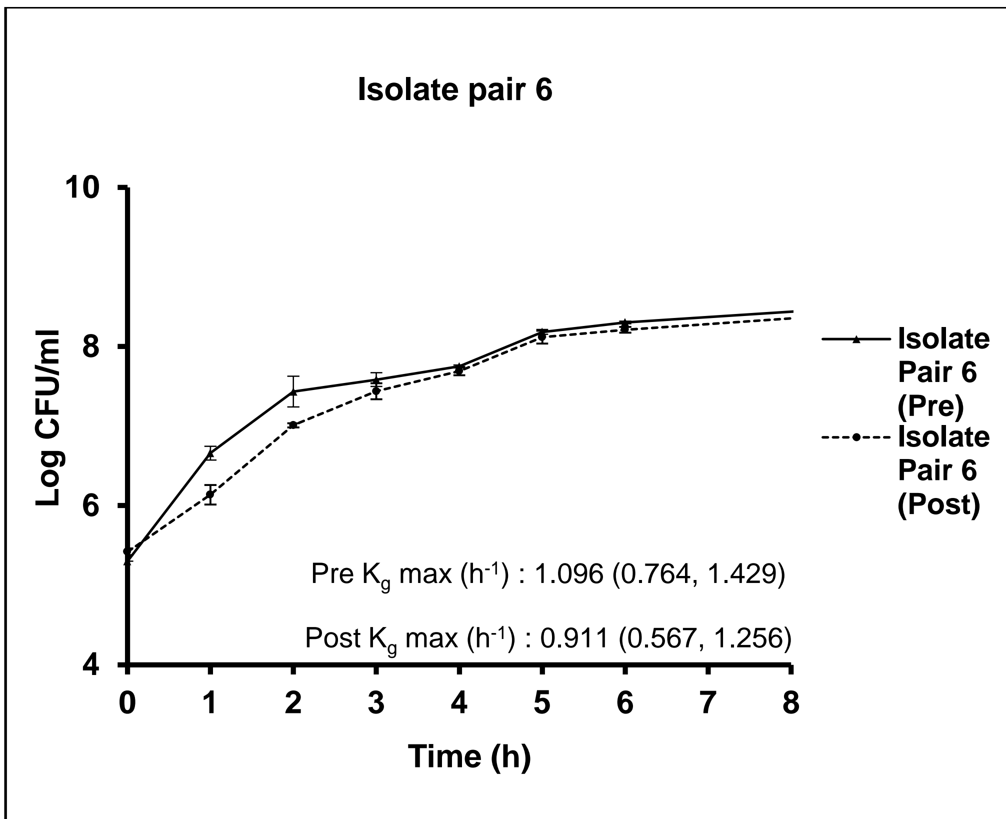



Isolate pair 3
10
8
6
4
Log CFU/ml
0
1
2
3
4
5
6
7
8
Time (h)
Isolate 3 (Pre)
Isolate 3 (Post)
Pre K_g max (h^{-1}) : 1.240 (0.845, 1.636)
Post K_g max (h^{-1}) : 0.997 (0.794, 1.200)

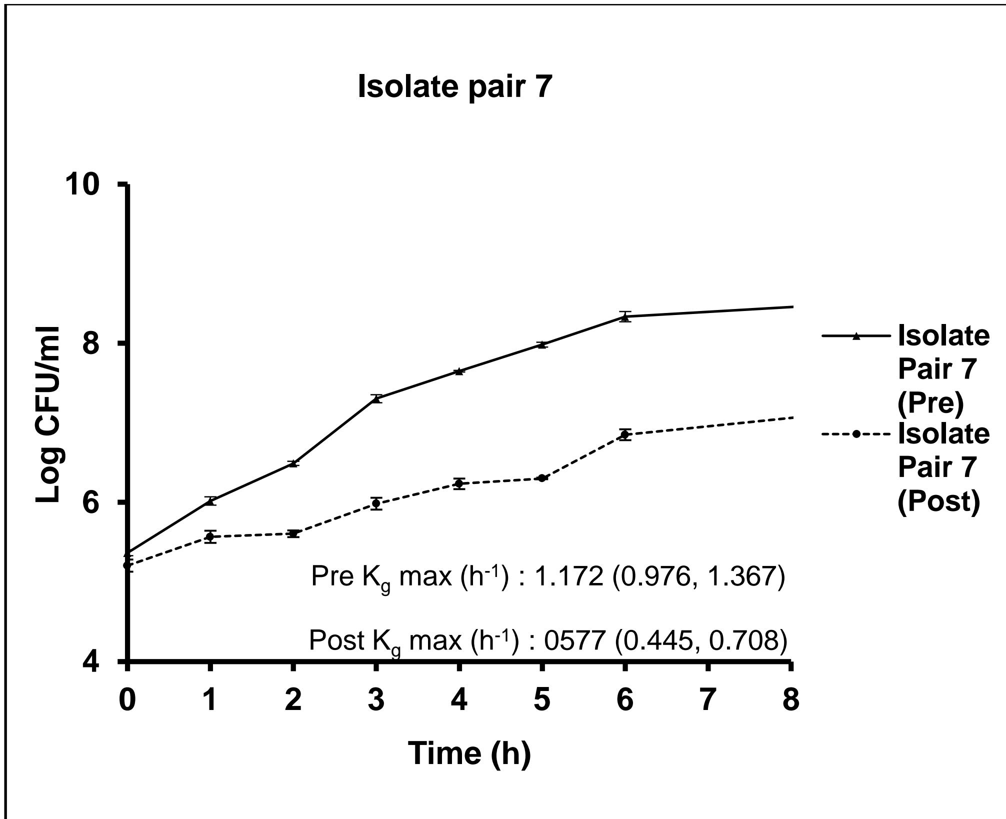

Isolate pair 4
10
8
6
4
Log CFU/ml
0
1
2
3
4
5
6
7
8
Time (h)
Isolate Pair 4 (Pre)
Isolate Pair 4 (Post)
Pre K_g max (h^{-1}) : 1.226 (0.784, 1.668)
Post K_g max (h^{-1}) : 1.141 (0.844, 1.439)

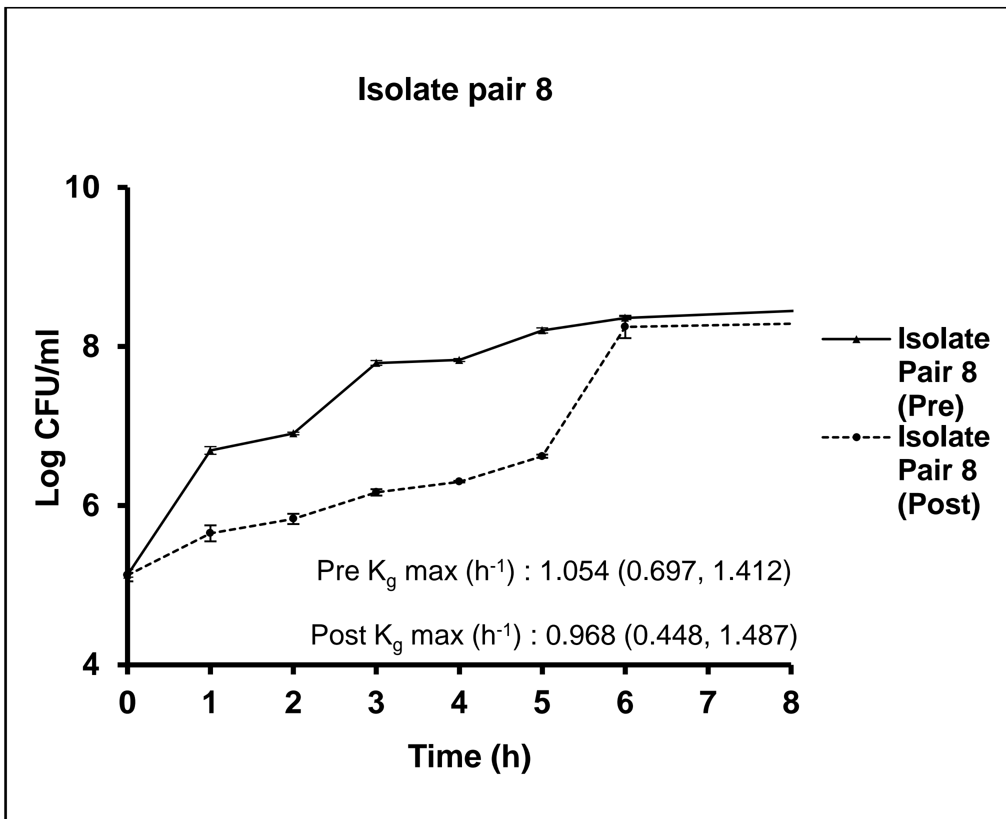


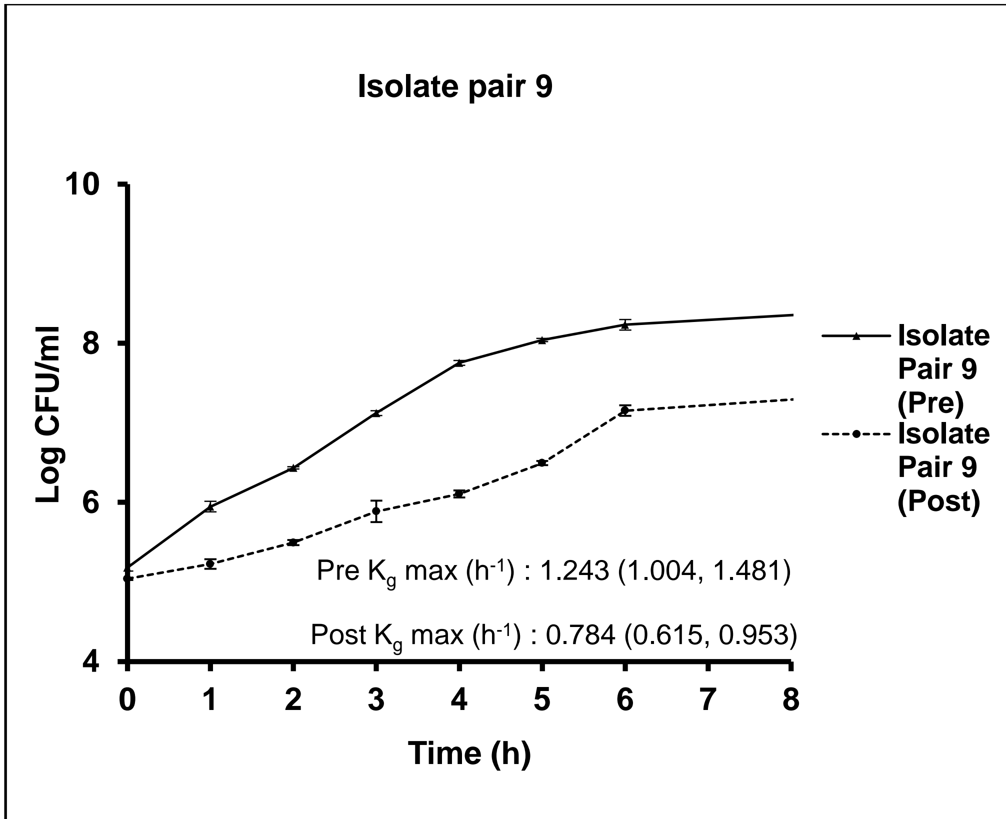
Isolate pair 5
Log CFU/ml
10
8
6
4
0
1
2
3
4
5
6
7
8
Time (h)
Isolate Pair 5 (Pre)
Isolate Pair 5 (Post)
Pre K$_g$ max (h^{-1}) : 1.249 (0.890, 1.607)
Post K$_g$ max (h^{-1}) : 0.843 (0.652, 1.034)

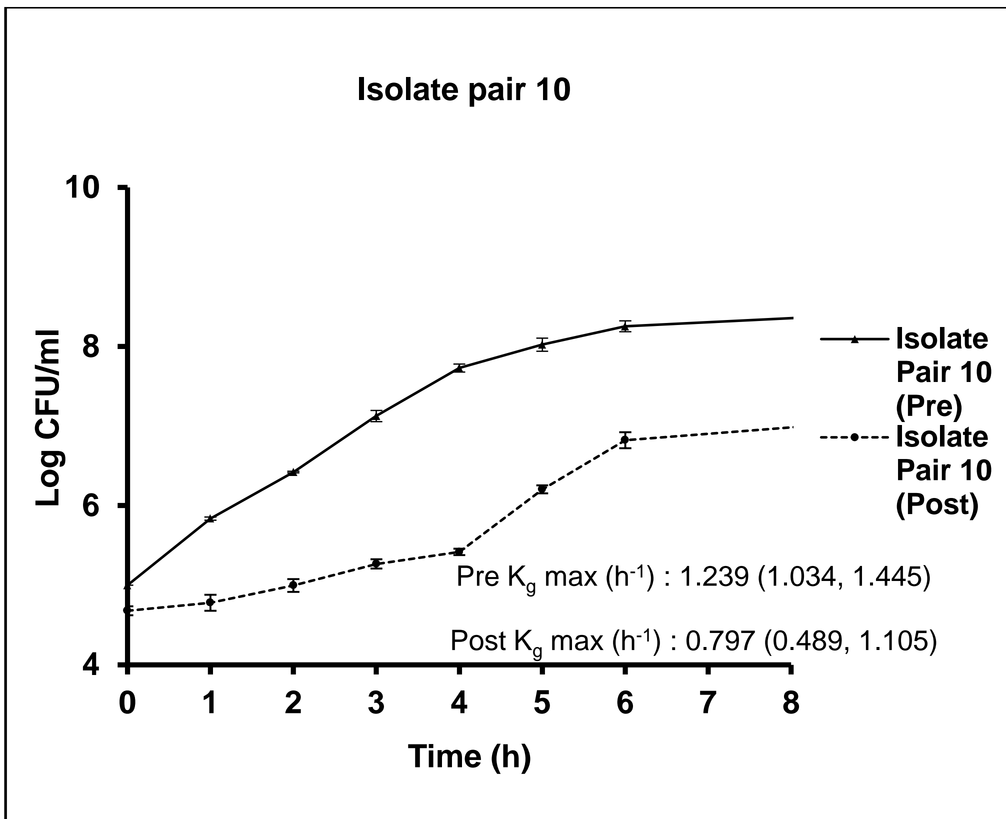


Isolate pair 6
Log CFU/ml
10
8
6
4
0
1
2
3
4
5
6
7
8
Time (h)
Isolate Pair 6 (Pre)
Isolate Pair 6 (Post)
Pre K$_g$ max (h^{-1}) : 1.096 (0.764, 1.429)
Post K$_g$ max (h^{-1}) : 0.911 (0.567, 1.256)


Isolate pair 7
Log CFU/ml
10
8
6
4
0
1
2
3
4
5
6
7
8
Time (h)
Isolate Pair 7 (Pre)
Isolate Pair 7 (Post)
Pre K_g max (h^{-1}) : 1.172 (0.976, 1.367)
Post K_g max (h^{-1}) : 0577 (0.445, 0.708)


Isolate pair 8
Log CFU/ml
10
8
6
4
0
1
2
3
4
5
6
7
8
Time (h)
Isolate Pair 8 (Pre)
Isolate Pair 8 (Post)
Pre K_g max (h^{-1}) : 1.054 (0.697, 1.412)
Post K_g max (h^{-1}) : 0.968 (0.448, 1.487)


Isolate pair 9
Log CFU/ml
Time (h)
Isolate Pair 9 (Pre)
Isolate Pair 9 (Post)
Pre K_g max (h^{-1}) : 1.243 (1.004, 1.481)
Post K_g max (h^{-1}) : 0.784 (0.615, 0.953)


Isolate pair 10
Log CFU/ml
Time (h)
Isolate Pair 10 (Pre)
Isolate Pair 10 (Post)
Pre K_g max (h^{-1}) : 1.239 (1.034, 1.445)
Post K_g max (h^{-1}) : 0.797 (0.489, 1.105)