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Supplementary Information for

Multi-drug resistant plasmids repress chromosomally encoded T6SS to enable their dissemination

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This PDF file includes:

Supplementary text
Figs. S1 to S4
Tables S1 to S2

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Supplementary Information Text

Materials and Methods

Bacterial strains and growth conditions.

The bacterial strains used in this study are listed in Table S1 in the supplemental material. Strains were routinely grown in lysogeny broth (LB) liquid medium at 37 °C with shaking (200 rpm). The antibiotics rifampicin (100 μg/ml), kanamycin (7.5 or 15 μg/ml), hygromycin B (200 μg/ml), chloramphenicol (12.5 μg/ml), sulfamethoxazole (30 μg/ml), trimethoprim (6 μg/ml) and gentamicin (15 μg/ml) were added when necessary. Spontaneous rifampicin-resistant mutant strains were obtained by plating an overnight culture on rifampicin. *Acinetobacter* strains were transformed with pBAV1K-t5-gfp plasmid (1) to obtain kanamycin resistance variants for conjugation and killing assays.

Diversity of *Acinetobacter* conjugative plasmids.

pAB3 was submitted to blastn against the nonredundant nucleotide collection to identify similar complete plasmid sequences (Table 1). Plasmids used by Nigro et al 2017 were also downloaded from NCBI (2) (Table 1). The complete sequences of all fasta files were annotated for ORFs using prokka (3). The GenBank output from prokka was viewed in EasyFig. Acquired antibiotic resistance genes and type IV secretion system components were specifically identified using ResFinder and AtlasT4SS, respectively (4, 5). Plasmid ORFs were ordered so that the tetR1 gene was first (Fig. S1).

Construction of *A. baumannii* mutant strains.

The primers used in this study are listed in Table S2 in the supplemental material. Mutants were constructed as described previously (6). Briefly, an antibiotic resistance cassette was amplified with 150 bp oligonucleotide primers (Integrated DNA Technologies) with homology to the flanking regions of the targeted gene with an additional 3'-18 to 25 nucleotides of homology to the FRT site-flanked kanamycin resistance cassette from plasmid pKD4 (7). This PCR product was

electroporated into competent *A. baumannii* ATCC 17978 carrying pAT04, which expresses the RecET recombinase. Mutants were selected on 7.5 µg/ml kanamycin, and integration of the resistance marker was confirmed by PCR. To remove the kanamycin resistance cassette, electrocompetent mutants were transformed with pAT03 plasmid, which expresses the FLP recombinase. PCR and sequencing were used to validate the mutants used in this study.

Transposon mutagenesis.

The protocol was adapted from Wither TR et al and Goodman AL et al (8, 9). The transposon library was created through bi-parental matting of 17978paB3ΔteR1,2 and *E. coli* BW19851 pSAM::OmpAp+Tn903. Plasmid pSAM::OmpAp+Tn903 was kindly donated by CM Harding, PhD. Overnight cultures were pelleted, washed three times in fresh LB, and resuspended at an OD_{600nm} of 1.0. The cultures were mixed at a 1.1 ratio and spotted on a dry LB agar plate. After 5 hr incubation at 37°C, the spot was resuspended in 1 ml of LB broth, and 10-fold dilutions were plated on kanamycin + chloramphenicol LB agar plates.

Hcp secretion screening.

The Hcp secretion screening was performed by colony blot as previously described (10). Bacterial colonies identified as positive in the screening, were isolated and Hcp secretion was confirmed by western blot. The presence of the transposon and absence of the plasmid was confirmed by PCR (pMAR2_seqF and pMAR2_seqR, pMAR5_seqF and pMAR5_seqR). In order to identify T6SS activating mutations harvested in the plasmid. Mutant plasmids were isolated upon conjugation onto a 17978 spontaneous rifampicin resistance strain. pAB3 presence was confirmed by PCR (11) and Hcp secretion was confirmed by Western blot. The whole genome of two strains carrying pAB3* and pAB3** respectively, was sequenced.

Generation of pAB3*, a pAB3 derivative that does not repress T6SS

To create a pAB3-derivative unable to repress T6SS, we mutated the pAB3-encoded TetR repressors. However, deletion of either pAB3 TetR gene (pAB3*ΔtetR1* and pAB3*ΔtetR2*), or both

genes (pAB3*ΔtetR1,2*) increased Hcp expression but did not restore Hcp secretion in 17978 strains carrying these plasmids (Fig. S3A). Employing subsequent random transposon mutagenesis on pAB3*ΔtetR1,2,* we obtained two derivatives, pAB3* and pAB3**, that were not able to repress T6SS. Surprisingly, the strain carrying pAB3* had a chromosomal transposon insertion, specifically in the peptide chain release factor 3 coding gene, *prfC*. However, an unmarked *prfC* deletion in 17878 strain carrying pAB3*ΔTetR1,2* was not able to secrete Hcp. Conjugation of pAB3* into 17978 did not block T6SS, indicating that the plasmid carries an additional mutation that relieves T6SS inhibition. Both plasmids, pAB3* and pAB3**, carry a spontaneous 6kb-deletion ("Locus1") found in a plasmid region unrelated to the TetR genes (Fig. S2). A 17978 strain carrying pAB3* or pAB3** secretes Hcp (Fig. S3A) and kills *E. coli* (Fig. S3B), consistent with an active T6SS. We verified that mutations in pAB3* do not affect T4SS activity, as we observed no difference in the conjugation efficiency of pAB3 and pAB3* between isogenic strains that are immune to T6SS-mediated killing (Fig. 2C). Thus, pAB3* can be used to investigate the impact of T6SS on LCP conjugation. Furthermore, this results suggests the presence of a third regulator on pAB3 plasmid involved in the regulation of T6SS. However the mechanistics insights of pAb3-mediated T6SS regulation is out of the scope of this paper.

Genomic comparison of pAB3* and pAB3** with wildtype pAB3.

Genomic DNA from *A. baumannii* ATCC 17978 with pAB3* was sequenced by the Genome Technology Access Center (https://gtac.wustl.edu/) on an Illumina HiSeq 2500 to obtain ~9 million 1 x 50 bp reads. 5 ng of Genomic DNA from *A. baumannii* ATCC 17978 with pAB3** was prepared for Illumina sequencing using the Nextera XT kit (Illumina, San Diego, CA) (12). The library was sequenced on an Illumina NextSeq 550 at the Edison Family Center for Genome Sciences and Systems Biology (https://genomesciences.wustl.edu) to obtain ~9 million 2 x 150bp paired end reads. Both sets of reads have been deposited in the NCBI Short Read Archive under BioProject ID PRJNA447986. Paired end reads were processed for quality using trimmomatic v.36 and deconseq v.4.3 (13, 14). Illumina reads for both samples were mapped to the complete sequence of pAB3 (GenBank entry CP012005.1) using bowtie2 v2.3.3 (15). Aligned reads were assembled

into contigs using SPAdes v3.11 (16). The complete sequence of pAB3 and de novo assemblies of pAB3* and pAB3** were annotated for open reading frames (ORFs) with prokka v1.12 (3).

Genes absent in pAB3* and pAB3** were identified by separately aligning the .ffn output from prokka for these assemblies with the .ffn from pAB3 in NCBI blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using default parameters (17). The pSAM insertion site was identified in pAB3** through a similar blastn comparison of the pSAM sequence (18). The genbank file produced by prokka annotation of pAB3 was viewed in DNAPlotter and annotated for ORFs absent in pAB3* and pAB3**, and the pSAM insertion site (19). The *tetR* deletion site and locus I containing genes, both absent in pAB3* and pAB3**, were specifically viewed in EasyFig (20). Identified ORFs were labeled using the notion from the GenBank annotation of pAB3.

SMPs transformation in 17978.

pRAY* and pABLAC2 plasmids were isolated with a commercial miniprep plasmid purification kit (Thermo) from *A. baumannii* D46 and LAC-4 strains, respectively. Plasmids were transformed to 17978, 17978pAB3 and 17978pAB3*, transformants were selected on kanamycin plates and the presence of the plasmid was confirmed by PCR.

Contact killing assay.

Competition assays were performed as previously described (11). Briefly, predator and prey overnight cultures were pelleted, washed three times in fresh LB, and resuspended at an OD_{600nm} of 1.0. The cultures were mixed at a predator:prey ratio of 5:1 or 1:10, and 5 µl drops in triplicate, were spotted on a LB agar plate. After 4 hr or overnight incubation at 37°C, the spots were harvested, resuspended in 1 ml of LB broth, serially diluted and plated on kanamycin or rifampicin LB agar plates in order to determine the number of surviving prey cells. Assays were done in duplicates and repeated at least 3 times.

Bacterial conjugation assay.

The serial dilutions from the previous assay were plated on the appropriate selection media to select for transconjugants. Colonies were only obtained from experiments where both strains were mixed together. For competitive conjugation assay, overnight cultures were processed as above, mixed at a 5:5:1 ratio (donor1:donor2:recipient) and incubated overnight at 37 ºC. The relative abundance of pAB3 and pAB3* plasmids in the population of transconjugants (100 colonies per assay, the assay was repeated 3 times) was determined by PCR with primers tetR_seq_F and tetR_seq_R (Table S2). Assays were done in duplicates and repeated at least 3 times. For M2 conjugation assay a ratio Donor:Recipient 1:10 was used. We found that conjugation can only be achieved in the presence of a larger number of *A. nosocomialis*, as 17978 can kill M2 in a T6-independent manner when present in higher numbers.

Hcp Western blotting.

Hcp western blot was performed as previously described (21). Supernatants and whole cells samples were obtained from mid-log cultures. Samples were resuspended in 1x Laemmli buffer and loaded in a 12% polyacrylamide gel for separation. The anti-Hcp (22) and anti-RNA polymerase (Biolegend, San Diego, CA) antibodies were both used at a concentration of 1:1000. Secondary IR dye antibodies from Licor were used at 1:10,000. All blots were blocked in TBS blocking buffer (Licor).

Statistical analysis

GraphPad Prism7 software was used for statistical analyses. Normal data distribution was checked with the Shapiro-Wilk normality test. For normally distributed datasets, parametric oneway ANOVA was performed with Tukey's correction for multiple comparisons. For non-normally distributed datasets, nonparametric Kruskal-Wallis test with Dunn's correction for multiple comparisons was used.

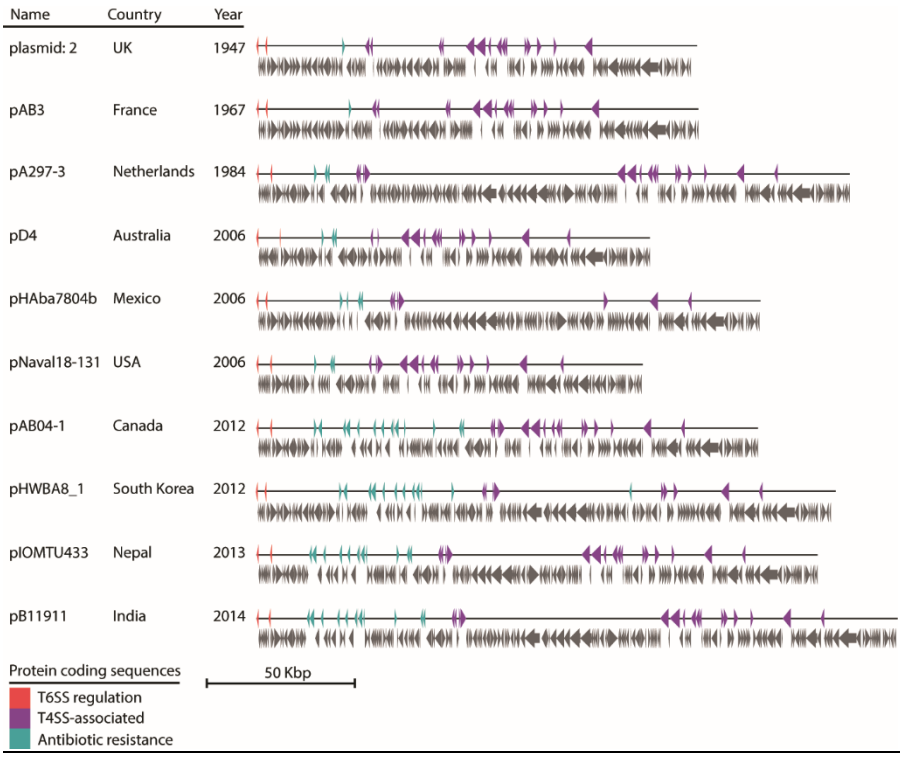

Fig. S1. LCPs are a family of globally distributed plasmids. LCPs share three common structural areas with genes coding for: putative T6SS regulation (red), antibiotic resistance (teal) and T4SS conjugation machinery (purple). ORFs are ordered so that the *tetR1* gene is first. It is noteworthy the increase in antibiotic resistance genes encoded in LCPs since 2010.

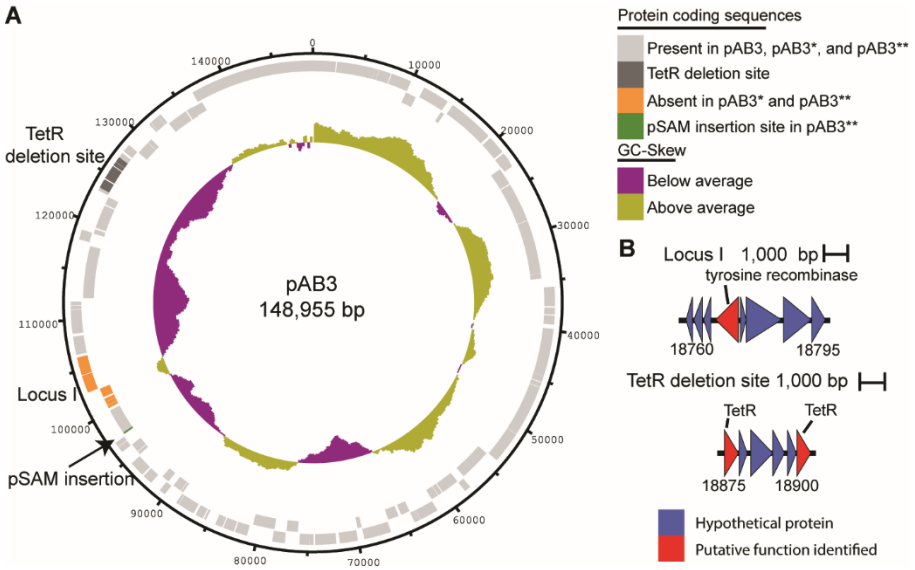

Fig. S2. Both T6+ pAB3 derivatives plasmids have the same 9 kb deletion. (A) pAB3 plasmid map. Outermost ring represents ORFs encoded in the forward reading direction. Second ring indicates ORFs encoded in the reverse reading direction. Innermost ring indicates the GC-Skew as calculated by DNAPlotter. Insertion or deletion areas in pAB3+ and pAB3** are shown in color. (B) EasyFig viewing of the ORFs encoded in the absent locus I and the TetR deletion site. ORFs are annotated in red if a putative function is identified via GenBank. The TetR deletion site was created during the construction of the unmarked double mutant.

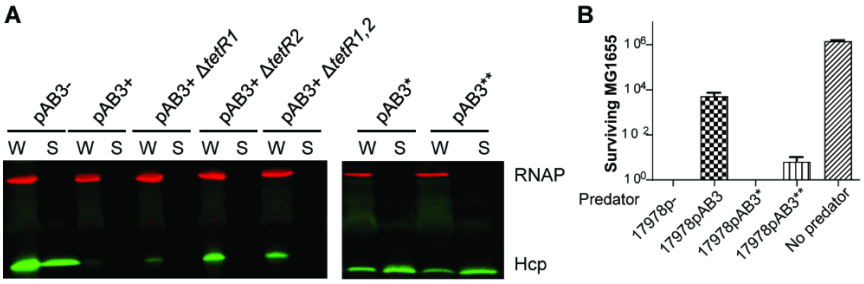

Fig. S3. pAB3* and pAB3 are not able to repress T6SS**. (A) Western blot assays probing for Hcp expression and secretion in 17978 harboring pAB3 and pAB3 mutant. RNA polymerase (RNAP) was used as loading control. W: whole cells, S: supernatants. (B) T6SS-dependent competition assay. Reported are the numbers of surviving *E. coli* MG1605 prey strain (indicated on the left), predator strains are indicated in the x axis. Data are mean ± SD, n=3 independent replicates.

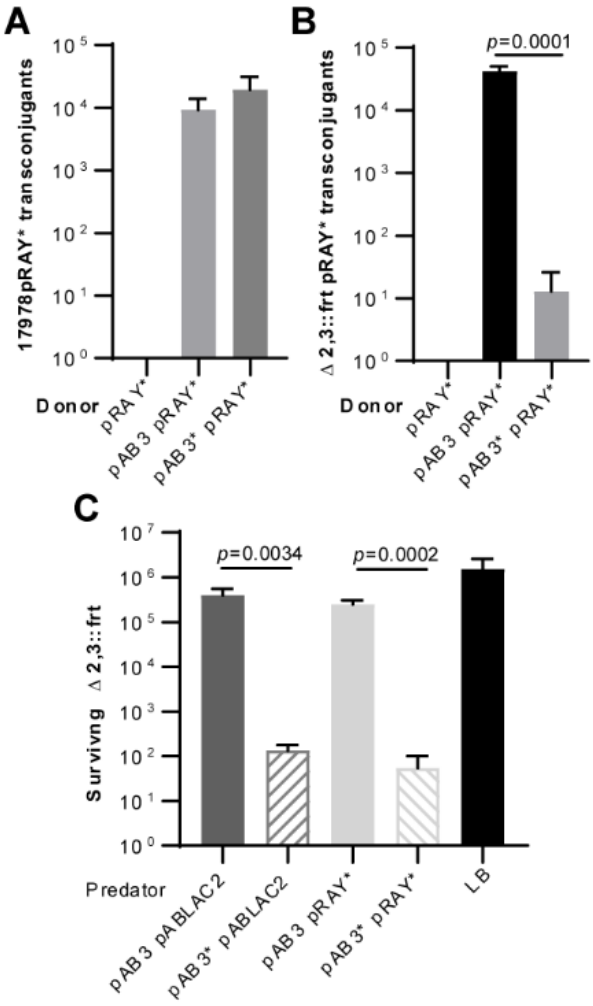

Fig. S4. pAB3 mediates pRAY* mobilization. Data are mean ± SD, n=3 independent replicates. Statistical analysis was performed using one-way ANOVA and the Tukey-Kramer multiple-comparison test. Conjugation assays were performed at a Donor:Recipient ratio 5:1 for 4 h. Quantification of recovered pRAY* transconjugants (indicated on the left of each panel), donor strains are indicated in the x axis. (A) pRAY* conjugation is non-detectable if pAB3 is not present in the donor strain. (B) pRAY* efficiency of conjugation to a T6SS-meadited killing susceptible recipient strain is highly reduced in the presence of an LCP unable to repress T6SS. (C) T6SS-dependent competition assay. Reported are the numbers of surviving 17978Δ*2,3::Km* prey strain (indicated on the left), predator strains are indicated in the x axis. Data are mean ± SD, n=3 independent replicates.

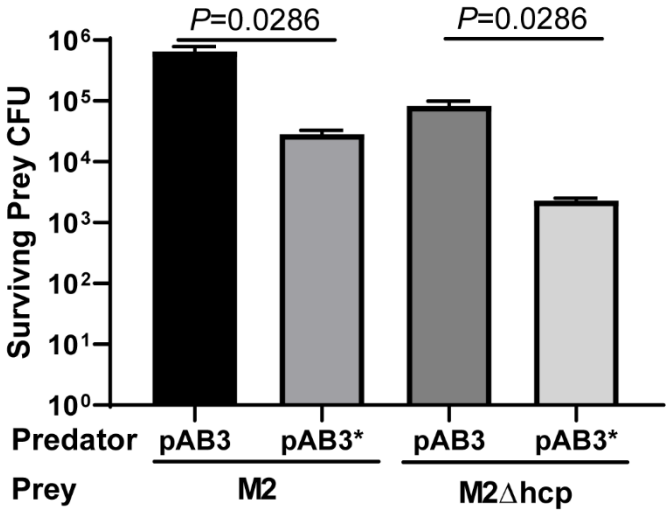

Fig. S5. *A. baumannii* 17978 can kill *A. nosocomialis* M2 in a T6SS-dependet manner. Data are mean ± SD, n=3 independent replicates. Statistical analysis was performed using one-way ANOVA and the Tukey-Kramer multiple-comparison test. T6SS-dependent competition assay was performed at a Predator:Prey ratio 1:10 for 4 h. Reported are the numbers of surviving prey strains. Predator strains 17978 with pAB3 (pAB3) or pAB3* (pAB3*) and prey strains *A. nosocomialis* M2 wild-type (M2) and Δhcp (M2Δhcp) are indicated in the x axis.

Table S1. Strains list

Strain or plasmid	Relevant characteristics	Reference
***A. baumannii* ATCC17978::Km**	T6SS active strain, Kanamycin resistant	(11)
***A. baumannii* ATCC17978 Rif^R**	spontaneous rifampicin resistant mutant	(11)
***A. baumannii* ATCC17978 pAB3**	T6SS inactive strain	(11)
***A. baumannii* ATCC17978 pAB3ΔtetR1**	mutant strain	This study
***A. baumannii* ATCC17978 pAB3ΔtetR2**	mutant strain	This study
***A. baumannii* ATCC17978 pAB3ΔtetR1,2**	mutant strain	This study
A. baumannii* ATCC17978 pAB3	mutant strain	This study
A. baumannii* ATCC17978 pAB3*	mutant strain	This study
***A. baumannii* ATCC17978Δ2::Km**	*A. baumannii* ATCC17978ΔvgrG-tse-tsi2::Km mutant strain	This study
***A. baumannii* ATCC17978 Δ3::Km**	*A. baumannii* ATCC17978ΔvgrG-tse-tsi3::Km mutant strain	This study
***A. baumannii* ATCC17978 Δ2,3::Km**	*A. baumannii* ATCC17978ΔvgrG-tse-tsi2, ΔvgrG-tse-tsi3::Km mutant strain	This study
***A. baumannii* ATCC17978 Δ2,3::frt**	*A. baumannii* ATCC17978ΔvgrG-tse-tsi2, ΔvgrG-tse-tsi3::frt unmarked mutant strain Spontaneous rifampicin resistant	This study
A. baumannii* ATCC17978 Δ*tssM* *pAB3*	mutant strain	This study
***A. baumannii* 1225**	T6SS inactive strain	(22)
***A. nosocomialis* M2**	T6SS active strain	(23)
A. nosocomialis* M2 Δ*tssD	Hcp mutant strain	(23)
***A. nosocomialis* M2 pAB3**	T6SS inactive strain	This study
*A. **baylyi* ADP1**	T6SS active strain	(24)
*A. **baylyi* ADP1 Δ*tssM***	T6SS mutant strain	(24)
*A. **baylyi* ADP1 pAB3**	T6SS inactive strain	This study
*A. **junii***	T6SS inactive strain	(22)
*A. **baumannii* UPAB1**	T6SS mutant strain	This study
*A. **baumannii* UPAB1 pAB3**	T6SS inactive strain	This study
***E. coli* BW19851 pSAM::OmpAb + Tn903**	Plasmid for random transposon mutagenesis	Harding, CM
A. baumannii* ATCC17978 pRAY	17978 transformed with pRAY*	This study
A. baumannii* ATCC17978 pAB3 pRAY	17978 with pAB3 transformed with pRAY*	This study
A. baumannii* ATCC17978 pAB3*pRAY	17978 with pAB3* transformed with pRAY* (Active T6SS)	This study
***A. baumannii* ATCC17978 pABLAC2**	17978 transformed with pABLAC2	This study
***A. baumannii* ATCC17978 pAB3 pABLAC2**	17978 with pAB3 transformed with pABLAC2	This study
***A. baumannii* ATCC17978 pAB3* pABLAC2**	17978 with pAB3* transformed with pABLAC2 (Active T6SS)	This study

Table S2. Primers list

Primer Name	Sequence (5'-3')	Description
tetR12_seq_F	AAACTTAACCCTGTATTATATTGTCTAA	for tetR1,2 region sequencing
tetR12_seq_R	CCGCATATTTTCCTAAAAAGT	for tetR1,2 region sequencing
vgrG_tse_tsi2_KO_F	CTTAACGCTCCAGCATTTCATTTGGCATTACAAATTCTAAAGTAATCGCATGTGAGGTCACGGTACGGGTACATGTCAGTTGAAATTGTAAATATCTAAATAGAAATGCCCTTATGGGCATTTAAAGCGATTGTGTAGGCTGGAGCTGCTTCG	for vgrG2-tse2-tsi2 whole locus deletion
vgrG_tse_tsi2_KO_R	TTTATTTTAGTGTATGGATAGGGTGTCCGACTAGCCATTAACATTGCAATTGATATAAAAAATAGACAGATCTGTACGCATTTTATAAAATATAATCGATTAAGTCTCAATTTATAACAAACGTACATATGAATATCCTCCTTAGTTCCTATTCCG	for vgrG2-tse2-tsi2 whole locus deletion
vgrG_tse_tsi3_KO_F	CCTGAAAATACCTTTTAATCATCTTAAACATCAACTCAATTGAATAACAAAAACTCTATTTATATCTTTTCTGATTCTAACTAGAATACTTATATCACAACTCAGCTATATGATTTTTAAATAAGAGCGATTGTGTAGGCTGGAGCTGCTTCG	for vgrG3-tse3-tsi3 whole locus deletion
vgrG_tse_tsi3_KO_R	TTGCGTAAATTCCTTTGCAACATAACCCATATTGAGAGCTCCCCATTTTGATCAGTATTACGATTTTCATAAAACGTAATATTGTTAAGCAAAATAGGTGCCATCTTTAAAGTGCATTAGATGAGCATATGAATATCCTCCTTAGTTCCTATTCCG	for vgrG3-tse3-tsi3 whole locus deletion
vgrG_tse_tsi2_seq_F	GATGATCTCTAAATTCATTCTCATC	for vgrG2-tse2-tsi2 deletion check
vgrG_tse_tsi2_seq_R	CCTATAAATCTGCTACAATTACGC	for vgrG2-tse2-tsi2 deletion check
vgrG_tse_tsi3_seq_F	GCTTAAACATGGCTTTAGATAA	for vgrG3-tse3-tsi3 deletion check
vgrG_tse_tsi3_seq_R	GTAAGTAAGAAACACTTCCCGAA	for vgrG3-tse3-tsi3 deletion check
rpoB_qPCR_F2	GCACAGGCCGACTCTG	for qPCR
rpoB_qPCR_probe_R	TCCGCACGTAAAGTAGGAAC	for qPCR
virB2_qPCR_F2	GCTGTTACTGGTCAGGTTG	for qPCR
virB2_qPCR_probe_R	TCTTGAACCCAATTTGTATTCG	for qPCR
pMAR2_seqF	CAGTTCCATAGGATGGCAAGA	for Mariner transposon
pMAR2_seqR	AACGCACTGAGAAGCCCTTAG	for Mariner transposon
pMAR5_seqF	ATGAAACATGGCTCCATCACT	for pSAM backbone
pMAR5_seqR	ACATAAACAGTAATACAAGGG	for pSAM backbone
TetR1UpFwdKO	TTCCGACCTAAAAGTCGAAGGAGC	for TetR1 deletion
TetR1UpRevKO	GGTACTGAGTATGGAGTTTGAAATCATGCGAAGCAGCTCCAGCCTACACAATCGCTCATGATTTCAAACTCCATACTCAGTACC	for TetR1 deletion

TetR1DwFwdKO	CGGAATAGGAACTAAGGAGGATATTCATATGGAG GCTTAAAGCCTCAAATAAGG	for TetR1 deletion
TetR1DwRevKo	CCAACAAGCTTTAAAGAAAGATCTATAGATCTTTCT TTAAAGCTTGTTGG	for TetR1 deletion
TetR1KOcheckFwd	TTGTAGTCATGCAACCAGATAAGACG	for TetR1 deletion check
TetR1KOcheckRev	GCAGTGATGCTTTAAGTGCTGTTTAAACAGCACTT AAAGCATCACTGC	for TetR1 deletion check
TetR2UpFwdKO	TTAGTGTTGTATGTGAATAACTCTGATGG	for TetR2 deletion
TetR2UpRevKO	CCTGAGAAATCACTGATGACAAATCGAAGCAGCTC CAGCCTACACAATCGCTATTTGTCATCAGTGATTT CTCAGG	for TetR2 deletion
TetR2DwFwdKO	CGGAATAGGAACTAAGGAGGATATTCATATGTTTT GAAAAAAAATAGTTTGAATCG	for TetR2 deletion
TetR2DwRevKo	GGCTGATCGAAAAGAAGCCATTATAATGGCTTCTT TTCGATCAGCC	for TetR2 deletion
TetR2KOcheckFwd	ATTCCTTAACGAGCACTGGTATTTCG	for TetR2 deletion check
TetR2KOcheckRev	GCGTGAATGTGTGGAAGCTTATATAAGCTTCCACA CATTCACGC	for TetR2 deletion check
pKD4_KanFRT_Fwd	AGCGATTGTGTAGGCTGGAGCTGCTTCG	for FRT-flanking Kanamycin cassete
pKD4_KanFRT_Rev	CATATGAATATCCTCCTTAGTTCCTATTCCG	for FRT-flanking Kanamycin cassete
MobA Fw240	GGTTCAGGTTTATCAAAAGGC	for SMPs detection
MobA Rv240	GTCTTTATCTAGTCCCGGG	for SMPs detection

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