Genome engineering in *Mycoplasma gallisepticum* using exogenous recombination systems

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Supplementary Information

Supplementary Figures

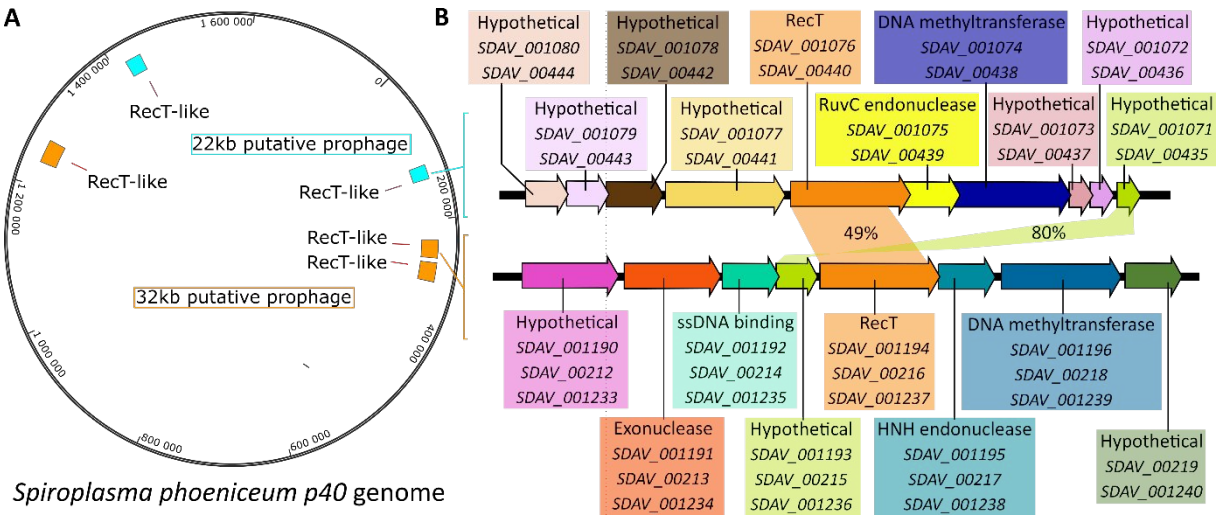

Figure S1. Putative prophage loci in *S. phoeniceum* p40 genome. **A-** Genome localization of putative prophages, as characterized by PHASTER analyses, in the *Spho* genome. Orange and blue boxes indicate the localization of the 32 kbp and 22 kbp predicted prophages. Genes encoding the RecT-like proteins are represented in red bar or purple bar. **B-** Comparison of loci around *recT*-like encoding genes predicted in 22 kbp (top) and 32 kbp (bottom) putative prophages families in *Spho* genome. Homologous genes between the two families are connected and protein similarity percentages are indicated. Predicted functions are indicated above the mnemonic list of *Spho* genes from the multiple prophage copies.

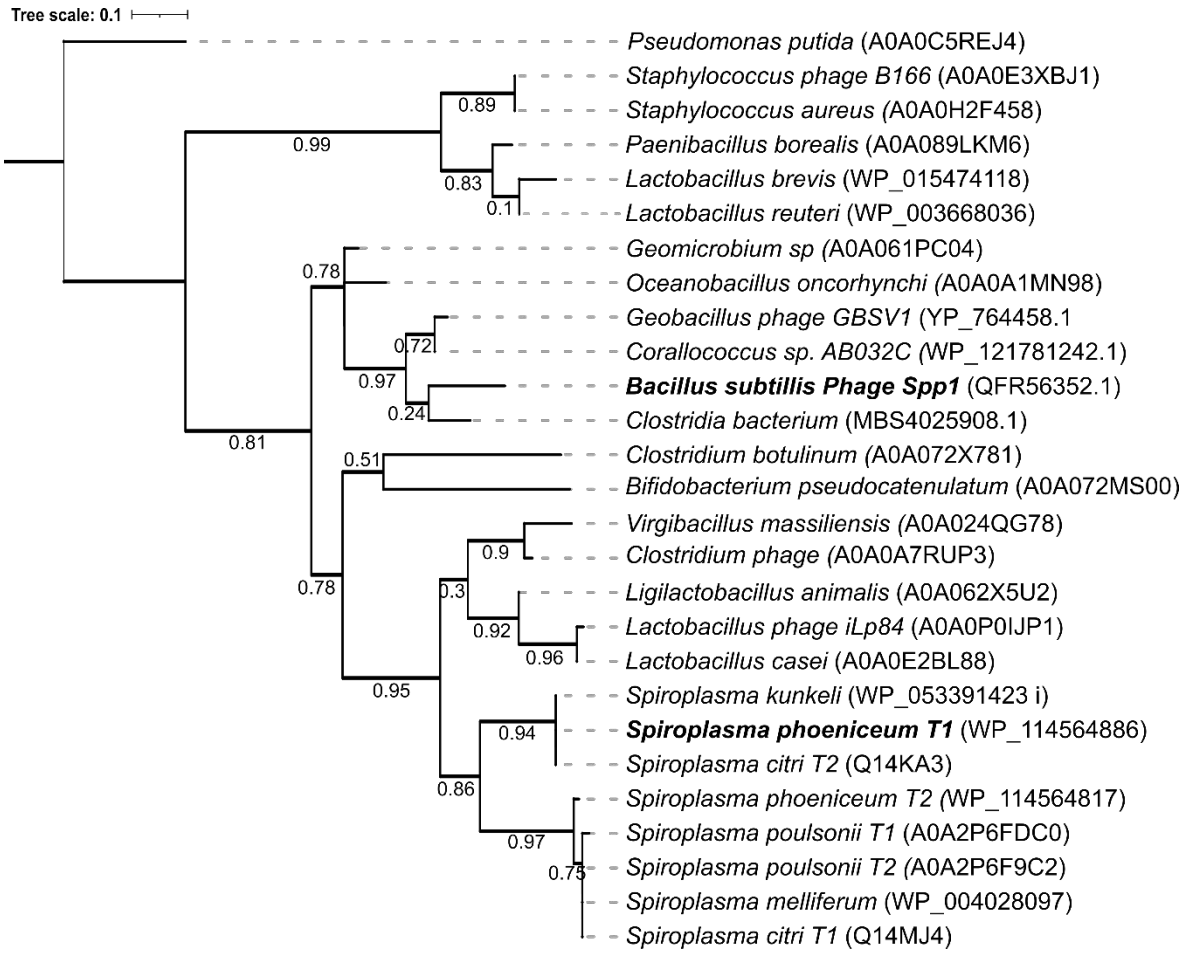

Figure S2. Phylogeny of RecT proteins. Phylogenetic tree was constructed using selected RecT homologs from the Interpro database (http://www.ebi.ac.uk/interpro/entry/InterPro/IPR004590/), using tools available http://www.phylogeny.fr/simple_phylogeny.cgi. Muscle was used for alignment of proteins sequences, Gblocks for curation and PhyML for maximum likelihood phylogenetic reconstruction. Final tree was designed using iTOL

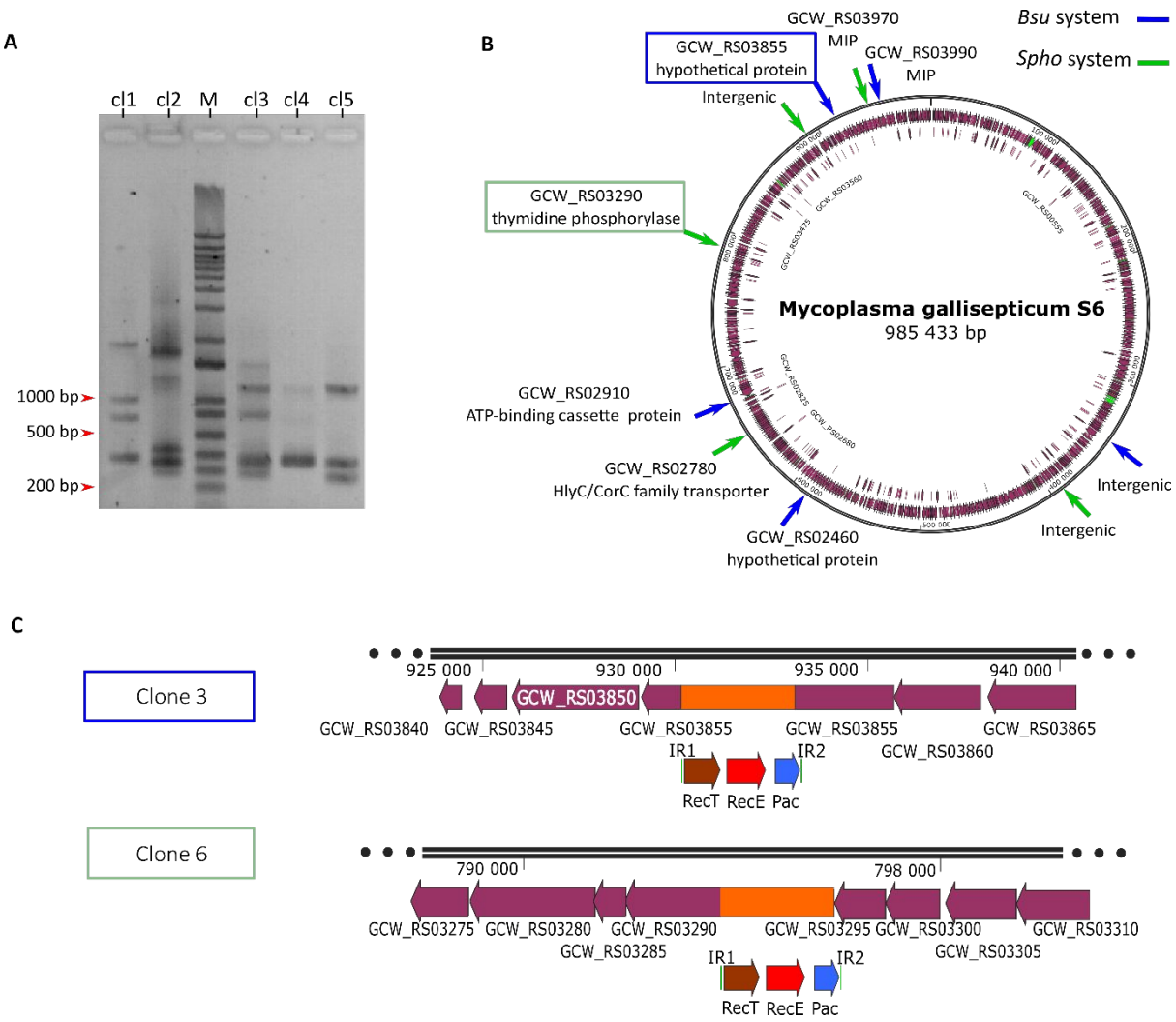

Figure S3. Characterization of transposon integration site in *Mgal* genome. **A**- Example of PCR profiles obtained after single primer PCR on 5 clones of *Mgal.* **B**- Transposon integration sites determined after Sanger sequencing and BLASTn against *Mgal* genome. Integration sites of the transposons harbouring the *Bsu* and the *Spho* RecET-like systems (5 clones each) are indicated with blue and green arrows, respectively. Genes where transposon integration occurred in clones 3 and 6 are framed in blue and green rectangles, respectively. **C**- Genomic context of transposon integration sites in selected clones 3 and 6.

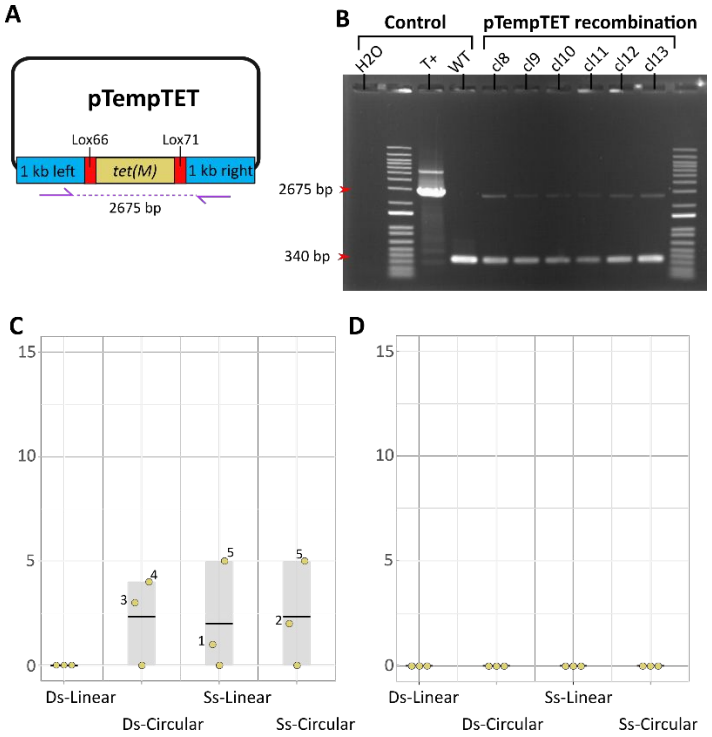

Figure S4. Targeted small deletion in *Mgal* genome using the *Bsu*RecET-like system. **A-** Schematic of the targeted region in *Mgal* genome and the pTempTET suicide plasmid harboring recombination cassette containing tetracycline resistance markers (*tet*(M)) flanked by 1 kbp homologous arms for recombination. Primers G55 and G56 were used to screen recombination events by PCR amplification with expected products that differed in size: WT DNA (340 bp) or recombinant DNA (2,675 bp). **B-** Example of colony screening using this scheme. Only mixed profiles were observed for all clones. **C and D-** Results of three assays of recombination on locus GCW_RS03665 using Tet template. Four template forms (Double stranded (ds)-linear, ds-circular, single stranded (ss)-linear and ss-circular were evaluated. Left graph (**D**) corresponds to all transformants obtained and the right one (**E**) corresponds to the number of isolated clones with a pure recombinant profile.

Supplementary text

SI-1. Material and Methods

Identification of RecET-like systems in Mollicutes genomes

Mollicutes orthologues of RecT-like proteins from the *E. coli*-derived Rac system (NP_415865), the *Bacillus* phage SPP1 (CAA66543.1) and *Lactobacillus* (WP_015474118 and WP_011101092) were searched using BLASTp against Tenericutes database (taxid: 544448). The InterPro (http://www.ebi.ac.uk/interpro/) database was also used to identify functional domains. Hits were found in *S. phoeniceum* p40 (CP031088), *S. poulsonii, S. kunkelii* CR2-3x (CP010899.1), *S. citri* R8A2 (CP013197.1) and *S. melliferum* AS576 (CP029202). Comparison between proteins were made using Blastp and protein alignement in MEGA-X (Kumar et al., 2018). Phages were further predicted in the *Spiroplasma* genome using PHASTER (http://phaster.ca/) (Arndt et al., 2016) and a final prediction of putative phages and RecT-like proteins was achieved manually.

Plasmid construction

Construction of Mini_SynMyco_RecET*Bsu* and Mini_SynMyco_RecET*Spho*. The Mini transposon vector derived from Tn*4001* (Clyde A. Hutchison et al., 1999) and the synthetized fragments provided by Twist biosciences (SI-2) were used as PCR template. Primers A1/A2, B4/B5 and B6/B7 and the Q5 High-Fidelity DNA Polymerase kit (NEB, M0491) were respectively used to amplify the plasmid backbone with puromycin resistance and the codon optimized sequences of *recT* and *recE* genes. PCR products were submitted to restriction with the DpnI enzyme (NEB, R0176S) following the manufacturer's protocol. Both products were purified using GFX™ PCR DNA or Gel Band Purification Kit (GE-healthcare). The NEBuilder HiFi DNA Assembly Cloning Kit (NEB, E5520S) was used to assemble the fragments into the Mini_SynMyco_RecET*Bsu* plasmid. Primers A3/A2, B8/B9 and B10/B11 and the procedure used for the assembly of the Mini_SynMyco_RecET*Spho* plasmid are described in Table S1. Two µL of the assembly was transformed into *E. coli* NEB 5-alpha (NEB, C2987H). Transformants were screened after DNA minipreps using NucleoSpin Plasmid kit (Macherey-nagel, 740588.50) and enzymatic digestion. Sanger sequencing was performed for final verification.

Construction of Mini_ p438_RecET*Bsu* and Mini_p438_RecET*Spho*. In order to change the promoters driving the expression of the Rec proteins, PCR amplifications of the *recT* genes were performed using the Mini_ SynMyco_RecET*Bsu* and Mini_SynMyco_RecET*Spho* plasmids as templates and primer C14 or C18 integrating the p438 promoter in pair with primer C15 or C19. PCR amplifications of the *recE* genes were performed using the Mini_ SynMyco_RecET*Bsu* and Mini_SynMyco_RecET*Spho* plasmids as templates and primer C16 or C20 integrating the p438 promoter in pair with primer C17 or C21. The backbone was PCR amplified using the primers C12/C13 and the Mini-SynPuro plasmid as template. Cloning was performed using the aforementioned procedure to generate the plasmids Mini_p438_RecETB*su* and Mini_p438_RecET*Spho*.

Construction of pTempCAT, pTempTET, pTempPI, pTempCAT-F1X0 and pTempTET-F1X0. Plasmids harboring the recombination templates were built using the pBlueScript II K- (Agilent, 212208) as backbone. The pBSK_loxCATlox or pBSK-_loxTetlox plasmid and the *Mgal* gDNA were used as PCR templates. Primers E32/E33, E26/E27, E30/E31 and E28/E29, were respectively used to amplify the backbone vector, the 1 kbp left recombination arms, the 1 kbp right recombination arms and the antibiotic resistance marker. Cloning was performed using the same procedure as for Mini_ SynMyco_RecETB*su* (see above) and resulted in the pTempCAT, pTempTET, pTempPI, pTempCAT-F1X0 and pTempTET-F1X0 recombinant plasmids.

For recombination template production, primers E26/E31, were used to amplify linear template using Q5® High-Fidelity DNA Polymerase (M0491). PCR products were purified using GFX™ PCR DNA and Gel Band Purification Kit (GE-healthcare) and eluted in water. To prepare single strand templates, DNAs resuspended in water were heated at 95°C for 15 min, and then placed immediately on ice before transformation.

Mycoplasma gallisepticum transformation

Mgal cells were grown for 36 h before transformation. At pH 6.2 to 6.5, aliquots of 10 mL were centrifuged during 15 min at 6,000 x *g*, 10°C. Cells were then resuspended with 5 mL of HBSS 1X wash buffer (ThermoFisher, 14065056) and centrifuged 15 min at 6000 x *g*, 10°C. The pellet was resuspended in 250 µL of $CaCl_2$ 0.1 M and incubated for 30 min on ice. Cold $CaCl_2$-incubated cells were gently mixed with plasmid DNA (10 µg) and 10 µg of yeast tRNA (ThermoFisher, AM7119). Then, 2 mL of 40 % polyethylene glycol 6000 (PEG) (Sigma, 11130) dissolved in HBSS 1X buffer were added to the cells. After 2 min of incubation at room temperature, contact with the PEG was stopped by addition of 20 ml of HBSS 1X wash buffer. Mixture was centrifuged 15 min at 6,000 x *g*, 10°C and the cells were resuspended in 1 mL of modified Hayflick medium pre-warmed at 37°C. After 2 hours of incubation at 37°C, cells were plated on Hayflick selective plates. For recombination assays, the incubation at 37°C was extended for two additional hours. During the Cre-Lox experiment, the suicide plasmid pCre-genta containing the Cre recombinase encoding gene under the control of p438 promoter and a gentamicin resistance marker was used. After transformation and incubation at 37°C during 2h, gentamicin was added along with 3 mL of fresh Hayflick medium. After a 96h culture, cells were collected by centrifugation, resuspended in 500 µL of Hayflick medium and spread on Hayflick plates without antibiotic. After 10-15 days at 37°C with 5 % CO_2, colonies were counted, picked and re-suspended in 1 mL of Hayflick for three passages (one passage ~48h).

Localization of transposon by single primer PCR

The genome sites of inserted transposons were determined as described by Rideau et al (Rideau et al., 2019). Briefly, DNA was extracted from 200 µL of transformant culture using genomic DNA extraction kit (Macherey-Nagel, 740952.50). Using primer D21, D22 or D23 and Taq DNA polymerase (NEB), single primer PCR reactions were performed (Buffer 1X, $MgCl_2$ 25 mM, primer D21, D22 or D23 100 µM, dNTPs 10 mM, Taq 0.5 U, gDNA 2.5 µL, H_2O for a total volume of 25 µL). The PCR program includes 20 cycles (95°C for 30 s, 60°C for 30 s and 68°C for 3 min), followed by 30 cycles (95°C for 30 s, 30°C for 30s and 68°C for 2 min), 30 cycles (95°C for 30 s, 60°C for 30 s, 68°C for 2 min) and final amplification at 68°C for 7 min. Amplification products were analyzed by electrophoresis on 1 % agarose gel and purified using GFX™ PCR DNA and Gel Band Purification Kit (GE-Healthcare). After Sanger sequencing (GENEWIZ), the localization of transposons was determined using BLASTn.

PCR screening of Mgal transformants

PCR screening was performed using the Advantage HF 2 PCR Kit (Takara, 639123). Primers used to screen recombinants on the different targets are listed in Table S1.

Whole genome sequencing of recombinant DNA

Genomic DNA of *Mgal* was extracted from a 10 mL culture using the Qiagen Genomic-Tips 100/G kit. Genome sequencing was performed by the Genome Transcriptome Facility of Bordeaux. Long reads and short reads were produced using a GridION (Oxford Nanopore) and a MiSeq (Illumina), respectively. For *Mgal* recombinant clone 3, ONT sequencing generated 31,164 reads and Illumina 1,637, 034 read pairs. Reads were submitted to SRA (accession number PRJNA769398). Analyses were performed on Galaxy (https://usegalaxy.eu/). Mutations were detected after mapping on the *Mgal* S6 genome (CP006916.3). Illumina reads were trimmed using Trimmomatic (V 0.38.1; Sliding Window 10,

20 ; Drop read below Minimal length 250), mapped using BWA-MEM (V 0.7.17.1), Samtools sort (V 2.0.3), MPileup (V 2.1.1), and variants were detected using VarScan mpileup (V 2.4.3.1; Minimum coverage 30, Minimum supporting read 20, Minimum Base quality 30, Minimum variant allele frequency 0,8, Minimum homozygous variants 0,75). Mutations are showed in Table S3. Genome assembly was performed using the following steps: Nanopore reads were filtered using Filter FASTQ (V 1.1.5, Minimum size 45000 bp), assembled using Flye Assembly (V 2.6), polished using 4 rounds of Pilon (1.20.1) combined with Illumina short reads. Assembled genome was compared to the *Mgal* S6 (CP006916.3) reference genome using MAUVE software (Darling et al., 2004). For the *Mgal* (ΔATPase) with a mixed PCR profile, the same procedure was used with 24,443 ONT long-reads and 1,776,420 Illumina short reads pairs.

References

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SI-2. Genes and plasmids sequences

*Nucleic sequence of original Bsu recE (*CAA66538.1)

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*Nucleic sequence of original Bsu recT (*CAA66543.1)

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Nucleic sequence of codon optimized Bsu recE

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Nucleic sequence of plasmid Mini_pSynMyco_recETBsu

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Nucleic sequence of plasmid Mini_p438_recETBsu

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*Nucleic sequence of original S. phoeniceum recE (*SDAV_001191)

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*Nucleic sequence of original S. phoeniceum recT (*SDAV_001194)

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Nucleic sequence of optimized S. phoeniceum recE

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Nucleic sequence of optimized S. phoeniceum recT

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Nucleic sequence of plasmid Mini_pSynMyco_recETSpiro

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Nucleic sequence of plasmid Mini_p438_recETSpiro

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Nucleic sequence of plasmid pTempCAT

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Nucleic sequence of plasmid pTempTET

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Nucleic sequence of plasmid pTempPI

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Nucleic sequence of plasmid pTempCAT-F1X0

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Nucleic sequence of plasmid pTempTET-F1X0

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SI-3. Supplementary results

Targeted small deletion in Mgal genome using the BsuRecET-like system and a tetM-containing recombination template.

In the same experiment, we also evaluated recombination templates containing the *tet(M)* marker (pTempTET plasmid) instead of the *cat* marker (Figure S4A). The experiment was repeated three times. Few transformants were obtained for most recombination template forms (0 to 5, depending on the replicate), with the exception of the ds linear template, which gave no transformants in any of the three replicates (Figure S4C). After PCR screening of recombinants, only mixed profiles were observed, indicating recombination events with a double cross-over had not occurred with these templates (Figure S4B, S4D). The difference in the results obtained with the *cat* and *tet(M)* markers could possibly be explained by the larger size of the *tet(M)* marker (1933 nt compared to 660 nt for *cat*), which could affect the frequency of double cross-over recombination. However, further experiments would be necessary to clarify this point.