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All-in-one construct for genome engineering using Cre-lox technology

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Abstract

Mycoplasma genitalium is an appealing model of a minimal cell and synthetic biology study, and it was one of the first organisms whose genome was fully sequenced and chemically synthesized. Despite its usefulness as a model organism, many genetic tools well established for other microorganisms are not currently available in mycoplasmas. We have developed several vectors to adapt the Cre-lox technology for genome engineering in *M. genitalium*, providing an all-in-one construct that could be also useful to obtain unmarked genetic modifications in many other slow growing microorganisms. This construct contains a modified promoter sequence based in TetR system that exhibits an enhanced control on Cre recombinase expression, virtually abolishing the presence of this recombinase in the absence of inducer. This allows to introduce the *Cre* recombinase gene and the desired genetic modification in a single transformation step. In addition, this inducible promoter may be a very promising tool for a wide range of molecular applications.

Key words: Cre-lox technology, mycoplasmas, genome engineering, TetR-TetO inducible promoters

1. Introduction

Site-specific recombination systems such as those based in Cre/lox from bacteriophage P1,[1,2] Xis/att from bacteriophage λ[3] or FLP/FRT from *Sacharomyces cerivisiae*[4] have been widely used for genome engineering in microorganisms, complex eukaryotic cells and mammals.[5–8] In a first step, the modification including the recombinogenic sequences is introduced in the genome. In second transformation or transfection step, a recombinase coded in a suicide plasmid, a curable plasmid or any other delivery vector catalyzes a recombination between two site-specific recognition sites, thus generating the desired DNA integration, excision or inversion. It is also possible to have the recombinase as a transgene in the genome of the organism of study and introduce the recombinase by genetic crossing. However, this approach has to deal with the recombinase off-site effects and toxicity which have been broadly described.[9–11] Among the different site-specific recombination systems, the Cre-lox shows a remarkable plasticity. Cre recombinase recognizes multiple loxP sites[1,2] allowing the design of variant sites such as lox66, lox2272, lox71, loxN, loxLE or loxRE.[12–16] All these variant lox sites have been tested for compatibility and recombination efficiency.

Mycoplasma genitalium is an emerging sexually transmitted human pathogen and is one of the free-living organisms with the smallest genome.[17] Besides being the causative agent of non-chlamydial and non-gonococcal urethritis,[18] this microorganism with a 580 kb genome is an appealing model of a minimal cell and synthetic biology studies, and it was one of the first organisms whose genome was fully sequenced[19] and chemically synthesized.[20] Multiple studies aiming to understand the cell function were developed with this microorganism, from the determination of non-essential genes in axenic culture[21] to modelling the phenotype prediction from a genotype.[22] This organism is also a model for system biology studies such as the ones performed to its close relative *Mycoplasma pneumoniae*.[23–28]

Many genetic tools well established for other microorganisms are not available in mycoplasmas.[29] Furthermore, most of the tools available, such as transposons, mini-transposons, replicative plasmids and suicide vectors for gene replacement rely on the incorporation of selective markers. These markers may interfere with the cell metabolism, and their use is a limiting factor for genome engineering given the low number of selection markers available. To circumvent these problems, we were aimed to implement the Cre-lox technology in *M. genitalium* by two different approaches. First, we used a suicide vector bearing *Cre* recombinase gene under the control of a constitutive promoter following similar procedures to those used in other organisms.[30] Finally, we implemented a time-saving method based on TetR repressor by engineering the inducible Pxyl/TetO2 promoter previously used in *Mycoplasma agalactie*.[31] This modified promoter tightly controls the expression of the *Cre* recombinase gene, which allows the introduction of both the desired mutation and Cre recombinase in a single step.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Escherichia coli XL-1Blue was grown at 37°C in LB medium or LB agar plates containing 100 μg/ml of ampicillin.[32] *M. genitalium* G37 wild-type strain (NCTC 10195) and its isogenic mutant derivatives (Supplementary Table S1) were grown at 37°C under 5% CO_2 in 75 cm^2 tissue culture flasks containing 20 ml of SP4 medium.[33] *M. genitalium* was transformed as previously described.[34] For mutant colony isolation, transformant cells were grown for 3 weeks in SP4 plates supplemented with 34 μg ml^{-1} chloramphenicol or 2 μg ml^{-1} tetracycline.

2.2. DNA manipulations

DNA manipulations were performed according to procedures in Sambrook & Russell.[35] Genomic DNA from *M. genitalium* was isolated using the E.Z.N.A. Bacterial DNA Kit (Omega BIO-TEK). Plasmid DNAs were obtained using the Fast Plasmid Mini Eppendorf Kit (VWR). Preparative amounts of plasmid DNAs were purified using the GenElute™ HP Plasmid Midiprep Kit (Sigma).

PCR amplification was performed using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) and adjusting the annealing temperature according to the primers used (Supplementary Table S2). Purification of PCR products and DNA digested fragments from agarose gels was performed by using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel) kits. FastDigest restriction enzymes (Thermo Scientific) and T4 DNA ligase (Roche) were used according to manufacturer instructions.

2.3. Plasmids construction

Primers used in this work are in Supplementary Table S2. All the constructs below were screened by restriction analysis and confirmed by Sanger sequencing.

pΔMG_217Cre (Supplementary Fig. S1A) was constructed as follows. First, TetR repressor under spiralin promoter control was amplified by PCR from pMT85-XTST[31] using the primers 5 KpnI Pspi-TetR and 3 SpeI lox 66 PstI TetR, which incorporates the lox66 sequence between SpeI and PstI restriction sites. This PCR fragment was then digested with SpeI and KpnI to generate cohesive ends. Xylose promoter including TetO regions (Pxyl/$TetO_2$) was amplified using the primers 5_KpnI_Pxyl/TetO2 and 3_NcoI_Pxyl/TetO2 from pMT85-XTST. This PCR product was KpnI–NcoI digested and ligated into a Litmus28 vector restricted with the same enzymes. Then, an ExSite mutagenesis PCR[36] was performed using the primers 5_NdeI_TetO2 and 3_NdeI_Litmus28 to modify the inducible promoter by deleting the region from nt 7,200 to nt 7,276 of pMT85-XTST, which includes the RBS sequence, obtaining in this way the Pxyl/$TetO_2$mod promoter cassette (Supplementary Fig. S3). Cre recombinase was amplified by PCR from pSH62[14] using the primers 5_NdeI_Cre and 3_MluI_Cre and digested with NdeI and MluI. Chloramphenicol resistance gene (*cat*) was amplified from pMTnCat[37] using the primers 5_MluI_pmg438_CmR and 3_ApaI_lox66_SalI_CmR, which incorporate the sequence of lox66 between the ApaI and SalI restriction sites. This PCR fragment was then digested with MluI and ApaI. Pxyl/$TetO_2$mod was excised from Litmus28 using the NdeI and MluI sites, mixed with the digested PCR products and ligated into a pBSK previously digested with SpeI and ApaI restriction enzymes. The resulting plasmid was named pBSK-TetR-Pxyl/TetO-Cre-cat. In a second step, a 919-bp PCR fragment containing the upstream homology region (UHR) and 60 bp of the 5′ end of *MG_217* was amplified by using the primers 5_XhoI_SacI_BE_mg217 and 3_SpeI_AscI_BE_mg217. The resulting PCR fragment was digested with XhoI and SpeI. Another 976 bp PCR fragment containing the last 139 bp of *MG_217* and the corresponding downstream homology region (DHR) was amplified using the primers 5_ApaI_BD_mg217 and 3_BamHI_NotI_BD_mg217 and further digested with ApaI and BamHI. Finally, both PCR fragments were mixed with the 2.9 kb SpeI-ApaI fragment from pBSK-TetR-Pxyl/TetO-Cre-cat and ligated to a pBSK previously digested with XhoI and BamHI.

pMTnTc66Cat66 (Supplementary Fig. S1B) was constructed as follows. *Cat* gene was amplified by PCR from pMTnCat using the 5_SpeI_lox66_PstI_pmg438_CmR and 3_ApaI_lox66_SalI_CmR primers, which incorporates the sequence of lox66 between ApaI and SalI the restriction sites. This PCR product was digested with SpeI and ApaI restriction enzymes, mixed with EcoRI-SpeI digested tetracycline resistance gene excised from pMTnTetM438[34] and ligated to the vector pMTn4001[34] previously digested with EcoRI and ApaI.

pGmR (Supplementary Fig. S1C) was constructed as follows. The *aac(6′)-aph(2″)* gene that confers gentamycin resistance was amplified by PCR from pMTnGm[34] using the primers 5_BamHI_pM438-GmRshort and 3_BamHI_GmR_short. The amplification with these primers only include the ORF region (excluding the inverted repeats flanking the *aac(6′)-aph(2″)* gene) and incorporate the *MG_438* promoter at the 5′ end. This PCR product was digested with BamHI digested and ligated to a BamHI-digested pBSK.

pGmRCre (Supplementary Fig. S1D) was constructed as follows. *Cre* recombinase gene was amplified by PCR from pSH62[14] using the primers 3_MluI_Cre and 5_pM438Cre, which incorporates the sequence of *MG_438* promoter. The PCR product was then ligated to vector pGmR previously digested with EcoRV.

2.4. Western immunobloting

Total mycoplasma protein extracts were subjected to electrophoresis through a 12% SDS-polyacrylamide gel, transferred electrophoretically to a PVDF membrane (Merck Millipore) previously activated in methanol and probed with a mouse polyclonal anti-MG217 antibodies[38] at a 1:1,000 dilution. After primary antibody incubation, the membrane was washed three times with PBS-0.05% Tween20 solution, incubated for 1 h at room temperature with a HRP-conjugated anti-mouse IgG (Sigma) at 1:5,000 dilution, washed three times with PBS-0.05% Tween20 and revealed using Luminata Forte (Millipore) in a Imaging System VersaDoc MP 4,000 (Bio-Rad). Then, the same membrane was incubated with a rabbit polyclonal anti-P41 antibodies[39] at

1:1,000 dilution, washed three times with PBS-0.05% Tween20 solution and incubated for 1 h at room temperature with a phosphatase alkaline (PA) conjugated anti-rabbit IgG (Sigma) at 1:5,000 dilution. After secondary antibody incubation, the membrane was washed three times with PBS-0.05% Tween20 and revealed using NBT/BCIP Solution (Roche) according to the manufacturer's instructions.

2.5. Real-time PCR

Real-time PCR (qPCR) reactions were carried out using iTaq Universal SYBR Green Supermix (Bio-rad) in a final volume of 10 µl containing 4 µl of a 10^{-4} dilution of genomic DNAs, 0.5 µM of each primer (Supplementary Table S2) and 5 µl of 2× SYBR Green Supermix. Reactions were run in a CFX384 PCR instrument (Bio-Rad) following a three-step PCR protocol including an initial denaturation at 95°C for 3 min and 40 cycles comprising a denaturing step at 95°C for 20 s, an annealing step at 56°C for 10 s and an extension step at 72°C for 10 s. Fluorescence readings were acquired at the end of each extension step, and the specificity of PCR products was verified by melting curve analyses at the end of each run.

Genome quantification was performed using primers qPCR216F and qPCR216R annealing to *MG_216*, a control gene located at the 5′ end of the target gene *MG_217*. Frequencies of genomes with excised cassettes were derived from qPCR reactions using primers qPCR_217BD_UPS and qPCR_217BE_DWS. Frequencies of non-excised genomes were derived from qPCR reactions using primers qPCR_217BD_UPS and qPCR_CmDWS. *T*-student statistical analyses were carried out using the SPSS software. For each condition, three biological repeats and two technical repeats were performed.

2.6. Cell viability, induction of Cre expression and genomic DNA sequencing

To test Cre induction and expression, cell cultures were grown for 72 h in the presence of 0, 0.1, 1, 10 and 100 ng ml^{-1} tetracycline. Then, cells were recovered and small samples were plated in SP4-agar and SP4-agar supplemented with 34 µg ml^{-1} chloramphenicol, incubated for 3 weeks as described above until colonies could be scored. *T*-student statistical analyses were carried out using the SPSS software. Genomic DNAs were purified from the remaining cells and used as template for qPCR assays. Five colonies from SP4 plates were also recovered and expanded to isolate genomic DNAs. Standard PCR reactions using the primers 5 screening D217 and 3 screening D217 were carried out to detect cassette excisions. All excisions were confirmed by Sanger sequencing performed at the Servei de Genòmica i Bioinformàtica, IBB-Parc de Recerca UAB.

3. Results

3.1. Stability of suicide vectors in *Mycoplasma genitalium*

Since no replicative vectors are available in *M. genitalium*, we tested whether a suicide plasmid delivered by electroporation and bearing a gentamicin resistance gene marker could remain inside the cells for limited periods of time. To this end, the plasmid pGmR was engineered (Supplementary Fig. S1C) and transformed on *M. genitalium* G37 WT cells. These cells were then incubated in the presence of gentamicin for 24, 48, 72, 96 and 168 h and were finally plated in SP4-agar in the absence of antibiotic to quantify the number of viable cfus remaining after the gentamicin treatment (Fig. 1A). About 10^4 colonies were recovered from cells electroporated with pGmR, with no appreciable differences between the different gentamicin incubation

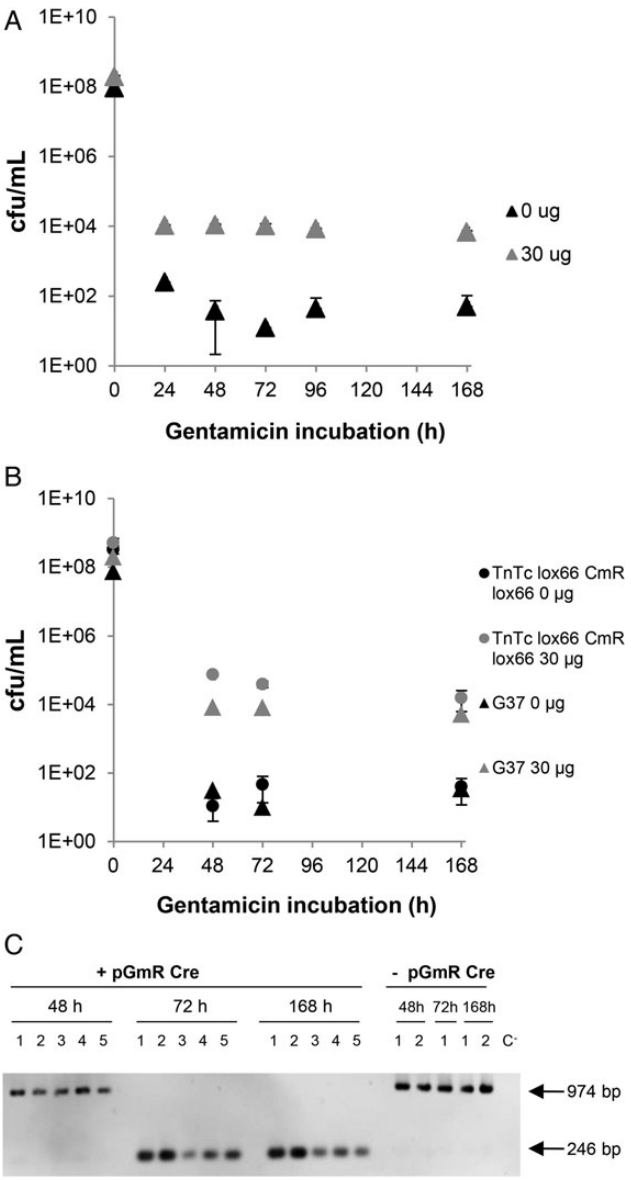

Figure 1. Persistence of a suicide vector inside *M. genitalium* cells and marker gene excision by Cre recombinase. (A) G37 strain was electroporated with pGmR or saline, and cells were incubated with gentamicin 100 µg ml^{-1} for 24, 48, 72, 96 and 168 h. Cells were then plated in SP4-agar medium and colonies were scored. Three independent repeats for each condition were performed, and the mean with its standard error (SE) is plotted. (B) G37 and TcloxCm strains were transformed with of pGmRCre and saline, and cells were incubated in SP4 medium containing gentamicin 100 µg ml^{-1} for 48, 72 and 168 h. Cells were then plated in SP4-agar medium and colonies were scored. Three independent repeats and two technical replicates were performed for each strain and condition, and the mean values with its standard error (SE) are plotted. (C) Colonies from TcloxCm strain electroporated with or without pGmRCre and incubated in SP4 medium containing gentamicin 100 µg ml^{-1} for 48, 72 and 168 h were recovered and expanded to isolate genomic DNAs. Genomic DNAs were amplified by PCR with the primers Seq tet DWS300 and Seq 5 IR-OR-Lox66 3′. A 1,118-bp PCR band is expected from genomic DNAs bearing the *cat* gene and a 246-bp PCR band is expected in those genomes with a excision of this selectable marker.

times tested. In contrast, only a few colonies were recovered from non-electroporated control cells, particularly after the longest gentamicin incubation times. These results suggest that 10^4 cells incorporate

the pGmR plasmid when delivered by electroporation, gentamicin marker remains functional inside these cells at least for several days, and gentamicin is killing most of the plasmid-free cells.

3.2. Evaluation of the Cre-Lox system

To evaluate the feasibility of Cre-lox system in *M. genitalium*, we first obtained a mutant strain including an antibiotic marker gene flanked by two lox66 sequences. To this purpose, we engineered the minitransposon pMTnTc66Cat66 which carries the tetracycline resistance gene and also the chloramphenicol acetyltransferase gene (*cat*) that was flanked by two lox66 sequences (Supplementary Fig. S1B). This minitransposon was transformed on *M. genitalium* cells and several recovered colonies were grown in liquid culture, and their DNAs were extracted and analysed by direct genome sequencing to confirm the presence of the minitransposon and to determine the genomic insertion points. One of the clones recovered was named TcloxCm and selected for further studies. On the other hand, we also constructed the suicide plasmid pGmRCre bearing the *Cre* recombinase gene under the control of *MG_438* constitutive promoter (Supplementary Fig. S1D). This construct was expected to promote the expression of Cre recombinase once introduced to *M. genitalium* cells.

Plasmid pMTnTc66Cat66 was designed to easily detect the *cat* cassette excision in TcloxCm mutants both by plating cells in a selective medium and by PCR amplification. Encouraged by the apparent stability of suicide vectors inside *M. genitalium* cells, we transformed a TcloxCm mutant with and without the pGmRCre plasmid and resulting cells were further incubated for 48, 72 and 168 h in the presence of gentamicin. TcloxCm viable cells remaining after the different gentamicin incubation times were also quantified, with results similar to those obtained when using pGmR plasmid (Fig. 1B). To assess the excision of cat cassette, five clones recovered from each experimental condition were checked by PCR. After 48 h of gentamicin incubation, all recovered clones showed a 1,118-bp PCR band consistent with the presence of the *cat* gene flanked by two lox66 sequences. Fortunately, after 72 and 168 h of gentamicin incubation, all recovered clones exhibited a single 246-bp PCR band consistent with the excision of *cat* cassette (Fig. 1C). These clones were able to grow in SP4 medium, but no growth was observed when they were cultured in SP4 medium supplemented with chloramphenicol, confirming also the absence of *cat* gene. None of the few clones recovered from control TcloxCm cells electroporated in the absence of pGmRCre plasmid and incubated with gentamicin showed the excision of the marker cassette (Fig. 1C). All these results indicate that Cre recombinase expression directed by pGmRCre plasmid has no noticeable toxic effects in *M. genitalium* cells, and this enzyme efficiently excises sequences flanked by lox in this microorganism.

3.3. Cre expression under the control of an inducible promoter

Genome engineering in a minimal cell model like *M. genitalium* would be greatly benefited from the existence of working constructs bearing an inducible *Cre* recombinase gene by providing a simple way to genetically modify cells in a single transformation step. As proof of concept, we chose to obtain an unmarked deletion of *MG_217* gene, which has been previously shown to be easily deleted by homologous gene replacement.[40] First, we designed the plasmid pΔMG_217Cre containing a cassette including the *tetR* repressor gene under the control of spiralin promoter,[31] the *Cre* recombinase gene under the control of a tailored version of the inducible Pxyl/TetO_2 promoter from pMT85-XTST[31] (named Pxyl/TetO_2mod) and *cat* gene as the selectable marker. Pxyl/tetO_2 was engineered to minimize the transcriptional leakage exhibited by this promoter simply by removing the sequence including the ribosome binding site at the 3′ end of this DNA fragment (Supplementary Fig. S3). With this modification, we expected a strong decrease in translation efficiency of Cre recombinase, greatly reducing the expression of this enzyme in the absence of inducer (Supplementary Fig. S4). The whole cassette, which was named Cre-cat cassette, was flanked by two lox66 sequences and enclosed by the same flanking regions previously used to obtain the *MG_217* null mutant[40] (Supplementary Fig. S1A). A double cross-over recombination event between plasmid pΔMG_217Cre and *M. genitalium* genome is expected to promote the deletion from bases 60 to 980 of the *MG_217* gene (82.2% of the coding region) and the replacement of this region by the cassette flanked by lox66 sequences (Fig. 2A).

To obtain the ΔMG_217Cre strain, WT cells were electroporated with pΔMG_217Cre plasmid and transformant colonies were isolated on SP4 medium containing chloramphenicol. Several colonies were expanded, and their genomic DNAs were analysed by PCR to check the presence of the intended replacement (Fig. 2A). All clones recovered showed a 5075-bp band consistent with the deletion of *MG_217* gene and its replacement by Cre-cat cassette (Fig. 2B). To further confirm the deletion of *MG_217* gene, a western blot was performed using the mouse polyclonal anti-MG217 antibodies.[40] A 60 kDa band corresponding to MG217 protein was detected in G37 wild-type strain, but it was not present in any of the recovered clones (Fig. 2C).

Preliminary time-course experiments revealed that most of the Cre-cat cassettes in *M. genitalium* genomes were found excised after 72 h of culture with 10 ng/ml tetracycline (data not shown). Next, several independent clones of ΔMG_217Cre strain were grown for 72 h at different tetracycline concentrations to optimize Cre expression. These induced cultures were then plated in SP4-agar to quantify all viable cells and also in chloramphenicol-SP4-agar plates to quantify the viable cells still bearing the *cat* gene (Fig. 3A). In the presence of the lowest inductor concentration tested 70% of cells could grow in the presence of chloramphenicol, and only around a 10% of cells incubated with the highest tetracycline concentrations were still found resistant to chloramphenicol. The excision of the Cre-cat cassette was also quantified by qPCR (Fig. 3B), obtaining similar results and indicating that >90% of the cell population excised this cassette when incubated with tetracycline at concentrations higher than 1 ng ml^{-1}. No statistically significant differences were found in the frequency of genomes with excised cassettes upon inducing Cre expression at 1, 10 or 100 ng ml^{-1} tetracycline (Fig. 3A and B). The presence of genomes with excised cassettes was finally checked by PCR in five isolated clones recovered after incubating with 10 ng ml^{-1} tetracycline for 72 h. These clones showed a 2195-bp PCR band consistent with the excision of Cre-cat cassette (Fig. 3C). We also determined the sequence of the excised cassettes by direct Sanger sequencing of genomic DNAs from these clones. Sequence analyses demonstrated that all the clones analysed have the same DNA sequence inside the excised cassettes (data not shown). All these data taken together indicate that Cre expression is efficiently induced in the presence of tetracycline, and the amounts of recombinase produced are enough to precisely excise DNA regions flanked by lox66 sequences.

3.4. Stability of lox66 cassettes in long-term uninduced cultures

Some applications using the Cre-lox technology may require a strict control on Cre recombinase expression. To obtain quantitative data about the transcriptional leakage of Pxyl/TetO2mod promoter

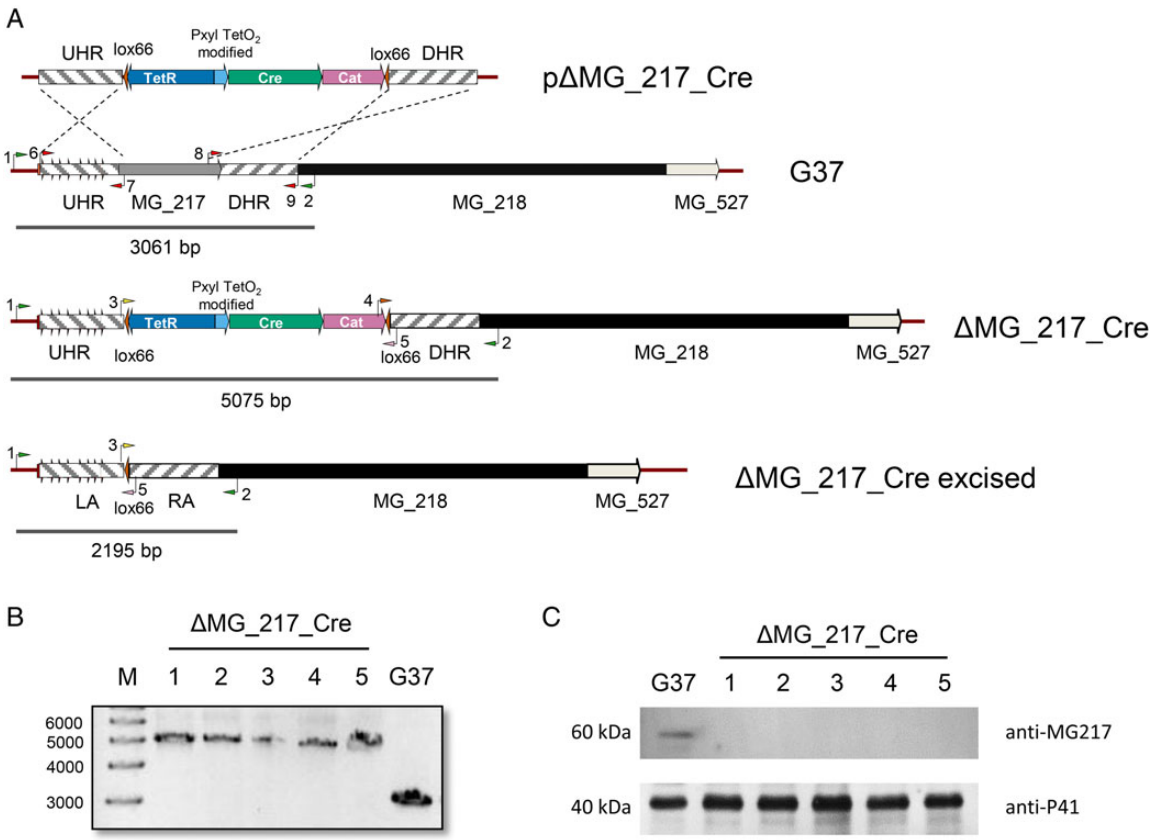

Figure 2. Obtaining a ΔMG_217Cre deletion mutant. (A) Schematic representation of recombination events between the pΔMG_217Cre suicide vector and the *MG_217* gene in G37 strain. PCR bands expected when using 5 screening D217 and 3 screening D217 primers to amplify G37, ΔMG_217_Cre and ΔMG_217_Cre genomic DNAs are also indicated. Primers are shown as coloured arrows in each strain and numbered as follows: (1) 5 screening D217; (2) 3 screening D217; (3) qPCR 217BE_DWS; (4) qPCR CmDWS; (5) qPCR 217BD_UPS; (6) 5 XhoI SacI BE mg217; (7) 3 SpeI AscI BE mg217; (8) 5 ApaI BD mg217 and (9) 3 BamHI NotI BD mg217. (B) Genomic DNAs from G37 and ΔMG_217_Cre strains were amplified by PCR using 5 screening D217 and 3 screening D217 primers (1 and 2). (C) Western Blot analyses of protein extracts from G37 and ΔMG_217_Cre strains. MG_217 protein was detected using a mouse anti-MG_217 polyclonal antibodies and a rabbit polyclonal antiserum anti-P41 was used as a loading control. This figure is available in black and white in print and in colour at *DNA Research* online.

in ΔMG_217Cre strain, the stability of Cre-cat cassette in this strain was tested after 1, 6 and 11 serial culture passages in the absence of inducer and chloramphenicol. Cells from these culture passages were plated in SP4-agar and chloramphenicol-SP4-agar, and no statistically significant differences in the number of viable cells plated in both conditions were observed at the different culture passages (Fig. 4A). A more precise quantification of the genomes with excised Cre-cat cassettes was made by qPCR. Only a 0.53% of the cell genomes isolated after the first culture passage exhibited excised cassettes. The number of excised cassettes slightly increased in the cell genomes isolated after 6 and 11 serial passages, showing frequencies of 1.36 and 4.50%, respectively (Fig. 4B) and indicating that Cre expression in ΔMG_217Cre cells is strongly repressed in the absence of inducer.

4. Discussion

Mycoplasmas are widely used for synthetic biology studies including modified genome transplantations.[21,22,41–43] Although their genomes can be synthesized chemically with the desired mutations and then assembled in yeast cells,[20,43] this is a difficult and time-consuming process that may not apply for high-throughput studies. Furthermore, transposition or gene replacement is nowadays a quicker, easier and cheaper way to obtain genetic modifications. Since Cre-lox technology is not currently available in mycoplasmas, we chose to adapt this friendly user technology as a new genetic tool for working with these microorganisms. Among multiple benefits, the Cre-lox system makes possible the removal of marker genes, allowing multiple rounds of genome editions. In this way, combining Cre technology and either homologous gene replacement or transposition may allow in minimal genome studies to introduce sequential gene deletions after removing the marker genes.

As the first step to implement the Cre-lox technology in *M. genitalium*, we tested the Cre expression when delivered by suicide plasmid[44] and demonstrated that cells transformed by a suicide plasmid remain viable in the presence of a selective medium for several days (Fig. 1A and B). Our data also indicate that >99% of viable cells recovered in the presence of a selective medium were transformed with the suicide plasmid, suggesting that the background of these experiments was very low, being the frequency of spontaneous gentamicin-resistant cells lower than 1% of viable cells. This suicide plasmid was also proved a very effective way to promote the Cre recombinase expression inside mycoplasma cells since all examined clones showed the excision of a lox66 cassette 48 h after plasmid delivering (Fig. 1C). Since no excision of this cassette was detected in the absence of the suicide vector, we can discard the presence of intrinsic Cre-like recombinases in *M. genitalium*.

Once it was demonstrated the Cre recombinase function in *M. genitalium*, we developed an all-in-one vector to introduce in a single

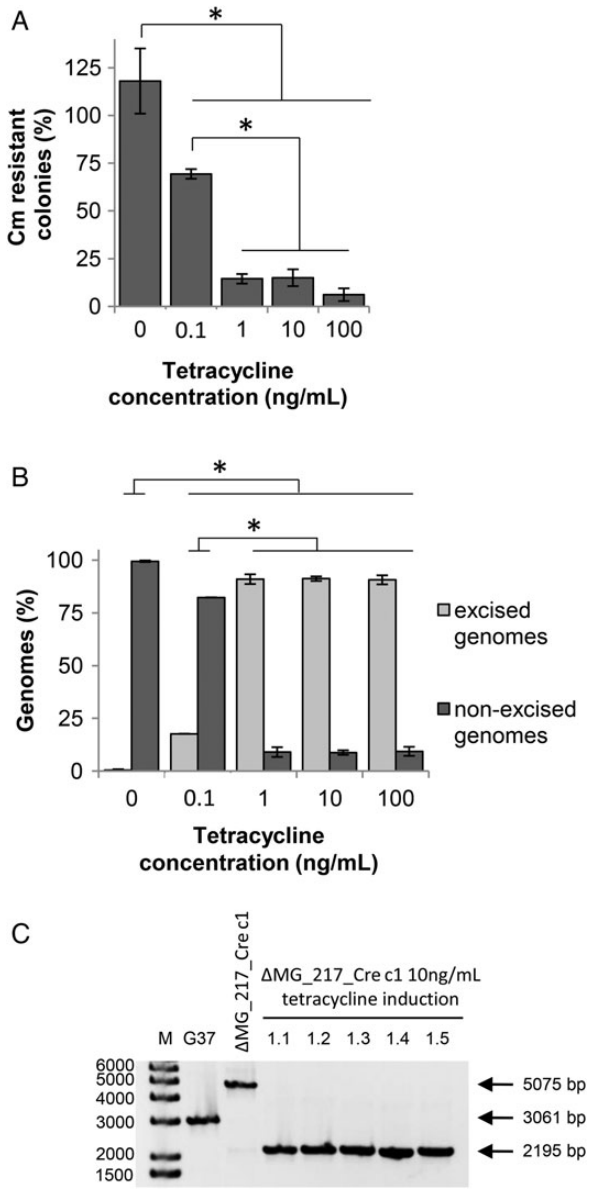

Figure 3. Cre recombinase expression tests. (A) Cell cultures containing several isolates of ΔMG_217Cre strain were grown with 0, 0,1, 1, 10 or 100 ng/ml tetracycline for 72 h and plated in SP4-agar plates and in SP4-agar plates supplemented with 34 μg/ml chloramphenicol. For each condition, three independent biological replicates and four technical repeats were performed. The frequency of chloramphenicol-resistant cfus was plotted for each condition with standard error bars. Asterisk symbol indicated statistical significant differences with a *P*-value <0.05. (B) Genomic DNAs from cell cultures in A were analysed by qPCR to quantify the Cre-cat cassette excision using primers qPCR CmDWS–qPCR 217BD_UPS and qPCR 217BE_DWS–qPCR 217BD_UPS. The frequency of the three biological repeats is plotted with its respective standard error bars. Asterisk symbol indicated statistical significant differences with a *P*-value <0.05. (C) A ΔMG_217_Cre mutant was grown in SP4 medium containing 10 ng m^{-1} tetracycline for 72 h, and cells were plated in SP4-agar medium. Five colonies were recovered and expanded to isolate genomic DNAs. Cre-cat cassette excision was inspected by PCR using primers 5 screening D217 and 3 screening D217. Outline and expected bands are shown in Fig. 2A.

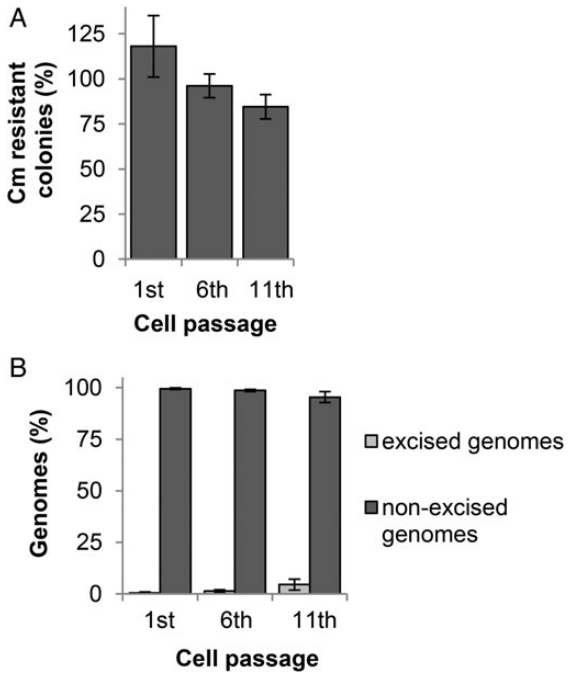

Figure 4. Stability of lox66 cassettes in long-term uninduced cultures. (A) ΔMG_217Cre strain was subjected up to 11 serial culture passages in the absence of tetracycline inducer and chloramphenicol. Each passage was started using 1% of cells of the previous passage. At passages 1, 6 and 11, cell samples were plated in SP4-agar medium and SP4-agar medium supplemented with chloramphenicol 34 μg ml^{-1}. For each passage, three independent biological replicates and four technical repeats were analysed. The frequency of chloramphenicol resistant cfus in the analysed passages is plotted with standard error bars. (B) Genomic DNAs from cell cultures in A were analysed by qPCR to quantify the Cre-catcassette excision using primers qPCR CmDWS–qPCR 217BD_UPS and qPCR 217BE_DWS–qPCR 217BD_UPS.

transformation step both a genome modification and an inducible version *Cre* recombinase gene. This saving-time technology would be especially interesting for those microorganisms with slow growing rates like mycoplasmas. To this end, a Cre-cat cassette was designed to include a Pxyl/TetO2 tetracycline-inducible system[31] which was modified to improve the control on Cre expression. The presence of Cre-cat cassette did not interfere in the gene replacement experiments, and the ΔMG_217Cre mutant was obtained with a transformation efficiency of 2×10^{-7} per viable cell, virtually the same transformation efficiency that was described in a previous report.[40] In addition, this mutant exhibited the same phenotype as previously described (data not shown) suggesting that the presence of Cre-cat cassette is not toxic for mycoplasma cells and no polar effects are produced on *MG_217* flanking genes. We also tested the effect of different tetracycline concentrations when inducing Cre expression by quantifying the frequency of the cassette excisions in genomes of ΔMG_217Cre cells. Cre expression was detected at tetracycline concentrations as low as 0.1 ng ml^{-1}, and the maximum expression rate was reached at concentrations close to 1 ng ml^{-1}, which is 200 times below the minimal inhibitory concentration of this antibiotic in *M. genitalium*.[34] When using Cre-cat cassette in microorganisms very sensitive to tetracycline, this antibiotic might be replaced by anhydrotetracycline, a less toxic tetracycline analogue[45] that can also be used to induce Tet promoters.

Implementing the Cre-lox system in mycoplasmas opens a new horizon in the study of these minimal microorganisms. With this technology a huge variety of genome modifications, such as insertions, deletions, translocations and inversions at specific sites, can be achieved now in mycoplasmas. Once demonstrated that lox66 sequences and

Cre recombinase are functional in *M. genitalium*, it seems feasible to use a combination of lox66 and lox71 sequences. Recombination between these two lox sites generates an inert lox72 sequence, which presents extremely reduced recombination efficiency.[12] Although a huge repertory of non-compatible lox sequences is currently available,[12,13,15,46] the lox72 seems the most suitable sequence to obtain an unlimited number of genetic modifications without interferences between different lox sequences.[12]

The RBS sequence at the 3′ end of Pxyl/TetO2 was removed to obtain a promoter with an improved control on the expression of target genes. The leakage of the modified version of this promoter was tested using a very sensitive assay. This assay was designed to detect and quantify by qPCR the frequency of excised Cre-cat cassettes in serial passage cultures of ΔMG_217Cre cells in the absence of inducer. After 11 serial passages, i.e. more than a month of continuous cell culture, only 4.5% of the total cell population underwent a cassette excision. As only four molecules of Cre recombinase are needed to perform a site-specific recombination,[47] our results suggest that Cre expression is tightly controlled. In addition, if the Cre-cat cassette is wanted to be strictly maintained, the addition chloramphenicol to the culture medium would kill those cells bearing excised the cassettes, resulting in a negligible cell mortality rate at every passage. Finally, the modifications introduced in Pxyl/TetO2 also make this inducible promoter a promising tool for other molecular applications. A tight control of the expressed genes is essential to develop conditional knock-out mutants to study the function of essential genes, the introduction of inducible counterselectable markers and the evaluation of mutant phenotypes after inducing the expression of target genes in time-course experiments.

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Supplementary data

Supplementary data are available at www.dnaresearch.oxfordjournals.org.

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