A useful gene cassette for conditional knock-down of essential genes by targeted promoter replacement in *Mycobacteria*

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A direct method to study essential genes is to construct conditional knock-down mutants by replacement of their native promoter by an inducible one. In *Mycobacteria*, replacement of an essential gene promoter with an anhydrotetracycline inducible one was successfully used but required a multi-step approach. In this work, we describe a gene cassette for the engineering of a conditional knock-down mutant, which allows the one-step targeted replacement of mycobacterial promoters by an anhydrotetracycline-inducible promoter. The functionality of this cassette was successfully tested by engineering conditional *clpP* and *SecA1* mutants of *Mycobacterium smegmatis*.

METHOD SUMMARY

The cKD gene cassette allows the one-step replacement of selected promoters in *Mycobacteria*, including those of essential genes, using the recombineering method. This hygromycin-resistant cassette contains a mycobacterial-adapted TetON promoter system and the Shine-Dalgarno sequence of the *dnaK* gene, allowing the anhydrotetracycline-dependent expression of a gene of interest. In this work, the cKD cassette was used to replace promoters of both *Mycobacterium smegmatis* *clpP* and *secA1* essential genes.

Various strategies were developed to study essential genes in *Mycobacterium*, including conditional expression of antisense RNA [1], CRISPRi-mediated transcriptional repression [2], insertion of a second copy of the essential gene under the control of an inducible promoter followed by deletion of the native copy [3], or replacement of the essential gene native promoter with an inducible one, such as the anhydrotetracycline (ATc)-inducible promoter $P_{myc1}tetO$ locked by the TetR10 repressor. This last method was achieved by recombination of a DNA fragment from either suicide plasmids [4] or temperature-sensitive mycobacteriophages [5]. In this study, we developed and used a new gene cassette, named conditional knock-down (cKD), which allows direct replacement of native mycobacterial promoters by an ATc inducible one, using homologous recombination of linear DNA fragment [6].

The cassette encodes a hygromycin-resistance gene and a TetON system composed of the TetR10 repressor and the $P_{myc1}tetO$ promoter (Figure 1A). This promoter contains 2 *tetO* surrounding the -35 sequence allowing efficient repression by TetR10 in absence of ATc [7,8]. This

repression is relieved by ATc that binds to TetR10. Notably, the TetR10 repressor used here, namely $B_{syn1-207}$, was previously adapted for mycobacterial codon usage to allow a more efficient expression and regulation of the $P_{myc1}tetO$ promoter [9]. Since the Shine-Dalgarno (SD) sequences are absent (leaderless mRNA) or difficult to determine in the GC-rich genome of *Mycobacteria* [10], we have included a part of 5'-UTR region of the *dnaK* gene of *M. tuberculosis*, containing the SD and the six nucleotides present between the SD and the initiation codon, at the 3' end of the cassette (Figure 1A). The cassette can be recombined directly upstream of the start codon of the gene of interest, using the recombineering method [6].

The 2678-nucleotide long cKD cassette was synthesized and cloned as an EcoRV fragment in pUC57 plasmid vector (Genscript, NJ, USA). The sequence is accessible at GenBank, accession number MG674207. The designed cassette was next used for promoter replacement of the *M. smegmatis* *clpP1/P2* operon encoding the AAA+ proteolytic subunit ClpP1/P2 [11]. We expected *clpP1* and *clpP2* to be essential [11]. The cassette was amplified as

a linear DNA fragment by a two-step fusion PCR in order to add 500-bp flanking regions of homology with the chromosomal region to recombine at each end (Figure 1B). To do so, three fragments corresponding to (i) 500 bp of the upstream genomic region (for *clpP1P2*:F1_PclpP1P2_For: 5'-caacgcgatc-cacggcctcg-3' and F1_PclpP1P2_Rev: 5'-ctgggccttctgtttattacggggcgctgcg-3'), (ii) the cKD cassette (for *clpP1P2*:F3_PclpP1P2_For: 5'-gcgaacgcgccccgtaataaacgaaggccag-3' and F3_PclpP1P2_Rev: 5'-gtgccacgcgtgctcagtcacgggtaatcctcctgagtg-3') and (iii) the first 500 bp of the gene of interest (for *clpP1P2*:F2_PclpP1P2_For: 5'-cacctcaggaggattcaccgtgactgacatgcgtggcac-3' and F2_PclpP1P2_Rev: 5'-gtccgaatcgccctcgatgc-3') were amplified by PCR and fused in a second PCR reaction, using F1_PclpP1P2_For and F2_PclpP1P2_Rev primers.

The resulting linear DNA was electroporated into *M. smegmatis* MC²155 strain containing plasmid pJV53K (kanR), which expresses the gp60 and gp61 recombinases ensuring an elevated level of homologous recombination of the cassette DNA into the chromosome [6]. *M. smegmatis* hygromycin-resistant clones were selected, the position of the insert into the genome was PCR verified using external primers and correct clones were subjected to successive cycles of growth in liquid 7H9 medium without kanamycin in order to cure the plasmid pJV53K, as described in [12].

The resulting mutant MC²155 *Ptet-clpP1/P2* was grown at 37°C in 7H9 medium supplemented with 0.05% Tween 20, 50 µg/mL hygromycin and 20 ng/mL of ATc inducer. At mid-log phase, the culture

Figure 1. Conditional knock-down mutants by promoter replacement using the cKD cassette. (A)

The cKD cassette (2,7 KB) encodes the TetR10 repressor (dark blue) under the control of the P_{imyc} promoter, which regulates the $P_{myc1}tetO$ promoter. The upstream UTR region of the *dnaK* gene covering the Shine-Dalgarno and the six downstream nucleotides (SDK; orange) was placed at the 3' end of the cassette allowing its insertion just before the start codon of the gene of interest (red [Figure 1B]). This cassette also encodes the hygromycin resistance gene (*hph*; light blue) followed by a transcriptional terminator (*ter*; dark grey) in order to limit polarity on downstream genes. **(B)** Using fusion PCR, the cKD cassette is flanked by two regions of homology of about 500 nucleotides long (in green) corresponding to the genomic region to recombine. The resulting linear DNA substrate is transformed into the *M. smegmatis* MC²155 containing the recombineering plasmid pJV53K, which promotes the subsequent recombination of the flanked cKD into *M. smegmatis* chromosome.

Figure 2. *M. smegmatis* MC²155 Ptet-clpP1/P2 conditional knock-down mutant. (A) Both MC²155 and MC²155 Ptet-clpP1/P2 were grown at 37°C to mid-log phase, serially diluted and spotted on 7H10 plates containing hygromycin with (+) or without (-) ATc as indicated. Plates were incubated 3 days at 37°C. (B) Western blot analysis of ClpP2 depletion. MC²155 Ptet-clpP1/P2 was grown for 3 days in the presence of 20 ng/mL ATc inducer, washed three times, diluted in fresh medium with (+) or without (-) inducer and grown to mid-log phase. Whole cell extracts were prepared and the presence of endogenous ClpP2 or RpoB (the RNA polymerase β subunit was used as loading control) was visualized by western blot using anti-ClpP2 (a kind gift of David Dougan; La Trobe University, Australia) and anti-RpoB (Neoclone W0023) antibodies. Whole cell extract of MC²155 wild type (MC²155) was used as a control. ATc: Anhydrotetracycline.

was serially diluted and spotted on agar plates with 7H10 growth medium containing hygromycin with or without ATc (Figure 2A). After 3 days at 37°C we could only detect a few colonies growing at dilution 10⁻¹, likely extragenous suppressors, when MC²155

Ptet-clpP1/P2 was plated without inducer, indicating that repression of clpP1/P2 in the absence of inducer leads to a severe growth inhibition. In sharp contrast, MC²155 Ptet-clpP1/P2 growth in the presence of inducer was comparable to that of MC²155

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wild type (Figure 2A). We next investigated ClpP2 expression in the conditional mutant by western blot analysis. As shown in Figure 2B, ClpP2 was not detected after 24 h of depletion in the absence of inducer, indicating that the gene was efficiently repressed. By contrast, ClpP2 level in the mutant was comparable to that of the wild-type strain in the presence of inducer (Figure 2B). Note that in addition to *clpP1/2*, this cassette was successfully used to replace the promoter of the *M. smegmatis* *secA1*, an essential gene encoding the Sec translocation motor (Supplementary Figure 1). In agreement with the efficiency of the recombineering procedure [6], all of the recombinant strains tested for both *clpP1/2* and *secA1* (ten in total) had the insertion at the correct position in the chromosome. Taken together, these results indicate that the newly developed cKD cassette is an efficient tool which allows the one-step engineering of *M. smegmatis* conditional knock-down mutants.

Author contributions

PT and MC performed experiments. MC and PB performed editing of the manuscript. PG acquired funding. PT and PG contributed to the writing of the manuscript. All the authors contributed to conception and design, analysis and interpretation of data.

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Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: <https://www.future-science.com/doi/suppl/10.2144/btn-2018-0074>

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