

Promoter mutagenesis for fine-tuning expression of essential genes in *Mycobacterium tuberculosis*

Francesca Boldrin,^{1,§} Giulia Degiacomi,^{1,§}
Agnese Serafini,^{1,†} Gaëlle S. Kolly,^{2,‡}
Marcello Ventura,¹ Claudia Sala,² Roberta Provvedi,³
Giorgio Palù,¹ Stewart T. Cole² and
Riccardo Manganeli^{1,*}

¹Department of Molecular Medicine, University of Padova, 35121 Padova, Italy.

²Ecole Polytechnique Fédérale de Lausanne, Global Health Institute, Station 19, 1015 Lausanne, Switzerland.

³Department of Biology, University of Padova, 35121 Padova, Italy.

Summary

A range of regulated gene expression systems has been developed for mycobacteria in the last few years to facilitate the study of essential genes, validate novel drug targets and evaluate their vulnerability. Among these, the TetR/Pip-OFF repressible promoter system was successfully used in several mycobacterial species both *in vitro* and *in vivo*. In the first version of the system, the repressible promoter was P_{ptr}, a strong Pip-repressible promoter of *Streptomyces pristinaespiralis*, which might hamper effective down-regulation of genes with a low basal expression level. Here, we report an enhanced system that allows more effective control of genes expressed at low level. To this end, we subjected P_{ptr} to targeted mutagenesis and produced 16 different promoters with different strength. Three of them, weaker than the wild-type promoter, were selected and characterized showing that they can indeed improve the performances of TetR/Pip-OFF repressible system both *in vitro* and *in vivo* increasing its stringency. Finally, we used

these promoters to construct a series of bacterial biosensors with different sensitivity to DprE1 inhibitors and developed a whole-cell screening assay to identify inhibitors of this enzyme.

Introduction

Tightly regulated gene expression systems are powerful tools widely used to study essential genes, validate drug targets and evaluate their vulnerability (Schnappinger, 2015). In recent years, several such systems were developed for mycobacteria [reviewed in (Schnappinger and Ehr, 2014; Choudhary *et al.*, 2016)]. The most widely used are those based on the TetR repressor (Carroll *et al.*, 2005; Ehr *et al.*, 2005), but recently other systems based on CRISPR interference (Choudhary *et al.*, 2015; Singh *et al.*, 2016), on the theophylline riboswitch (Seeliger *et al.*, 2012) or on dual-control switches which combine transcriptional regulation and regulated proteolysis (Kim *et al.*, 2011, 2013) were shown to be highly effective.

In previous work, we reported the development of a repressible promoter system (TetR/Pip-OFF) based on two chromosomally encoded repressors and the tunable promoter P_{ptr} (Boldrin *et al.*, 2010). In this system, entirely integrated in single copy in the mycobacterial genome, the gene encoding the tetracycline-responsive repressor TetR is constitutively expressed, while the gene coding for the pristinamycin-responsive repressor Pip is expressed from a TetR-dependent promoter, and the gene of interest is expressed from P_{ptr}, a strong promoter repressible by Pip. In the absence of ATc, TetR represses *pip* expression and the target gene is expressed; in the presence of ATc, TetR releases the promoter of the Pip gene resulting in repression of P_{ptr}. The system can be further modulated by the addition of pristinamycin I, which can alleviate P_{ptr} repression by Pip (Boldrin *et al.*, 2010).

Since then, the TetR/Pip-OFF system has been successfully used by several groups to study essential genes and validate drug targets *in vitro* and *in vivo* in different mycobacterial species (Serafini *et al.*, 2009, 2013; Cortes *et al.*, 2011; Di Luca *et al.*, 2012; Mondino *et al.*, 2013; Ventura *et al.*, 2013; Ahmed *et al.*, 2014, 2016; Bazet Lyonnet *et al.*, 2014; Bhowmick *et al.*, 2014; Boldrin *et al.*, 2014; Kolly *et al.*, 2014a,b; Pandey and Rodriguez, 2014; Verma and Chatterji, 2014; Gola *et al.*,

Received 18 May, 2017; revised 5 September, 2017; accepted 25 September, 2017.

*For correspondence. E-mail riccardo.manganeli@unipd.it; Tel. +39 8272366; Fax +39 827 2355.

†Present address: Mycobacterial Metabolism and Antibiotic Research Laboratory, The Francis Crick Institute, 1 Midland Road, London NW1 1AT, UK.

‡Present address: Institut Pasteur de Lille, 1, rue du professeur Calmette, 59019 Lille Cedex, France.

§These two authors contributed equally to this work.

Microbial Biotechnology (2018) 11(1), 238–247
doi:10.1111/1751-7915.12875

Funding Information

This work was supported by the European Community's Seventh Framework Programme under grant agreement 260872.

© 2017 The Authors. *Microbial Biotechnology* published by John Wiley & Sons Ltd and Society for Applied Microbiology.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

2015; Gupta *et al.*, 2015; Mori *et al.*, 2015; Hu *et al.*, 2016; Degiacomi *et al.*, 2017). This broad experience taught us that the main drawback of this otherwise very successful system was the strength of the P_{ptr} promoter, which causes overexpression of the gene of interest, when this is physiologically expressed at low level, with resulting accumulation of its product. As a consequence of such accumulation, the effect of gene repression in these cases was often visible only after a number of serial passages of the bacterial culture in the presence of the repressor (ATc). This step was needed to titrate down the protein product. Moreover, we failed to obtain a phenotype in conditional mutants of genes expressed at very low levels (such as transcriptional regulators), probably because even when repressed their residual expression, due to promoter leakage, produced sufficient target protein to effect its physiological function (unpublished data). In this manuscript, we describe the construction and characterization of a series of P_{ptr} promoter mutants of different strength and show that these promoters can be used in the context of the Tet/Pip-OFF repressible system thus overcoming the problems outlined above.

Results

Promoter mutagenesis

The TetR/Pip-OFF repressible promoter system has been successfully used to construct several conditional mutants in *Mycobacterium tuberculosis* (Boldrin *et al.*, 2010). However, the high strength of the P_{ptr} promoter, on which the system is based, may represent a problem when the target gene is physiologically expressed from a weak promoter. To solve this problem, we designed a

mutagenic strategy to weaken the promoter by introducing point mutations in the -10 or -35 consensus sequences or by modifying the length of the spacer region. Using PCR, we obtained eight promoters with mutations in the -10 region, six promoters with mutations in the -35 region and two promoters where the length of the spacer region was modified (Fig. 1). Mutated and wt promoters were cloned upstream of a promoterless *egfp* gene in the integrative plasmid pFRA61, containing the TetR/Pip-OFF system (Boldrin *et al.*, 2010). The resulting plasmids were transformed in both *Mycobacterium smegmatis* mc²155 and in *M. tuberculosis* H37Rv (Table S1).

Characterization of the mutant promoters

To evaluate promoter strength, *M. tuberculosis* and *M. smegmatis* strains harbouring the integrative plasmids containing the *egfp* transcriptional fusions were grown to exponential phase, and their fluorescence was measured using a fluorometer. Results clearly showed that the mutations resulted in promoters with a wide range of strength and that almost all of the new promoters behaved similarly in *M. tuberculosis* and in *M. smegmatis* (Fig. 2). Interestingly, mutations in the spacer region and in the -35 consensus sequence always caused strong attenuation of the promoter, whereas mutations in the -10 consensus sequence resulted in partial attenuation or even an increase in promoter activity (P_{ptr9} , P_{ptr13} , P_{ptr14}). Some promoters showed an activity which was barely over the background. To determine whether their activity was totally abrogated or so low as to be undetectable when present in a single chromosomal copy, some of them were subcloned together with the *egfp*

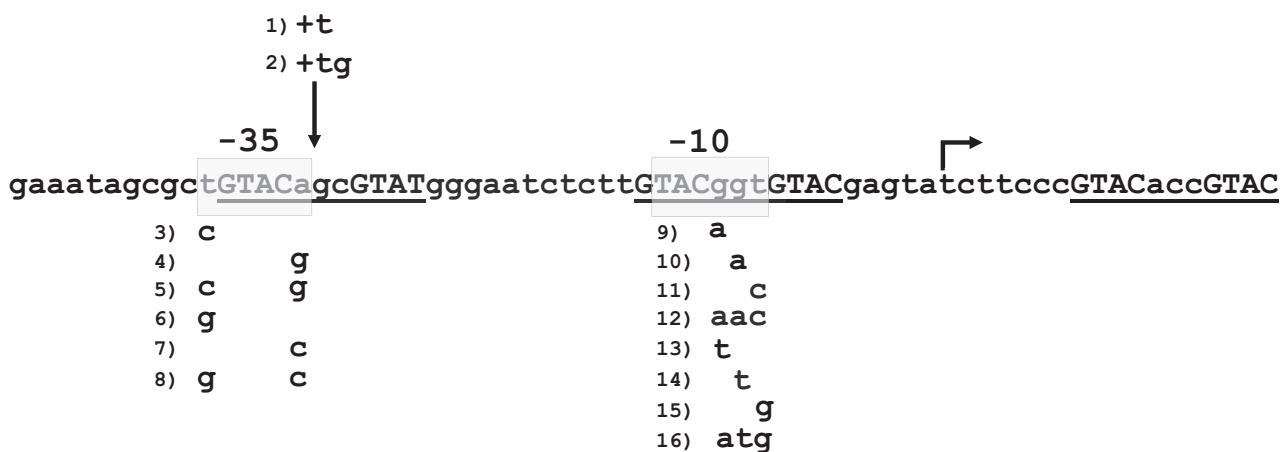


Fig. 1. Mutations introduced in the P_{ptr} promoter. Putative -10 and -35 are boxed, while the three Pip-binding regions are underlined. Conserved repeats within the Pip-binding regions are in capital letters. Mutations are numbered (1–16), and the nucleotides replacing the original are shown below the wt sequence. Mutations 1 and 2 are insertions; the nucleotides replacing the original are shown below the original. The transcriptional start site is indicated by an arrow.

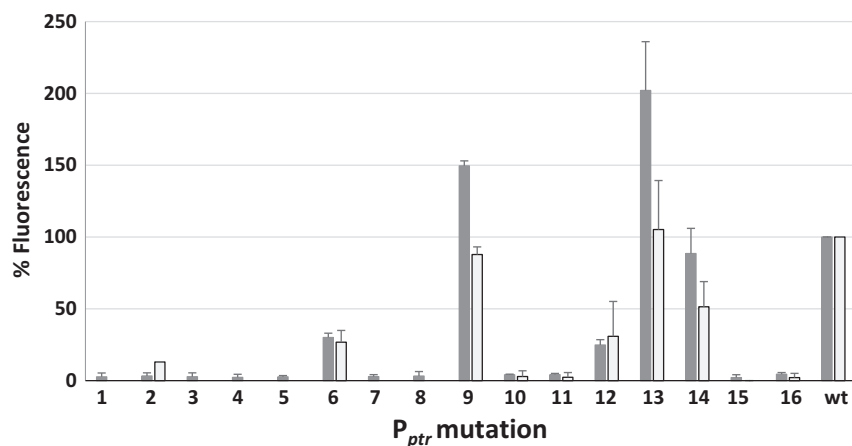


Fig. 2. Relative fluorescence of *Mycobacterium tuberculosis* (dark grey) and *M. smegmatis* (light grey) strains harbouring integrative plasmids encoding the *egfp* gene downstream of the different P_{ptr} promoter mutants (1–16). Fluorescence of the strain expressing *egfp* from wt P_{ptr} was considered as 100%. For unknown reasons, we could not introduce the plasmids containing the P_{ptr} mutations 5 and 8 in *M. smegmatis*.

gene in a replicative plasmid and again introduced in *M. tuberculosis*. Figure 3 reports the relative fluorescence of the resulting strains, indeed confirming that in these conditions their activity was detectable.

Starting from these data, we chose three promoters with different relative strength for further characterization: P_{ptr11} , P_{ptr12} and P_{ptr14} . To define whether these promoters were still repressible by Pip, the *M. tuberculosis* strains containing transcriptional fusions to *egfp* were grown to exponential phase with or without ATc 500 ng ml⁻¹, and fluorescence was measured. As shown in Fig. 4, all of them were still repressible and suitable for use in the TetR/Pip-OFF system.

Construction and characterization of conditional mutants expressing *dprE1* from promoters with different strength

Previously, we reported the construction and characterization of a *dprE1* conditional mutant based on the TetR/

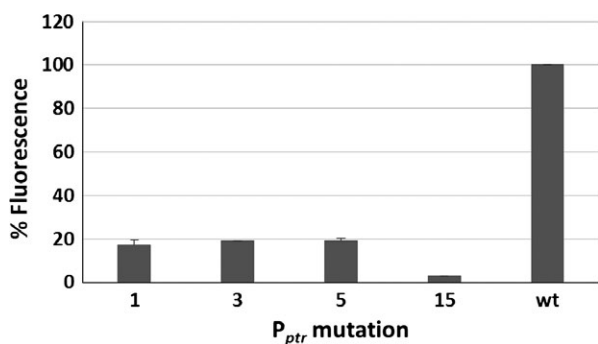


Fig. 3. Relative fluorescence of *Mycobacterium tuberculosis* strains harbouring replicative plasmids encoding the *egfp* gene downstream P_{ptr} promoters shown to have undetectable activity in Fig. 2. Fluorescence of the strain expressing *egfp* from wt P_{ptr} was considered as 100%.

Pip-OFF system (Kolly *et al.*, 2014a). We demonstrated that *dprE1* is an essential gene, as its repression upon exposure to ATc resulted in growth arrest. However, the strain had to be subcultured several times in the presence of ATc to deplete the gene product effectively. This was due to the higher strength of P_{ptr} with respect to the physiological promoter of *dprE1*, which resulted in its overexpression in the conditional mutant (Kolly *et al.*, 2014a). To rectify this, we constructed three conditional mutants expressing *dprE1* from P_{ptr11} , P_{ptr12} , and P_{ptr14} (TB386, TB387, and TB388 respectively; Table 1). The three conditional mutants were grown in parallel with the original conditional mutant TB110 expressing *dprE1* from the wt P_{ptr} in the presence of ATc 500 ng ml⁻¹. As shown in Table 2, the number of passages needed to observe the growth arrest was directly correlated to the strength of the promoter.

The protein encoded by *dprE1* is the target of benzothiazinone (BTZ), a new antimycobacterial compound currently under development (Makarov *et al.*, 2009, 2014; Piton *et al.*, 2017). As the level of DprE1 has been

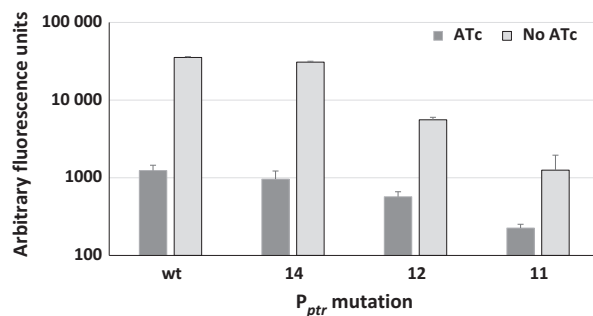


Fig. 4. Fluorescence of *Mycobacterium tuberculosis* strains harbouring integrative plasmids encoding the *egfp* gene downstream of different P_{ptr} promoter mutants grown with or without ATc.

Table 1. *dprE1* conditional mutants based on the TetR/Pip-OFF or the Pip-ON systems.

| Mutation | PlasmidTetR/ Pip-OFF | Strain | Plasmid Pip-ON | Strain |
|----------|-------------------------|--------|-------------------|--------|
| 11 | pFRA184 | TB386 | pFRA212 | TB434 |
| 12 | pFRA186 | TB387 | pFRA213 | TB435 |
| 14 | pFRA188 | TB388 | pFRA214 | TB436 |

Table 2. Passages in fresh ATc needed to stop the growth of *dprE1* conditional mutants.

| Strains | P _{ptr} strength ^a (%) | Passages in ATc 500 ng ml ^{-1b} |
|---------|--|--|
| TB386 | 4 | 3 |
| TB387 | 25 | 4 |
| TB388 | 89 | 7 |
| TB110 | 100 | > 7 |

a. Referred to P_{ptr} wt which was considered as 100%.

b. Strains were grown in standing condition with or without ATc. Every 72–96 h, bacteria were diluted into fresh media containing ATc.

shown to correlate with the sensitivity to BTZ, we measured the sensitivity of the new conditional mutants to this molecule: as illustrated in Fig. 5, there was a clear correlation between the level of promoter activity and the MIC to BTZ. The strain expressing *dprE1* from P_{ptr12} (TB387) showed the same MIC for BTZ as H37Rv, suggesting that in these two strains, *dprE1* is expressed at a similar level and that P_{ptr12} and the physiological promoter of *dprE1* are similarly active (Table 3). To confirm this hypothesis, the *dprE1* natural promoter was cloned upstream of a promoterless *egfp* gene in the same integrative plasmid used to evaluate the P_{ptr} mutants. The

resulting plasmid was introduced into *M. tuberculosis* to obtain TB419, whose fluorescence was compared with that of the *M. tuberculosis* strain expressing *egfp* from P_{ptr11}, P_{ptr12}, or P_{ptr14}. Results in Fig. 6 confirmed that the strength of the *dprE1* promoter was comparable to that of P_{ptr12}.

Finally, the *M. tuberculosis* conditional mutants expressing *dprE1* from wt P_{ptr} (TB110) or from P_{ptr11} (TB386) were used to infect two groups of mice, one of which was treated with doxycycline. As visible in Fig. 7, at day 15 post-infection, mice infected with TB110 showed a higher bacterial burden than those infected with TB386 suggesting that expression of *dprE1* from P_{ptr11} results in suboptimal intracellular DprE1 concentration for *in vivo* growth. Moreover, 15 days after infection treatment with doxycycline resulted in a 10-fold decrease in the bacterial burden of TB110 and in TB386 levels decreasing below the limit of detection, thus demonstrating the essentiality of *dprE1* during infection.

Development of a whole-cell screening assay to identify inhibitors of DprE1

The development of whole-cell screenings based on bacterial biosensors sensitized to inhibitors of specific targets represents a novel strategy to identify new antibacterial drugs (Hughes and Karlen, 2014; Schnappinger, 2015). As DprE1 has been shown to be a valid and vulnerable target for *M. tuberculosis* (Makarov *et al.*, 2009), we decided to use our set of promoters to develop an assay to identify molecules able to inhibit its activity. To this end, we constructed a set of *dprE1* conditional mutants in which *dprE1* was controlled by P_{ptr11}, P_{ptr12}, or P_{ptr14} under the control of the Pip-ON inducible

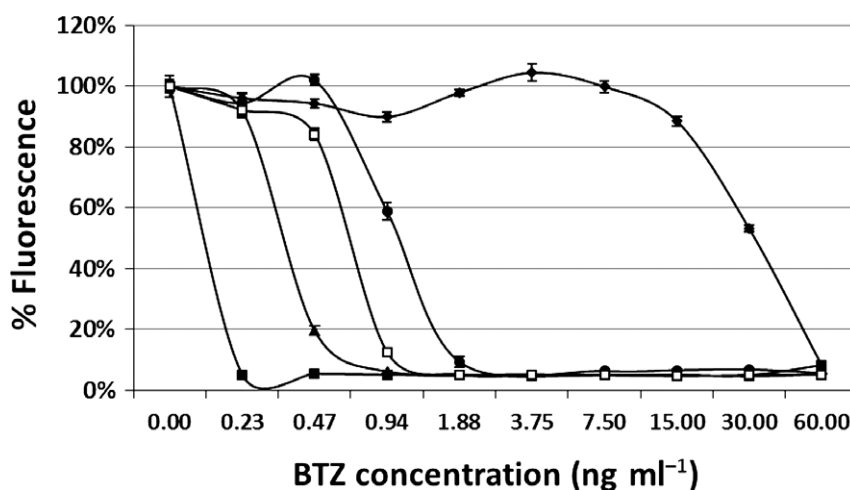


Fig. 5. Resazurin microplate assay (REMA) to determine the BTZ sensitivity of *Mycobacterium tuberculosis* conditional mutants expressing *dprE1* from different promoters. Filled squares, TB386 (P_{ptr11}); empty squares, H37Rv; triangles, TB387 (P_{ptr12}); circles, TB388 (P_{ptr14}); diamonds, TB110 (P_{ptr}).

system (Forti *et al.*, 2009). These plasmids were then introduced into the conditional *dprE1* mutant TB110 by plasmid switching (Pashley and Parish, 2003) obtaining the strains TB434, TB435 and TB436 respectively (Table 1). The resulting strains were pristinamycin-dependent, as their only functional copy of *dprE1* was under transcriptional control of one of the P_{ptr} promoters, which are not active in the absence of pristinamycin due to Pip-mediated repression (Fig. S1).

Finally, using REMA, we determined the MIC to BTZ of these three strains grown in the presence of different concentrations of pristinamycin. As shown in Table 4 and Fig. 8, the MIC was proportional to the strength of

Table 3. BTZ MIC₈₅ obtained in the experiment shown in Fig. 5 compared with promoter strength.

| Strain | Promoter strength (%) | MIC ₈₅ (ng ml ⁻¹) |
|--------|-----------------------|--|
| TB386 | 4 | 0.23 |
| TB387 | 25 | 0.94 |
| TB388 | 89 | 1.88 |
| TB110 | 100 | 60 |

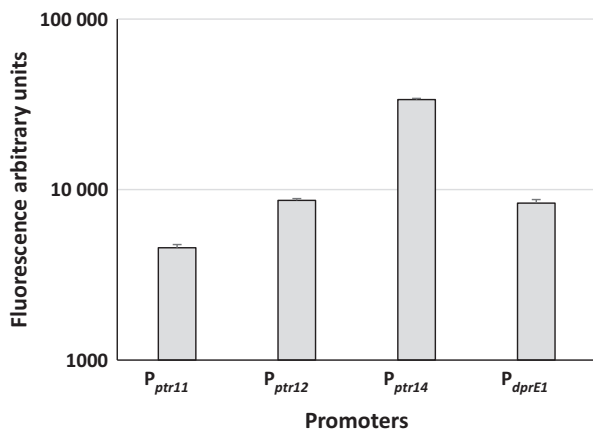


Fig. 6. Fluorescence of *Mycobacterium tuberculosis* strains harbouring integrative plasmids encoding the *egfp* gene downstream of the different P_{ptr} promoter mutants and of the *dprE1* promoter.

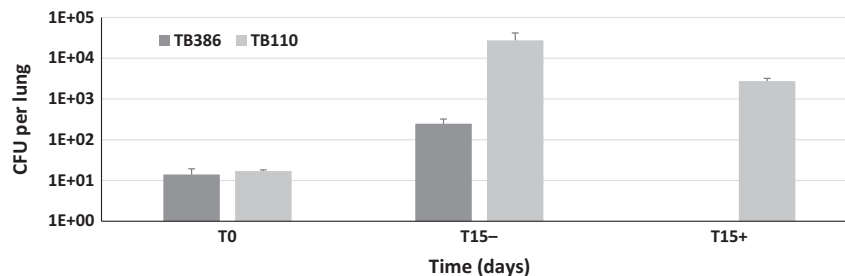


Fig. 7. Bacterial burden of TB110 and TB386 in mouse lungs. One group of mice was treated with doxycycline (+) from the beginning of the experiment. Mice were sacrificed at time 0 and 15 days after infection.

the promoter transcribing *dprE1* and to the concentration of pristinamycin with a range going from 0.12 ng ml⁻¹ (TB434, 25 ng ml⁻¹ pristinamycin) to 15 ng ml⁻¹ (TB436, 100 ng ml⁻¹ pristinamycin), while the MIC of H37Rv was 1.88 ng ml⁻¹.

Discussion

The main aim of this work was to mutagenize the repressible promoter P_{ptr} to increase the tunable range of the TetR/Pip-OFF repressible system (Boldrin *et al.*, 2010). The *pip* promoter encloses three Pip operators each containing two directly repeated 4 bp palindromes separated by a 3 bp spacer. Two of these operators overlap the putative -10 and -35 consensus sequences, while the third is located 10 nucleotides after the *ptr* transcriptional start site (Fig. 1; Blanc *et al.*, 1995). As our aim was to change P_{ptr} strength without interfering with its regulation by the pristinamycin-dependent transcriptional repressor Pip, mutations were introduced either before the operators or within the spacers (Fig. 1). Two insertions increased the distance between the putative -10 and -35 sequences (mutations 1 and 2), and most of the changes in the putative -35 sequence (mutations 3–8) resulted in very strong attenuation of transcription. However, mutations in the putative -10 sequence showed a wide variation of transcriptional activity: some of them, similarly to mutations in the -35 sequence, caused a dramatic decrease in activity (mutations 10, 11, 15 and 16), others (mutations 12 and 14) resulted in decreased, but still appreciable level of activity, or in an increased level of activity (mutations 9 and 13). It should be noted; however, that only mutations of the first and last base of the -35 hexamer were designed and tested, given the overlap of this sequence with the first Pip operator (Fig. 1). Mycobacterial promoters usually have well-conserved -10 regions, while -35 are less conserved and hardly recognizable, probably because of the high number of sigma factors operating in these bacteria (Bashyam *et al.*, 1996; Newton-Foot and Gey van Pittius, 2013; Manganelli, 2014). Moreover, several

Table 4. MIC (ng ml⁻¹) to BTZ of H37Rv and different *Mycobacterium tuberculosis dprE1* conditional mutants upon the addition of different amounts of pristinamycin.

| Strain | Pristinamycin concentration (ng ml ⁻¹) | | | | |
|--------|--|------|------|------|------|
| | 0 | 25 | 50 | 75 | 100 |
| H37Rv | 1.88 | | | | |
| TB434 | | 0.12 | 0.12 | 0.23 | 0.23 |
| TB435 | | 0.23 | 0.47 | 0.47 | 0.47 |
| TB436 | | 3.75 | 7.5 | 7.5 | 15 |

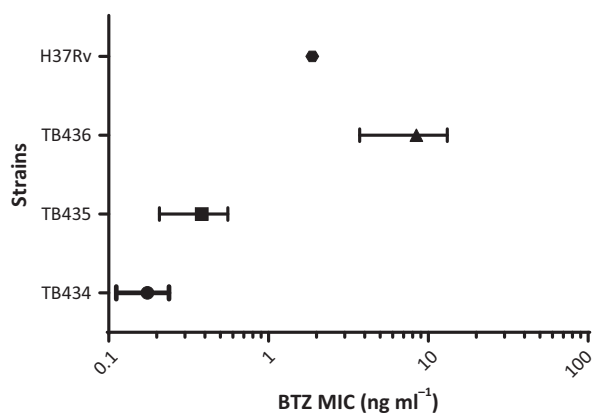


Fig. 8. MIC range of different *Mycobacterium tuberculosis* conditional mutants in the presence of different pristinamycin concentrations.

mycobacterial promoters present extended -10 regions (containing a TGn sequence at the 5'-end) which increase their potency, and it was proposed that these promoters do not require the presence of a -35 sequence (Kenney and Churchward, 1996). It is not clear if the P_{ptr} promoter has an extended -10, as the borders of its -10 consensus sequence are not easily recognizable, but a TG sequence is present just before its locus (Fig. 1).

Three mutant promoters with variable strengths were selected, and the possibility for Pip to repress their activity was confirmed. To demonstrate that these promoters could be used to improve the performances of the TetR/Pip-OFF repressible promoter system, we constructed three conditional *dprE1* mutants using these promoters and compared them to the previously characterized *dprE1* mutant obtained with wt P_{ptr} . DprE1 is an essential decaprenylphosphoryl D-ribose epimerase, an enzyme involved in arabinogalactan and lipoarabinomannan biogenesis (Kolly *et al.*, 2014a) and is the target of BTZ, one of the most potent and best characterized candidates in the TB drug development pipeline (Makarov *et al.*, 2009; Brecik *et al.*, 2015). The *dprE1* conditional mutant was functional to show the essentiality of this gene, but due to the higher strength of the P_{ptr} promoter

in comparison with its physiological promoter, several passages in the presence of ATc were needed to titrate DprE1 down and stop cells growing when cultivated without aeration (Kolly *et al.*, 2014a) and data not shown). Indeed, when this mutant was cultivated in parallel with the new mutants in these conditions, it was clear that the weakest promoters required fewer passages in the presence of ATc to stop bacterial growth. Moreover, this agreed well with the MIC to BTZ of the various mutants which increased with promoter potency (and thus with DprE1 intracellular concentration; Table 3). The MIC of the conditional mutant expressing *dprE1* from P_{ptr12} was equal to that of the wt strain, suggesting a similar potency of these two promoters, which was confirmed when the two promoters were compared (Fig. 6). However, careful inspection of the data obtained by REMA shows that H37Rv is slightly more resistant to BTZ than the conditional mutant (Fig. 5); this may be explained either by the existence of a metabolic burden in the mutant strain due to the presence of the integrated plasmid and the TetR/Pip-OFF system which might increase its sensitivity, or more interestingly by the possibility that in the presence of a drug inhibiting DprE1, the *dprE1* promoter might be induced thereby increasing DprE1 intracellular concentration.

When the original mutant and the mutant in which *dprE1* was transcribed from the weakest of the selected promoters (P_{ptr11}) were used to infect mice, we proved that the effect of *dprE1* repression was more dramatic in the strain expressing *dprE1* at the lower level (more than two log reduction in the number of cfu in 2 weeks vs one log reduction for the other strain). Interestingly, this strain grew more slowly than the other one in the lungs even in the absence of doxycycline treatment. As the two strains grow similarly *in vitro* (not shown), this suggests that bacteria require more DprE1 expression during infection than during growth in axenic culture, probably for the faster protein turnover in this environment generated by exposure to stress. It is worth noting that as the strength of P_{ptr11} is lower than that of P_{dprE1} (Fig. 6), this strain probably contains a suboptimal concentration of DprE1 which might well explain its attenuation even in the absence of doxycycline treatment.

Finally, we wanted to investigate the possibility to use our promoters to develop bacterial biosensors sensitized to inhibitors of specific targets to optimize target-based whole-cell screenings. As proof of principle, we used the *dprE1* gene. As whole-cell screenings with *M. tuberculosis* usually need long incubation times, we reasoned that ATc, which in our experience has a short half-life, was not the molecule of choice to develop such a system. We therefore used the Pip-ON inducible system, based on the more stable pristinamycin (Forti *et al.*, 2009). In this system, the pristinamycin-sensitive transcriptional

repressor Pip is constitutively expressed thereby inhibiting transcription at P_{ptr} , but in the presence of pristinamycin, it releases its operators in a dose-dependent manner and allows transcription. Measurement of MIC to BTZ of the three Pip-ON-based conditional mutants in the presence of two different concentrations of pristinamycin clearly showed that each strain was more vulnerable to the drug when the expression of *dprE1* was lower due to a lower concentration of pristinamycin. However, the differences due to pristinamycin-dependent *dprE1* expression modulation were smaller than those due to the strength of the different promoters. It is worth noting that the strain with the lower MIC (TB434 grown in the presence of 25 ng ml⁻¹ of pristinamycin) was 16-fold more sensitive than H37Rv, while the strain with the highest MIC (TB436 grown in the presence of 100 ng ml⁻¹ of pristinamycin) was 8-fold more resistant than H37Rv (Fig. 8). Consequently, the 128-fold MIC range, from the most resistant to the most sensitive strain, provides a very sensitive window to screen for active compounds.

In conclusion, the construction and characterization of different P_{ptr} promoter mutants widely expanded the potential of Pip-based tunable promoter systems, thus increasing the range of genes whose expression can be effectively manipulated for the construction of bacterial biosensors.

Experimental procedures

Strain and culture conditions

The following bacterial strains were used in this study: *Escherichia coli* Top10 (Invitrogen, Carlsbad, CA, USA), DH5 α and HB101 (laboratory stock), *M. smegmatis* mc²155 (laboratory stock), and *M. tuberculosis* H37Rv (laboratory stock). *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth or on LB agar plates. Mycobacterial strains were grown at 37°C in Middlebrook 7H9 broth (Difco, Franklin Lakes, NJ, USA) in 150 ml roller bottles with slow rotation (3 rpm), in 10 ml screw-cap tubes without agitation, or 7H10 agar plates (Difco), supplemented with 0.2% glycerol and 0.05% Tween-80. For growth of *M. tuberculosis*, the medium was supplemented with 10% albumin-dextrose-sodium chloride complex (ADN; Jacobs *et al.*, 1991). When needed, antibiotics were added to the media at the following concentrations: streptomycin (Sm) 20 μ g ml⁻¹, kanamycin (Km) 50 μ g ml⁻¹ (*E. coli*) or 20 μ g ml⁻¹ (*M. smegmatis* and *M. tuberculosis*), hygromycin (Hyg) 150 μ g ml⁻¹ (*E. coli*) or 50 μ g ml⁻¹ (*M. smegmatis* and *M. tuberculosis*). Anhydrotetracycline (ATc; Sigma, St. Louis, MO, USA) or pristinamycin (Molcan Corporation, Richmond Hill, ON, Canada) were added when required at the concentrations indicated in the text. Benzothiazinone 043

(BTZ) was dissolved in DMSO at a concentration of 9 μ g ml⁻¹. Preparation of electrocompetent cells, electroporation and preparation of mycobacterial genomic DNA were performed as previously described (Maciag *et al.*, 2007).

Plasmid construction and PCR-mutagenesis

The sequence encoding eGFP was PCR amplified from pMV4-36 (Cascioferro *et al.*, 2010) using primers RP1335/RP1336 (Table S2) and subcloned in the suicide vector pFRA50 downstream of the repressible promoter P_{ptr} (Boldrin *et al.*, 2010). The P_{ptr} -*egfp* fragment was then subcloned in TOPO (Invitrogen). This plasmid was used as a template for PCR-mutagenesis using upper primers overlapping the P_{ptr} sequence and containing the different point mutations and a lower primer overlapping the final part of *egfp* (Fig. S2 and Table S2). The amplified mutagenized P_{ptr} -*egfp* fragments were cloned in TOPO and, after confirmation of the presence of the correct mutation by sequencing, subcloned in both pFRA61 (integrative plasmid harbouring the TetR/Pip-OFF system; Kolly *et al.*, 2014a) and in pMV261 (replicative plasmid) (Cooksey *et al.*, 1993). The resulting integrative plasmids were introduced into *M. smegmatis* mc²155 and *M. tuberculosis* H37Rv while replicative plasmids were electroporated into *M. smegmatis* MS212 and *M. tuberculosis* TB262 (mc²155- and H37Rv-derivatives harbouring the TetR/Pip-OFF system). Vectors and bacterial strains are listed in Table S1.

Construction of a *dprE1*-reporter plasmid

A fragment of 518 bp immediately upstream of *dprE1* containing its putative promoter was amplified with primers RP1629/RP1630 using Phusion[®] High-Fidelity DNA Polymerase and cloned upstream of a promoterless *egfp* generating pFRA194. The P_{dprE1} -*egfp* fragment was excised from this plasmid and subcloned into the integrative plasmid pFRA61 (Kolly *et al.*, 2014a) obtaining pFRA206. This plasmid was introduced in *M. tuberculosis* H37Rv to obtain TB419.

Construction of *M. tuberculosis dprE1* conditional mutants

The gene encoding DprE1 was amplified using primers RP806/RP807 (Table S2) using Phusion[®] High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) and cloned into pCR-Blunt II-TOPO (Invitrogen). The amplified fragment was then excised and subcloned (i) into three integrative plasmids containing the TetR/Pip-OFF system and one of the three mutated P_{ptr}

(mutations 11, 12, and 14) to obtain pFRA183, pFRA186, and pFRA188 respectively and (ii) into three integrative plasmids containing the Pip-ON system and one of the three mutated P_{ptr} (mutations 11, 12, and 14) to obtain pFRA212, pFRA213, pFRA214 respectively. These six plasmids were introduced into the *dprE1* conditional mutant TB110 (Kolly *et al.*, 2014a) by plasmid switching (Pashley and Parish, 2003) generating TB386, TB387, TB388 (TetR/Pip-OFF) and TB434, TB435, TB436 (Pip-ON) respectively (Table 1).

Fluorescence assay

eGFP fluorescence was measured in Nunclon 96-well Flat Bottom Black Polystyrol FluoroNunc microplates (Thermo Scientific, Carlsbad, CA, USA) to minimize background fluorescence. Bacterial strains were inoculated in 5 ml of 7H9/ADC, supplemented with appropriate antibiotics, and grown to $OD_{540} = 0.5$. Cells were harvested by centrifugation (5 min, 10 000 rpm), and pellets were resuspended in a volume of 0.1 ml of Middlebrook 7H9 to obtain a concentration of about 10^9 cfu ml^{-1} . Then, 10^8 cfu were added in the microplates, and fluorescence values were measured with Infinite 200Pro microplate reader (Tecan Group, Mannendorf, Switzerland), using excitation and emission wavelengths of 485 and 535 nm respectively. Wells containing only medium or bacterial cultures of *M. smegmatis* and *M. tuberculosis* strains not expressing GFP were used as controls to determine bacterial autofluorescence that was used to subtract the background from the measurements. All the experiment were repeated at least twice in triplicate. Data were reported as mean \pm standard deviation.

Benzothiazinone susceptibility test

Bacterial strains were grown in 3 ml of Middlebrook 7H9 supplemented with the appropriate antibiotics to OD_{540} 0.2–0.4. Cultures were diluted to a theoretical OD_{540} of 0.0005 and 200 μ l of the resulting suspension were spotted into each well of the first column of a Nunclon 96-well Flat Bottom Black Polystyrol FluoroNunc microplates (Thermo Scientific). Starting from the second column, 100 μ l of bacteria were added to the other wells of the plate. BTZ (stock concentration = 9 μ g ml^{-1}) was added to a final concentration of 60 ng ml^{-1} in each well of the first column and then serial dilutions (1:1) were made. Plates were incubated 7 days at 37°C, and then 10 μ l of AlamarBlue (Invitrogen) was added to each well and incubated at 37°C. The detection was performed after 24 h of incubation using an Infinite 200Pro microplate reader (Tecan Group), with excitation and emission wavelengths of 535 and 590 nm respectively. The lowest drug concentration that resulted in growth inhibition of at

least 85% was considered as the MIC. The experiments were repeated twice in triplicate. Data were reported as mean \pm standard deviation.

In vivo experiments

Female C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA) were infected by low-dose aerosol with the *dprE1* conditional knock-down strains. Mice were fed with doxycycline-containing food (2000 ppm, Harlan) starting from 5 days before infection. Control groups were fed with regular diet, equal in composition to the doxycycline-containing food except for the antibiotic. Four mice per group and time point were sacrificed at day 0 and 2 weeks post-infection. Lung homogenates were plated on 7H10 plates supplemented with ampicillin (100 μ g ml^{-1}) and cycloheximide (10 μ g ml^{-1}). The experiment was performed once. Data were calculated as mean \pm standard deviation. Experimental procedures involving animals were approved by the Swiss Cantonal and Federal Authorities (Authorization number 2658).

Acknowledgements

This work was supported by the European Community's Seventh Framework Programme under grant agreement 260872.

Conflict of interest

None of the authors have any potential conflict of interest to declare.

References

- Ahmed, W., Menon, S., Godbole, A.A., Karthik, P.V., and Nagaraja, V. (2014) Conditional silencing of topoisomerase I gene of *Mycobacterium tuberculosis* validates its essentiality for cell survival. *FEMS Microbiol Lett* **353**: 116–123.
- Ahmed, W., Menon, S., Karthik, P.V., and Nagaraja, V. (2016) Autoregulation of topoisomerase I expression by supercoiling sensitive transcription. *Nucleic Acids Res* **44**: 1541–1552.
- Bashyam, M.D., Kaushal, D., Dasgupta, S.K., and Tyagi, A.K. (1996) A study of mycobacterial transcriptional apparatus: identification of novel features in promoter elements. *J Bacteriol* **178**: 4847–4853.
- Bazet Lyonnet, B., Diacovich, L., Cabruja, M., Bardou, F., Quemard, A., Gago, G., and Gramajo, H. (2014) Pleiotropic effect of AccD5 and AccE5 depletion in acyl-coenzyme A carboxylase activity and in lipid biosynthesis in mycobacteria. *PLoS ONE* **9**: e99853.
- Bhowmick, T., Ghosh, S., Dixit, K., Ganesan, V., Ramagopal, U.A., Dey, D., *et al.* (2014) Targeting *Mycobacterium*

- tuberculosis* nucleoid-associated protein HU with structure-based inhibitors. *Nat Commun* **5**: 4124.
- Blanc, V., Salah-Bey, K., Folcher, M., and Thompson, C.J. (1995) Molecular characterization and transcriptional analysis of a multidrug resistance gene cloned from the pristinamycin-producing organism, *Streptomyces pristinaespiralis*. *Mol Microbiol* **17**: 989–999.
- Boldrin, F., Casonato, S., Dainese, E., Sala, C., Dhar, N., Palu, G., et al. (2010) Development of a repressible mycobacterial promoter system based on two transcriptional repressors. *Nucleic Acids Res* **38**: e134.
- Boldrin, F., Ventura, M., Degiacomi, G., Ravishankar, S., Sala, C., Svetlikova, Z., et al. (2014) The phosphatidylyl-myo-inositol mannosyltransferase PimA is essential for *Mycobacterium tuberculosis* growth in vitro and in vivo. *J Bacteriol* **196**: 3441–3451.
- Brecik, M., Centarova, I., Mukherjee, R., Kolly, G.S., Huszar, S., Bobovska, A., et al. (2015) DprE1 is a vulnerable tuberculosis drug target due to its cell wall localization. *ACS Chem Biol* **10**: 1631–1636.
- Carroll, P., Muttucumar, D.G., and Parish, T. (2005) Use of a tetracycline-inducible system for conditional expression in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. *Appl Environ Microbiol* **71**: 3077–3084.
- Cascioferro, A., Boldrin, F., Serafini, A., Provvedi, R., Palu, G., and Manganelli, R. (2010) Xer site-specific recombination, an efficient tool to introduce unmarked deletions into mycobacteria. *Appl Environ Microbiol* **76**: 5312–5316.
- Choudhary, E., Thakur, P., Pareek, M., and Agarwal, N. (2015) Gene silencing by CRISPR interference in mycobacteria. *Nat Commun* **6**: 6267.
- Choudhary, E., Lunge, A., and Agarwal, N. (2016) Strategies of genome editing in mycobacteria: achievements and challenges. *Tuberculosis* **98**: 132–138.
- Cooksey, R.C., Crawford, J.T., Jacobs, W.R. Jr, and Shinnick, T.M. (1993) A rapid method for screening antimicrobial agents for activities against a strain of *Mycobacterium tuberculosis* expressing firefly luciferase. *Antimicrob Agents Chemother* **37**: 1348–1352.
- Cortes, M., Singh, A.K., Reytrat, J.M., Gaillard, J.L., Nassif, X., and Herrmann, J.L. (2011) Conditional gene expression in *Mycobacterium abscessus*. *PLoS ONE* **6**: e29306.
- Degiacomi, G., Benjak, A., Madacki, J., Boldrin, F., Provvedi, R., Palu, G., et al. (2017) Essentiality of *mmpL3* and impact of its silencing on *Mycobacterium tuberculosis* gene expression. *Sci Rep* **7**: 43495.
- Di Luca, M., Bottai, D., Batoni, G., Orgeur, M., Aulicino, A., Counoupas, C., et al. (2012) The ESX-5 associated *eccB-EccC* locus is essential for *Mycobacterium tuberculosis* viability. *PLoS ONE* **7**: e52059.
- Ehrt, S., Guo, X.V., Hickey, C.M., Ryou, M., Monteleone, M., Riley, L.W., and Schnappinger, D. (2005) Controlling gene expression in mycobacteria with anhydrotetracycline and Tet repressor. *Nucleic Acids Res* **33**: e21.
- Forti, F., Crosta, A., and Ghisotti, D. (2009) Pristinamycin-inducible gene regulation in mycobacteria. *J Biotechnol* **140**: 270–277.
- Gola, S., Munder, T., Casonato, S., Manganelli, R., and Vicente, M. (2015) The essential role of SepF in mycobacterial division. *Mol Microbiol* **97**: 560–576.
- Gupta, S., Banerjee, S.K., Chatterjee, A., Sharma, A.K., Kundu, M., and Basu, J. (2015) Essential protein SepF of mycobacteria interacts with FtsZ and MurG to regulate cell growth and division. *Microbiology* **161**: 1627–1638.
- Hu, Y., Wang, Z., Feng, L., Chen, Z., Mao, C., Zhu, Y., and Chen, S. (2016) SigE-dependent activation of RbpA controls transcription of the *furA-katG* operon in response to oxidative stress in mycobacteria. *Mol Microbiol* **102**: 107–120.
- Hughes, D., and Karlen, A. (2014) Discovery and preclinical development of new antibiotics. *Ups J Med Sci* **119**: 162–169.
- Jacobs, W.R. Jr, Kalpana, G.V., Cirillo, J.D., Pascopella, L., Snapper, S.B., Udani, R.A., et al. (1991) Genetic systems for mycobacteria. *Methods Enzymol* **204**: 537–555.
- Kenney, T.J., and Churchward, G. (1996) Genetic analysis of the *Mycobacterium smegmatis* *rpsL* promoter. *J Bacteriol* **178**: 3564–3571.
- Kim, J.H., Wei, J.R., Wallach, J.B., Robbins, R.S., Rubin, E.J., and Schnappinger, D. (2011) Protein inactivation in mycobacteria by controlled proteolysis and its application to deplete the beta subunit of RNA polymerase. *Nucleic Acids Res* **39**: 2210–2220.
- Kim, J.H., O'Brien, K.M., Sharma, R., Boshoff, H.I., Rehren, G., Chakraborty, S., et al. (2013) A genetic strategy to identify targets for the development of drugs that prevent bacterial persistence. *Proc Natl Acad Sci USA* **110**: 19095–19100.
- Kolly, G.S., Boldrin, F., Sala, C., Dhar, N., Hartkoorn, R.C., Ventura, M., et al. (2014a) Assessing the essentiality of the decaprenyl-phospho-d-arabinofuranose pathway in *Mycobacterium tuberculosis* using conditional mutants. *Mol Microbiol* **92**: 194–211.
- Kolly, G.S., Sala, C., Vocat, A., and Cole, S.T. (2014b) Assessing essentiality of transketolase in *Mycobacterium tuberculosis* using an inducible protein degradation system. *FEMS Microbiol Lett* **358**: 30–35.
- Maciag, A., Dainese, E., Rodriguez, G.M., Milano, A., Provvedi, R., Pasca, M.R., et al. (2007) Global Analysis of the *Mycobacterium tuberculosis* Zur (FurB) Regulon. *J Bacteriol* **189**: 730–740.
- Makarov, V., Manina, G., Mikusova, K., Mollmann, U., Ryabova, O., Saint-Joanis, B., et al. (2009) Benzothiazinones kill *Mycobacterium tuberculosis* by blocking arabinan synthesis. *Science* **324**: 801–804.
- Makarov, V., Lechartier, B., Zhang, M., Neres, J., van der Sar, A.M., Raadsen, S.A., et al. (2014) Towards a new combination therapy for tuberculosis with next generation benzothiazinones. *EMBO Mol Med* **6**: 372–383.
- Manganelli, R. (2014) Sigma factors: key molecules in *Mycobacterium tuberculosis* physiology and virulence. *Microbiol Spectr* **2**: MGM2-0007-2013. doi: 10.1128/microbiolspec.MGM2-0007-2013
- Mondino, S., Gago, G., and Gramajo, H. (2013) Transcriptional regulation of fatty acid biosynthesis in mycobacteria. *Mol Microbiol* **89**: 372–387.
- Mori, G., Chiarelli, L.R., Esposito, M., Makarov, V., Bellinzoni, M., Hartkoorn, R.C., et al. (2015) Thiophenecarboxamide derivatives activated by EthA kill *Mycobacterium tuberculosis* by inhibiting the CTP synthetase PyrG. *Chem Biol* **22**: 917–927.

- Newton-Foot, M., and Gey van Pittius, N.C. (2013) The complex architecture of mycobacterial promoters. *Tuberculosis* **93**: 60–74.
- Pandey, R., and Rodriguez, G.M. (2014) IdeR is required for iron homeostasis and virulence in *Mycobacterium tuberculosis*. *Mol Microbiol* **91**: 98–109.
- Pashley, C.A., and Parish, T. (2003) Efficient switching of mycobacteriophage L5-based integrating plasmids in *Mycobacterium tuberculosis*. *FEMS Microbiol Lett* **229**: 211–215.
- Piton, J., Foo, C.S., and Cole, S.T. (2017) Structural studies of *Mycobacterium tuberculosis* DprE1 interacting with its inhibitors. *Drug Discov Today* **22**: 526–533.
- Schnappinger, D. (2015) Genetic approaches to facilitate antibacterial drug development. *Cold Spring Harb Perspect Med* **5**: a021139.
- Schnappinger, D., and Ehrh, S. (2014) Regulated expression systems for mycobacteria and their applications. *Microbiol Spectr* **2**: MGM2-0018-2013. doi: 10.1128/microbiolspec.MGM2-0018-2013
- Seeliger, J.C., Topp, S., Sogi, K.M., Previti, M.L., Gallivan, J.P., and Bertozzi, C.R. (2012) A riboswitch-based inducible gene expression system for mycobacteria. *PLoS ONE* **7**: e29266.
- Serafini, A., Boldrin, F., Palu, G., and Manganelli, R. (2009) Characterization of a *Mycobacterium tuberculosis* ESX-3 conditional mutant: essentiality and rescue by iron and zinc. *J Bacteriol* **191**: 6340–6344.
- Serafini, A., Pisu, D., Palu, G., Rodriguez, G.M., and Manganelli, R. (2013) The ESX-3 secretion system is necessary for iron and zinc homeostasis in *Mycobacterium tuberculosis*. *PLoS ONE* **8**: e78351.
- Singh, A.K., Carette, X., Potluri, L.P., Sharp, J.D., Xu, R., Pristic, S., and Husson, R.N. (2016) Investigating essential gene function in *Mycobacterium tuberculosis* using an efficient CRISPR interference system. *Nucleic Acids Res* **44**: e143.
- Ventura, M., Rieck, B., Boldrin, F., Degiacomi, G., Bellinzoni, M., Barilone, N., et al. (2013) GarA is an essential regulator of metabolism in *Mycobacterium tuberculosis*. *Mol Microbiol* **90**: 356–366.
- Verma, A.K., and Chatterji, D. (2014) Dual role of MsRbpA: transcription activation and rescue of transcription from the inhibitory effect of rifampicin. *Microbiology* **160**: 2018–2029.

Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Pristinamycin-dependent growth of Pip-ON based *dprE1* conditional mutants.

Fig. S2. Strategy to construct promoter mutants.

Table S1. Mutagenized promoters and corresponding strains.

Table S2. Primers used in this study.