

Development of a repressible mycobacterial promoter system based on two transcriptional repressors

Francesca Boldrin¹, Stefano Casonato¹, Elisa Dainese¹, Claudia Sala², Neeraj Dhar², Giorgio Palù¹, Giovanna Riccardi³, Stewart T. Cole² and Riccardo Manganelli^{1,*}

¹Department of Histology, Microbiology and Medical Biotechnologies, University of Padova, Via Gabelli, 63 35100 Padova, Italy, ²Global Health Institute, Ecole Polytechnique Fédérale de Lausanne, CH-1015 Lausanne, Switzerland and ³Department of Genetics and Microbiology, University of Pavia, Via Ferrata, 1, 27100 Pavia, Italy

Received November 3, 2009; Revised March 1, 2010; Accepted March 21, 2010

ABSTRACT

Tightly regulated gene expression systems represent invaluable tools for studying gene function and for the validation of drug targets in bacteria. While several regulated bacterial promoters have been characterized, few of them have been successfully used in mycobacteria. In this article we describe the development of a novel repressible promoter system effective in both fast- and slow-growing mycobacteria based on two chromosomally encoded repressors, dependent on tetracycline (TetR) and pristinamycin (Pip), respectively. This uniqueness results in high versatility and stringency. Using this method we were able to obtain an *ftsZ* conditional mutant in *Mycobacterium smegmatis* and a *fadD32* conditional mutant in *Mycobacterium tuberculosis*, confirming their essentiality for bacterial growth *in vitro*. This repressible promoter system could also be exploited to regulate gene expression during *M. tuberculosis* intracellular growth.

INTRODUCTION

Mycobacterium tuberculosis is still one of the most deadly microorganisms as it is responsible for ~2-million human deaths every year (1). The lack of an efficient vaccine makes antimicrobial treatment the principal strategy to fight tuberculosis, but the emergence of multidrug-resistant (MDR) (2), and, more recently, extensively drug-resistant (XDR) *M. tuberculosis* strains (3), is endangering this strategy and makes the development of new antitubercular drugs a global urgency.

Tightly regulated gene expression systems are powerful tools for studying gene function and for validating drug targets in bacteria. Moreover, they are required to characterize the function of essential genes, which cannot be deleted without obtaining a lethal phenotype. While many regulated promoters have been characterized in both Gram positive and Gram negative bacteria, only a few of them have been successfully used in mycobacteria. The first regulated gene expression system to be widely exploited in mycobacteria is based on the acetamide-inducible promoter of *Mycobacterium smegmatis*. It is very complex, based on two positive and one negative regulators, and can be induced by the addition of acetamide to the growth medium (4). It has been successfully used to overexpress proteins and to characterize conditional mutants in both *M. smegmatis* and *M. tuberculosis*, but it presents some major weaknesses such as the large size of its operator (~1.4 kb), sensitivity to catabolite repression, low stringency, and unfeasibility in experimental models of infection (5,6). Alternative systems for modulating gene expression in *M. tuberculosis* are those based on the TetR repressor (7–10), the *Streptomyces coelicolor* pristinamycin I repressor Pip (11), and the *Rhodococcus rhodochrous* nitrilase repressor NitR (12).

TetR-based systems are the most widely used in mycobacteria: the gene under investigation is placed under transcriptional control of a promoter containing one or two TetR operators (*tetO*). In the absence of tetracycline (Tc), TetR binds to *tetO* preventing the expression of the gene under investigation. On addition of Tc, TetR is inactivated and dissociates from *tetO*, leading to expression of the downstream gene. Since Tc and its less toxic derivative anhydrotetracycline (ATc) can cross biological membranes, while their analog doxycycline can be easily administered to animals, TetR-based systems can be successfully employed to control gene expression during

*To whom correspondence should be addressed. Tel: +39 049 827 2366; Fax: +39 049 827 2355; Email: riccardo.manganelli@unipd.it

intracellular growth and experimental infections. In 2005, three different groups independently reported the development of a Tc-inducible system for mycobacteria, and showed that it could be used to construct conditional mutants of *M. tuberculosis* as well as to characterize them during experimental infection (7–9). Even though these tools proved to be quite useful, they have the disadvantage that Tc functions as an inducer of gene expression and has therefore to be removed to silence the gene under investigation. For this reason Guo *et al.* (13) developed a modified system using a mutated TetR able to bind *tetO* only in the presence of Tc (revTetR). In this case repression could be achieved by addition, rather than removal, of Tc from the culture medium. This approach, even if less stringent than the classical TetR system, was successfully applied to study a *secA1* conditional mutant of *M. smegmatis* (13) and a conditional mutant of *M. tuberculosis* affected in its ability to express the α and β subunits of the proteasome (14). This approach was recently improved by adapting the revTetR coding sequence to mycobacterial codon usage, but still requires the repressor gene to be provided on a multicopy plasmid to obtain efficient gene repression in slow growing mycobacteria (15).

Here, we report the development of an alternative promoter repressible system based on two different repressors (TetR and Pip) encoded at the chromosomal level. In this approach Tc-based induction of the Pip-encoding gene allows tight repression of the gene under investigation. We also show that this system is suitable for the construction of conditional mutants in essential genes of both *M. smegmatis* and *M. tuberculosis*.

MATERIALS AND METHODS

Bacterial strains, growth media and transformation conditions

The following bacterial strains were used: *Escherichia coli* Top10 (Invitrogen), *E. coli* DH5 α and HB101 (laboratory stock), *M. smegmatis* mc²155 and *M. tuberculosis* H37Rv (laboratory stock).

Escherichia 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) in 150 ml roller bottles with slow rotation (3 rpm) 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% ADN (Albumin, Dextrose, NaCl) (16).

When needed, antibiotics were added to the media at the following concentrations: streptomycin (Sm): 20 $\mu\text{g ml}^{-1}$; kanamycin (Km): 50 $\mu\text{g ml}^{-1}$; hygromycin (Hyg) 150 $\mu\text{g ml}^{-1}$ (*E. coli*) or 50 $\mu\text{g ml}^{-1}$ (*M. smegmatis* and *M. tuberculosis*). ATc (Sigma) was added when required at final concentrations from 1 to 200 ng/ml. Pristinamycin I, a gift of Florence Bordon-Pallier (Sanofi-Aventis), was added when required at concentrations ranging from 20 to 2000 ng/ml.

Preparation of electrocompetent cells, electroporation and preparation of mycobacterial genomic DNA were performed as previously described (17).

Plasmid construction

pMY718 is an integrative plasmid containing the $P_{furA102}$ promoter (11) upstream of the gene encoding the Pip repressor, and the Pip-dependent P_{ptr} promoter upstream of a promoterless *lacZ* gene. The region containing $P_{furA102}$ and *pip* was subjected to oligonucleotide-directed PCR mutagenesis as previously described (7) to introduce two TetR operators using the following primers (underlined sequences represent the operator sequences; HindIII restriction sites in RP558 and RP557 are shown in bold, while the PaeI site of RP558 is shown in italics) (Supplementary Figure S1):

RP555 (upper), 5'-GATCAACTGATCCCTATCAGTG
ATAGACATATTGTCTAGTGTGGCGGCCG-3';
RP558 (lower), 5'-AAGCTTTTAATTAATCAGGCCTG
TTCGACCATCGC-3';
RP557 (upper), 5'-AAGCTTCCATCCTGACGGATGG
CCTCCACCATGCAGGCCCGGCTCAT-3';
RP556 (lower), 5'-CTGATAGGGATCAGTTGATCTA
TCACTGATAGGGAGGCGTCTGCTAGGACCCG
A-3'.

The mutagenized PCR product was cloned into pCR-Blunt II-TOPO (Invitrogen) and subcloned into pMY718 (an integrative vector able to integrate at the unique *attB* site of mycobacteriophage L5) (18) using HindIII to replace the $P_{furA102}$ -*pip* region to obtain pFRA40 (Supplementary Figure S2, Supplementary Table S1).

The Tn10-derived *tetR* gene, obtained by NotI/EcoRI digestion of pMC1s (7) was cloned into the unique PaeI site present in pFRA40, to obtain pFRA42A and pFRA42B, differing only for *tetR* orientation (Supplementary Figure S3, Table S1). The correct orientation of the two plasmids was verified by restriction analysis and DNA sequencing. Both plasmids were finally introduced in *M. smegmatis* and *M. tuberculosis* to obtain MS82, MS83, TB38.1 and TB38.2 respectively (Supplementary Table S2).

Construction of conditional mutants in *M. smegmatis* and *M. tuberculosis*

The 180-bp DNA fragment containing the *ptr* promoter region was amplified from pMY718 using primers RP707: 5'-AGATCTCCATCCTGACGGATGGCCTT-3' and RP708: 5'-AGATCTATGCATTCAGGCTCCTTGAC
GGTGATC-3' and cloned in pCR-Blunt II-TOPO. The resulting plasmid was digested with BglII and the fragment containing the promoter was inserted into a Hyg cassette-containing pBend-derived suicide vector (19) to obtain pFRA50. Subsequently, the first 704 bp of *M. smegmatis ftsZ*, and the first 754 bp of *M. tuberculosis fadD32* were cloned downstream of the *ptr* promoter region in the suicide plasmid pFRA50 to obtain pFRA53, and pFRA51, respectively (Supplementary Table S1).

M. smegmatis mc²155 and *M. tuberculosis* H37Rv were electroporated with 2 µg of the two plasmids, in order to replace by insertional duplication the promoters of *ftsZ* or *fadD32* with the Pip controlled promoter P_{ptr} (Supplementary Figure S4). Recombinants were selected on 7H10 agar plates containing Hyg (50 µg ml⁻¹). Integration of the plasmid via insertional duplication was confirmed by PCR. The resulting conditional mutant strains were finally transformed with the integrative plasmid pFRA42B (containing the TetR/Pip system) to obtain MS98 (*M. smegmatis ftsZ* conditional mutant) and TB47 (*M. tuberculosis fadD32* conditional mutant) (Supplementary Table S2).

Analysis of stability

A single colony of MS98 was grown for ~20 generations on solid medium. Bacteria were resuspended, diluted and then plated with or without ATc to determine the viable counts. The proportion of mutants escaping ATc repression/generation was calculated using the following formula: percentage of mutants/(100 × number of generations) (20).

β-Galactosidase activity assay

The level of repression after addition of ATc was determined in *M. smegmatis* MS82 and MS83. Their parental strain mc²155 was used as a control.

- (i) Cultures were grown overnight (ON) in 7H9 with or without ATc (50 ng/ml). The following morning, cultures grown in ATc were diluted in 7H9 with ATc, while cultures grown without ATc were diluted both in medium containing ATc (50 ng/ml) and in medium without ATc. After 6 h (approximately three generations) cells were collected and β-galactosidase assays performed.
- (ii) For dose-response experiments cultures were grown ON with ATc (50 ng/ml), and then diluted into fresh media with different ATc concentrations (from 0 to 50 ng/ml). Every 24 h each culture was diluted into fresh medium maintaining the same ATc concentration. Samples were collected at 24, 48 and 72 h, and β-galactosidase activities were measured.

Similar experiments were performed with *M. tuberculosis* TB38.1 and TB38.2 using their parental strain H37Rv as a control. In this case the ATc concentration used ranged from 0 to 200 ng/ml.

β-galactosidase was assayed as previously described (21) and enzymatic activity expressed in Miller units (22) using the following formula: $A = (377.77 \times OD_{420}) / (\text{time of incubation at } 37^\circ\text{C} \times \text{sample volume} \times \text{protein concentration in mg/ml})$.

RNA extraction

Mycobacterium tuberculosis TB47 was grown to A₆₀₀ 0.1 and a sample (20 ml) was taken as reference for day 0. The culture was then split into two samples: 200 ng/ml ATc was added to one of them, whereas the other served as untreated control. Both were incubated at 37°C with

gentle shaking (45 rpm) and samples (20 ml) were taken at 24 and 48 h after addition of ATc. Each cell pellet was resuspended in 0.3 ml Trizol (Invitrogen) and transferred to screw-cap tubes containing 150 µl zirconia-silica beads (Biospec Products) for disruption by bead-beating. The cell suspension was then transferred to a new tube, where chloroform-isoamylalcohol (24:1) extraction was performed. RNA was precipitated by adding 1/10 volume of sodium acetate 2 M pH 5.2 and 0.7 volume of isopropanol, washed with 70% ethanol, air-dried and resuspended in DEPC-treated water. DNase treatment was carried out using RQ1 RNase-free DNase (Promega), following the manufacturer's recommendations and the reactions were subsequently stopped by phenol-chloroform extraction and ethanol precipitation. RNA was stored at -80°C in DEPC-treated water. The amount and purity of RNA were determined spectrophotometrically.

Reverse transcription

Mycobacterium tuberculosis RNA (1 µg) was incubated with specific reverse primers for *sigA* and *fadD32* (1 µM each) at 70°C for 5 min. After cooling on ice, 1 mM dNTPs, 40 U RNase inhibitor and 25 U Transcriptase Reverse Transcriptase (Roche) were added in a final volume of 20 µl. The reaction was incubated at 55°C for 1 h and at 85°C for 5 min to inactivate reverse transcriptase. Samples without reverse transcriptase were also set up as negative controls.

Quantitative PCR

All PCR primers and probes (molecular beacons) were designed by using Primer3 software (<http://frodo.wi.mit.edu/primer3/>), according to previously published guidelines (23,24), and their sequences are reported in Table 1. The 20 µl PCR reaction consisted of 1 × GeneAmp PCR Gold buffer (Applied Biosystems), 2.5 mM MgCl₂, 0.25 mM each dNTP, 0.5 µM each primer, 2.5 ng/µl molecular beacon, 1 × ROX Reference Dye (Invitrogen), 1 U of AmpliTaq Gold DNA Polymerase (Applied Biosystems) and 2 µl of cDNA.

Table 1. Oligonucleotides and probes used in quantitative RT-PCR assays

Primer or probe	Sequence 5'→3'
Reverse transcription primers	
<i>fadD32</i>	AGT CCG AAG TGG CGA AGA C
<i>sigA</i>	CTG ACA TGG GGG CCC GCT ACG TTG
PCR primers	
<i>fadD32</i> -fwd	CAA GGA CCT CGT CAT CAT CG
<i>fadD32</i> -rev	CGT CGA ACA CTG TCT GAG GA
<i>sigA</i> -fwd	TGC AGT CGG TGC TGG ACA C
<i>sigA</i> -rev	CGC GCA GGA CCT GTG AGC GG
Molecular beacons (FAM-labeled)	
<i>fadD32</i>	GCA GCC ACA GGA TCT CGA GTG CAC GGG CTG C
<i>sigA</i>	CCT CGC GTC GAA GTT GCG CCA TCC GAG CGA GG

Bases constituting the hairpin stem are indicated in bold.

Reactions were carried out in triplicate in an Applied Biosystems 7900HT Sequence Detection System with the following protocol: denaturation at 95°C for 10 min, 15 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 30 s with 0.5°C step down each cycle, extension at 72°C for 30 s, 25 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s with data collection, extension at 72°C for 30 s.

Parallel reactions using different amounts of H37Rv chromosomal DNA were performed for each primer-probe set in order to obtain the standard curve correlating the threshold cycle with the number of template molecules. The resulting equation was used to quantify the target cDNA for *fadD32* and *sigA* at each time point. Results were expressed as the ratio between the number of cDNA copies of the gene of interest and the number of *sigA* cDNA copies in the culture exposed to ATc divided by the same ratio in an RNA preparation obtained from cultures without ATc ($(fadD32 \text{ cDNA} + \text{ATc}/sigA \text{ cDNA} + \text{ATc})/(fadD32 \text{ cDNA} - \text{ATc}/sigA \text{ cDNA} - \text{ATc})$) (23,24).

Infection of THP-1-derived macrophage

THP-1 cells were grown at 37°C in a 5% CO₂ atmosphere and were maintained in RPMI medium (Gibco) supplemented with 10% fetal bovine serum (Gibco). After expansion, THP-1 cells were differentiated into macrophages and infected with *M. tuberculosis* in 96-well plates with a multiplicity of infection of 1:20 cfu/macrophage as previously described (25). Bacteria were pre-grown for 48 h in ATc-containing medium before infection. After 90 min of incubation at 37°C, the medium was removed, and cells were washed twice with 100 µl of warm PBS to remove extracellular bacteria. Finally, 100 µl of warm RPMI with or without ATc (200 ng/ml), was added to each well and the plate was incubated at 37°C. RPMI with or without ATc was replaced every 48 h.

For 8 days, every 24 h, starting from 90 min after the initial washes, the medium was removed from three wells, and then intracellular bacteria were released by lysing the macrophages with 100 µl of 0.05% SDS. The suspensions obtained from the lysed macrophages were immediately diluted in 7H9 and plated to determine viable counts. About 95% of macrophages remained viable during the entire experiment, as determined by Trypan blue exclusion.

RESULTS

Construction of the TetR/Pip OFF repressible promoter system

In order to develop a repressible mycobacterial promoter system, we constructed two integrative plasmids containing (i) the Tn10-derived gene encoding the tetracycline-sensitive transcriptional repressor TetR; (ii) the *S. coelicolor* gene encoding the pristinamycin I-sensitive transcriptional repressor Pip, and (iii) the reporter gene *lacZ*. In these constructs *tetR* is transcribed from a constitutive promoter, *pip* from a TetR-dependent

promoter, and *lacZ* from a Pip-dependent promoter (Supplementary Figure S3). The *tetR* repressor was obtained from the TetR-dependent inducible system previously developed by Ehrt *et al.* (7), while *pip* and the Pip-dependent promoter were taken from the pristinamycin I-inducible system previously developed by Forti *et al.* (11). The TetR-dependent promoter was constructed by introducing two TetR operators (*tetO*) into the constitutive promoter P_{furA102} (11,26) (Supplementary Figure S5). The two integrative plasmids (pFRA42A and pFRA42B), differing in the orientation of the genes encoding the two repressors, were introduced by electroporation into both *M. smegmatis* and *M. tuberculosis* to obtain the strains MS82, MS83, TB38.1 and TB38.2, respectively.

In the absence of Tc (or its less toxic derivative ATc), TetR can bind to its operators turning off *pip* transcription, and allowing *lacZ* expression. However, in the presence of Tc (or ATc), inactivation of TetR allows transcription of *pip*, resulting in repression of *lacZ* (Figure 1).

Characterization of the TetR/Pip OFF repressible promoter system

In order to evaluate the TetR/Pip OFF regulatory circuit, the *M. smegmatis* strains MS82 and MS83, containing the two different constructs, were plated on 7H10 plates containing X-gal and increasing concentrations of ATc. As shown in Supplementary Figure S6, both strains displayed clear β-galactosidase activity when grown in the absence of ATc, which decreased in the presence of 10 ng/ml of ATc and almost disappeared when the concentration was 50 ng/ml.

The system was subsequently tested in liquid medium. Bacteria were first grown for 12 h with or without 50 ng/ml ATc. Cultures grown with ATc were then diluted into fresh media containing the same ATc concentration,

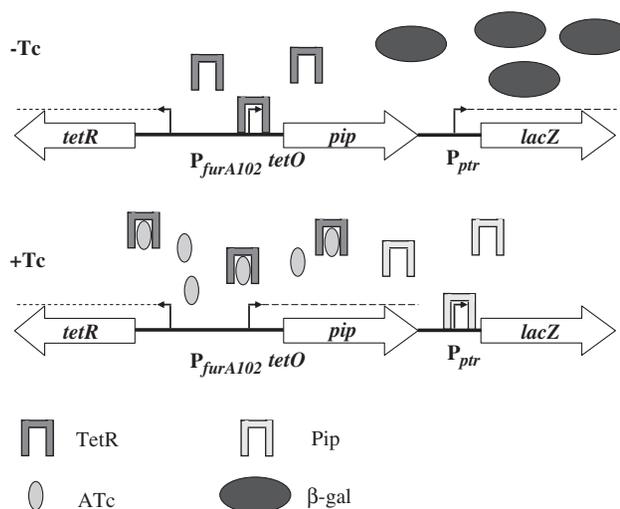


Figure 1. Model of the TetR/Pip OFF system. In the absence of tetracycline (Tc), TetR binds to its operators turning off *pip* transcription, and allowing *lacZ* expression. In the presence of Tc, transcription of *pip* is allowed. As a result, Pip represses *lacZ* expression.

while cultures grown without ATc were diluted into fresh media with or without ATc. This allowed us to obtain cultures continuously exposed to ATc for 6 or 18 h, or not exposed at all. Bacteria were lysed and their β -galactosidase activity was determined. Results, shown in Figure 2A, clearly indicate that after 18 h of repression (~ 7.2 generations), β -galactosidase activity decreased ~ 65.6 - (MS82) or 24.5-fold (MS83), with respect to the untreated sample, and was comparable to that of the negative control (*M. smegmatis* mc²155), while after 6 h of growth in the presence of ATc (~ 2.4 generations), it decreased ~ 5.1 - (MS82) or 4.8-fold (MS83). These data, while suggesting that some time is needed to titrate out β -galactosidase, clearly indicate that tight *lacZ* repression is obtained since the beginning of exposure to ATc.

A similar experiment was performed in *M. tuberculosis*. In this case, bacteria pre-grown without ATc were diluted and grown with or without 200 ng/ml of ATc for 48 h (~ 2.6 generations). Here, we detected repression of β -galactosidase activity of ~ 7 -fold for one construct and ~ 12 -fold for the other (Figure 2B).

In both species, the activity of pFRA42A was higher than that of pFRA42B suggesting that the different orientation of *pip* and *tetR* interferes with *pip* expression.

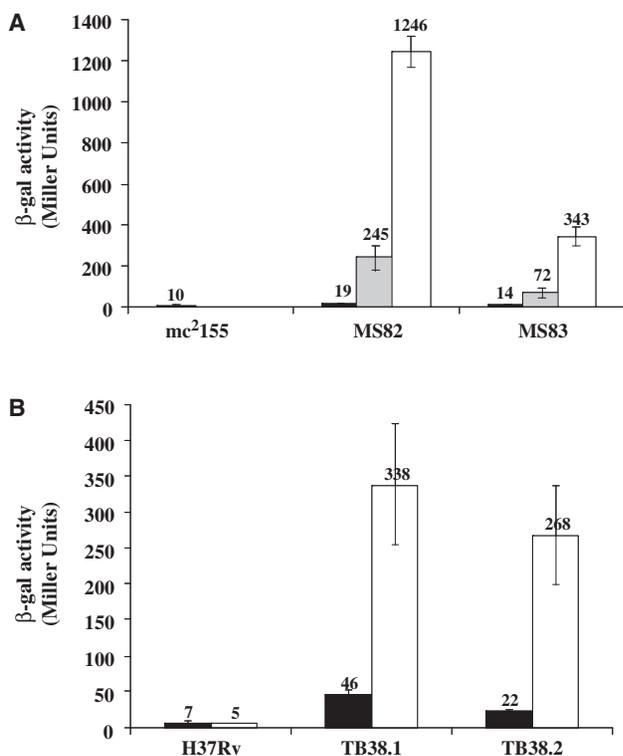


Figure 2. Characterization of the TetR/Pip OFF system. (A) β -galactosidase assay in *M. smegmatis* in liquid media: MS82 and MS83 were grown in Middlebrook 7H9 medium with or without 50 ng/ml ATc. White bars: bacteria grown without ATc; grey bars: bacteria exposed to ATc for 6 h; black bars: bacteria exposed to ATc for 18 h. (B) β -galactosidase assay in *M. tuberculosis* in liquid media: TB38.1 and TB38.2 were grown in Middlebrook 7H9 medium with or without 200 ng/ml ATc. White bars: bacteria grown without ATc; black bars: bacteria exposed to ATc for 48 h. Results are expressed in Miller units.

ATc concentration-dependent repression of the TetR/Pip OFF system

In order to evaluate the ATc concentration-dependent repression of β -galactosidase, the *M. smegmatis* strain MS82 was grown overnight with 50 ng/ml of ATc, washed and then diluted into fresh media with different ATc concentrations. Every 24 h, an aliquot of the culture was collected for β -galactosidase activity measurement, while the culture was diluted into fresh media containing the same ATc concentration. Results, shown in Figure 3A, clearly indicate that 24 h after ATc removal some β -galactosidase activity was detectable only in cultures with ATc concentrations lower than 9 ng/ml. After 48 h this activity increased in all samples to remain stable for at least another 24 h. The level of activity was dependent on the ATc concentration. Activity in cultures grown with ATc concentrations higher than 3 ng/ml was comparable with that of the negative control (*M. smegmatis* mc²155) during the whole experiment showing that the TetR/Pip OFF system is extremely sensitive to very low doses of ATc and can be used to fine-tune gene expression.

A similar experiment was run with *M. tuberculosis* TB38.2. In this case bacteria were grown for 72 h with 200 ng/ml of ATc, washed and then diluted into fresh media containing different ATc concentrations. Every 48 h an aliquot of the culture was collected for β -galactosidase activity measurement, while the culture was diluted into fresh media containing the same ATc concentration. As shown in Figure 3B, in cultures exposed to 200 ng/ml of ATc, β -galactosidase activity remained low and, at least at the two later time points (96 and 144 h), when steady state was reached, it was comparable to that of the negative control (*M. tuberculosis* H37Rv). However, the culture exposed to 50 ng/ml of ATc showed higher β -galactosidase activity, which remained stable for the entire experiment. In cultures exposed to 10 ng/ml of ATc, β -galactosidase activity increased during the entire course of the experiment, suggesting that 10 ng/ml of ATc are not enough to obtain gene repression in *M. tuberculosis*. Finally in cultures grown in the absence of ATc β -galactosidase activity increased between 48 and 96 h to reach steady state. The β -galactosidase regulatory window observed at the two later time points of this experiment was ~ 60 -fold.

In strains containing the TetR/Pip OFF system, the possibility of a leaky expression of Pip could result in partial repression of P_{pir} even in the absence of ATc. To investigate this possibility, we performed an experiment in which we compared the β -galactosidase activity of TB38.2 to that of TB29, a strain containing pMY696 (11) integrated into the chromosome. This plasmid is a progenitor of pFRA42B (present on TB38.2 chromosome) and carries *lacZ* under P_{pir} transcriptional control in the absence of both *tetR* and *pip* (Supplementary Table S1). Cultures pregrown in the absence of ATc were diluted in fresh media without ATc (TB38.2 and TB29) or containing 200 ng/ml of ATc (TB38.2). Every 48 h and for 6 days, one aliquot of each culture was diluted into fresh medium containing the same amount of ATc as before. β -galactosidase activity was measured 96 and 144 h from

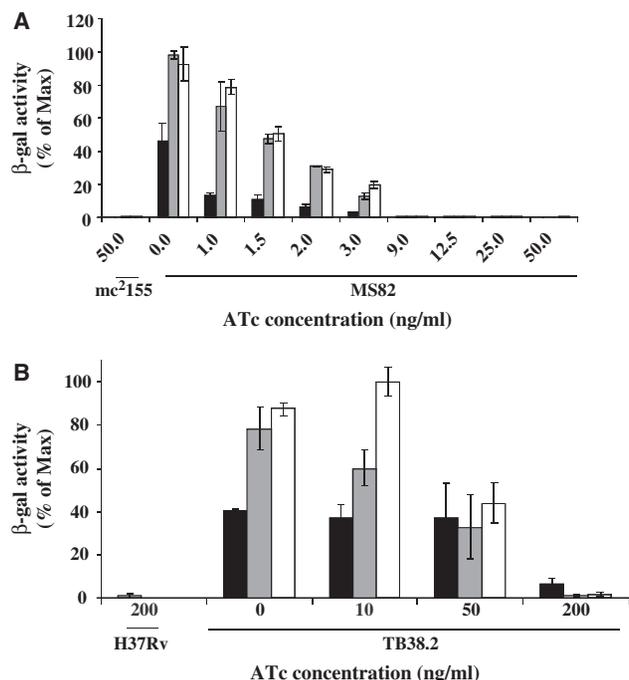


Figure 3. β -Galactosidase assay in liquid media. (A) *M. smegmatis* MS82 was pre-grown in Middlebrook 7H9 containing 50 ng/ml ATc, and diluted into fresh media containing different ATc concentrations. Every 24 h and for 3 days, one aliquot of each culture was used to measure β -galactosidase activity and one aliquot was diluted into fresh medium containing the same amount of ATc as before. Black bars: 24 h; gray bar: 48 h; white bars: 72 h. (B) TB38.2 was pre-grown in Middlebrook 7H9 containing 200 ng/ml ATc, and diluted into fresh media containing different ATc concentrations. Every 48 h and for 6 days, one aliquot of each culture was used to measure β -galactosidase activity and one aliquot was diluted into fresh medium containing the same amount of ATc as before. Black bars: 48 h; grey bar: 96 h; white bars: 144 h. Values are indicated as percentages of maximal activity.

the beginning of the experiment. As shown in Figure 4, TB29 level of β -galactosidase activity was higher than that of TB38.2 grown without ATc (slightly more than 2-fold) suggesting that indeed some level of P_{ptr} repression due to leaky expression of Pip may occur in this strain. It is worth noting that in TB38.2 cultures supplemented with ATc, the levels of β -galactosidase activity were comparable at 96 and 144 h, suggesting that at these time points β -galactosidase activity reaches steady state.

Construction of an *ftsZ* conditional mutant in *M. smegmatis*

One of the most important features of a repressible promoter system is the possibility to construct conditional mutants allowing the study of essential genes. To demonstrate that the TetR/Pip OFF system was suitable for this purpose, we constructed an *M. smegmatis* mutant where *ftsZ*, a well-known essential gene involved in cellular division (7), was placed under transcriptional control of the Pip-dependent promoter P_{ptr} (Supplementary Figure S4). The mutant was then transformed with the integrative plasmid pFRA42B carrying the TetR/Pip OFF system to obtain the conditional mutant MS98. When this strain

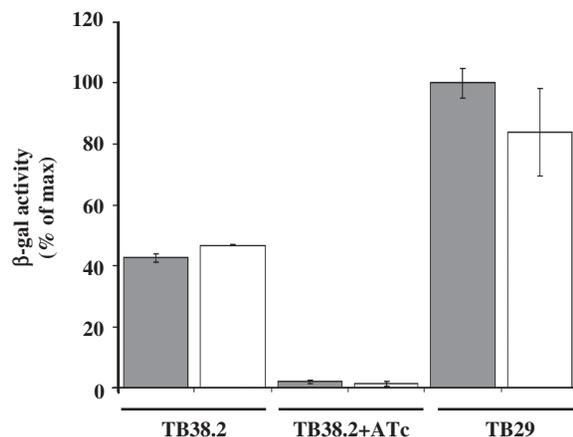


Figure 4. β -Galactosidase assay in liquid media. TB38.2 and TB29 were pre-grown in 7H9 in the absence of ATc and diluted into fresh media with (TB38.2) or without ATc (TB38.2 and TB29). Every 48 h and for 6 days, one aliquot of each culture was diluted into fresh medium containing the same amount of ATc as before. β -Galactosidase activity was measured at 96 (grey bars) and 144 h (white bars).

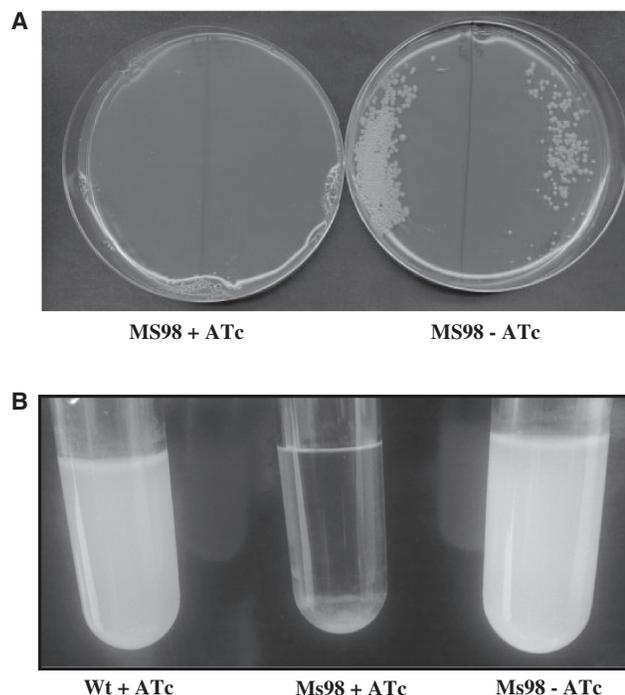


Figure 5. Characterization of the *M. smegmatis* *ftsZ* mutant MS98. (A) MS98 was plated on Middlebrook 7H10 medium with or without 50 ng/ml ATc. (B) MS98 and its parental wild-type strain were grown in Middlebrook 7H9 with or without ATc.

was plated on solid medium containing 50 ng/ml of ATc, no growth was detected confirming that *ftsZ* repression was stringent enough to prevent growth (Figure 5A). Moreover, when this conditional mutant was grown in liquid media with or without ATc, after an initial phase during which optical density of the two cultures increased at the same rate (data not shown), bacteria growing in the presence of ATc started to clump and settled at the

bottom of the tube (Figure 5B). When we looked at this culture under the microscope, the presence of very long and filamentous bacilli was observed, as expected for FtsZ-depleted bacteria (Supplementary Figure S7).

Mutants escaping ATc repression appeared at a frequency of $\sim 2.3 \times 10^{-5}$ or 1.2×10^{-8} /generation depending on the absence/presence of streptomycin in the culture medium, respectively, suggesting a low level of instability of the integrative plasmid carrying *pip* and *tetR* in the absence of selection.

Construction of a *fadD32* conditional mutant in *M. tuberculosis*

The same method used to obtain the *ftsZ* conditional mutant in *M. smegmatis* was used to obtain a conditional mutant in *M. tuberculosis* H37Rv where the *fadD32* operon was placed under transcriptional control of the Pip-dependent promoter (TB47). The *fadD32* operon, involved in mycolic acid biosynthesis, was previously demonstrated to be essential in mycobacteria (11,27). When plated on solid media containing 200 ng/ml of ATc, TB47 was unable to grow, confirming that the *fadD32* operon is indeed essential in *M. tuberculosis* (data not shown). The mutant was then grown in liquid media containing different ATc concentrations ranging from 50 to 200 ng/ml: as shown in Figure 6A, under these conditions the *fadD32* mutant stopped growing

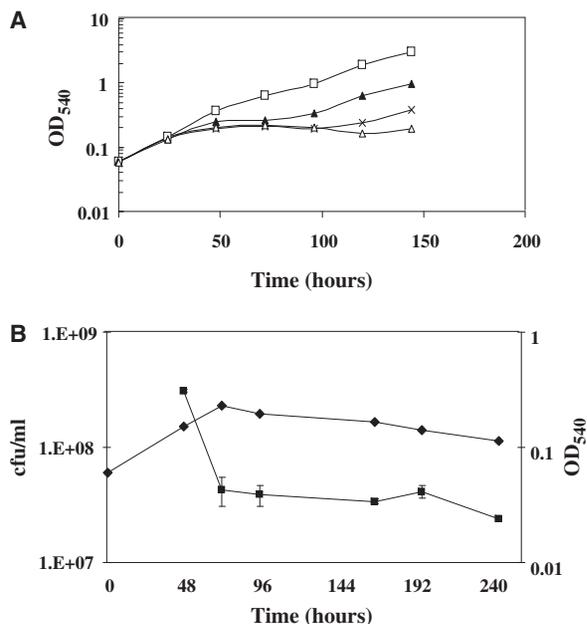


Figure 6. Characterization of the *M. tuberculosis* *fadD32* conditional mutant TB47. (A) Growth curve in the presence of different concentrations of ATc. TB47 was grown in Middlebrook 7H9 medium containing 200 ng/ml ATc (empty triangles), 100 ng/ml ATc (crosses), 50 ng/ml ATc (filled triangles) or No ATc (empty squares). (B) Viable counts variation during growth inhibition due to *fadD32* depletion. TB47 was grown in Middlebrook 7H9 medium containing 200 ng/ml ATc. An aliquot of the bacterial culture was collected at different time points starting from 48 h after the beginning of the experiment, diluted and plated for cfu determination. Squares: cfu/ml; diamonds: optical density at 540 nm.

after 48 h of incubation. Growth was restored at different times, depending on ATc concentration in the media (Figure 6A). When these cultures were plated on solid media containing 200 ng/ml of ATc, we found no colonies, suggesting that growth resumption was due to ATc instability in these conditions and not to selection of mutants (data not shown). Interestingly, during the growth inhibition phase, bacteria lost their acid fastness (Supplementary Figure S8). Finally, we repeated this experiment by exposing the culture to 200 ng/ml of ATc for 10 days, followed by viable counts. While the presence of ATc was able to inhibit TB47 growth during the entire course of the experiment, a sharp decrease (~ 10 -fold) in viable counts was detected between 48 and 72 h from the time of addition of ATc and it remained stable until the end of the experiment (Figure 6B).

In order to correlate cell growth and expression of the *fadD32* operon, the relative amount of *fadD32* mRNA was measured by quantitative RT-PCR during the first two days of exposure to ATc, before cells stopped growing and viable counts decreased. As shown in Figure 7, *fadD32* was repressed ~ 12 - and 37 -fold 24 and 48 h after the beginning of exposure to ATc, respectively. These data indicate that the TetR/Pip OFF system works efficiently in *M. tuberculosis* by shutting down transcription from the *P_{pir}* promoter in the presence of ATc.

Ex-vivo application of the TetR/Pip OFF system

In order to determine if the TetR/Pip OFF repressible system could be exploited in controlling gene expression in intracellular bacteria, we infected THP-1-derived human macrophages with TB47. The strain was pre-grown for 48 h in 200 ng/ml ATc before the infection. When ATc was added to the cell culture medium, intracellular growth of the *fadD32* mutant TB47 was strongly inhibited (Figure 8). These findings confirmed that ATc can penetrate macrophages and switch off transcription of the *fadD32* operon, which is essential for intracellular growth of *M. tuberculosis*.

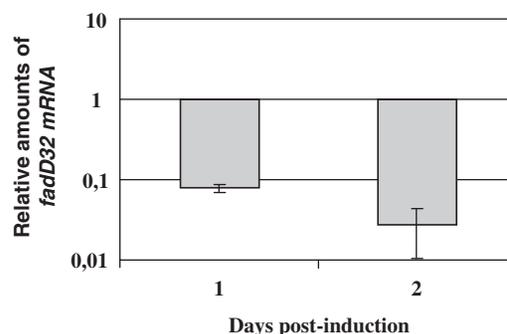


Figure 7. Changes in the relative amounts of *fadD32* mRNA measured by quantitative RT-PCR. The values are expressed as the ratio between the number of cDNA copies detected in a TB47 culture grown in the absence of ATc, and the number of cDNA copies detected in cultures grown with 200 ng/ml ATc. The values are normalized to *sigA*. Samples for RNA extraction were collected at 24 and 48 h after addition of ATc.

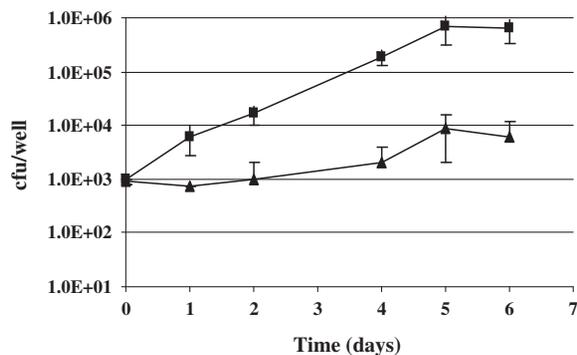


Figure 8. Growth of *M. tuberculosis* conditional mutant TB47 in THP-1-derived human macrophages in the presence (triangles) or in the absence (squares) of 200 ng/ml ATc.

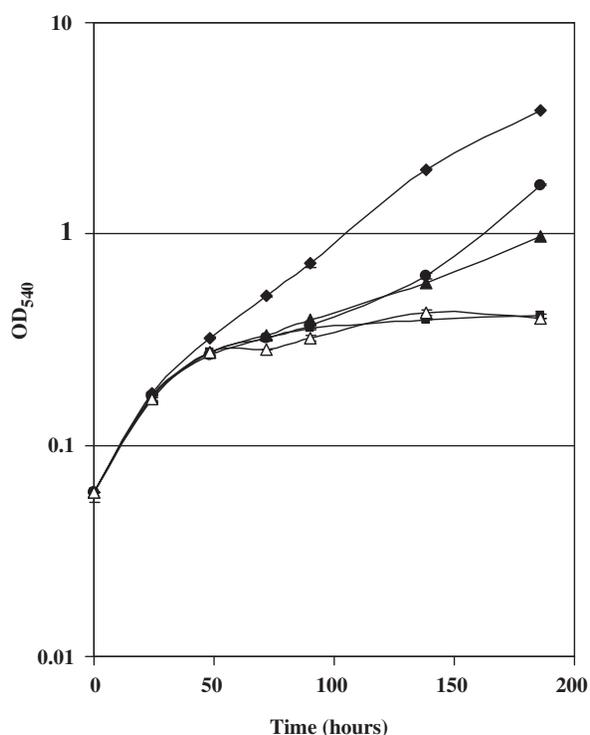


Figure 9. Growth curve of the *fadD32* conditional mutant TB47 in the presence of ATc and pristinamycin I. Bacteria were grown in Middlebrook 7H9 without ATc (diamonds) or with 200 ng/ml ATc (all the others). Forty-eight hours after the beginning of the experiment cultures were supplemented with 20 (filled triangles), 200 (circles) or 2000 ng/ml (open triangles) of pristinamycin I. As a control, one culture grown in 200 ng/ml ATc did not receive pristinamycin I (squares).

Pristinamycin I can restore growth of ATc-treated TB47 cultures

In the TetR/Pip OFF system the addition of ATc leads to induction of *pip* whose gene product represses the gene under P_{pir} control (*fadD32*, in case of TB47). Since Pip is released from its operator in the presence of pristinamycin I (28), the addition of this drug to TB47 ATc-treated cultures should be able to restore growth by alleviating Pip-mediated *fadD32* repression.

To validate this hypothesis we grew TB47 in the presence of 200 ng/ml ATc, and after 48 h we added different concentrations of pristinamycin I (20, 200 and 2000 ng/ml). As shown in Figure 9, in cultures which received 20 or 200 ng/ml of pristinamycin I growth was rescued in a dose-dependent manner despite the presence of ATc in the media. However, no growth restoration was seen in the culture with the highest amount of pristinamycin I (2000 ng/ml) suggesting that at this concentration pristinamycin I is toxic (further experiments confirmed this hypothesis, unpublished observation). These findings clearly confirmed our hypothesis, underlining the extreme versatility of the TetR/Pip OFF system to fine-tune gene expression in slow-growing mycobacteria.

DISCUSSION

In this article we describe the development of a novel repressible promoter system effective in both fast- and slow-growing mycobacteria. This approach is based on two chromosomally encoded repressors dependent on tetracycline (TetR) or pristinamycin I (Pip). The gene of interest is placed under transcriptional control of P_{pir} , a Pip-dependent promoter from *Streptomyces pristinaespiralis* (28). The Pip structural gene is placed under transcriptional control of a TetR-dependent promoter. Addition of Tc (or ATc) to the culture induces *pip* leading to repression of transcription from P_{pir} (Figure 1).

In order to characterize this repressible system we constructed two *M. smegmatis* strains in which the reporter gene *lacZ* was placed under P_{pir} transcriptional control in slightly different genetic backgrounds: in one strain *tetR* and *pip* were integrated in the same orientation, while in the other one they were integrated in opposite orientation (head-to-head, Supplementary Figure S3). In both strains β -galactosidase activity was strongly repressed in the presence of ATc both in liquid and solid media (Figure 2A and Supplementary Figure S6). Similar data were obtained when the constructs carrying *pip*, *tetR* and the reporter gene were introduced in *M. tuberculosis* (Figure 2B). The strains carrying *pip* and *tetR* in the same orientation (MS83 and TB38.2), when grown in the absence of ATc, had a lower basal activity, demonstrating that the reciprocal position of the two genes has an effect on the system. These strains were chosen for further experiments and the plasmid used to generate them (pFRA42B) was used to construct the conditional mutants.

A dose-response experiment in which bacteria were exposed to different ATc concentrations showed that repression of *lacZ* was inducer concentration-dependent in both slow- and fast-growing mycobacteria. This is of great interest, since it demonstrates that the TetR/Pip OFF system can be used to fine-tune gene expression (Figure 3).

Two mutant strains were constructed, one in *M. smegmatis* (*ftsZ*) and the other in *M. tuberculosis* (*fadD32*). Addition of ATc affected the growth of the

M. smegmatis ftsZ mutant even at very low concentrations (Figure 5), leading to the filamentous phenotype typical of FtsZ-depleted cells (Supplementary Figure S7), thus confirming the predicted essentiality of *ftsZ*, and showing that regulation of the TetR/Pip OFF system is tight enough to obtain conditional mutants. The same strain was also used to determine the rate at which mutants escaping ATc-mediated *ftsZ* repression could arise. Our data indicate that the number of mutants increased if cells were not subjected to antibiotic selection for the integrative plasmid carrying *tetR* and *pip*, suggesting that its excision was the main reason for their occurrence. Since the *int* gene present on L5-based integrative plasmids was previously shown to cause their intrinsic instability (29), the construction of an integrative plasmid with the *int* gene provided *in trans* by a replicative, curable vector should overcome this drawback. In this way, the TetR/Pip OFF circuit would be more suitable for long-term *in vivo* experiments in the absence of any kind of selection.

The system also performed well in *M. tuberculosis* as the *fadD32* mutant stopped growing 2 days after ATc addition. In this mutant, the P_{prt} promoter is placed upstream of the operon encoding *fadD32* and two additional genes, *pks13* and *accD4*. Therefore, addition of ATc likely shuts off the whole cluster. The growth arrest may thus be due to essentiality of *fadD32* or of any other gene in the operon. When ATc concentrations lower than 200 ng/ml were used in the experiment, growth of the *fadD32* mutant resumed after 4 (50 ng/ml) or 5 days (100 ng/ml) from the initial exposure (Figure 6A). However, when the bacterial culture was exposed to 200 ng/ml of ATc, growth was inhibited for at least 10 days (Figure 6B), suggesting that this concentration should be regarded as the most effective in guaranteeing long-lasting performance. These data also imply that ATc is not stable in this condition and that if its concentration falls below a critical point, bacteria that survived *fadD32* depletion could resume their growth. Forti *et al.* (11) recently reported the construction of a similar *fadD32* conditional mutant in which the operon was positively regulated by Pip. The phenotype shown by this mutant was slightly different from that displayed by our mutant: in that strain *fadD32* down-regulation caused clumping and a continuous decrease in optical density from day 3–13 after pristinamycin I removal. This difference could be due either to different kinetics of *fadD32* silencing between the two strains or to a different stringency of the two systems. The first hypothesis is the most probable, since a preliminary experiment showed that the level of expression of the target gene in the repressed state is the same in the two systems (data not shown). Finally, since Forti *et al.* grew the mutant in medium with 2 μ g/ml of pristinamycin I, a concentration that we showed to be slightly toxic for *M. tuberculosis* (Figure 9), the cells were already suffering when pristinamycin I was removed to repress *fadD32*, and this could have exacerbated their phenotype.

Our data confirmed that in *M. smegmatis* TetR-based regulated promoter systems are more sensitive to ATc than in *M. tuberculosis* (7). This could be due to several reasons: the cell wall of *M. tuberculosis* could be more

permeable to ATc than that of *M. smegmatis*; moreover, *M. tuberculosis* encodes several efflux pumps, and at least two of them have been shown to increase the MIC to tetracycline when expressed in *M. smegmatis* (7): these pumps could reduce the ATc intracellular concentration. Finally, experiments with *M. tuberculosis* are usually longer than those performed with *M. smegmatis*, making them more susceptible to the decrease of ATc concentration due to its instability at 37°C.

When we analyzed cell viability during the growth inhibition phase, we found that viable counts dropped ~10-fold between the second and the third day of incubation in the presence of ATc, but then remained stable for seven additional days, when the experiment was terminated (Figure 6B). Interestingly, after 72 h of incubation in ATc, most bacteria lost their acid-fastness clearly supporting the data indicating involvement of the *fadD32* operon in mycolic acid biosynthesis (Supplementary Figure S8) (27). Our findings suggest that *fadD32* depletion is deleterious for most of the cells, but a small percentage of them can adapt and survive, even in the absence of active replication. However, additional data are required to confirm this hypothesis.

Growth of the *fadD32* mutant stopped ~48 h after exposure to ATc, thus indicating that some time is required to reduce the concentration of the operon gene products below a critical point. This is most likely the result of several factors such as the rate of ATc penetration into the cells, *fadD32* mRNA half-life and protein turnover. Indeed, quantitative RT-PCR showed that the level of the *fadD32* transcript was reduced ~12- and 37-fold after 24 and 48 h from ATc addition, respectively, confirming that some time is required to completely ablate the mRNA, but that some repression occurs since the beginning of the experiment (Figure 7).

Finally, to verify that the TetR/Pip OFF system could be used to silence genes during macrophage infection, the *fadD32* conditional mutant was used to infect THP-1-derived human macrophages. The growth of the *fadD32* conditional mutant was strongly inhibited by the presence of ATc, but we could not detect the decrease in viable counts previously observed *in vitro*, suggesting that repression of *fadD32* might be more detrimental for *M. tuberculosis* during growth in axenic culture than during growth in macrophages (Figure 8). Experiments to address this issue are in progress.

One of the appealing aspects of the TetR/Pip OFF system is that, being based on two repressors sensing different inducers, it is possible to turn it off and on by adding the two molecules in sequence. We verified this possibility by designing an experiment in which the *fadD32* mutant was first exposed to ATc, and then pristinamycin I was added to the culture media. Results showed that indeed the effect of ATc on the *fadD32* conditional mutant could be reversed by addition of pristinamycin I (without ATc removal), and that this effect is dose-dependent (Figure 9). This novelty could be extremely useful to fine-tune gene expression without perturbing the bacterial culture with centrifugation and washing steps.

In conclusion, we have developed a stringent, stable, chromosomally located, repressible promoter system, suitable for constructing conditional mutants in both fast- and slow-growing mycobacteria. The level of repression that was obtained in *M. tuberculosis* was ~60-fold. This is clearly lower than that reported by Forti *et al.* (11) for the Pip ON system. Since in the TetR/Pip OFF system the level of β -galactosidase activity after addition of ATc at steady state reaches the value of the negative control not encoding *lacZ* (Figure 3B), the smaller regulatory window showed by this system with respect to the Pip ON system is probably due to a lower level of expression in its ON state (in the absence of ATc) due to leaky expression of *pip*. This hypothesis is supported by the fact that an *M. tuberculosis* strain carrying *lacZ* under P_{ptr} transcriptional control, but not encoding Pip, has higher β -galactosidase activity than TB38.2 (Figure 4). While this might complicate the construction of conditional mutants, if the target gene requires higher expression levels, this seems to be a minor problem since we have already used this system successfully to obtain 10 conditional mutants in *M. tuberculosis* (including *fadD32*) and two in *M. smegmatis* (including *ftsZ*) without difficulty (unpublished results).

The main advantage of the TetR/Pip OFF system with respect to the other repressible promoter systems that have been previously developed for mycobacteria (13,15) is that it does not have to be harbored by a replicative plasmid to obtain tight repression in slow-growing mycobacteria. This is probably due to amplification of the signal, thanks to the presence of two repressors working in series. This characteristic is likely to result in higher stability of the system, making it more suitable for long-term *in vivo* experiments. Moreover, its uniqueness, based on two different repressors, sensing different inducer molecules, results in a better versatility with respect to the other regulated promoter systems developed in the past for mycobacteria, likely facilitating the fine-tuning of gene expression.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

The authors thank Sabine Ehrt for pMC1s, Daniela Ghisotti for pMY696 and pMY718, Florence Bordon-Pallier (Sanofi-Aventis) for pristinamycin I and Issar Smith for helpful discussion and reading the manuscript.

FUNDING

European Commission (LHSP-CT-2005-018923). Funding for open access charge: European Commission, VI Framework (contract No. LHSP-CT-2005-018923).

Conflict of interest statement. None declared.

REFERENCES

- Vitoria, M., Granich, R., Gilks, C.F., Gunneberg, C., Hosseini, M., Were, W., Raviglione, M. and De Cock, K.M. (2009) The global fight against HIV/AIDS, tuberculosis, and malaria: current status and future perspectives. *Am. J. Clin. Pathol.*, **131**, 844–848.
- Wright, A., Zignol, M., Van Deun, A., Falzon, D., Gerdes, S.R., Feldman, K., Hoffner, S., Drobniewski, F., Barrera, L., van Soolingen, D. *et al.* (2009) Epidemiology of antituberculosis drug resistance 2002–07: an updated analysis of the Global Project on Anti-Tuberculosis Drug Resistance Surveillance. *Lancet*, **373**, 1861–1873.
- Gandhi, N.R., Moll, A., Sturm, A.W., Pawinski, R., Govender, T., Laloo, U., Zeller, K., Andrews, J. and Friedland, G. (2006) Extensively drug-resistant tuberculosis as a cause of death in patients co-infected with tuberculosis and HIV in a rural area of South Africa. *Lancet*, **368**, 1575–1580.
- Parish, T., Mahenthalingam, E., Draper, P., Davis, E.O. and Colston, M.J. (1997) Regulation of the inducible acetamidase gene of *Mycobacterium smegmatis*. *Microbiology*, **143**(Pt 7), 2267–2276.
- Triccas, J.A., Parish, T., Britton, W.J. and Gicquel, B. (1998) An inducible expression system permitting the efficient purification of a recombinant antigen from *Mycobacterium smegmatis*. *FEMS Microbiol. Lett.*, **167**, 151–156.
- Manabe, Y.C., Chen, J.M., Ko, C.G., Chen, P. and Bishai, W.R. (1999) Conditional sigma factor expression, using the inducible acetamidase promoter, reveals that the *Mycobacterium tuberculosis sigF* gene modulates expression of the 16-kilodalton alpha-crystallin homologue. *J. Bacteriol.*, **181**, 7629–7633.
- 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.
- Carroll, P., Muttucumaru, 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.
- Blokpoel, M.C., Murphy, H.N., O'Toole, R., Wiles, S., Runn, E.S., Stewart, G.R., Young, D.B. and Robertson, B.D. (2005) Tetracycline-inducible gene regulation in mycobacteria. *Nucleic Acids Res.*, **33**, e22.
- Hernandez-Abanto, S.M., Woolwine, S.C., Jain, S.K. and Bishai, W.R. (2006) Tetracycline-inducible gene expression in mycobacteria within an animal host using modified *Streptomyces ttp830* regulatory elements. *Arch. Microbiol.*, **186**, 459–464.
- Forti, F., Crosta, A. and Ghisotti, D. (2009) Pristinamycin-inducible gene regulation in mycobacteria. *J. Biotechnol.*, **140**, 270–277.
- Pandey, A.K., Raman, S., Proff, R., Joshi, S., Kang, C.M., Rubin, E.J., Husson, R.N. and Sasseti, C.M. (2009) Nitrile-inducible gene expression in mycobacteria. *Tuberculosis (Edinb)*, **89**, 12–16.
- Guo, X.V., Monteleone, M., Klotzsche, M., Kamionka, A., Hillen, W., Braunstein, M., Ehrt, S. and Schnappinger, D. (2007) Silencing *Mycobacterium smegmatis* by using tetracycline repressors. *J. Bacteriol.*, **189**, 4614–4623.
- Gandotra, S., Schnappinger, D., Monteleone, M., Hillen, W. and Ehrt, S. (2007) In vivo gene silencing identifies the *Mycobacterium tuberculosis* proteasome as essential for the bacteria to persist in mice. *Nat. Med.*, **13**, 1515–1520.
- Klotzsche, M., Ehrt, S. and Schnappinger, D. (2009) Improved tetracycline repressors for gene silencing in mycobacteria. *Nucleic Acids Res.*, **37**, 1778–1788.
- Jacobs, W.R. Jr, Kalpana, G.V., Cirillo, J.D., Pascopella, L., Snapper, S.B., Udani, R.A., Jones, W., Barletta, R.G. and Bloom, B.R. (1991) Genetic systems for mycobacteria. *Methods Enzymol.*, **204**, 537–555.
- Maciag, A., Dainese, E., Rodriguez, G.M., Milano, A., Provvedi, R., Pasca, M.R., Smith, I., Palù, G., Riccardi, G. and Manganelli, R. (2007) Global Analysis of the *Mycobacterium tuberculosis* Zur (FurB) Regulon. *J. Bacteriol.*, **189**, 730–740.
- Hatfull, G.F. and Sarkis, G.J. (1993) DNA sequence, structure and gene expression of mycobacteriophage L5: a phage system for mycobacterial genetics. *Mol. Microbiol.*, **7**, 395–405.

19. Kim, J., Zwieb, C., Wu, C. and Adhya, S. (1989) Bending of DNA by gene-regulatory proteins: construction and use of a DNA bending vector. *Gene*, **85**, 15–23.
20. Bloor, A.E. and Cranenburgh, R.M. (2006) An efficient method of selectable marker gene excision by Xer recombination for gene replacement in bacterial chromosomes. *Appl. Environ. Microbiol.*, **72**, 2520–2525.
21. Donà, V., Rodrigue, S., Dainese, E., Palù, G., Gaudreau, L., Manganelli, R. and Proveddi, R. (2008) Evidence of complex transcriptional, translational, and posttranslational regulation of the extracytoplasmic function sigma factor SigE in *Mycobacterium tuberculosis*. *J. Bacteriol.*, **190**, 5963–5971.
22. Miller, J.H. (1992) *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor Laboratory Press, New York.
23. Manganelli, R., Dubnau, E., Tyagi, S., Kramer, F.R. and Smith, I. (1999) Differential expression of 10 sigma factor genes in *Mycobacterium tuberculosis*. *Mol. Microbiol.*, **31**, 715–724.
24. Manganelli, R., Tyagi, S. and Smith, I. (2001) Real-Time PCR using molecular beacons: a new tool to identify point mutations and to analyze gene expression in *Mycobacterium tuberculosis*. In Parish, T. and Stoker, N.G. (eds), *Mycobacterium tuberculosis Protocols*. Humana Press, Totowa, NJ, pp. 295–310.
25. Manganelli, R., Voskuil, M.I., Schoolnik, G.K. and Smith, I. (2001) The *Mycobacterium tuberculosis* ECF sigma factor SigE: role in global gene expression and survival in macrophages. *Mol. Microbiol.*, **41**, 423–437.
26. Sala, C., Forti, F., Di Florio, E., Canneva, F., Milano, A., Riccardi, G. and Ghisotti, D. (2003) *Mycobacterium tuberculosis* FurA autoregulates its own expression. *J. Bacteriol.*, **185**, 5357–5362.
27. Portevin, D., de Sousa-D'Auria, C., Montrozier, H., Houssin, C., Stella, A., Laneelle, M.A., Bardou, F., Guillhot, C. and Daffe, M. (2005) The acyl-AMP ligase FadD32 and AccD4-containing acyl-CoA carboxylase are required for the synthesis of mycolic acids and essential for mycobacterial growth: identification of the carboxylation product and determination of the acyl-CoA carboxylase components. *J. Biol. Chem.*, **280**, 8862–8874.
28. Folcher, M., Morris, R.P., Dale, G., Salah-Bey-Hocini, K., Viollier, P.H. and Thompson, C.J. (2001) A transcriptional regulator of a pristinamycin resistance gene in *Streptomyces coelicolor*. *J. Biol. Chem.*, **276**, 1479–1485.
29. Springer, B., Sander, P., Sedlacek, L., Ellrott, K. and Bottger, E.C. (2001) Instability and site-specific excision of integration-proficient mycobacteriophage L5 plasmids: development of stably maintained integrative vectors. *Int. J. Med. Microbiol.*, **290**, 669–675.