

Controlling gene expression in mycobacteria with anhydrotetracycline and Tet repressor

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ABSTRACT

Gene expression systems that allow the regulation of bacterial genes during an infection are valuable molecular tools but are lacking for mycobacterial pathogens. We report the development of mycobacterial gene regulation systems that allow controlling gene expression in fast and slow-growing mycobacteria, including *Mycobacterium tuberculosis*, using anhydrotetracycline (ATc) as inducer. The systems are based on the *Escherichia coli* Tn10-derived tet regulatory system and consist of a strong tet operator (*tetO*)-containing mycobacterial promoter, expression cassettes for the repressor TetR and the chemical inducer ATc. These systems allow gene regulation over two orders of magnitude in *Mycobacterium smegmatis* and *M.tuberculosis*. TetR-controlled gene expression was inducer concentration-dependent and maximal with ATc concentrations at least 10- and 20-fold below the minimal inhibitory concentration for *M.smegmatis* and *M.tuberculosis*, respectively. Using the essential mycobacterial gene *ftsZ*, we showed that these expression systems can be used to construct conditional knockouts and to analyze the function of essential mycobacterial genes. Finally, we demonstrated that these systems allow gene regulation in *M.tuberculosis* within the macrophage phagosome.

INTRODUCTION

Mycobacterium tuberculosis, the etiologic agent of tuberculosis, infects about one-third of the world's population and kills two million people every year (1,2). Successful therapy of tuberculosis requires continuous treatment for at least 6 months with multiple drugs (2). Shortening the duration of chemotherapy selects for drug-resistant tuberculosis, which is difficult to treat and has a high mortality (3). The development of novel anti-mycobacterial drugs that would allow shorter chemotherapies and allow treatment of drug resistant tuberculosis is urgently needed.

Inducible gene expression systems are powerful tools for studying gene function and for the validation of drug targets in bacteria (4,5). Unfortunately, only a few regulated expression systems are available to control gene expression in mycobacteria (6–8). The most widely used system is controlled by two positive regulators (AmiC and AmiD) and one negative regulator (AmiA) and can be induced by the addition to the growth medium of short aliphatic amides, acetamide or butyramide (7–9). Acetamide-controlled expression systems have been used to analyze conditional mutants of *Mycobacterium smegmatis* (10,11) and *M.tuberculosis* (12) and for the over-expression of mycobacterial antigens in *M.smegmatis* (13). The currently available regulated expression systems are valuable for certain experiments that can be performed in liquid culture. None of the available systems, however, allows controlled regulation of mycobacterial gene expression during an infection.

Tet repressor (TetR) proteins regulate the expression of a family of tetracycline (Tc)-exporting proteins (14). In the absence of Tc, TetR tightly binds the tet operators (*tetO*) in

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the *tetA* promoter and suppresses transcription of *tetA*, which encodes the Tc exporter. Once Tc enters the cell it binds TetR and induces a conformational change that results in dissociation of TetR from *tetO* and thus induces expression of TetR-controlled genes. Induction of TetR occurs prior to inhibition of the ribosome by tetracyclines because affinity of TetR for these drugs is 10^3 - to 10^5 -fold higher than the affinity of the drugs for the ribosome (14). Tetracyclines can cross biological membranes by diffusion, enabling these inducers to penetrate most bacterial and eukaryotic cells. Efficient TetR-controlled expression systems have been developed for Gram-positive and Gram-negative bacteria and can be used to regulate the bacterial gene expression during infection of mice (15–18). Here, we describe the development of a TetR-controlled expression system that allows the efficient regulation of gene expression in mycobacteria in liquid culture and during infection of mammalian cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, media and culture conditions

Table 1 lists the strains and plasmids used in this study. Mycobacteria were grown in Middlebrook 7H9 medium (Difco/VWR) with 0.2% glycerol and 0.05% Tween-80. For growth of *M.tuberculosis* H37Rv, the medium was supplemented with 0.5% BSA, 0.2% dextrose and 0.085% sodium chloride (ADN). For selection of recombinant mycobacteria, kanamycin and hygromycin were used at 30 and 50 $\mu\text{g/ml}$, respectively. *Escherichia coli* DH5 α was grown in Luria-Bertani broth (LB) and kanamycin and hygromycin were used at 60 and 200 $\mu\text{g/ml}$, respectively.

Promoter trap library

Chromosomal DNA from *M.tuberculosis* H37Rv and *M.smegmatis* mc²155 was isolated as described previously (19) and partially digested with Sau3A. Digested DNA was

analyzed using 5% polyacrylamide gels; the 50–500 bp fraction was purified (20) and ligated into dephosphorylated, BamHI-digested pMGuvK. Ligated DNA was electroporated into *M.smegmatis* and fluorescent colonies were selected on LB plates containing 50 $\mu\text{g/ml}$ hygromycin. Plasmids were isolated and transformed into *E.coli* DH5 α for amplification. Preparation of competent *M.smegmatis* and electroporation were as described previously (21).

Primer extension

Primer extension was as described previously (20) using a synthetic oligonucleotide *gfp2* (5'-gcataccttcaccctctccactgac-3') and 10 μg RNA from recombinant *M.smegmatis*. RNA was isolated as described previously (22).

Reporter gene constructs

Two *gfp* genes, *gfpuv* (23) and the more recently developed *gfp+* (24), were used. The *gfpuv* gene was used as reporter in plasmid pMGuvK to isolate mycobacterial promoters in *M.smegmatis* on LB plates. The *gfp+* gene was used in plasmid pMGK to quantify green fluorescent protein (GFP) activity of mycobacteria growing in liquid cultures. The *lacZ* gene was cloned from pSVlacZ (Promega) into pMS2 (25) leading to the plasmid pME0L0.

Reporter gene assays

GFP activity. *M.smegmatis* was transformed by electroporation, plated onto selective (50 $\mu\text{g/ml}$ hygromycin) agar plates and incubated at 37°C. After 3 days of incubation, three colonies from each transformation were used to inoculate 3 ml liquid cultures and incubated for ~60 h on a shaker (250 r.p.m.) at 37°C. The bacteria were then diluted 100-fold into fresh medium and grown as before for ~15 h to reach the logarithmic growth phase. Optical density was measured, and bacteria were harvested by centrifugation and resuspended in phosphate-buffered saline (PBS) to a density of 2 OD₅₈₀/0.1 ml. Then, 0.1 ml of the bacterial suspension was transferred into black 96-well plates, and fluorescence at 515 nm after

Table 1. Bacterial strains and plasmids used in this work

Strains	Genotype	Source or reference
<i>E.coli</i> DH5 α	F ⁻ ϕ 80dlacZ Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>deoR recA1 endA1 hsdR17</i> (<i>r_K</i> ⁻ <i>m_K</i> ⁺) <i>phoA supE44 λ thi-1 gyrA96 relA1</i>	Gibco, BRL
<i>M.tuberculosis</i> H37Rv		ATCC 25618
<i>M.smegmatis</i> mc ² 155		(54)
Plasmids		
pMS2	Hyg ^r ; pAL500 origin, ColE1 origin, contains multiple cloning site and 2 transcriptional terminators	(25)
pMGuvK	pMS2 derivative, Hyg ^r , promoterless <i>gfpuv</i> , promoterless Km ^r	This work and (25)
pMGK	pMS2 derivative, Hyg ^r , promoterless <i>gfp+</i> , promoterless Km ^r	This work and (24)
pWH520		(27)
pUV15	pMS2 derivative, Hyg ^r , Km ^r , p _{smyc} - <i>gfp</i>	This work and (25)
pUV15 <i>tetORs</i>	pUV15 derivative, Hyg ^r , Km ^r , p _{smyc} - <i>tetR</i> , p _{myc1} <i>tetO-gfp+</i>	This work
pUV15 <i>tetORm</i>	pUV15 derivative, Hyg ^r , Km ^r , p _{myc} - <i>tetR</i> , p _{myc1} <i>tetO-gfp+</i>	This work
pME0L0	pMS2 derivative, Hyg ^r , promoterless <i>lacZ</i>	This work
pME0L1	pMS2 derivative, Hyg ^r , p _{myc1} <i>tetOlacZ</i>	This work
pME1sL1	pMS2 derivative, Hyg ^r , p _{smyc} - <i>tetR</i> (B), p _{myc1} <i>tetO-lacZ</i>	This work
pME1mL1	pMS2 derivative, Hyg ^r , p _{myc} - <i>tetR</i> (B), p _{myc1} <i>tetO-lacZ</i>	This work
pMV306Km	Km ^r , int, attP integrates at <i>attB</i> site on mycobacterial chromosome	(32)
pMC1s	pMV306Km derivative, Km ^r , p _{smyc} - <i>tetR</i>	This work

excitation at 485 nm was measured in a fluorescence microplate reader (Molecular Devices). Experiments with *M.tuberculosis* were performed as described for *M.smegmatis* with the exceptions that plates were incubated for 18 days and liquid cultures were not shaken and incubated for 8–10 days, then diluted 1:10 and grown for 2–3 days to reach the logarithmic growth phase.

β-galactosidase activity. Bacteria were cultivated as for GFP measurements. An aliquot of 10 μl of a 330 μM solution of the fluorogenic β-galactosidase substrate C2FDG [Molecular Probes, (26)] was mixed with 0.1 ml of bacteria in a black 96-well plate. The plate was incubated in the dark for 3 h (*M.smegmatis*) or 1 h (*M.tuberculosis*) after which the cells were excited at 485 nm and fluorescence emission was measured at 515 nm in a fluorescence microplate reader (Molecular Devices). Incubation with the substrate was shorter for *M.tuberculosis* because higher absolute β-galactosidase activities were observed with this organism. For all GFP and β-galactosidase measurements, triplicate bacterial cultures were grown and measurements are representatives of at least three independent transformations. The fluorescence intensity was normalized to the cell density and expressed in relative fluorescence units (RFUs). Mycobacteria transformed with plasmids pMS2 containing no *gfp/lacZ* gene and pMGK or pMEOL0 containing a promoterless *gfp+* or *lacZ* gene were used to measure background fluorescence of the cells and background expression.

Construction of P_{myc1tetO} and P_{s/myc1tetR}

Tet operators were inserted into P_{smyc} using oligonucleotide-directed PCR mutagenesis (20). The PCR oligonucleotides were smyc-tet1 (5'-gagttgtcctcctatcagtgatagataggctctgggag-taccgctcg-3'), smyc-tet2 (5'-ctgataggaggacaaactctatcactgat-aggagttctcccctcgtcagagaccct-3'), *gfp2* (5'-gcatcacctcaccct-cctcact gac-3') and poly3 (5'-gaactagttgattagtaagcagaagg-3'). A PCR product containing P_{myc1tetO}, the *tetO* containing P_{smyc} derivative, was digested with XbaI and SphI and cloned into pUV15 to replace P_{smyc}. A Tn10-derived *tetR* gene was amplified by PCR from pWH520 (27) and cloned into pMS2 for extrachromosomal expression or into pMV306Km for integration onto the mycobacterial chromosome. The promoters P_{smyc} or P_{imyc} were used for the expression of TetR in mycobacteria. All constructs were verified by restriction analysis and DNA sequencing.

Construction of a conditional *ftsZ* knockout in *M.smegmatis*

The first 700 bp of *M.smegmatis ftsZ* were cloned downstream of P_{myc1tetO} in a pMS2 derivative in which the pAL5000 origin of replication was deleted. The resulting plasmid, pKIfsZ, cannot replicate as an episome in mycobacteria. *M.smegmatis* that carried a P_{smyc-tetR} fusion in the *attB* site of the chromosome was electroporated with 1 μg of pKIfsZ and recombinants were selected on 7H11 agar plates that contained 50 μg/ml hygromycin and either no anhydrotetracycline (ATc) or 50 ng/ml ATc. Integration of the plasmid via homologous recombination was confirmed by PCR using primers that hybridized to P_{myc1tetO} and to the 3' region of *ftsZ* that was not part of pKIfsZ.

Macrophage infections

Murine bone marrow-derived macrophages were prepared as described previously (22,28) and seeded onto coverslips in 24-well plates. The cells were infected with live *M.tuberculosis* at a multiplicity of infection (MOI) of 5–10 bacteria to 1 macrophage. Four hours post infection, the macrophage monolayers were washed three times with warm PBS, followed by the addition of complete tissue culture medium containing 100 μg/ml amikacin to kill extracellular bacteria. Eight hours post infection the monolayers were washed again and medium was replaced. Twenty-four hours post infection, 100 ng/ml ATc were added where indicated and 72 h later the coverslips were analyzed by microscopy. Widefield images of infected cells were obtained using a Leica DMIRB fluorescence microscope equipped with a 40× 1.25 numerical aperture objective and a 1.5× magnification changer. Digital images were captured with a Photometrics CoolSnap HQ camera (Photometrics/Roper Scientific, Tucson, AZ) driven by MetaMorph image acquisition software (Universal Imaging, Downingtown, PA). Fluorescence images of all samples within one experiment were acquired under identical conditions, and the images are displayed at the same contrast so that all images are directly comparable. For the transmitted light images, phase contrast optics was used to allow visualization of the bacteria.

RESULTS

Isolation of strong mycobacterial promoters

50–500 bp long fragments of chromosomal DNA from *M.smegmatis* and *M.tuberculosis* were prepared and ligated into an *E.coli*-mycobacteria shuttle vector upstream of a promoterless *gfp* gene. Plasmid ligations were directly transformed into *M.smegmatis* and colonies with GFP activity were identified on agar plates. The GFP activities of 77 fluorescent clones, 34 from the *M.tuberculosis* library and 43 from the *M.smegmatis* library, were measured in liquid culture and compared with the activity of GFP that was transcribed from the *Mycobacterium bovis* BCG *hsp60* promoter. The *hsp60* promoter has strong activity in *M.smegmatis* and *M.tuberculosis* and has been frequently used to overexpress proteins in mycobacteria (29–32). Clones isolated from the two DNA libraries had GFP activities that ranged from 5-fold less to 10-fold higher than that of *hsp60-gfp* containing bacteria. Plasmids isolated from the eight clones with the highest GFP activities in *M.smegmatis* were also analyzed in *M.bovis* BCG (Figure 1). These plasmids produced GFP activities in BCG that were similar to those detected in *M.smegmatis*. The plasmid that produced the highest GFP activity in *M.smegmatis* and *M.bovis* BCG, named pUV15, was chosen for further characterization.

Insertion of tet operators into P_{smyc} generated a strong mycobacterial promoter that can be efficiently repressed by TetR

Sequencing of pUV15 revealed that it contains 290 bp of *M.smegmatis* DNA upstream of *gfp*. Of this fragment 189 bp are 100% identical to the upstream region of the putative

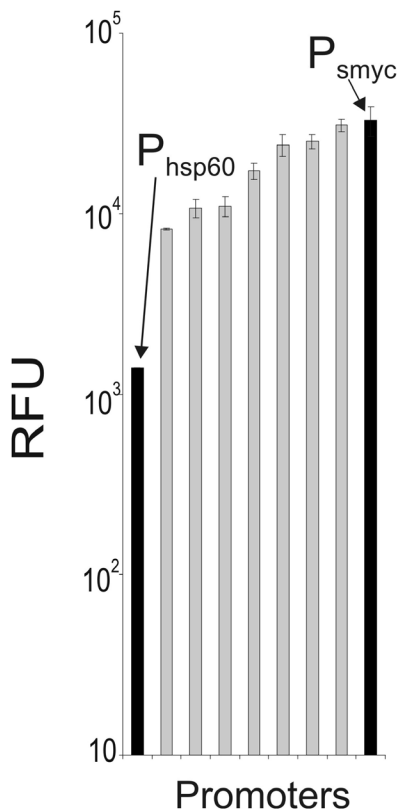


Figure 1. Isolation of DNA fragments with promoter activity in *M.bovis* BCG. *M.bovis* BCG was transformed with plasmids from the promoter library or control plasmid and GFP activity was measured in log phase cultures. The eight promoters with the highest activity in *M.smegmatis* (data not shown) were measured in *M.bovis* BCG. All measurements were carried out in triplicate. The fluorescence intensity was normalized to the cell density and expressed in RFUs. Data are averages and error bars represent standard deviations.

rpsA gene of *M.smegmatis*. The 5' end of the *gfp* mRNA encoded by pUV15 was identified by primer extension and located within the 189 bp fragment (Figure 2A). pUV15 produced no GFP activity in *E.coli* and the sequence elements that are located upstream of the putative transcriptional start point are only marginally similar to the *E.coli* -10 and -35 promoter consensus sequences. However, replacement of the putative -10 region TAGGCT with the sequence GGCTGG reduced GFP activity in *M.smegmatis* by 98%, suggesting that primer extension correctly identified the transcriptional start point within the cloned *M.smegmatis* DNA. This promoter was named P_{smyc} and is identical to that described by Kaps *et al.* (25). The 17 nt upstream and downstream of the putative -35 consensus sequence of P_{smyc} were replaced with tet operators (*tetO*) to construct a P_{smyc} derivative, P_{myc1tetO} (Figure 2B), that might be susceptible to inhibition by the transcriptional repressor TetR. P_{myc1tetO} had only 2-fold lower activity than P_{smyc} (Figure 2C). The effect of TetR on P_{myc1tetO} activity was tested using an expression cassette, in which the strong promoter P_{smyc} was used to transcribe a derivative of the Tn10 *tetR* gene (27). Figure 2D demonstrates that the expression of TetR led to efficient, 200-fold repression of GFP activity in *M.smegmatis*.

Induction of P_{myc1tetO} with ATc

ATc, a tetracycline derivative with a very high affinity to Tn10 TetR and low toxicity (33), was used to measure induction of P_{myc1tetO}. Addition of 50 ng/ml ATc to *M.smegmatis* transformed with P_{myc1tetO} and P_{smyc-tetR} resulted in 150-fold induction of GFP (Figure 2D), but the GFP activity did not reach that observed in the absence of TetR. An expression cassette in which a weaker promoter, P_{imyc} (25), transcribed *tetR* was constructed and used to test whether reducing the intracellular amount of TetR could increase the efficiency of induction. Transformation of *M.smegmatis* with P_{imyc-tetR} produced amounts of TetR that were lower than those produced by P_{smyc-tetR} (data not shown) but sufficient to repress P_{myc1tetO} by a factor of 170 (Figure 2D). Addition of 50 ng/ml ATc to *M.smegmatis* transformed with P_{myc1tetO} and P_{imyc-tetR} resulted in complete induction of GFP (Figure 2D).

Induction of P_{myc1tetO} by ATc in *M.smegmatis* is time- and dose-dependent and complete at a concentration that is 10-fold below the minimal inhibitory concentration of ATc

M.smegmatis was transformed with an episomal plasmid containing P_{myc1tetO-gfp} and P_{imyc-tetR} and cultivated in the presence of different concentrations of ATc. Quantification of GFP activities after 15 h of cultivation demonstrated that concentrations of 50 and 100 ng/ml ATc gave maximal GFP activities (Figure 3A). Induction with 25 ng/ml ATc led to 1.6-fold lower GFP activities. Cultivation of *M.smegmatis* in the presence of 250 or 500 ng/ml ATc also led to reduced GFP activities. Only 500 ng/ml ATc caused significantly reduced growth of *M.smegmatis* (Figure 3B). These experiments demonstrated that induction is dose-dependent and complete at concentrations that are 10-fold below the minimal inhibitory concentration (MIC) of ATc. The kinetics of GFP induction was measured using P_{imyc-tetR} and P_{smyc-tetR} (Figure 3C). Maximal GFP activities in P_{imyc-tetR} containing bacteria were observed 4 h after the addition of ATc. P_{smyc-tetR} containing bacteria showed strong but incomplete and delayed induction. This induction was time-dependent and the kinetics of induction depended on the expression level of TetR. Analyses using a more sensitive reporter gene, *lacZ*, confirmed that regulation of almost two orders of magnitude was achieved with P_{myc1tetO} (Figure 4A) and demonstrated that complete induction of P_{myc1tetO-lacZ} resulted in high β -galactosidase protein levels (Figure 4B).

P_{myc1tetO} allows construction of TetR-controlled, conditional knockouts in *M.smegmatis*

FtsZ is a bacterial tubulin homolog that is essential for cell division in most bacteria (34,35). A mycobacterial suicide plasmid was constructed, in which the first 700 bp of the *M.smegmatis* *ftsZ* gene were cloned downstream of P_{myc1tetO}. Integration of this plasmid into the chromosome of *M.smegmatis* via homologous recombination replaced the native *ftsZ* promoter(s) with P_{myc1tetO} (Figure 5A). *M.smegmatis* that contained *tetR* constitutively expressed by P_{smyc} in the chromosomal *attB* site (*attB:tetR*) was used to replace the *ftsZ*

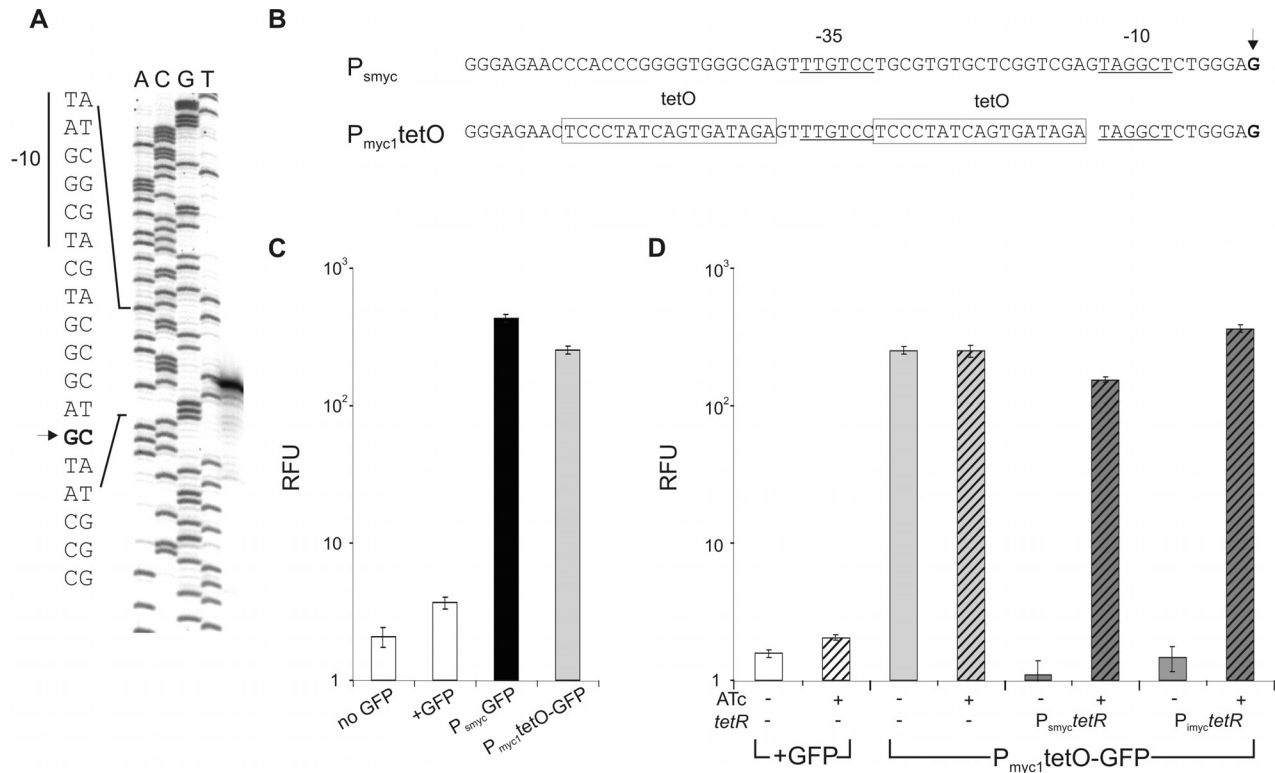


Figure 2. TetR-controlled gene expression in *M. smegmatis*. (A) Sequence of transcriptional start point of P_{smyc} determined by primer extension. Primer extension reactions were performed with RNA isolated during logarithmic growth. (B) Nucleotide sequence of P_{smyc} and $P_{myc1tetO}$. Putative -10 and -35 promoter consensus sequences are underlined. Tet operator sequences are indicated by boxes. (C) Effect of incorporation of Tet operator sites on P_{smyc} activity. The bar labeled 'no GFP' contains the *gfp* reporter without P_{smyc} or $P_{myc1tetO}$ and shows background promoter activity of the plasmid; P_{smyc} -GFP shows GFP activity driven by P_{smyc} (black bar), and $P_{myc1tetO}$ -GFP shows GFP activity driven by $P_{myc1tetO}$ (gray bar). (D) Effect of Atc and *tetR* expression levels on activity of $P_{myc1tetO}$. Crosshatched bars indicate the addition of 50 ng/ml Atc. Light gray bars show constitutive $P_{myc1tetO}$ activities. Dark gray bars indicate the expression of *tetR* by P_{smyc} (strong promoter see Figure 1) and by P_{myc1} (intermediate strength promoter). In (D), all fluorescence intensities were corrected for background fluorescence of the bacteria. All measurements were carried out in triplicate. The fluorescence intensity was normalized to the cell density and expressed in RFUs. Data are averages and error bars represent standard deviations.

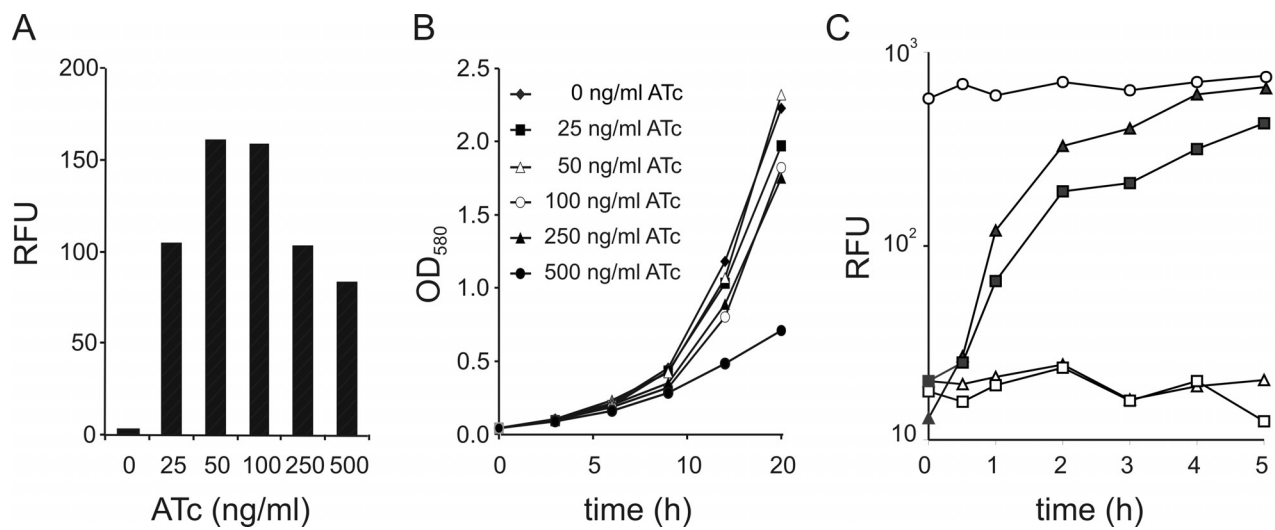


Figure 3. Determination of the optimal inducer concentration, growth inhibition by Atc and kinetics of induction. (A) Inducer concentration. *M. smegmatis* cultures transformed with plasmids carrying $P_{myc1tetO}$ -*gfp* and P_{smyc} -*tetR* were grown into log phase ($OD_{580} \sim 0.5$) and increasing amounts of Atc were added. Fifteen hours later, GFP activities were determined. (B) Growth curves in the presence of Atc. *M. smegmatis* was grown in 7H9 medium without and with different concentrations of Atc and optical densities of the cultures were recorded every 3 h. (C) Kinetics of induction. *M. smegmatis* transformed with plasmids containing $P_{myc1tetO}$ -*gfp* (circles), $P_{myc1tetO}$ -*gfp* + P_{smyc} -*tetR* (squares) and $P_{myc1tetO}$ -*gfp* + P_{myc1} -*tetR* (triangles) were grown into log phase ($OD_{580} \sim 0.5$), then 50 ng/ml Atc was added ($t=0$) to duplicate cultures of *M. smegmatis* transformed with $P_{myc1tetO}$ -*gfp* + P_{myc1} -*tetR* (filled squares) and $P_{myc1tetO}$ -*gfp* + P_{myc1} -*tetR* (filled triangles). GFP activity was determined 0.5, 1, 2, 3, 4 and 5 h after the addition of Atc.

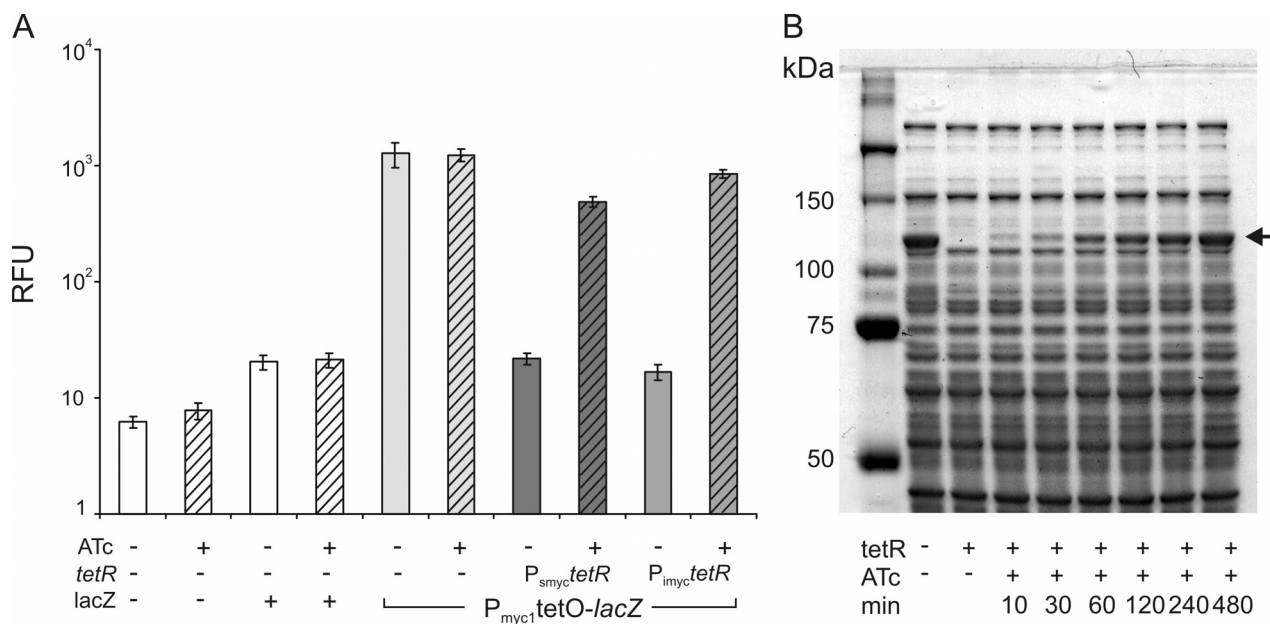


Figure 4. TetR controlled expression of *lacZ* in *M. smegmatis*. (A) TetR-controlled β -galactosidase activity. *M. smegmatis* was transformed as indicated underneath the bars. Bacteria were grown in 7H9 medium with 50 μ g/ml hygromycin to an OD_{580} of ~ 1.5 . Cultures were diluted 1:100 into fresh medium without and with the addition of 50 ng/ml ATc (cross-hatched bars). After 15 h induction time, β -galactosidase activity was determined using the fluorescent substrate C2FDG. All fluorescence measurements were carried out in triplicate. The fluorescence intensity was normalized to the cell density and expressed in RFUs. Data are averages and error bars represent standard deviations. (B) Time course of TetR-controlled β -galactosidase expression. Lysates from *M. smegmatis* (18 μ g protein) expressing *lacZ* with the $P_{myc1}tetO$ promoter were separated on a 9% SDS-PAGE gel. Lane 1, molecular weight marker; lane 2, constitutively expressed *lacZ* (no *tetR*); lane 3, repressed *lacZ* (+*tetR*); lanes 4–9, time course of induction of TetR-controlled *lacZ* by 50 ng/ml ATc. The arrow indicates the β -galactosidase protein band.

promoter with $P_{myc1}tetO$. No recombinants were obtained with TetR expressing *M. smegmatis* unless ATc was included in the plates used for selection. Integration of $P_{myc1}tetO$ in front of *ftsZ* was confirmed by PCR (data not shown). The requirement of ATc for the growth of *M. smegmatis attB:tetR P_{myc1}tetO-ftsZ* recombinants suggested that TetR efficiently repressed the expression of *ftsZ* and inhibited cell division. To test this hypothesis, *M. smegmatis attB:tetR P_{myc1}tetO-ftsZ* was taken from ATc containing plates, grown in liquid culture in the presence of ATc until they reached the logarithmic growth phase, harvested and used to inoculate liquid media with different ATc concentrations. Removal of ATc led to the inhibition of growth and aggregation of *M. smegmatis attB:tetR P_{myc1}tetO-ftsZ* but not of wt *M. smegmatis* (Figure 5B and C). Both phenotypes were delayed or reversed by the addition of ATc; low amounts of ATc delayed occurrence of the phenotypes and *M. smegmatis attB:tetR P_{myc1}tetO-ftsZ* grew normally in the presence of 2–40 ng/ml ATc. Removal of ATc from *M. smegmatis attB:tetR P_{myc1}tetO-ftsZ* cultures also resulted in the morphological changes that are expected to occur as a result of the inhibition of FtsZ (10): the bacteria became elongated, showed bud-like structures and formed large aggregates (Figure 6). Addition of 40 ng/ml ATc to the growth medium prevented these effects and the bacteria were indistinguishable from wt cells.

TetR-controlled gene expression in *M. tuberculosis*

A replicative plasmid containing $P_{myc1}tetO-lacZ$ and $P_{imyc-tetR}$ was transformed into *M. tuberculosis* H37Rv, and

β -galactosidase activities were analyzed during *in vitro* growth. The β -galactosidase activities of *M. tuberculosis* containing $P_{myc1}tetO-lacZ$ and $P_{imyc-tetR}$ were ~ 400 times lower than the β -galactosidase activities of *M. tuberculosis* containing only $P_{myc1}tetO-lacZ$ and no *tetR* (Figure 7A). Addition of 50 or 200 ng/ml ATc led to a 150- and 160-fold increase in β -galactosidase activities after 96 h. Quantification of β -galactosidase activities 24, 48 and 72 h after the addition of ATc showed that full induction of β -galactosidase occurred 72 h after the addition of 50 ng/ml ATc (Figure 7B). Lowering the ATc concentration below 25 ng/ml resulted in a dose-dependent decrease in β -galactosidase expression levels (Figure 7C). Growth curves of *M. tuberculosis* H37Rv with different concentrations of ATc revealed that concentrations of up to 1 μ g/ml did not decrease the growth rate (Figure 7D). These experiments demonstrated that the combination of $P_{myc1}tetO$ and $P_{imyc-tetR}$ allows efficient gene repression and ATc concentration-dependent induction of gene expression in virulent *M. tuberculosis*. Induction can be achieved with ATc concentrations that are at least 20-fold below the MIC of the antibiotic.

TetR-controlled gene expression in intraphagosomal *M. tuberculosis*

An important application of a TetR-controlled mycobacterial gene expression system is to regulate gene expression in mycobacteria that reside within macrophages or mice. We tested whether the TetR-controlled expression system allows the regulation of GFP expression within intraphagosomal

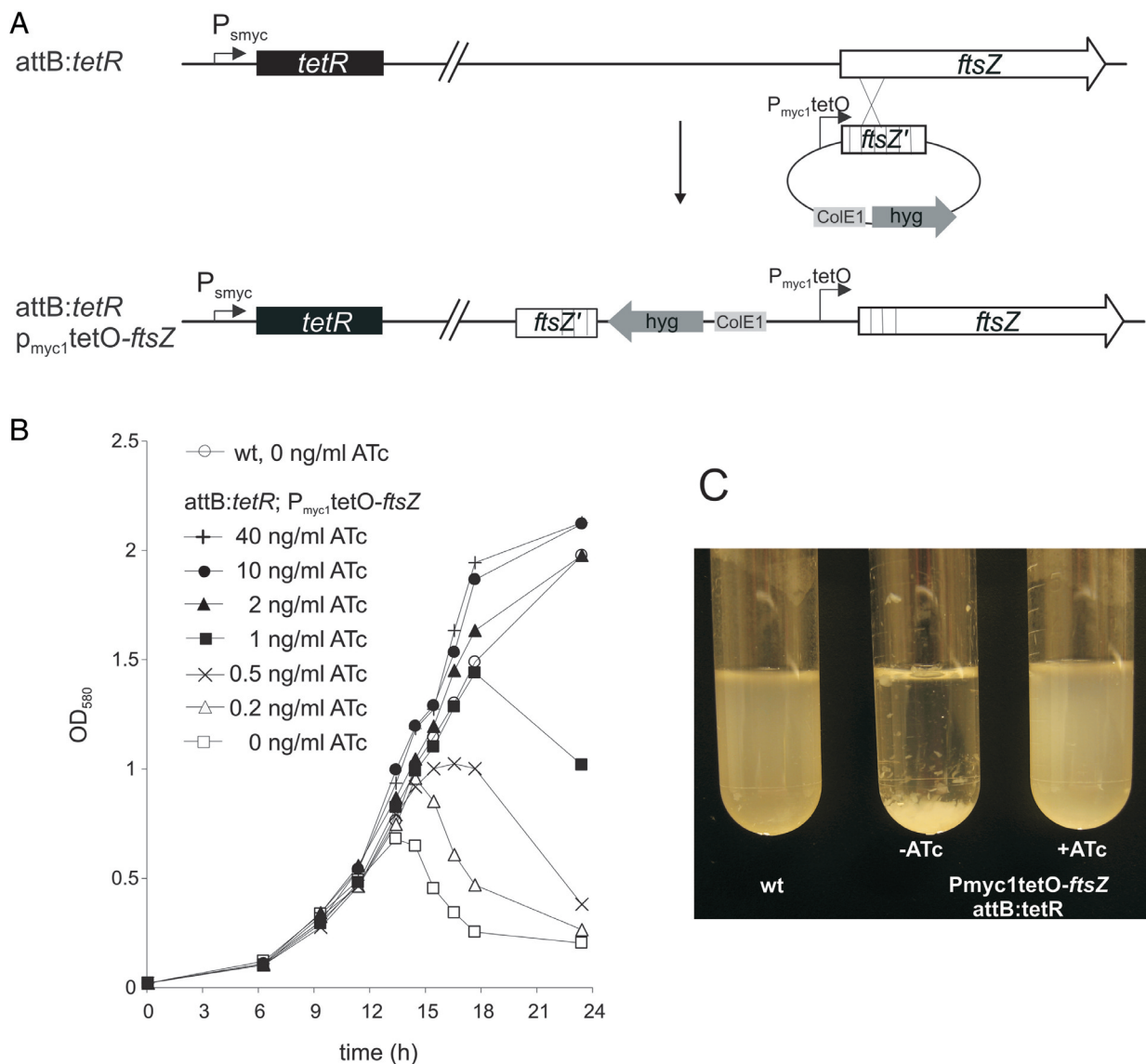


Figure 5. TetR-controlled regulation of *ftsZ* expression in *M. smegmatis*. (A) Strategy for construction of the conditional *ftsZ* knockout. A suicide plasmid was used to replace the *ftsZ* upstream region with $P_{myc1}tetO$ in an *M. smegmatis* strain that had $P_{smyc}tetR$ integrated in the *attB* site. (B) Impact of *ftsZ* silencing on growth of *M. smegmatis*. Wt *M. smegmatis* and the conditional *ftsZ* knockout were grown in the presence of 40 ng/ml ATc into the mid log phase. The cultures were washed and diluted to an OD_{580} of 0.02 into fresh medium without and with different concentrations of ATc ($t = 0$). Growth in the presence and absence of ATc was followed by measuring optical densities of the cultures. (C) Photographs of wt *M. smegmatis* and the conditional *ftsZ* knockout cultures 20 h after growth without and with 40 ng/ml ATc.

M. tuberculosis. Bone marrow-derived murine macrophages were infected with *M. tuberculosis* carrying either constitutively expressed *gfp* ($P_{myc1}tetO-gfp$) or TetR repressed *gfp* ($P_{myc1}tetO-gfp + P_{imyc}-tetR$) on a replicative plasmid. Extracellular bacteria were killed using amikacin. ATc was added 24 h post infection. Treatment of infected macrophages with 100 ng/ml ATc for 72 h led to the induction of *gfp* in intracellular *M. tuberculosis* (Figure 8). This demonstrated that both promoters, $P_{myc1}tetO$ and P_{imyc} , are active in intracellular *M. tuberculosis* and that ATc gains access to the intraphagosomal bacteria to induce gene expression. Thus, the TetR-controlled expression system allows regulation of gene expression in intracellular *M. tuberculosis*.

DISCUSSION

The ideal bacterial expression system should be silent in the absence of inducer, quickly respond to non-toxic concentrations of inducer, allow regulation over a range of expression levels and, if used for the analysis of bacterial pathogens, permit gene regulation during infection of cells in tissue culture or animals. In an attempt to develop such a system for mycobacteria, we made use of the *Tn10* encoded tet regulatory system of *E. coli* and adapted it for use in mycobacteria. A TetR responsive promoter was constructed by insertion of tet operators into regions of a strong mycobacterial promoter that are not essential for promoter activity. This promoter,

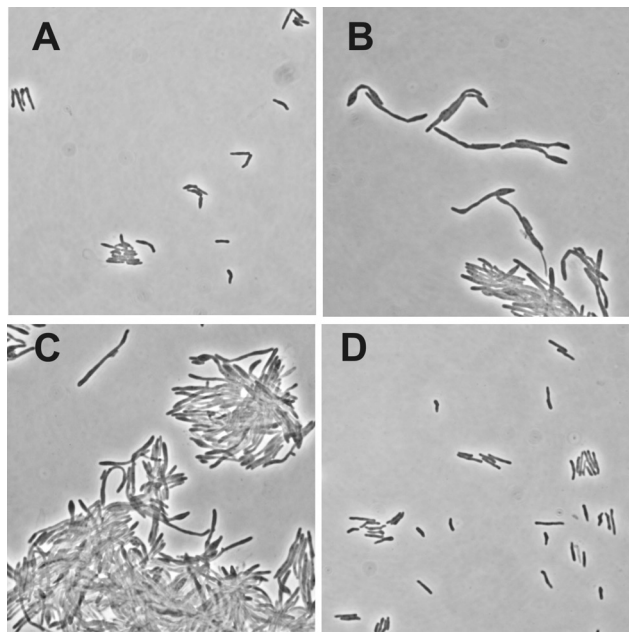


Figure 6. Morphology of the *M. smegmatis* conditional *ftsZ* knockout. Cultures grown in the presence or absence of ATc were examined by phase contrast microscopy with a 100 \times objective. (A) Wt *M. smegmatis*. (B, C and D) the conditional *ftsZ* knockout strain 9 h after removal of ATc (B), 12 h after removal of ATc (C) and in the presence of 40 ng/ml ATc (D).

$P_{myc1}tetO$, mediated strong gene expression in *M. smegmatis* and *M. tuberculosis* and was efficiently repressed by TetR. However, some β -galactosidase activity was detected in the presence of $P_{myc1}tetO$ and TetR (Figure 4A). A similar level of β -galactosidase activity was measured in *M. smegmatis* using a vector that contained *lacZ* without $P_{myc1}tetO$ (Figure 4A). This background activity was therefore most likely due to an unidentified promoter within the plasmid that is not controlled by TetR. $P_{myc1}tetO$ independent β -galactosidase activity was also observed in *M. tuberculosis*. Elimination of this activity might further improve the expression system. However, the low level of gene expression that was detected in the presence of TetR did not interfere with effective silencing of *ftsZ* in *M. smegmatis*.

The efficient regulation of promoter activities by transcriptional repressors depends on several factors; one of them is the concentration of free repressor in the cell. Overexpression of a repressor can interfere with the efficient induction of promoter activity (17), as observed for induction of $P_{myc1}tetO$ in *M. smegmatis* and *M. tuberculosis*. In *M. smegmatis*, transcription of *tetR* by P_{imyc} allowed maximal induction, while P_{smyc} led to TetR levels that were incompletely induced. This effect was even more pronounced in *M. tuberculosis*. When *tetR* was expressed by the strong promoter P_{smyc} , induction of β -galactosidase expression with 200 ng/ml for 96 h was only 2-fold in *M. tuberculosis* (data not shown). In contrast, when *tetR* transcription was driven by P_{imyc} , a promoter of intermediate strength, induction was 160-fold under the same conditions. Thus, the use of promoters of different strength to drive transcription of *tetR* provides an additional mechanism to control expression levels of the regulated gene in the repressed and induced state.

The kinetics of induction of $P_{myc1}tetO$ with ATc was different in *M. smegmatis* and *M. tuberculosis*. The slower induction kinetics in *M. tuberculosis* may be explained by the increased resistance of this species to tetracycline. The MIC of tetracycline for *M. smegmatis* is 0.5 μ g/ml (36) and identical to the concentration of ATc that inhibited growth of *M. smegmatis* in our experiments. In contrast, we did not detect any significant impact on the growth of *M. tuberculosis* after the addition of up to 5 μ g/ml ATc. This is consistent with reports that tetracyclines have little activity against *M. tuberculosis* (37–39). A tetracycline resistance determinant, Tet(V), has been identified in *M. smegmatis* (40). *M. tuberculosis* contains two mycobacterial multidrug resistance efflux pumps, TapA and P55, that when expressed in *M. smegmatis* increase its MIC to tetracycline 4- to 8-fold (36,41). These efflux pumps may contribute to the increased tetracycline resistance of *M. tuberculosis* and explain the delayed kinetics of tetracycline-induced gene expression. It is also possible that *M. tuberculosis* is less permeable for tetracyclines than non-tuberculous mycobacteria. Nevertheless, access of ATc to TetR in *M. tuberculosis* was sufficient to allow full induction of TetR in response to 50 ng/ml ATc after 72 h (three generations).

A tetracycline-dependent, conditional *ftsZ* knockout was constructed in *M. smegmatis* by integrating $P_{myc1}tetO$ directly upstream of the start codon of *ftsZ* and simultaneously expressing TetR. Removal of ATc from this *M. smegmatis* strain resulted in phenotypes that are typical for inactivation of FtsZ: growth inhibition, filamentation, formation of bud-like structures and bacterial aggregation. Expression of *ftsZ* by $P_{myc1}tetO$ presumably led to normal FtsZ levels, because overexpression of FtsZ is toxic (42). Different genes most likely require different expression levels during normal growth. $P_{myc1}tetO$ is a strong promoter, and full induction may, for some genes, lead to growth inhibition. However, the use of different ATc concentrations (Figures 5B and 7C) showed that β -galactosidase and FtsZ expression levels were inducer concentration-dependent. This demonstrated that the TetR-controlled expression system can be used to fine-tune gene expression and suggests that promoter replacement is a generally applicable strategy to generate conditional knockouts in mycobacteria.

Gene expression systems that allow turning mycobacterial genes on and off during an infection will be valuable tools for the characterization of genes that are important for *in vivo* growth and persistence of *M. tuberculosis*. The system described here allows the control of *M. tuberculosis* gene expression within phagosomes of primary macrophages and may therefore also allow control of *M. tuberculosis* gene expression during an animal infection. The strategy that we used to construct $P_{myc1}tetO$ should be applicable to other mycobacterial promoters. Increasing knowledge of mycobacterial gene expression within the host (22,43–45) should allow the selection of promoters with robust *in vivo* activities and the construction of TetR-controlled promoters that can be induced during all phases of an *M. tuberculosis* infection. A TetR-controlled expression system for mycobacteria based on the *tetZ* resistance determinant from *Corynebacterium glutamicum* has also been constructed and has been successfully used to downregulate *ftsZ* via an antisense approach (M.C.J. Blokpoel, personal communication).

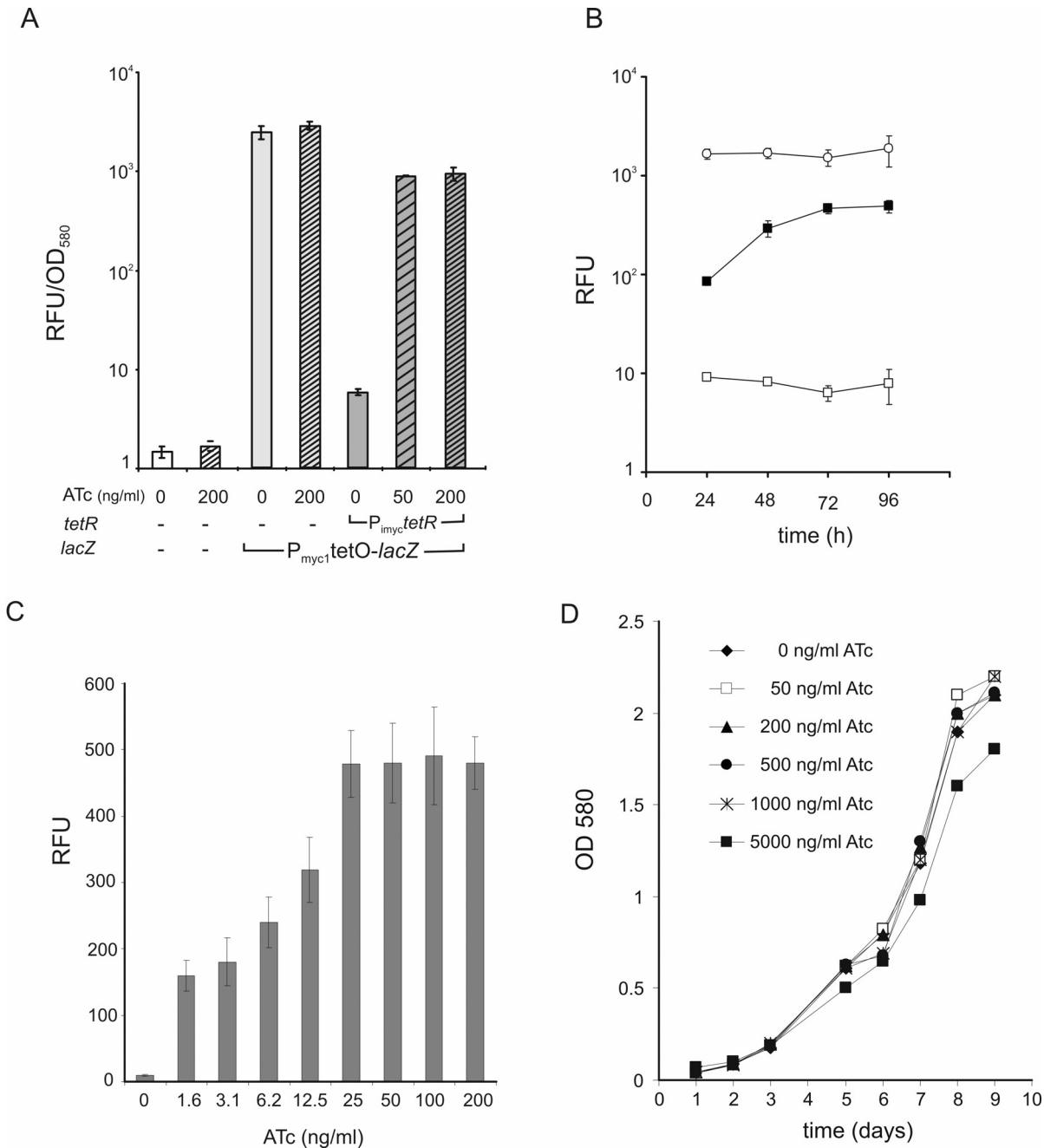


Figure 7. TetR controlled regulation of *lacZ* expression in *M.tuberculosis* H37Rv. (A) *M.tuberculosis* was transformed as indicated underneath the bars. Bacteria were grown at 37°C in 7H9 medium with 10% ADNaCl enrichment and 50 µg/ml hygromycin to an OD₅₈₀ of ~0.5. Cultures were then diluted 1:5 into fresh medium without and with addition of 50 or 200 ng/ml ATc (cross-hatched bars). After 96 h induction time, β-galactosidase activity was determined using the fluorescent substrate C2FDG. All fluorescence measurements were carried out in triplicate. The fluorescence intensity was normalized to the cell density and expressed in RFUs. Data are averages and error bars represent standard deviations. (B) Kinetics of β-galactosidase induction in H37Rv. H37Rv transformed with plasmids containing P_{myc1}tetO-lacZ (circles), P_{myc1}tetO-lacZ + P_{myc1}tetR (squares) were grown into early log phase (OD₅₈₀ ~ 0.2), then 50 ng/ml ATc was added (t = 0) to cultures of H37Rv transformed with P_{myc1}tetO-lacZ + P_{myc1}tetR (filled squares). β-galactosidase activities were measured after 24, 48, 72 and 96 h induction time as described in (A). (C) Concentration-response curve of β-galactosidase induction in H37Rv. TetR-controlled *lacZ* was induced with different concentrations of ATc for 72 h, and β-galactosidase activities were determined as described in (A). (D) Growth curves in presence of ATc. *M.tuberculosis* was grown in enriched 7H9 medium without and with increasing concentrations of ATc and optical densities of the cultures were recorded every 24 h.

We used a Tn10-derived TetR for our studies because it and its close relatives are by far the best characterized TetRs. Mutants with altered specificities for operator binding (46,47), dimerization (48) and induction (49–51) as well

as mutants with an altered response to ATc binding (52,53) provide an excellent basis for the rational improvement of tetracycline-inducible expression systems in mycobacteria.

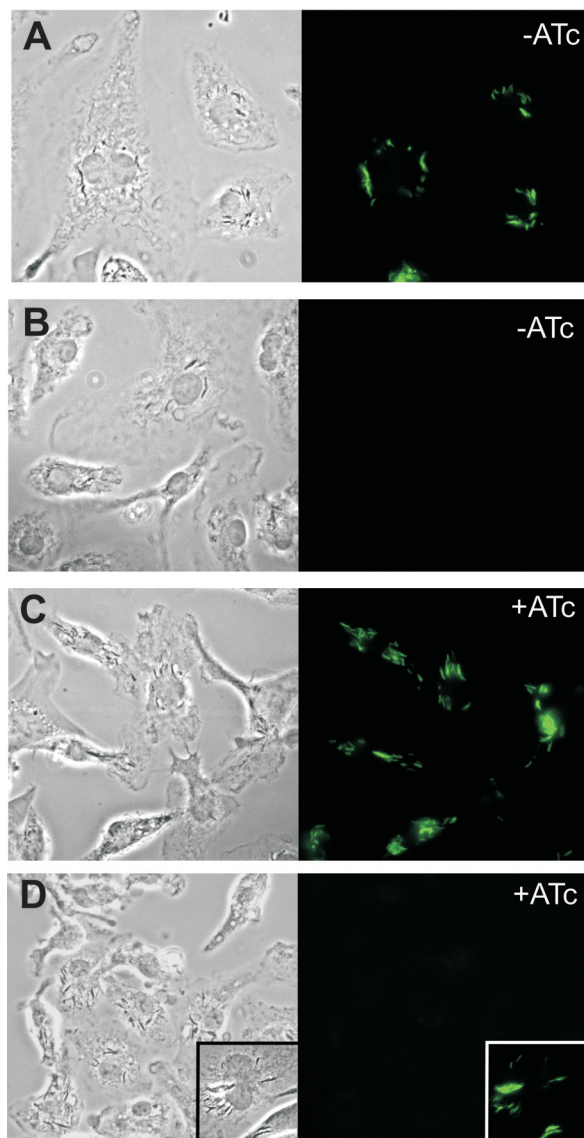


Figure 8. TetR-controlled GFP expression in intraphagosomal *M.tuberculosis* H37Rv. Murine bone marrow-derived macrophages grown on coverslips in 24 well plates were infected with live bacteria at an MOI of 5–10 bacteria: 1 macrophage. Four hours post infection, the macrophage monolayers were washed three times with warm PBS, followed by the addition of complete tissue culture medium containing 100 µg/ml amikacin to kill extracellular bacteria. The monolayers were washed again 8 h post infection. Twenty-four hours post infection, 100 ng/ml ATc were added to wells shown in (C) and 72 h later the coverslips were analyzed by microscopy as described in Materials and Methods. The left panels depict the cell monolayers in phase contrast and the right panels show the corresponding fluorescence image. Macrophages were infected with *M.tuberculosis* transformed with $P_{myc1}tetO$ -GFP (A); $P_{myc1}tetO$ -GFP + P_{imyc} -tetR (B and C). (D) Cells that are infected with Mtb lacking GFP and treated with ATc. Samples were prepared on a different day than those shown in (A–C), but the images were acquired under the exact same conditions. The relative brightness of control samples was similar on the two different days (insert: macrophages infected with Mtb constitutively expressing GFP).

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