

# Tetracycline-inducible gene regulation in mycobacteria

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Received November 22, 2004; Revised and Accepted January 18, 2005

## ABSTRACT

A system for the tetracycline-inducible regulation of gene expression in mycobacteria has been developed. We have sub-cloned the *tetRO* region from the *Corynebacterium glutamicum* *TetZ* locus into a mycobacterial shuttle plasmid, making expression of genes cloned downstream of *tetRO* responsive to tetracycline. Using the *luxAB*-encoded luciferase from *Vibrio harveyi* as a reporter (pMind-Lx), we observed a 40-fold increase in light output from *Mycobacterium smegmatis* cultures 2 h after adding 20 ng ml<sup>-1</sup> of tetracycline. Similarly, exposure to the drug resulted in up to 20-fold increase in relative light units from *M.bovis* BCG carrying the reporter construct, and a 10-fold increase for *M.tuberculosis*. Tetracycline induction was demonstrated in log and stationary phase cultures. To evaluate whether this system is amenable to use *in vivo*, J774 macrophages were infected with *M.bovis* BCG[pMind-Lx], treated with amikacin to kill extracellular bacteria, and then incubated with tetracycline. A 10-fold increase in light output was measured after 24 h, indicating that intracellular bacteria are accessible and responsive to exogenously added tetracycline. To test the use of the tetracycline-inducible system for conditional gene silencing, mycobacteria were transformed with a pMind construct with *tetRO* driving expression of antisense RNA for the *ftsZ* gene. Bacterial cells containing the antisense construct formed filaments after 24 h exposure to tetracycline. These results demonstrate the potential of this tetracycline-regulated system for the manipulation of mycobacterial gene expression inside and outside cells.

## INTRODUCTION

An estimated 2 billion people carry a latent infection with *Mycobacterium tuberculosis*, the causative agent of tuberculosis (1). There are 8 million new cases of disease every year and 2 million deaths, many of those in patients already infected with HIV. There is a need for new drugs and vaccines to treat active disease, and also to prevent development of disease in individuals with latent infection. Progress is restricted by the lack of information about the physiological status of tuberculosis bacteria within infected tissues; particularly during latent infection. The current consensus is that the bacteria persist predominantly in a non-dividing form which may be partially mimicked by *in vitro* models involving exposure to low oxygen tension or nutrient depletion (2,3). Recent experiments with non-human primates suggests that these may provide a useful model of latent tuberculosis, with ~50% of *Macaca fascicularis* given a low bronchial dose of *M.tuberculosis* developing a stable asymptomatic infection that can be reactivated to cause clinical disease by immuno-suppressing the animals (4,5). A major challenge is to identify genes that are essential for maintaining bacterial viability during latent infection in such models, and to test their suitability as drug targets. To identify such genes, we need to be able to switch them on or off at selected times during infection.

Research into the molecular microbiology of mycobacteria has been hampered by a lack of the tools that most bacterial geneticists take for granted. Members of the *M.tuberculosis* complex are neither naturally transformable nor do they commonly exchange DNA by conjugation, therefore many of the systems used for enteric bacteria have had to be extensively re-engineered for use in mycobacteria. Inducible systems have remained problematic, with reports in the literature limited to the use of the heat-shock promoter (6), the *Ptra* promoter and temperature-sensitive TraR repressor from *Streptomyces* (7), and the inducible acetamidase gene from *Mycobacterium smegmatis* (8,9). The acetamidase system has been used to

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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control gene expression (10–14) and to make a conditional antisense system (15). However the inducer, acetamide requires special growth media for optimum activity, is not suitable for use *in vivo*, and depends on the presence of a large 1.4 kb operator region, which lies 1.5 kb upstream of the promoter (9,16). The *hsp60* promoter has also been used for protein over-expression (6,12,17,18), but it is not tightly repressed, with a high basal level of activity in multi-copy and only limited induction after heat shock.

The aim of the present study was to develop an inducible system for mycobacteria that meets the criteria of tight repression in the absence of inducer, low likelihood of gratuitous induction, and with an inducer that shows good bioavailability *in vitro* and *in vivo*. These requirements have been met in a range of eukaryotes and prokaryotes by regulation of gene expression by tetracyclines, based on Tet efflux systems such as that encoded by *Tn10*, (19–21). Ehrt *et al.* have recently described the potential use of the *Tn10* system in mycobacteria (22). In the present study, we have explored the use of an alternative Tet efflux system identified on a plasmid isolated from *Corynebacterium glutamicum* (23). We reasoned that the close taxonomic relation between corynebacteria and mycobacteria might allow us to avoid problems often encountered in adapting *E.coli* expression systems for use in high GC organisms. Sequence analysis

of the R-plasmid pAG1 from *C.glutamicum* identified a novel tetracycline-resistance determinant, TetZ, comprising a predicted *tetA*-like efflux pump divergently transcribed from a *tetR*-like transcriptional repressor (Figure 1A). This is the first repressor-regulated Tet efflux system to be found in gram-positive bacteria (23), and it is of interest to determine whether similarities identified at the level of sequence comparison are borne out by functional analysis.

In this study, we report the first application of the corynebacterial Tet-efflux system and describe the development of a tetracycline-inducible system for use in mycobacteria. We demonstrate its application to the regulation of gene expression in extracellular and intracellular mycobacteria.

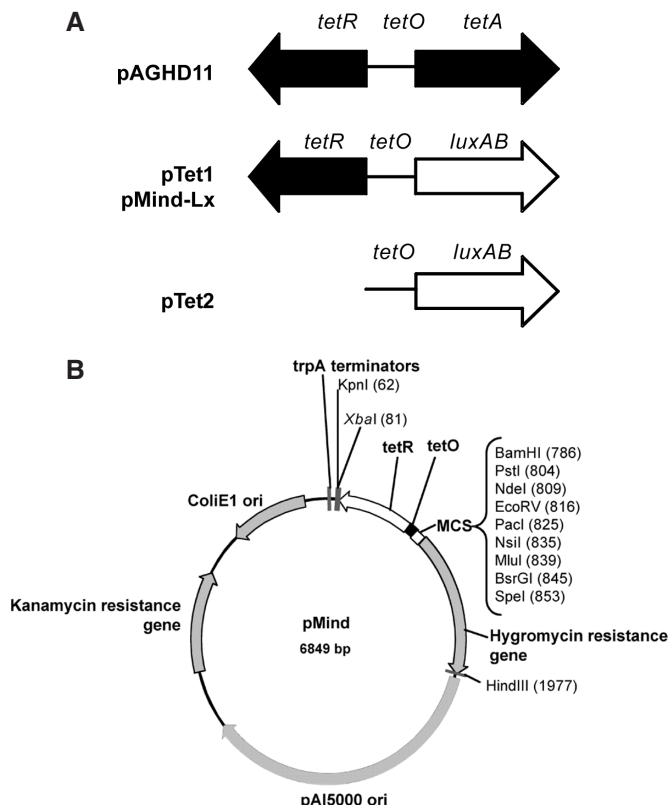
## MATERIALS AND METHODS

### Bacterial strain and growth conditions

*Mycobacterium smegmatis* mc<sup>2</sup>155 was grown at 37°C, in Hartmans–de-Bont minimal medium (supplemented with 0.08% glycerol and 0.05% Tween-80) as described previously (3), or on Luria-agar plates. *Mycobacterium bovis* BCG (Pasteur) and *M.tuberculosis* H37Rv were grown in Middlebrook 7H9 liquid broth or on Middlebrook 7H11 solid media, prepared according to the manufacturer's instructions, and supplemented with OADC (Difco). All *E.coli* strains were grown on LB-agar plates or in LB broth. Hygromycin was added as required at a concentration of 250 µg ml<sup>-1</sup> for *E.coli* and 50 µg ml<sup>-1</sup> for mycobacteria. Growth curves were performed using triplicate cultures in Hartmans–de-Bont minimal medium (3) (supplemented with 0.08% glycerol and 0.05% Tween-80) at 37°C in 125 ml conical flasks shaken at 100 or 200 r.p.m. in an orbital shaker for BCG and *M.smegmatis*, respectively. Tetracycline (Tc) was added as appropriate. Samples were processed for luminescence as described below.

### Plasmid construction

Plasmid pAGHD1 containing the 11 kb HindIII fragment of pAG1 encompassing the TetZ determinant was a kind gift from Dr Andreas Tauch, Department of Genetics, University of Bielefeld, Germany (23). The *tetRO* region from TetZ was amplified by PCR using primers TetRFor (5'-CGGG-ATCCTCACGATTCGCTCGAGGTC-3') and TetORev1 (5'-CGCATATGTGTCAGGATTCCACGATGAG-3'); the *tetO* determinant was amplified using TetOFor (5'-CGGGATC-CAGTTGCACTTTATCATCGATAAC-3') and TetORev1. The forward primers contain BamHI restriction sites and the reverse primer contains an NdeI restriction site (underlined). Both products were cloned upstream of the *luxAB* genes from *Vibrio harveyi* in the vector pSMT1 (24), to make pTet1 and pTet2 (Figure 1A). For further work, the *tetRO* region was amplified by PCR using TetRO-F (5'-GCTCTAGATCAC-GATTCGCTCGAGGTC-3') and TetRO-R (5'-CGGGATCC-TGTCAGGATTCCACGATGAG-3') containing XbaI and BamHI sites (underlined) respectively, and cloned into the XbaI–BamHI sites upstream of the *luxAB* genes in pSHKLx (25) to generate pMindLx (Figure 1A). Plasmid pSHKLx is an *E.coli*–mycobacteria shuttle plasmid containing kanamycin and hygromycin selectable markers and the *luxAB* genes from *Vibrio harveyi* as a reporter. In pMind the *luxAB* genes have been replaced by a multiple cloning site (Figure 1B) to



**Figure 1.** (A) The organization of the TetZ region from pAGHD1. pTet1 and pMind-Lx contained the *tetR* and *tetO* regions cloned in front of the *luxAB* reporter; *tetR* was omitted from pTet2. (B) A map of the pMind vector, illustrating origins of replication and selectable markers for both *E.coli* and mycobacteria, and the *tetRO* operator–repressor region upstream of a multiple cloning site.

facilitate cloning of other genes of interest. Vectors were introduced into mycobacteria by electroporation (26).

### Luciferase assay

Bioluminescence of *M. smegmatis* and *M. bovis* BCG was measured in a Berthold AutoLumat LB953 tube luminometer; for *M. tuberculosis* a Turner-Designs 20/20 tube luminometer was used. Previous work has shown that the Turner-Designs reader gives relative light unit (RLU) values approximately 1000-fold lower than the Berthold instrument, but the study confirmed linearity between machines (24). To measure RLUs, 0.1 ml of the substrate, 1% *n*-decyl aldehyde (Sigma) in ethanol, was injected automatically into tubes containing cells in a final volume of 1 ml in phosphate-buffered saline (PBS). Raw data were collected in duplicate or triplicate over a 20 s period and mean values calculated. The luminescent output was normalized to the cell density and expressed as RLU. Samples were diluted 10-fold in PBS to minimize quenching of luminescence.

### Construction of an *ftsZ* antisense knock-down in *M. smegmatis*

The *M. tuberculosis ftsZ* gene (Rv2150c) was amplified by PCR using primers *ftsZ*antisense1 (5'-GGACTAGTATGACCCCCCGCACAACTA-3') and *ftsZ*antisense2 (5'-CGGGATCCTCAGCGGCGCATGAAGGGCG-3'), containing *SpeI* and *BamHI* restriction sites (underlined), respectively. This product was cloned into the *BamHI*-*SpeI* sites of pMind, to generate pMind-*FtsZ*-antisense, which was transformed into *M. smegmatis*. Tetracycline (20 ng ml<sup>-1</sup>) was added to mid-log phase bacteria (OD<sub>600nm</sub> ~0.5) and culture continued for 24 h (OD<sub>600nm</sub> ~ 1.6). Samples were acid fast stained and slides were viewed at 100× magnification with a Nikon Eclipse E600 microscope. Images were captured using a Nikon DXM1200 camera with ACT-1 software.

### Infection of macrophages *in vitro* and induction of *luxAB* reporter

The J774A.1 murine macrophage-like cell line was seeded overnight at  $5 \times 10^5$  cells per well in NUNC 12-well plates at 37°C and 5% CO<sub>2</sub> in HI-glucose DMEM (Sigma), supplemented with 10% heat-inactivated foetal calf serum and 2 mM L-glutamine. Log-phase cultures of mycobacteria were centrifuged at 3000 g for 10 min, and resuspended in DMEM to obtain the required multiplicity of infection (MOI). Infections were performed at an MOI of 10–20 bacteria per cell for 6 h at 37°C, and then the monolayer was washed three times with Dulbecco's PBS (Invitrogen). Cells were treated for 2 h with 200 µg ml<sup>-1</sup> amikacin in DMEM to eliminate extracellular mycobacteria (27). The cells were washed again three times with Dulbecco's PBS and incubated in fresh DMEM. After 24 h of further incubation, 100 ng ml<sup>-1</sup> Tc was added to the medium. Samples were taken from triplicate wells at 24, 48 and 72 h after the addition of tetracycline. RLUs and colony forming units (CFUs) were determined at each time point; eukaryotic cells were lysed by the addition of 1800 µl of sterile Dulbecco's PBS containing 0.1% Triton X-100 per well.

## RESULTS

### Functional activity of the repressor-regulated *C. glutamicum TetZ*

During sequence analysis of pAG1 plasmid from *Corynebacterium glutamicum*, Tauch *et al.* identified a set of genes with homology to tetracycline-regulated efflux systems previously characterized in gram-negative bacteria (Figure 1A) (23). This comprises the gene predicted to encode a transcriptional repressor (TetR) together with a divergently transcribed efflux pump (TetA). The intervening operator region (*tetO*) is predicted to include the promoter sequence for each gene, as well as repressor sites for binding of TetR. By analogy with the *Tn10* system, it is anticipated that binding of TetR to *tetO* will block expression from the *tetA* and *tetR* promoters in the absence of tetracycline, and that this inhibition will be relieved when drug is added to the system.

To test this model, we amplified the gene encoding the transcriptional regulator (*tetR*) along with the operator region (*tetO*). This region was then cloned upstream of the *luxAB* genes encoding the luciferase enzyme from *Vibrio harveyi*, in the mycobacteria-*E. coli* shuttle vector pSMT1 (24) to construct pTet1 (Figure 1A), which was introduced into *Mycobacterium smegmatis* by electroporation. A second construct, pTet2, was generated in the same way, but without the *tetR* gene (Figure 1A). We anticipated that luciferase activity conferred by pTet2 would provide a measure of gene expression from the *tetA* promoter, and that pTet1 expression would provide a measure of the efficiency of repression by TetR. In fact, the luminescence output for both constructs was ~10<sup>5</sup> RLU per ml, a similar level to that obtained with *luxAB* in the absence of any added promoter. This background expression is thought to be due to readthrough from sequences within the plasmid backbone (24). The absence of enhanced luminescence in pTet2 suggested either that the *tetA* promoter was inactive in mycobacteria, or that promoter activity required sequence elements present within the *tetR* gene. Results obtained by addition of tetracycline are consistent with the second explanation. Cultures containing the two constructs were grown in the presence or absence of 10 ng ml<sup>-1</sup> Tc for 2.5 h and luminescence was compared. Addition of Tc to the pTet1 cultures resulted in a 68-fold increase in luminescence output (~10<sup>7</sup> RLU ml<sup>-1</sup>). Tc had no effect on luminescence of pTet2 cultures. These results show that TetR is expressed as a functional repressor in mycobacteria, and that it regulates activity from a promoter region that includes *tetO* and part of the *tetR* gene itself.

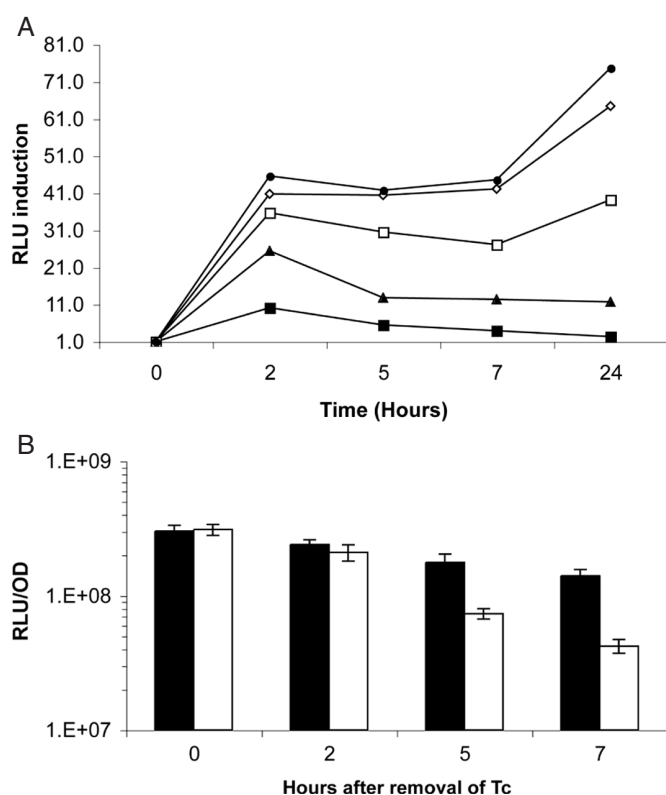
### Dose-dependent Tc regulation of gene expression in mycobacteria

For further work, the *tetRO* region was re-cloned upstream of *luxAB* in pSHKLx (25) to construct pMind-Lx, or without the luciferase genes to make pMind (Figure 1B). A dose-response curve was established by adding doubling dilutions of Tc from 20 to 1.25 ng ml<sup>-1</sup> to triplicate samples from a log phase culture of *M. smegmatis* harbouring pMind-Lx (OD<sub>600nm</sub> = 0.5, ~10<sup>9</sup> CFU ml<sup>-1</sup>). Figure 2A illustrates the kinetics of induction over a 24 h time period. At the 24 h time point, luminescence in the absence of Tc was  $6.3 \times 10^5$  RLU per OD unit; this rose to  $4.6 \times 10^7$  RLU per OD unit after



24 h growth in the presence of  $20 \text{ ng ml}^{-1}$  of Tc. Background luminescence from a promoterless *luxAB* in this vector is  $2 \times 10^5$  RLU per OD unit, indicating repression is incomplete. A concentration of  $20 \text{ ng ml}^{-1}$  gave the maximum levels of induction and was chosen for future experiments. This is well below the minimal inhibitory concentration of Tc for *M. smegmatis* ( $80 \text{ ng ml}^{-1}$ ; data not shown), and no effect on mycobacterial growth was observed in the dose-response experiment.

As observed previously (24), luminescence output was lower in stationary phase bacteria. To test the effect of Tc-induction in non-dividing *M. smegmatis*[pMind-Lx],  $20 \text{ ng ml}^{-1}$  Tc was added to a culture that had been allowed to grow into stationary phase ( $\text{OD}_{600\text{nm}} = 1.6$ ,  $\sim 8 \times 10^9 \text{ CFU ml}^{-1}$ ). A 10-fold increase in light output was observed after 2 h.



**Figure 2.** (A) The kinetics of luciferase induction following addition of varying concentrations of Tc to *M. smegmatis* [pMind-Lx]. Tc was added to bacterial cultures and luminescence was measured after different time intervals. Tc was added at  $1.25 \text{ ng ml}^{-1}$  (filled square),  $2.5 \text{ ng ml}^{-1}$  (filled triangle),  $5 \text{ ng ml}^{-1}$  (open square),  $10 \text{ ng ml}^{-1}$  (open diamond) and  $20 \text{ ng ml}^{-1}$  (filled circle). Y-axis values are the ratio of RLU measured in the presence and absence of Tc. (B) The decrease in luminescence over time after Tc has been removed. Open bars show the loss of luminescence from induced *M. smegmatis* [pMind-Lx] that were washed and resuspended in fresh medium. The corresponding induced culture was washed then resuspended in its original Tc-containing medium (dark bars). Y-axis values are RLU per OD unit.

Restoration of repression of the system once Tc is removed was tested by inducing bacteria with  $20 \text{ ng ml}^{-1}$  of Tc for 18 h, washing the cells, then continuing growth in fresh broth. There was a progressive reduction in luminescence, with a 70% decrease in light output after 7 h (Figure 2B).

### Activity of tetracycline derivatives

Other members of the tetracycline family are able to induce the *Tn10*-based Tet system (19). We tested six tetracyclines for their ability to induce light production by *M. smegmatis* [pMind-Lx] at a concentration of  $20 \text{ ng ml}^{-1}$ . After 18–24 h exposure, Tc showed the highest level of induction (Table 1), with relatively poor levels of induction by all the other derivatives, including doxycycline and anhydrotetracycline, both of which have proved successful in *Tn10*-based systems (19). Doxycycline and chlortetracycline were found to be toxic in this system, and bacterial death, as indicated by a decrease in optical density, was observed at both time points.

### Tetracycline-dependent luminescence in slow-growing mycobacteria

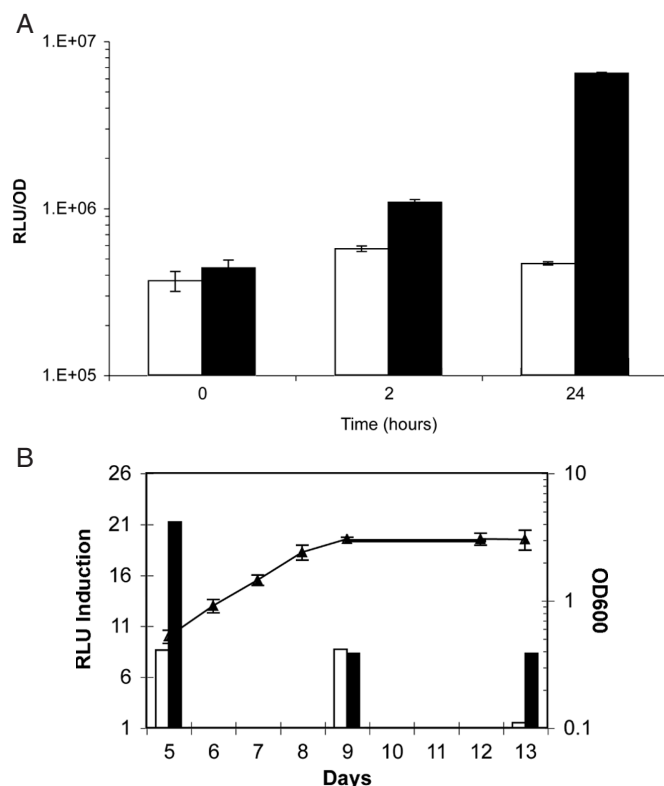
*M. smegmatis* is a rapid-growing saprophytic mycobacterium with a doubling time of  $\sim 3 \text{ h}$ . To test the function of this system in slow-growing mycobacteria (which have a doubling time of  $\sim 24 \text{ h}$ ),  $20 \text{ ng ml}^{-1}$  Tc was added to a log phase culture ( $\text{OD}_{600\text{nm}} 0.4\text{--}0.5$ ) of *M. tuberculosis* harbouring the pMind-Lx plasmid, and light output was measured after 2 and 24 h. Luminescence of the treated culture was 2-fold higher than that in the control culture at the 2 h time point, rising to 13-fold after 24 h (Figure 3A). Similarly, Tc induction of an *M. bovis* BCG[pMind-Lx] culture during log phase (day 5), resulted in 8-fold and 21-fold increase in luminescence after 2 and 24 h (from a level of  $1.8 \times 10^5$  RLU per OD unit in the uninduced culture). Luminescence output and Tc induction decreased as cultures entered stationary phase with a maximum 8-fold induction observed after 24 h in day 9 and day 13 cultures (from a level of  $4.3 \times 10^4$  and  $6.1 \times 10^3$  RLU per OD unit, respectively, in the uninduced cultures) (Figure 3B). These results show that the Tc regulated promoter is active in logarithmic and stationary phase cultures of slow-growing as well as fast-growing mycobacteria.

### Tetracycline-dependent luminescence of intracellular *M. bovis* BCG

Pathogenic mycobacteria are able to persist during infection within a phagosomal compartment of host macrophages, and an important property of a tool for mycobacterial gene regulation is the ability to control gene expression within this *in vivo* environment. To test the bioavailability of tetracycline to mycobacteria inside phagosomes, we infected J774 macrophages with *M. bovis* BCG[pMind-Lx]. After 6 h, cells were treated for 2 h with  $200 \mu\text{g ml}^{-1}$  of amikacin. This procedure has been shown to be effective in killing the majority of

**Table 1.** Luciferase induction in response to  $20 \text{ ng ml}^{-1}$  different tetracycline-related compounds compared to non-induced control

Time	Control	Tetracycline	Minocycline	Doxycycline	Chlortetracycline	Oxytetracycline	Anhydrotetracycline
18 h	1	38	1	3	8	1	1
24 h	1	108	1	6	41	1	2

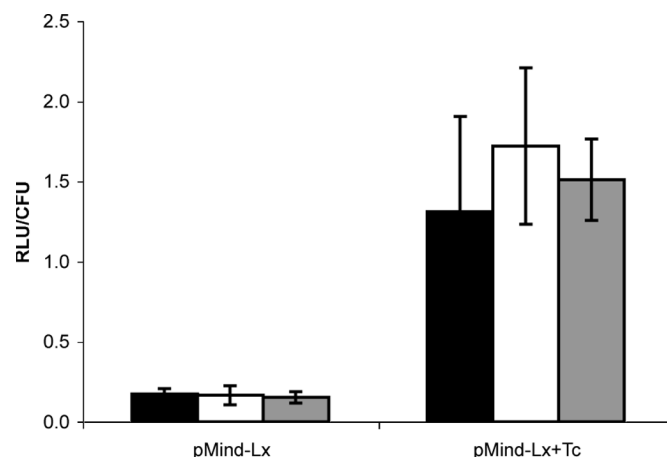


**Figure 3.** (A) The light output from *M. tuberculosis* [pMind-Lx] after 2 and 24 h induction with Tc. Samples were incubated with 20 ng ml<sup>-1</sup> Tc (filled bars) and controls without Tc (open bars). Y-axis values are RLU per OD unit. (B) Luciferase induction from *M. bovis* BCG[pMind-Lx] at various times during growth. Replicate cultures were induced with 20 ng ml<sup>-1</sup> Tc on day 5 (log phase growth), day 9 (early stationary phase) and day 13 (late stationary phase) and the light output measure after 2 h (open bars) and 24 h (filled bars). The ratio of RLU in the presence and absence of Tc induction is plotted on the Y-axis. The mean OD<sub>600nm</sub> for all the replicate cultures is also shown (filled triangles).

extracellular mycobacteria with minimal effect on bacteria that have already been taken up by the macrophage (27). Medium containing amikacin and dead bacteria was then removed and replaced with fresh tissue culture medium and incubation continued. After 18 h, the medium was removed and replaced with fresh medium containing 100 ng ml<sup>-1</sup> Tc. This procedure allowed us to focus predominantly on the luminescence signal from intracellular bacteria. Infected cultures were then lysed, and the number of viable bacteria (CFU) and light output (RLU) was measured from triplicate wells after 24, 48 and 72 h induction (Figure 4). The number of bacteria was similar in all cultures (~10<sup>4</sup> CFU ml<sup>-1</sup>). There was a 10-fold increase in luminescence 48 h after exposure to Tc. As a control, we also measured light output from BCG carrying a construct in which luciferase is constitutively expressed under control of the *hsp60* promoter (24). Luminescence output from this construct (1 × 10<sup>2</sup> RLU per CFU after 24 h) was unaffected by addition of Tc (data not shown).

#### Tetracycline-regulated expression of antisense mRNA

A further important property of a tool for gene regulation is its application in experiments involving down regulation of selected genes. One way to achieve this is to induce expression of

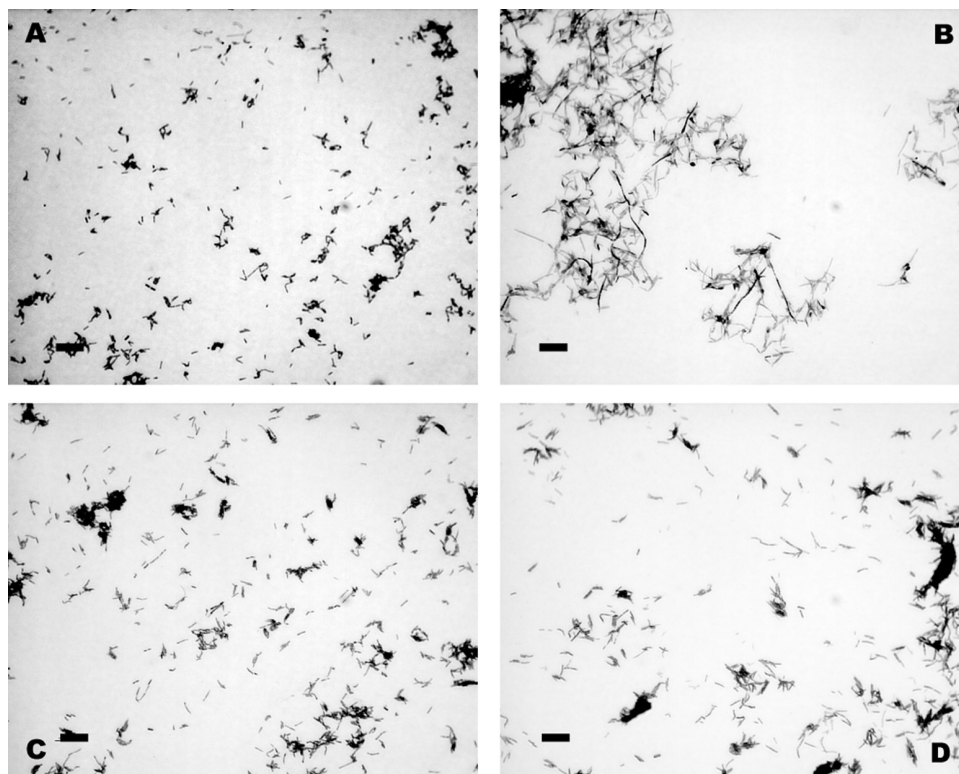


**Figure 4.** Luminescence produced by intra-phagosomal *M. bovis* BCG[pMind-Lx]. J774 macrophages infected with *M. bovis* BCG[pMind-Lx] were exposed to 100 ng ml<sup>-1</sup> Tc and light output was measured in samples taken after 24, 48 or 72 h. Addition of Tc resulted in an ~10-fold increase in light output from BCG[pMind] but had no effect on output from the constitutively expressed *luxAB* in BCG[pSHK-Lx] (data not shown). There was no significant difference in the number of viable bacteria present in drug-treated or control cultures. Y-axis values are mean RLU per CFU from triplicate wells; error bars represent SD values. Filled bars, 24 h; open bars, 48 h; grey bars, 72 h induction.

antisense mRNA. To test the function of the pMind construct in this system, we focused on regulation of the *ftsZ* gene in mycobacteria. The FtsZ protein forms a ring at the site of bacterial cell division that acts as a key focus for many of the proteins involved in septum formation and cell division. Decreased production of FtsZ means that the septum fails to form and results in a filamentous phenotype that is easily assessed by visual examination (14). We constructed a strain of *M. smegmatis* carrying pMind-FtsZ-antisense, which contains the whole of the *ftsZ* gene in the antisense orientation under the control of the *tetRO* region. Logarithmic phase cultures were grown for 24 h in the presence or absence of Tc, and then examined by microscopy. Panel A of Figure 5 illustrates the normal morphology of *M. smegmatis* in the absence of antisense induction, and panel B shows the characteristic filamentation associated with reduced expression of *ftsZ* after antisense induction. Panels C and D illustrate results with control cultures of *M. smegmatis* without the inducible construct, demonstrating that filamentation is not simply a consequence of exposure to Tc. This experiment shows that the TetZ system has application in the construction of conditional phenotypes for otherwise essential genes.

#### DISCUSSION

Repressor-regulated efflux systems for tetracycline resistance have been extensively characterized from gram-negative bacteria and have been widely used in development of tools for conditional gene regulation. In the present study, we have demonstrated the functional activity of an analogous regulatory system from a gram-positive bacterium, *Corynebacterium glutamicum*, and demonstrated its potential as a tool for gene regulation in mycobacteria.



**Figure 5.** Micrographs showing the filamented morphology of *M.smegmatis* after induction of *ftsZ* antisense. (A) *M.smegmatis* [pMind-FtsZ-antisense] after 24 h without Tc; (B) *M.smegmatis* [pMind-FtsZ-antisense] after 24 h induction with 20 ng ml<sup>-1</sup> Tc; (C) *M.smegmatis* without Tc; (D) *M.smegmatis* with 20 ng ml<sup>-1</sup> Tc. Images taken under a 100× objective, the scale bar is 2 μm.

In contrast to *Tn10*-based Tet-systems we found that tetracycline, rather than other members of the family such as doxycycline, was the most efficient inducer of the *C.glutamicum* TetZ-based system. The TetR(Z) protein may have a different Tc-binding specificity to the corresponding *Tn10* repressor, and some support for this comes from an examination of the protein sequence. TetR(Z) shares 43% identical amino acid residues with the two other members of the Tet-efflux family TetR(A) and TetR(G), but TetR(Z) is missing alpha-helix 9, which contains 2 out of 15 amino acids residues that are in contact with the magnesium-Tc inducer (28). It may be that these residues are important for efficient binding of other tetracyclines.

An important feature of an inducible system is the tightness of the control of expression in the absence of inducer. Incomplete repression of *tetA* expression by TetR is indicated by the fact that luminescence output from the pMind-Lx construct in the absence of Tc was ~10-fold higher than that observed for *luxAB* in the absence of *tetRO* insert. A potential explanation for incomplete repression is suggested by comparison of the TetR(Z) and TetR(B) sequences. TetR(Z) contains a mutation in the DNA binding α2 helix–turn–helix motif; Thr40 of TetR(B), which contacts the GC base pair at position 6 of the operator, is changed to Gly in TetR(Z), a substitution previously shown to lead to 40-fold less efficient repression in a *tetR-lacZ* system in *E.coli* (29). It is possible that re-engineering the *tetR(Z)* gene to reverse this change will enhance repressor binding.

A second important feature is the magnitude of the induced response, which is in turn a function of the selected promoter

region. In the present study, we have relied on the *tetA* promoter present in the original *C.glutamicum* plasmid. Luminescence output from unrepressed *tetA* in BCG was significantly lower than that obtained with the strong mycobacterial *hsp60* promoter, and it is likely that substitution of *tetA* by alternative mycobacterial promoter sequences could be used to enhance the induced response.

While there is clearly scope to improve the TetZ regulatory system, in its present form it represents a significant advance on previous acetamide-based inducible expression systems available for mycobacterial genetics. It does not require specific growth media and functions in slow-growing as well as rapid-growing mycobacteria. The magnitude of induction (~10-fold) was lower in *M.tuberculosis* and *M.bovis* BCG than in *M.smegmatis* (~50-fold), and the kinetics of induction were slower. This difference may be due to a combination of factors such as the metabolic status of the cell and its permeability to Tc, and may reflect differences both in gene expression and in the enzymic activity of the luciferase reporter resulting from differences in cofactor availability. A reduction in magnitude and speed of the induction response was also observed as bacteria entered the stationary phase of growth.

We have demonstrated two features of the TetZ system that will be of particular importance in future applications to studying mycobacterial pathogenesis. First, it can be used to regulate the expression of genes in mycobacteria that are present inside eukaryotic cells during infection. The initial studies presented here demonstrate Tc-induced expression of luciferase by BCG in macrophage cultures, and comparison



with other systems suggests that an extension of this approach can be used to regulate *M. tuberculosis* gene expression during infection in whole animal models (30–33). Second, we have shown that TetZ can be used to regulate expression of antisense mRNA, allowing knockdown of selected gene products. Using the essential *ftsZ* gene as proof of principle, our results show that the level of background expression in the absence of Tc is sufficiently low that it does not have a phenotypic effect, and also that the level of induction achieved by the TetZ system is sufficiently high that it results in a very marked biological phenotype. The combination of these two features of the TetZ system—allowing us to switch off target genes during infection in animal models—will have broad application in studying the biology of latent tuberculosis.

## ACKNOWLEDGEMENTS

The authors thank Sabine Ehrt and Dirk Schnappinger for helpful discussions and a critical review of the manuscript. This work was funded by the Biotechnology and Biological Sciences Research Council and the Wellcome Trust. Funding to pay the Open Access publication charges for this article was provided by the Biotechnology and Biological Sciences Research Council.

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