Arch Microbiol (2006) 186:459–464
DOI 10.1007/s00203-006-0160-2

ORIGINAL PAPER

Tetracycline-inducible gene expression in mycobacteria within an animal host using modified *Streptomyces tcp830* regulatory elements

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Received: 6 June 2006 / Revised: 21 July 2006 / Accepted: 25 July 2006 / Published online: 30 August 2006

Abstract Inducible expression systems are powerful tools for studying gene function. Though several inducible expression systems are now available for mycobacteria, none have been used to modulate bacterial gene expression during an animal infection. A tetracycline-inducible expression system from *Streptomyces coelicolor* was successfully adapted for use in mycobacteria. To prevent baseline expression without induction, *S. coelicolor tetR* gene was overexpressed using the acetamidase promoter and regulatory gene block. Target gene expression was controlled by the *S. coelicolor tcp830* promoter and operator allele. The −10 promoter consensus sequence of the *tcp830* promoter was modified to better resemble known strong mycobacterial promoters. Using this system, induction of *tetR* fully repressed tcp830-dependent expression of green fluorescent protein (GFP) to baseline levels. Addition of anhydrotetracycline led to a 62-fold induction of GFP expression in vitro and 15-fold induction in a mouse mycobacterial peritonitis model in the presence of maximal *tetR* expression. Chemically regulatable gene expression during animal infection may be a useful tool in studying mycobacterial pathogenesis.

Keywords Anhydrotetracycline · Acetamide · Mycobacteria

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Introduction

Tuberculosis is currently the seventh leading cause of disability and death globally and is expected to remain so if current tools and patterns of control prevail (Murray and Salomon 1998). The 2004 World Health Organization (WHO) report on global TB control estimates that 8.8 million people acquire new tuberculosis disease each year (WHO 2004). One of the major reasons for the success of *Mycobacterium tuberculosis* as a pathogen is its ability to adapt to a wide range of conditions inside the human host. This includes its ability to stay dormant in a latent phase in the host for years or even decades. WHO estimates that one-third of the world's population is latently infected with *M. tuberculosis*, and many develop reactivation disease, years after the initial exposure (Lurie 1942; Stead 1967). The mechanisms that allow *M. tuberculosis* to adapt to these wide ranges of conditions and/or reactivate are not clearly understood. Studying mycobacterial genes involved in this adaptive response is essential to understand the mechanisms involved in latency and other mycobacterial adaptive responses.

Inducible expression systems are powerful tools for studying gene function and validating drug targets in bacteria (Carroll et al. 2005; Raghunand et al. 2006). Though inducible expression systems are now available for mycobacteria (Blokpoel et al. 2005; Carroll et al. 2005; Ehrt et al. 2005), none have been used in animal infections. We developed a tetracycline-inducible expression system for use in mycobacteria which was adapted from *Streptomyces coelicolor*, an Actinomycete closely related to mycobacteria with a similarly high GC-content (Rodriguez-Garcia et al. 2005). In this system, tetracycline repressor protein (TetR)

regulates the transcription of its targets by binding with high affinity to the operator *tetO*, thus repressing gene expression. In the presence of nanomolar levels of tetracycline or various analogs, TetR dissociates from *tetO* and allows transcription to proceed. The *S. coelicolor tcp830* promoter-operator sequence has recently been shown to offer up to 270-fold chemically inducible expression in *Streptomyces* (Rodriguez-Garcia et al. 2005). Using the *tcp830* promoter, we demonstrated chemically inducible expression in mycobacteria. We added tighter regulation by placing the *tetR* gene under control of the mycobacterial acetamidase promoter, and we enhanced expression by modifying the -10 region of the promoter to better resemble strong mycobacterial promoters. Finally, we tested the activity of this system within a murine host using an intraperitoneal cavity infection.

Materials and methods

Bacterial strains and media

Mycobacterium smegmatis and its transformants were grown in Middlebrook 7H9 liquid or 7H10 agar medium (Difco Laboratories, Detroit, MI, USA) supplemented with OADC enrichment (Difco). *Escherichia coli* DH5α was grown on liquid or agar Luria-Bertani medium. When required, the antibiotic kanamycin (Sigma) was added at concentrations of 50 μg/ml for *E.coli* and 20 μg/ml for *M. smegmatis*. Hygromycin B (Roche) was added at a concentration of 200 μg/ml for *E. coli* and 50 μg/ml for *M. smegmatis*.

Flow cytometry and fluorescence microscopy of *M. smegmatis* transformants

Ten thousand mycobacteria from each sample were analyzed using fluorescence activated cell sorting (FACS-Calibur, Becton Dickinson) and all the events recorded to ensure statistical significance. Levels of fluorescence were plotted as the geometric mean of the histograms and analysis of the data was performed by the CellQuest software (version 3.1f) and statistical analysis was performed using STATA software (version 8.0, College Station, TX, USA).

Fluorescence microscopy analysis was performed using Eclipse E600 (Nikon) microscope with 420–490 nm excitation and 515-nm emission filters. Two hundred microliters of the samples prepared for flow cytometry were concentrated by micro-centrifugation, and placed on slides under coverslips. The images were recorded with 1 or 5 s exposure time with Nikon ACT-1 (Version 2.63, Nikon Corporation) and SPOT (Version 4.1, Diagnostic Instruments, Inc.) software.

Construction of the tetracycline-inducible expression system

A synthetic tetracycline-inducible promoter, *tcp830*, derived from *Streptomyces coelicolor* and the *S. coelicolor* tetR gene homolog SCO0253 were obtained from Dr. M. Smith (Rodriguez-Garcia et al. 2005). The DNA fragment (*tetR*) encoding the TetR repressor was cloned into an acetamide-inducible mycobacterial integrating vector pSCW54 to produce pART and confirmed by sequencing. pSCW54 is an *E. coli* plasmid capable of mycobacterial chromosomal integration which is derived from pMH94 (Lee et al. 1991). It lacks a kanamycin-resistance conferring cassette, but contains a *hyg* (hygromycin-resistance conferring) cassette as well as the *M. smegmatis* acetamidase promoter and regulatory gene block (Parish et al. 1997). pART was used to generate mc^{2}155::pART by transformation of *M. smegmatis* using Gene Pulser II electroporator (Bio-Rad). The green fluorescent protein (GFP) constructs were made as shown in Fig. 1a. The promoter-operator sequence of *tcp830*, P_{tcp830}, was optimized for maximal expression in mycobacteria by modifying the -10 box to match either the S16 (*M. smegmatis*) or T101 (*M. tuberculosis*) mycobacterial promoters (Bashyam et al. 1996), producing $P_{tcp830\text{-}S16}$ and $P_{tcp830\text{-}T101}$, respectively. All three of these promoters were directionally cloned into the promoterless GFP vector pFPV27 (Valdivia and Ramakrishnan 2000) and confirmed by sequencing. The resulting plasmids were named pMHA1 (P_{tcp830}::*gfp*), pMHA2 ($P_{tcp830\text{-}S16}$::*gfp*) and pMHA3 ($P_{tcp830\text{-}T101}$::*gfp*). *M. smegmatis* mc^{2}155 and recombinant *M. smegmatis* mc^{2}155::pART cultures were transformed separately with pFPV27, pMHA1, pMHA2 and pMHA3 as described above.

Growth curves in the presence of different concentrations of anhydrotetracycline and acetamide were obtained by measuring the OD_{600} of *M. smegmatis* mc^{2}155::pART + pMHA2 cultures in 7H9 media every 4 h.

Mycobacterium smegmatis mc^2::pART + pFVP27 and mc^2::pART + pMHA2 transformants were grown to mid-log phase (OD_{600} = 0.7 to 0.9), washed and resuspended in fresh 7H9 medium containing (1) no additions, (2) with 0.2% acetamide, (3) with 50 ng/ml anhydrotetracycline and (4) with 0.2% acetamide plus 50 ng/ml anhydrotetracycline. After exposure to these conditions for approximately 13 h, GFP expression

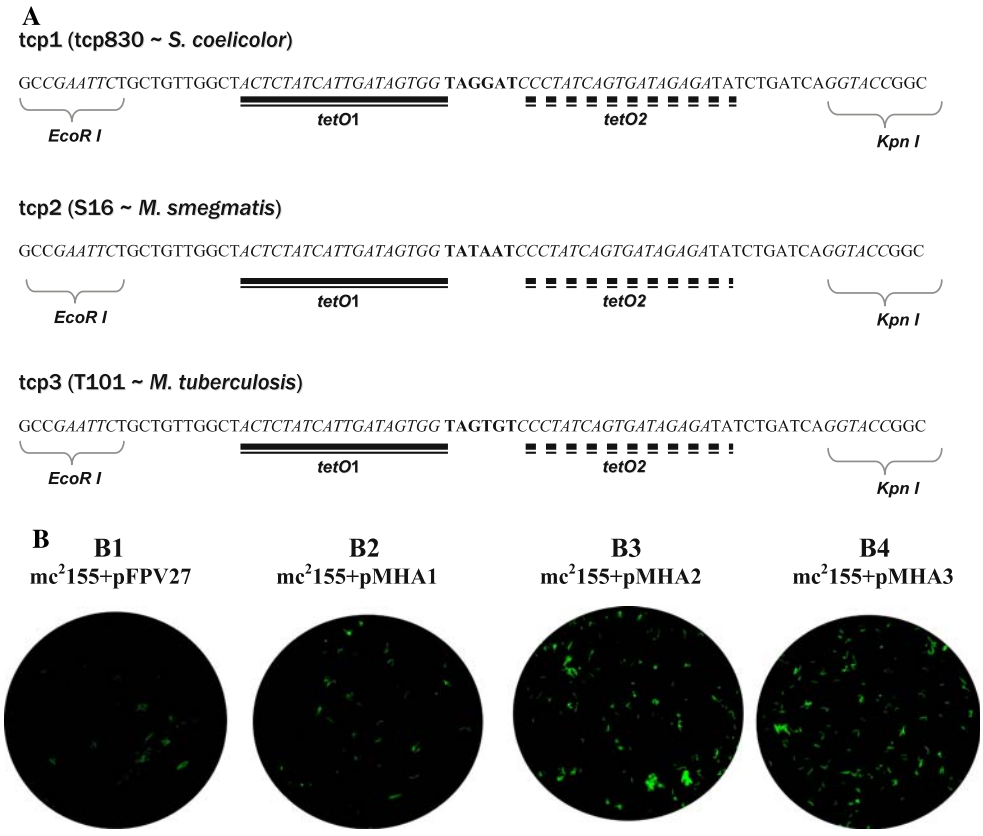

Fig. 1 Optimization of the P_{tcp830} promoter: modifying the -10 region (between *tetO*1 and *tetO*2) of the P_{tcp830} promoter to better resemble strong mycobacterial promoters S16 and T101 (panel **a**) enhances *gfp* expression in *M. smegmatis* (panel **b**)

levels were analyzed by flow cytometry and fluorescence microscopy.

Tetracycline-inducible gene expression in mycobacteria within a murine host

Mycobacterium smegmatis mc²::pART + pFVP27 and mc²::pART + pMHA2 transformants were grown to mid-log phase (OD_{600} = 0.7 to 0.9), washed and resuspended in PBS at a concentration of 10^8 mycobacteria/ml. BALB/c mice (5–6 weeks old) were injected intraperitoneally with 250 µl of this mycobacterial suspension. Four hours after infection, the mycobacteria were induced with (1) 250 µl of PBS (control), (2) 250 µl of 2.4% acetamide (final concentration 0.6%), (3) 250 µl of 1.0 µg/ml anhydrotetracyline (final concentration 250 ng/ml), and (4) 250 µl of 2.4% acetamide plus 250 µl of 1.0 µg/ml anhydrotetracycline. The final concentrations of acetamide and anhydrotetracycline were calculated for a 1 ml peritoneal cavity free space volume. Four mice were used for each group. One milliliter of PBS was used to recover mycobacteria from the peritoneal cavity by peritoneal lavage 16 h post-induction. Cells from peritoneal lavages were washed twice in PBS and then analyzed by flow cytometry and fluorescence microscopy as described above.

Results

We adapted the *tcp830* tetracycline-inducible expression system from *Streptomyces coelicolor* (Rodriguez-Garcia et al. 2005) for use in mycobacteria and studied the system using a murine intraperitoneal infection model.

Construction of the tetracycline-inducible expression system

As shown in Table 1, *M. smegmatis* transformants harboring the *S. coelicolor tcp830* promoter (pMHA1) produced a 63-fold higher level of fluorescence than the vector control pFPV27 which displayed minimal fluorescence (Fig. 1b), indicating the recognition of *Streptomyces* promoter-operator element by the mycobacterial transcription machinery. As expected, the mycobacterial-optimized $P_{tcp830\text{-}S16}$ (pMHA2) and $P_{tcp830\text{-}T101}$ (pMHA3) promoters produced more fluorescence than was observed with the native *tcp830* promoter with levels 136- and 131-fold above baseline, respectively. *M. smegmatis* transformed with pMHA2 had the highest fluorescence, and we therefore chose it for further study in a recombinant *M. smegmatis* strain expressing the *S. coelicolor tetR* repressor under the control of the acetamidase promoter (mc²155::pART). *M. smegmatis* mc²155::pART transformed with pFPV27 was used as a negative control.

Table 1 (a) Fluorescence intensities of *M. smegmatis* mc^{2}155 transformed with mycobacterial-optimized P_{tcp830} constructs: *M. smegmatis* mc^{2}155 harboring the P_{tcp830} (pMHA1) promoter produce much higher level of fluorescence than the vector control pFPV27. Mycobacterial-optimized $P_{tcp830\text{-}S16}$ (pMHA2) and $P_{tcp830\text{-}T101}$ (pMHA3) promoters produce more fluorescence than observed with the P_{tcp830} promoter. (b and c) Fluorescence intensities of *M. smegmatis* mc^{2}155::pART + pFPV27 and *M. smegmatis* mc^{2}155::pART + pMHA2 after induction under the different conditions in vitro and in vivo: fluorescence of *M. smegmatis* mc^{2}155::pART + pMHA2 incubated with acetamide is negligible and indistinguishable from that of a transformant containing promoterless *gfp*. Addition of the inducer anhydrotetracycline releases the repression. No such effects are seen for the negative control *M. smegmatis* mc^{2}155::pART + pFPV27

(a) Fluorescence intensities of *M. smegmatis* mc^{2}155 transformed with mycobacterial-optimized P_{tcp830} constructs

Construct	Fluorescence mean (±SD)
pFPV27 (control)	3.4 (±2.2)
pMHA1 (P_{tcp830})	214.3 (±133.1)
pMHA2 ($P_{tcp830\text{-}S16}$)	462.7 (±297.4)
pMHA3 ($P_{tcp830\text{-}T101}$)	384.6 (±243.2)

(b) Fluorescence intensities of *M. smegmatis* mc^{2}155::pART + pFPV27 and *M. smegmatis* mc^{2}155::pART + pMHA2 after induction under the different conditions in vitro

Condition	*M. smegmatis* mc^{2}155::pART + pFPV27	*M. smegmatis* mc^{2}155::pART + pMHA2
No additions	5.3 (±3.1)	243.9 (±137.3)
Acetamide (0.2%)	3.2 (±2.5)	3.6 (±3.9)
Anhydrotetracycline (50 ng/ml)	7.6 (±3.5)	280.0 (±166.3)
Acetamide (0.2%) + anhydrotetracycline (50 ng/ml)	4.6 (±3.0)	221.5 (±128.5)

(c) Fluorescence intensities of *M. smegmatis* mc^{2}155::pART + pFPV27 and *M. smegmatis* mc^{2}155::pART + pMHA2 after induction under the different conditions in vivo

Condition	*M. smegmatis* mc^{2}155::pART + pFPV27	*M. smegmatis* mc^{2}155::pART + pMHA2
No additions	6.1 (±2.9)	144.5 (±70.0)
Acetamide (0.6%)	5.0 (±2.6)	5.0 (±2.6)
Anhydrotetracycline (250 ng/ml)	6.4 (±3.1)	188.6 (±86.9)
Acetamide (0.6%) + anhydrotetracycline (250 ng/ml)	5.3 (±2.9)	73.9 (±31.7)

M. smegmatis growth curves showed that up to 250 ng/ml anhydrotetracycline and 0.5% acetamide did not significantly affect the growth rate of this recombinant strain (Fig. 2). We tested this final system by measuring the *gfp* expression after incubation with acetamide and anhydrotetracycline. As expected, the fluorescence of *M. smegmatis* mc^{2}155::pART + pMHA2 incubated with 0.2% acetamide was negligible and was indistinguishable from that of a transformant containing promoterless *gfp*. Thus, acetamide-induced *tetR* expression was highly effective in 'switching off' P_{tcp830} -driven GFP expression. As shown in Table 1, addition of the inducer anhydrotetracycline at 50 ng/ml released the repression and led to a fluorescence increase of 62-fold (acetamide present). No such effects were seen for the negative control *M. smegmatis* mc^{2}155::pART::pFPV27.

Tetracycline-inducible gene expression in mycobacteria within a murine host

We tested the activity of this system within an animal host using a murine intraperitoneal cavity infection model. Mice were treated with the inducers as described in Materials and methods 4 h after bacterial implantation. Mycobacteria recovered from the mice 16 h after induction were analyzed. As shown in Table 1, *M. smegmatis* mc^{2}155::pART + pMHA2 recovered from peritoneal washings of mice treated with acetamide displayed fluorescence intensities that were the same as that of background, while induction treatment of mice with 250 ng/ml of anhydrotetracycline intraperitoneally resulted in the recovery of bacilli with fluorescence intensities 15-fold higher (acetamide added). No such effects were seen for the negative control *M. smegmatis* mc^{2}155::pART::pFPV27.

Discussion

Inducible expression systems are now available for mycobacteria (Blokpoel et al. 2005; Carroll et al. 2005; Ehrt et al. 2005). Blokpoel et al. (2005) developed a tetracycline responsive system adapted from *Corynebacterium glutamicum*. Using a luciferase reporter, they

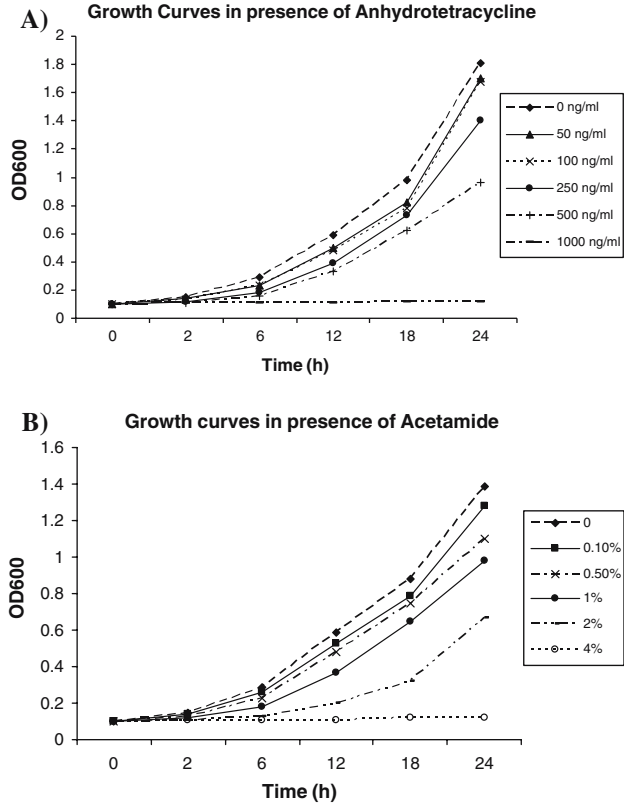

Fig. 2 In vitro growth curves in the presence of different concentrations of anhydrotetracycline (panel **a**) and acetamide (panel **b**) show that up to 250 ng/ml anhydrotetracycline and 0.6% acetamide do not significantly affect *M. smegmatis* growth

showed 10 and 40 times increase in the expression in response to tetracycline in *M. smegmatis* and *M. tuberculosis*, respectively. They further tested this system in J774 macrophages infected with *M. bovis* BCG with their construct and showed a 10-fold increase in expression on induction with tetracycline. Carroll et al. (2005) developed a second tetracycline responsive system and showed its activity in *M. smegmatis* and *M. tuberculosis*. Finally, Ehrt et al. (2005) developed a third tetracycline inducible system and demonstrated its function in *M. tuberculosis* within the macrophage phagosome.

It is clear that tetracycline and its analogs have good penetration into both mammalian cells and mycobacteria and therefore can be used in animal hosts (Bocker et al. 1981). Since none of the current systems have been used in animal infections, we tested our system in mouse peritoneal cavity infection. Like the previous tetracycline-responsive systems, our chemically inducible clones demonstrated significant expression increases in the presence of anhydrotetracycline; in this study induction ratios as high as 62-fold were observed. Improved induction was partly achieved by substituting mycobacterial elements in the -10 box of the promoter while tight control was achieved by placing TetR repressor under the control of an acetamide-inducible mycobacterial promoter. Of note, higher concentrations of acetamide and the inducer anhydrotetracycline were used for animal infections since we expected rapid absorption of both these compounds from the highly vascular peritoneal cavity, lowering their actual concentration.

The murine intraperitoneal cavity infection model is artificial and does not reflect natural mycobacterial infection. However, it is proof of concept that our system can indeed be used in animal models. Additionally, all plasmids designed for our system can be transformed into *M. tuberculosis* and are expected to yield similar results. Our system requires the concurrent use of acetamide for tight control of the reporter gene. Though acetamide is a relatively non-toxic compound we expect that it will have a short half-life in a mammalian host limiting its use. However, placement of TetR repressor under the control of the strong mycobacterial constitutive promoter *hsp60* (Stover et al. 1991) will eliminate the need for acetamide repression. Since tetracycline and its analogs are extensively distributed in mammalian hosts, it is expected that this system would work in most animal models of tuberculosis such as the mouse, guinea pig, rabbit and non-human primate models (Bocker et al. 1981; Kelly et al. 1992; Wrightson et al. 1998; Altboum et al. 2002; Chong et al. 2002).

In summary, we have adapted and optimized a tetracycline-inducible expression system from *S. coelicolor* and demonstrated chemically inducible mycobacterial gene expression in an animal host. We expect that our system would work in *M. tuberculosis*, and with modifications in most animal models of tuberculosis representing natural mycobacterial infection. Such inducible systems may offer considerable value for evaluating the virulence of specific mycobacterial genes in animal hosts.

The vector system described in this study can be obtained through the Tuberculosis Animal Research and Gene Evaluation Taskforce website at http://www.hopkinsmedicine.org/TARGET/info.htm

Acknowledgments This work was supported in part by NIH grants AI36973, AI37856, AI43846, AI51668, and NIH contract N01-30036. The authors thank Dr. Maggie Smith for donating the plasmid pAR933a containing the *tcp830* promoter, Sandeep Tyagi for assistance with the mice inoculations, and Norman Morrison for assistance in manuscript preparation.

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