Use of a Tetracycline-Inducible System for Conditional Expression in
*Mycobacterium tuberculosis* and *Mycobacterium smegmatis*

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A number of essential genes have been identified in mycobacteria, but methods to study these genes have not been developed, leaving us unable to determine the function or biology of the genes. We investigated the use of a tetracycline-inducible expression system in *Mycobacterium tuberculosis* and *Mycobacterium smegmatis*. Using a reporter gene which encodes an unstable variant of GFP, we showed that tetracycline-inducible expression occurred in *M. smegmatis* and that expression levels were titratable to some extent by varying the concentration of tetracycline. The removal of tetracycline led to cessation of GFP expression, and we showed that this was a controllable on/off switch for fluorescence upon addition and removal of the antibiotic inducer. The system also functioned in *M. tuberculosis*, giving inducible expression of the reporter gene. We used homologous recombination to construct a strain of *M. tuberculosis* that expressed the only copy of the tryptophan biosynthetic enzyme, TrpD, from the tetracycline-inducible promoter. This strain was conditionally auxotrophic, showing auxotrophy only in the absence of tetracycline, confirming that *trpD* was tightly controlled by the foreign promoter. This is the first demonstration of the use of an inducible promoter to generate a conditional auxotroph of *M. tuberculosis*. The ability to tightly regulate genes now gives us the possibility to define the functions of essential genes by switching them off under defined conditions and paves the way for in vivo studies.

The mycobacteria include a number of human pathogens of global importance, such as *Mycobacterium tuberculosis*, *Mycobacterium leprae*, and *Mycobacterium avium*. *M. tuberculosis* is still a major killer and poses a serious threat to worldwide health. In order to develop new drugs and/or vaccines, a better understanding of the biology of the organism is required. Molecular techniques have been at the forefront of the methods for studying bacteria, and the mycobacteria are no exception. However, there are still a number of areas in which there is a paucity of available tools. Mycobacteria have a GC-rich genome and are slow growing and difficult to lyse because of their paucity of available tools. Mycobacteria have a GC-rich genome and are slow growing and difficult to lyse because of their lipid-rich cell wall. In addition, *M. tuberculosis* is a pathogenic organism which requires specialized handling facilities. These problems partially explain why the development of genetic systems for mycobacteria has lagged behind the development of genetic systems for other bacteria.

Defined mutations are central to the understanding of individual gene function in any organism. However, essential genes cannot be deleted since a lethal phenotype is generated, so the ability to elucidate their functional roles is more difficult. Increasing numbers of essential genes are being discovered in *M. tuberculosis* (22, 25–28, 37). Thus, methods for studying essential genes in mycobacteria are urgently required.

One technique that has been used is the generation of conditional mutants which can express a gene only under defined conditions. Often this has been achieved using inducible promoters which can be switched on and off in a controlled manner. To date, the acetamide-inducible system of *Mycobacterium smegmatis* has been the most widely used controllable promoter in mycobacteria (7, 20). However, this system cannot be tightly switched off and therefore may not be suitable for the study of an essential gene since there is always a basal level of expression.

Numerous inducible promoter systems in other organisms have been developed. In *Escherichia coli* these include Ptet (2, 18), PBAD (14), Plac (36), P lac (6), P lac (4), P l, PR (9), and P T7 (32). Additionally, inducible promoters have been described in several gram-positive species, including P sk (8) and PM (33) in *Lactococcus lactis*, P xyI and P syst in *Staphylococcus aureus* (1, 38), Pan,k in *Streptococcus pneumoniae* (5), and P nit in *Streptomyces* (17).

Tetracycline-inducible promoter systems (P tet) have been developed for use in multiple organisms, including *E. coli*, *Bacillus subtilis* (11), *Staphylococcus aureus* (1), and *S. pneumoniae* (30), and are widely utilized in eukaryotic models (10, 12). The P tet system has been shown to regulate gene expression both in vitro and in vivo (1, 13, 38), making it a versatile system. The P tet system consists of two divergent promoters, one which drives expression of the TetR repressor and one which drives expression of the target gene (Fig. 1). Two operator sites which are the target of TetR binding overlap the promoters. In the absence of tetracycline, TetR binds to the operator sites and prevents transcription. In the presence of tetracycline, TetR binds to tetracycline, which induces a conformational change, making it unable to interact with the operator, so that target gene expression can occur (2).

We investigated the use of the P tet system in mycobacteria and found that it can be used to inducibly express foreign genes in *M. smegmatis* and *M. tuberculosis*. We also used this system to construct the first conditionally auxotrophic mutant of *M. tuberculosis*. 
MATERIALS AND METHODS

Media and strains. M. smegmatis was grown in Lemo medium (10 g liter\(^{-1}\) peptone, 5 g liter\(^{-1}\) Lemo powder, 5 g liter\(^{-1}\) NaCl) containing 0.05% (wt/vol) Tween 80 for liquid cultures or 15 g liter\(^{-1}\) agar for agar plates. Liquid cultures were incubated at 37°C with shaking at 100 rpm. M. tuberculosis H37Rv (ATCC 25618) and Tame 5 (AppD) (21) were grown in Middlebrook 7H9 medium plus 10% (vol/vol) oleic acid-albumin-dextrose-catalase supplement (Becton Dickinson) and 0.05% (wt/vol) Tween 80 or on Middlebrook 7H10 agar plus 10% (vol/vol) oleic acid-albumin-dextrose-catalase. Hygromycin was added at a concentration of 100 μg/ml, kanamycin was added at a concentration of 20 μg/ml, tetracyclin was added at a concentration of 50 μg/ml, and L-tryptophan was added at a concentration of 50 μg/ml when required. Minimal medium for acetamide-inducible expression was prepared as previously described (20).

Construction of vectors. For construction of vectors (Table 1), Ptet was cloned from pALC2073 (1) as an EcoRI-PstI fragment into pMV206 to generate pTACT1 and into pUC18 to generate pTACT2. The antigen 85A (Ag85A) promoter from pEM37 (Edith Machowski, University of the Witwatersrand, South Africa) was cloned as a BamHI-BglII fragment into pTACT2 to generate the P\(_{\text{Ag85A}}\) version (pTACT7). The P\(_{\text{Ag85A}}\) fragment was amplified from pTACT7 and cloned into pGEM Easy T to make pTACT18. pTACT24 was then excised as an EcoRI fragment and cloned upstream of the unstable gfp gene in the pFLAME3 and pFLAME4 vectors to make pHLEM3 and pHLEM4. pFLAME3 carries the GFP-LAA variant (LAA is the tag), and pFLAME4 carries the GFP-ASV variant. Similarly, the P\(_{\text{Ag85A}}\) fragment was excised and cloned into the pFLAME3 and pFLAME4 vectors to generate pHLEM2 and pHLEM4. A promoterless trpD gene was amplified from M. tuberculosis genome DNA using primers Tap1 (5' AAG CIT AGC AGA GGT TGT CAG TCA 3') and Tap10 (5' CAT GGG ACC ATC AGC GCG TGG 3'), cloned into pGEM Easy T, excised as an EcoRI fragment, and cloned into pTACT1 to generate pTACT3. A NotI fragment containing P\(_{\text{Ag85A}}\) and the 5' end of trpD was excised from pTACT3 and cloned into pNiNL to generate pTACT12.

Construction of vectors for green fluorescent protein (GFP) assays. Electrocompetent M. smegmatis and M. tuberculosis cells were prepared as described previously (24) and electroporated with plasmid DNA, and transformations were selected with kanamycin. Overnight cultures of M. smegmatis transformations were inoculated into media (1/10) and grown for 24 h before preparation of cell extracts. For the on/off experiments, 5-ml cultures were washed in 10 mM Tris (pH 8) and resuspended in 2 ml of Lemo broth at 24-h intervals. One milliliter of each suspension was used to inoculate fresh growth media with or without tetracycline as required, and the remainder was used to prepare cell extracts. M. tuberculosis transformations were subcultured (1/10) and grown without agitation for 2 weeks before preparation of cell extracts.

GFP assays. M. smegmatis cells were washed twice in 10 mM Tris (pH 8) and 0.05% (wt/vol) Tween 80, resuspended in 10 mM Tris (pH 8), and added to 0.1-mm glass beads on ice. Cells were disrupted using a MiniBead Beater by using two 1-min cycles at 4,200 rpm with a 2-min rest between cycles on ice. Samples were centrifuged for 2 min, and the supernatants were recovered and filter sterilized. M. tuberculosis cells were washed and resuspended in 1 ml 10 mM Tris (pH 8). Cell extracts were generated using a FastPrep (QBIogene) at setting 6.0 for 30 s. The supernatant was recovered after a brief pulse in a microcentri-
inducible system (Ptet) that had previously been shown to function in S. aureus (1) (Table 1). We cloned the fragment carrying the repressor with divergent promoters and overlapping operator sites upstream of the gfp reporter genes (Fig. 1). In addition, we made a second version (PAtet) in which the repressor was expressed from a constitutive mycobacterial promoter, the antigen 85A promoter. Both versions were tested for the ability to drive expression of gfp in mycobacteria.

pFLAME-ace carrying the acetamide-inducible system and the empty pFLAME vectors were used as positive and negative controls, respectively. Plasmids were electroporated into M. smegmatis and M. tuberculosis, and GFP expression was analyzed by measuring fluorescence in cell extracts.

Inducible expression of gfp in M. smegmatis. The vectors tested for activity were pHLEG1 (PAtet in pFLAME3 [Gfp-LAA]), pHLEG2 (Ptet in pFLAME3), pHLEG3 (PAtet in pFLAME4 [GFP-ASV]), and pHLEG4 (Ptet in pFLAME4). We observed tetracycline-dependent induction of GFP in M. smegmatis strains carrying all four pHLEG vectors (Fig. 2) with high levels of GFP induction at all concentrations of tetracycline except 100 ng/ml, at which growth inhibition started to become a problem (data not shown), and pHLEG4 (Ptet-GFP-ASV) at a concentration of 30 ng/ml. The basal level of expression of GFP was low and was not significantly higher than that of the empty pFLAME vectors. Interestingly, for the two vectors containing PAtet (pHLEG1 and pHLEG3) there appeared to be a biphasic titration pattern with a peak of expression at 20 to 30 ng/ml, followed by a second peak at 100 ng/ml, and these vectors gave high levels of inducible expression. For the two Ptet vectors (pHLEG2 and pHLEG4), there was a lower level of expression generally and a single peak of expression around 40 ng/ml; pHLEG2 (Ptet-GFP-LAA) exhibited a broad peak at 30 to 50 ng/ml, whereas pHLEG4 (PAtet-GFP-LAA) had a single peak at 40 ng/ml. Thus, for all the vectors, we observed induction of GFP expression after addition of tetracycline and a low level of expression in the noninduced state.

Temporal expression of gfp. Once we confirmed that inducible expression occurred, we determined whether tetracycline-dependent expression could be easily switched on and off by subculturing transformants and only including tetracycline on alternate days (Fig. 3). Since 20 ng/ml tetracycline had been effective in inducing expression without any detrimental affect on growth, we used this concentration. As Fig. 3 shows, gfp expression was clearly induced when tetracycline was present (on) and was repressed when it was absent (off) (Fig. 3). This was the case for all pHLEG vectors. Similarly, gfp induced by the ace promoter could be turned off by passing into growth media without acetamide. The Ptet and PAtet vectors had much higher expression levels than Pace, indicating that they are relatively well expressed, since Pace has previously been shown to be a very strong promoter. The experiment was also repeated with 10 ng/ml tetracycline, and the same results were obtained. As in our initial experiments, the vectors carrying the PAtet version showed higher levels of induction than the vectors carrying the Pton versions. These results gave a very good indication that the tet system functioned as expected in mycobacteria, so we subsequently extended our study to M. tuberculosis.

Inducible expression of gfp in M. tuberculosis. We next assayed whether the same vectors functioned in M. tuberculosis (Fig. 4). Transformants with pHLEG vectors all showed...
We constructed plasmid pTACT3 carrying the *M. tuberculosis* trpD gene and thus requires tryptophan supplementation for growth. Tetracycline (200 ng/ml) was added to most ON cultures; the only exception was pFLAME3-ace, which was cultured in the presence of acetamide. The bars indicate the averages for three independent transformant cultures, and the error bars indicate standard deviations.

**FIG. 4.** Inducible expression of *gfp* in *M. tuberculosis*. *M. tuberculosis* transformants carrying different plasmids were grown for 2 weeks, and *gfp* expression was assayed by measuring fluorescence in cell extracts. Tetracycline (200 ng/ml) was added to most ON cultures; the only exception was pFLAME3-ace, which was cultured in the presence of acetamide. The bars indicate the averages for three independent transformant cultures, and the error bars indicate standard deviations.

Clear tetracycline-dependent induction of fluorescence. *gfp* expression was also induced by acetamide in the pFLAME-ace transformants. The induction of GFP was much higher in the *Pte* vector than in the *Patet* vector in the pFLAME3 background (pHLEG1 and pHLEG2), and although there was no difference between the two promoters in the pFLAME4 background (pHLEG3 and pHLEG4), pHLEG3 transformants did not grow as well on solid media or in liquid media. The basal level for all the vectors was very low as well, indicating that we were getting controllable as well as inducible expression of GFP. Of the four vectors, pHLEG1 was the least effective in induction ratios. pHLEG2 appeared to be the most useful as the induction ratios were higher. This was in marked contrast to *M. smegmatis*, in which pHLEG1 and pHLEG2 (P *Patet* version) functioned better. The basal level of expression was the same for all the pHLEG vectors.

**Inducible expression of *trpD* in *M. tuberculosis*.** Once we confirmed that *Pte* was inducible by tetracycline in *M. smegmatis* and *M. tuberculosis*, we then assayed whether it could be used to direct the conditional expression of a mycobacterial gene. Our aim was to generate a system which allowed us to completely switch off gene expression, so we wanted to use an assay that allowed us to sensitively determine whether gene expression was occurring.

We used a previously constructed strain (Tame 5) (21) which is auxotrophic for tryptophan by virtue of an unmarked deletion of *trpD* and thus requires tryptophan supplementation for growth. We constructed plasmid pTACT3 carrying the *M. tuberculosis* *trpD* gene under the control of *Pte* in a mycobacterial replicating vector and transformed this plasmid into Tame 5. Thus, if *trpD* expression were occurring, we would see growth in the absence of tryptophan.

The growth of the wild type and Tame 5 transformants in the presence and absence of tetracycline and tryptophan is shown in Fig. 5. As Fig. 5 shows, the presence of tetracycline did not inhibit growth of the wild type, nor did the presence of either a control (pTACT1) or test (pTACT3) plasmid greatly affect growth. As expected, the tryptophan auxotroph carrying the control plasmid was unable to grow in the absence of tryptophan, and tetracycline did not inhibit growth in the presence of tryptophan. Tame 5:pTACT3 was able to grow in the absence of tryptophan, indicating that expression of *trpD* from *Pte* was occurring; in the presence of tetracycline prototrophy was restored, and the cells could grow normally without any exogenous tryptophan. In the absence of tetracycline (i.e., noninduced *Pte*) there was some growth, although there was less growth than in the tetracycline-induced state. These data indicate that inducible expression of *trpD* occurred but that there was a basal level of expression in the noninduced state. We have shown previously that the *trpD* promoter is not very active, as assessed by a reporter gene (21), and therefore, the gene may be expressed at a fairly low level in normal cultures. If this is correct, then very low levels of TrpD may be enough to permit slow growth, and this may account for the nonsupplemented growth in the absence of induction. However, these results showed that we could get expression of a mycobacterial gene and that it was inducible to some extent by tetracycline.

**Construction of a conditional auxotroph in *M. tuberculosis*.** The expression of *trpD* from *Pte* on a low-copy-number plasmid showed that we could obtain induction using tetracycline as an inducer. However, the basal level of expression was not zero. It is well known that having multiple copies of plasmids can lead to an increase in the basal level of transcription from even tightly controlled promoters. Thus, we decided to assay whether *trpD* expression from *Pte* in a single copy could be completely switched off to give us a conditional auxotroph. We constructed plasmid pTACT21 carrying the 5′ end of the *trpD* gene downstream of *Pte*. This plasmid was introduced into the chromosome by a single homologous recombination event, giving rise to a strain (Fig. 6) in which the only functional copy of *trpD* was expressed from *Pte*. The resulting strain was analyzed by Southern blotting to confirm the expected genotype (Fig. 6).

We looked at auxotrophy to confirm that the strains did not express *trpD* in the noninduced state (i.e., without tetracycline). Twelve individual transformants were tested for auxotrophy on solid plates, and all had the expected phenotype (i.e., no growth in the absence of tryptophan supplementation) (data not shown).

**FIG. 5.** Inducible expression of *trpD* using the *tetRPO* system in *M. tuberculosis*: growth of transformants carrying pTACT1 and pTACT3. Wild-type (WT) and auxotrophic strains carrying both pTACT3 (P *te*), *trpD*) and pTACT1 (P *te*, empty control) were grown in various media, as indicated; growth was read after 9 days. The results are the averages ± standard deviations for three independent transformants.
FIG. 6. Construction of conditional auxotrophs. (A) The recombination vector pTACT21 was constructed by cloning the 5' region of \textit{trpD} downstream of \textit{tetRPO} into a vector which does not replicate in mycobacteria. Single-crossover recombinants were generated by homologous recombination. The resulting strains had one complete functional copy of \textit{trpD} downstream of \textit{P}_{tet}, as well as the 5' end of \textit{trpD} only. EcoRI sites are indicated by numbers in parentheses. WT, wild type; kan, kanamycin resistance; hyg, hygromycin resistance. (B) Southern analysis of the recombinants. DNA was digested with EcoRI and probed with the complete \textit{trpD} gene indicated in panel A. The two SCO bands and the resulting loss of the wild-type band (wt) expected in the Southern analysis are also shown as black lines in panel A. Lanes 1 to 5 contained five independent mutants, and lane 6 contained wild-type strain H37Rv.
Growth of Tact 21 strains in liquid cultures. We looked at the growth of six independent recombinants (Tact 21:A to Tact 21:F), and the results for three of these recombinants are shown in Fig. 7. As Fig. 7 shows, all the strains behaved similarly in terms of growth and showed normal growth in the presence of tryptophan. There was very little increase in the OD$_{580}$ in the absence of tetracycline and tryptophan. However, when tetracycline was added to induce expression of trpD, substantial growth could be seen, although the strains did not grow as well as the supplemented strains. These data confirmed again that tetracycline-inducible expression of trpD occurred and also that the gene was essentially switched off in the absence of tetracycline (i.e., that the control of expression was much tighter when only a single copy of $P_{tet}$ was present).

Titration of trpD expression. One of the advantages of the tetRPO system has been the ability to titrate expression by varying the concentration of tetracycline. In order to determine whether we could titrate trpD expression, we looked at growth of the wild-type and Tact 21 strains in response to concentrations of tetracycline ranging from 2 to 2,000 ng/ml (Fig. 8). The wild-type strain showed no inhibition of growth in the presence of 2,000 ng/ml. However, interestingly, there was growth inhibition at the lower concentrations of tetracycline. With the Tact 21 strain, a clear increase in growth rather than inhibition could be seen, suggesting that there was an increase in trpD expression, although this was only seen as increased growth compared with the control at the highest concentration of tetracycline. This showed that there was an increase in gene expression with increasing concentrations of tetracycline, confirming our previous results with *M. smegmatis*.

**DISCUSSION**

We showed that the tetracycline-inducible system functions in mycobacteria and that it can be used to generate conditional mutants. Initial experiments indicated that $P_{A_{tet}}$ induced higher levels of GFP than $P_{tet}$ induced in *M. smegmatis*, but this was not true in *M. tuberculosis*. In addition, *M. tuberculosis* strains carrying the $P_{A_{tet}}$ derivatives grew more slowly than *M. tuberculosis* strains carrying the $P_{tet}$ derivatives, as determined by colony size and OD$_{580}$ after 2 weeks (data not shown). It is possible that the extra copy of the Ag85A promoter may interfere with natural expression of the native Ag85A protein, a mycolyl transferase involved in cell wall biosynthesis. We consider this to be unlikely as other vectors with this promoter expressing alternative genes do not show the same slow growth (unpublished results). Alternatively, constitutive expression of the TetR repressor may be detrimental or stressful to the cell in some way. It is also possible that the TetR repressor may interfere with the expression of the native tetracycline-inducible efflux pump. In any case the levels of expression from both the tet promoters were much higher than those for the acetamide system, indicating that these promoters could be useful for overexpression of genes.

**Induction of trpD and conditional mutant.** The ability to construct conditional mutants with mutations in essential genes and the ability to turn gene expression on and off using a simple inducer are extremely useful tools. We developed the tetracycline-inducible system for use in mycobacteria, particu-
larly *M. tuberculosis*, as a tool for studying essential genes. This is the first system which has been shown to function in such a way to produce a conditional mutant of *M. tuberculosis*. Previous work by us and others has demonstrated the use of anti-sense to generate a conditional auxotroph in *M. smegmatis* (23) or to reduce gene expression in *M. tuberculosis* (15, 16, 34, 35), but this is the first report of switching a gene completely off. It is likely that for different genes the minimal level of expression for a functional phenotype is different, and we purposefully chose a gene with a normally low expression level to test our system in a robust manner.

We also demonstrated that gene expression levels can be controlled by various tetracycline concentrations, although the dynamic range is not as great as that seen in other bacteria. Further optimization of the system, including the addition of extra operator sites, expression of the repressor from alternative promoters, and/or the use of single-copy integrating vectors may help to extend the operational range of this system to give a larger range of expression levels.

**Tetracycline efflux.** We saw inhibition of growth of *M. tuberculosis* by tetracycline only at the midrange of the concentrations that we used (20 to 200 ng/ml), whereas at higher or lower concentrations there was no growth inhibition. This apparent contradiction could be explained if there is an inducible efflux system which is only expressed at the higher concentration of tetracycline. It has previously been reported that *M. tuberculosis* has a multidrug efflux pump that can exclude tetracycline from the cell (29), and our results suggest that this system or another similar one may be inducible by tetracycline in a manner similar to that of the P* tet* system that we used. Our results suggest that the tetracycline concentration should be optimized for each construct to get the most appropriate expression levels.

The same situation may also explain why we saw differences in the induction profiles of the two different P* tet* promoters in *M. smegmatis*, in particular the biphasic nature of induction for P* Act*, i.e., at higher concentrations, tetracycline efflux reduces the intracellular concentration, and so at 100 ng/ml there may only be the same level of tetracycline inside the cell as there is at 20 to 30 ng/ml. These data also demonstrate that *M. smegmatis* and *M. tuberculosis* do not have the same tetracycline sensitivity profile and that there are differences in how each organism transcribes the same promoters.

Although tetracycline is light sensitive and so may degrade over the time scale of the experiments in *M. tuberculosis* (2 weeks), we were still able to observe expression of the GFP reporter gene. Since the GFP reporters that we used are unstable reporters, this does not represent accumulation of the protein but represents recent expression. Therefore, we are confident that there is sufficient tetracycline available to act as an inducer even over the longer incubation times. In addition, although tetracycline may lose its antibiotic activity, it may still act as an inducer. However, the decrease in antibiotic activity over time could well explain some of the differences between the growth inhibition of *M. tuberculosis* and the growth inhibition of *M. smegmatis*.

Other applications for this system include the expression of recombinant mycobacterial proteins in mycobacterial hosts. This is particularly important since a number of proteins are posttranslationally modified in mycobacteria and thus cannot be expressed in a native form in *E. coli*. A tightly controlled promoter system would also be invaluable for the overexpression of toxic genes in mycobacteria. At present there is no system that allows toxic genes to be cloned and expressed, and the P* tet* system is a good candidate for this.

**Conclusion.** We show here that the tetRPO system is both inducible and controllable in *M. smegmatis* and *M. tuberculosis* and that we can use it to express genes and, more importantly, switch them off. Our work should allow us to look at the function of other essential genes by constructing conditionally expressing strains. One advantage of this system, which has been widely made use of in eukaryotic systems, is the ability of tetracycline to enter eukaryotic cells. This opens up the exciting possibility of being able to control mycobacterial gene expression in vivo as well as in vitro.

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