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A *lacZ* Reporter-Based Strategy for Rapid Expression Analysis and Target Validation of *Mycobacterium tuberculosis* Latent Infection Genes

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Abstract We report a novel *lacZ* fusion vector and demonstrate its utility for expression analysis of genes associated with *Mycobacterium tuberculosis* latent infection. The vector contains *E. coli* (*oriE*) and mycobacterial (*oriM*) origins of replication, a kanamycin resistance gene (Km^r) as selection marker, and a *lacZ* reporter gene in fusion with MCS for cloning of upstream regulatory sequence of the desired genes. β-galactosidase activity of the vector was standardized for expression analysis under latent mycobacterial conditions using P*hsp60*, a constitutive mycobacterial promoter, utilizing *Mycobacterium smegmatis* as model organism. Validation of the vector was done by cloning and expression analysis of P*hspX* (alpha crystalline) and P*icl* (isocitrate lyase), promoters from two of the genes shown to be involved in *M. tuberculosis* persistence. Both genes showed appreciable levels of β-galactosidase expression under hypoxia-induced persistent conditions in comparison to their actively replicating state. Expression analysis of a set of hypothetical genes was also done, of which Rv0628c showed increased expression under persistent conditions. The reported fusion vector and the strategy can be effectively used for short listing and validation of drug targets deduced from various non-conclusive approaches such as bioinformatics and microarray analysis against latent/persistent form of mycobacterial infection.

Introduction

Mycobacterium tuberculosis remains one of the world's most lethal and resilient organisms accounting for about 9 million infections and 1.3 million deaths in 2013 alone [3]. Much success of the pathogen can be attributed to its adaptive ability to lie dormant in the human body for extended periods, thus escaping the host immune system. According to WHO estimates, one-third of the world population harbors latent tuberculosis (TB) infection. Only 10 % of the individuals exposed to the etiological agent develop an active form of the disease, while in the rest, the bacilli are present as an asymptomatic, persistent, or latent infection [3]. Such latent carriers have 2–23 % risks of reactivation to active TB and this risk ascends with immunosuppression. The presence of ample reservoirs of latent infection and ability of the bacilli to reactivate into an active form pose a major hurdle in global tuberculosis control programs.

Molecular pathogenesis of *M. tuberculosis* under latent or persistent condition is still to be unveiled completely. One of the major mechanisms known to control dissemination of TB infection is the formation of 'granulomas'. Granulomas are aggregates of host immune cells, and are believed to be the seat of latent *M. tuberculosis* bacteria [27]. Latent bacilli are exposed to a set of unfavorable physiological conditions of hypoxia, nutrition depletion, and acidic pH inside the granulomatous structures [27]. Since mechanisms leading to latency may take several

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years to manifest their symptoms in human hosts, various in vitro and in vivo models have been proposed utilizing different physiological conditions encountered by the latent bacilli inside the granuloma to mimic *M. tuberculosis* latent infection.

In vitro models employing gradual oxygen depletion induced-hypoxia, leading to non-replicating persistence (NRP), have been most commonly used for the study of latent infection [8, 10]. Although hypoxia-induced model for persistent *M. tuberculosis* infection is also known [25], *Mycobacterium smegmatis*, a non-pathogenic mycobacterial *sps.*, has been commonly exploited for rapid investigation of genes under in vitro latency conditions [10, 12]. The present study also exploits obvious advantages associated with the use of *M. smegmatis* persistence model, owing to its short generation time and ease of handling.

The current regimen of antimycobacterials is by and large ineffective in eliminating the *M. tuberculosis* latent infection [1, 28]. Although a pool of new drug and vaccine targets has been reported against tubercle bacilli over the years [23, 30], little or no success has been achieved in drug development efforts against latent form of infection, emphasizing the need for further studies in this direction. In addition to enriching the pool of target candidates, better and swift methods are also required for analysis and validation of novel drug targets against such form of infection enabling better tackling of *M. tuberculosis* latent infection.

In the present study, we report a *lacZ* fusion vector for expression analysis of genes under hypoxia-induced mycobacterial latency. Far-red reporters have been used for expression analysis under hypoxic conditions in mycobacterial system [2], but no study has been published utilizing reporter system for expression analysis of genes under mycobacterial latent infection. This is the first report of utilizing a *lacZ*-based vector for expression analysis under hypoxia-induced NRP state of mycobacteria.

β-galactosidase activity of the reporter was standardized and validated under hypoxia-induced NRP conditions of *M. smegmatis*. Utility of the vector and the strategy was further established by expression analysis of a set of hypothetical genes. This strategy can be effectively used for expression analysis of genes along with rapid and easy validation of potential drug targets under different stress conditions related to mycobacterial latency.

Materials and Methods

Bacterial Strains, Culture Conditions, and Antibiotics

All experiments were carried on *E. coli* DH5α and *Mycobacterium smegmatis* mc^2 155 strains. *E. coli* DH5α cells were grown in Luria–Bertani (Difco) broth or agar as required. *M. smegmatis* cells were grown in Luria–Bertani (Difco) broth supplemented with 0.5 % glycerol (Fischer Scientific) and 0.2 % Tween80 (Bio Basic Inc) (LBGT) or plated on Nutrient Agar (Hi-Media) supplemented with 0.05 % Tween80 (NAT). All cultures were grown at 37 °C with constant shaking at 200 rpm to obtain actively growing aerobic cultures.

Antibiotics—rifampicin, isoniazid, ethambutol, and kanamycin were procured from Hi-Media. All antibiotics were dissolved in water except rifampicin which was dissolved in DMSO. Metronidazole used was injection-based market preparation available in 5 mg/ml concentration (J. B. chemicals and pharmaceuticals).

Establishment and Validation of Non-replicating Persistent (NRP) Conditions of *M. smegmatis*

M. smegmatis was used as model organism for expression analysis of genes under hypoxia-induced NRP condition, according to the method described by Dick et al. [8]. Viability and CFU (colony-forming units) of the cells were checked at regular intervals till 3 weeks post anaerobiosis. Morphological characterization in terms of change in shape and size of the bacilli was done by transmission electron microscopy (TEM), while deviation in staining properties was confirmed by Ziehl–Neelsen (ZN) staining, according to standard protocols. Physiological tolerance of *M. smegmatis* towards common antimycobacterial drugs was determined for rifampicin, metronidazole, isoniazid, and ethambutol. MICs were determined for actively replicating culture according to CLSI guidelines, and the same concentrations were applied on NRP culture. Percentage survival of the *M. smegmatis* cells was compared when exposed to the drugs under replicating and persistent conditions.

Construction of *Mycobacterium–E. coli* Shuttle Vector with *lacZ* Reporter Gene

A pMV206::*lacZ* vector was constructed to facilitate the cloning of upstream regulatory sequence of the genes, so as to assay their expression by β-galactosidase activity of the reporter gene. The structural gene coding for *lacZ* (β-galactosidase) was PCR amplified from pMV261*lacZ* [21] using forward primer GCTCTAGAATGGCCAAGA CAATTGCG and reverse primer CCCAAGCTTCTTATT TTTGACACCAGAC having *Xba*I and *Hind*III sites, respectively. This PCR-amplified *lacZ* fragment was cloned into pMV206 Mycobacterium–*E. coli* shuttle vector [21]. The multiple cloning site upstream of *lacZ* reporter was retained to facilitate insertion of individual upstream regulatory sequence of *M. tuberculosis* genes.

Cloning of Upstream Regulatory Sequence and Electroporation of the Recombinant Constructs into *M. smegmatis*

Upstream sequences of the genes were retrieved from tuberculosis database (http://tuberculist.epfl.ch/) and PCR amplified from *M. tuberculosis* H37Rv genomic DNA using primers having *Kpn*I and *Xba*I sites at 5′ ends of forward and reverse primers, respectively (Supplementary file 1 and Table 1), to facilitate directional cloning into pMV206::*lacZ* vector. PCR-amplified fragments were cloned into pGEM-Teasy TA cloning vector (Promega), followed by sequencing for confirmation of their sequence. Restriction-digested and gel-extracted inserts were ligated to pMV206::*lacZ* vector. These constructs were transformed into *E. coli* DH5α for amplification of the plasmid DNA, followed by electroporation into *M. smegmatis* according to standard protocol [17]. Recombinant *M. smegmatis* cells for each construct were obtained by selection on NAT plates containing kanamycin.

p-Nitrophenyl Phosphate (*p*NPP) Assay for Expression Analysis of Genes

Wild-type and recombinant strains were analyzed and compared for β-galactosidase activity under aerobic and hypoxia-induced NRP conditions using *p*-nitrophenyl phosphate (*p*NPP) assay [14], and the reporter activity observed was expressed as Miller units.

For β-galactosidase activity of cultures subjected to hypoxia-induced persistent conditions, the cultures were centrifuged and washed twice with PBST (PBS pH7.2, supplemented with 0.05 % Tween80) to remove blue color due to addition of methylene blue, which could interfere with the absorbance values of the culture. The cells were vortexed to break clumps and resuspended in fresh LBGT medium containing lysozyme (3 mg/mL). The suspension was incubated at 37 °C for 1–2 h to allow complete cell lysis. Following lysis, *p*NPP assay was performed as described above for actively replicating cultures of *M. smegmatis*.

Standardization and Validation of *lacZ* Expression for *M. smegmatis* Latency Conditions

Standardization of the β-galactosidase activity is required, as during persistent state, the number of cells per mL of the media decreases, and the cells enter a state of metabolic shut down resulting in an overall decrease in the expression of genes. For standardization of *lacZ* expression under replicating and hypoxia-induced NRP conditions, a promoterless pMV206::*lacZ* vector was used as negative control, and β-galactosidase activity of the negative control was compared with that of a vector containing P*hsp60* (a constitutive mycobacterial promoter) [5].

To validate whether the pMV206::*lacZ* vector could be used for expression analysis of genes under mycobacterial persistence conditions, β-galactosidase activity of two genes—*hsp*X (Rv2031c) [13] and *icl* (Rv0437) [20] known to be up-regulated under hypoxia-induced persistent conditions was determined. Miller units obtained on expression analysis of different genes were normalized with respect to constitutive P*hsp*60 expression values obtained under NRP and actively replicating conditions.

Expression Analysis of a Set of Hypothetical Genes

To further establish the utility of the vector, expression analysis of a set of hypothetical genes chosen from a microarray-based study [15] was done. Recombinant *M. smegmatis* strains with individual constructs containing upstream regulatory sequence of Rv0227c, Rv0430, Rv0883c, Rv0628c, or Rv0347 were subjected to actively replicating and hypoxia-induced persistent conditions. Expression of the genes was determined by *lacZ* reporter activity using *p*NPP assay and normalized as discussed above.

Statistical Analysis

Data were analyzed with the ANOVA test using GraphPad Prism software. Values of $P < 0.0001$ and $P < 0.05$ were considered to be statistically significant.

Results

M. smegmatis Showed Characteristic Features of a Persistent Mycobacterial Infection Under Hypoxia-Induced Stress Condition

Non-replicating persistent state of *M. smegmatis* was achieved and validated as reported earlier [8]. *M. smegmatis* cultures when subjected to complete anaerobiosis showed constant CFU over an observation period of 3 weeks (Supplementary file 1 and Fig. 1). Bacteria adapt themselves to hypoxic condition and survive anaerobiosis leading to a state of persistence. NRP state bacteria were shorter, unevenly stained, and exhibited beaded appearance, in comparison to rod-shaped red/dark pink-stained actively replicating cells (Supplementary file 1 and Fig. 2). The structural changes were further confirmed by transmission electron microscopic (TEM) studies (Supplementary file 1 and Fig. 3).

Active state cultures were sensitive to commonly used antimycobacterial drugs and simultaneously exhibited

resistance to metronidazole, a common drug effective under anaerobic condition. The drug susceptibility profile was reversed for the bacteria under anaerobiosis-induced persistent state. Bacilli under NRP state expressed tolerance to common antimycobacterial drugs and were sensitive to metronidazole (Supplementary file 1 and Table 2), a characteristic feature of persistent mycobacteria.

pMV206::*lacZ* Vector for Expression Analysis of *M. tuberculosis* Genes Under Hypoxia-Induced Latency Conditions

A pMV206::*lacZ* shuttle vector was obtained containing *E. coli* (*col*E1) and mycobacterial (*oriM*) origins of replication, kanamycin resistance gene (Km^r) as selection marker, and a *lacZ* reporter gene. The presence of a multiple cloning site upstream to the *lacZ* reporter gene facilitates cloning of upstream regulatory sequence of the genes. Size of the vector was estimated to be 7610 bp (Fig. 1). The vector was used for expression analysis of genes under actively replicating and hypoxia induced-persistent conditions of mycobacterial infection, using *M. smegmatis* model established above.

Recombinant *M. smegmatis* Strains were Obtained on Cloning and Electroporation of Vector Constructs

Recombinant pMV206::*lacZ* constructs containing individual upstream regulatory sequence of the genes under study were obtained after cloning. The constructs were electroporated into *M. smegmatis* to obtain recombinant *M. smegmatis* strains. Each strain was individually subjected to actively replicating and hypoxia-induced NRP conditions to determine expression of the respective gene using *p*NPP assay.

Expression of pMV206::*lacZ* was Standardized Using Constitutive Promoter (P*hsp60*), and Validated by Upregulation of Positive Control Genes (P*hspX* and P*icl*)

M. smegmatis containing promoterless vector showed *lacZ* expression values similar to that of wild-type *M. smegmatis* strain, suggesting negligible basal activity of the reporter. P*hsp60* showed positive and significant expression in comparison to *M. smegmatis* containing promoterless vector and wild-type *M. smegmatis* under actively replicating and hypoxia-induced persistent conditions, indicating that the vector is functional under both conditions. P*hsp60* showed significant values ($P \leq 0.0001$) of *lacZ* expression in comparison to wild-type and promoterless construct. *P* denotes significant statistical difference between expression of P*hsp60* in comparison to promoterless construct and wild-type, promoterless constructs under actively replicating and NRP states. Expression values of constitutive P*hsp60* were used for normalization of expression of other genes.

Promoters from the control gens, P*hspX* (alpha crystalline) and P*icl* (isocitrate lyase), showed increased expression of 91- and 19-fold, respectively, under hypoxia-induced persistence in comparison to their actively replicating state (Fig. 2a), validating utility of the vector under NRP conditions.

Rv0628c may be a Potential Drug Target for *M. tuberculosis* Latent Infection

Expression analysis of a set of hypothetical genes showed significant upregulation of Rv0628c (5.8 fold) under hypoxia-induced persistent conditions in comparison to its actively replicating state (Fig. 2b). Promoters of Rv0430 and Rv0347 showed an upregulation of 1.4- and 1.6-fold, respectively, under NRP conditions, while Rv0883c (0.6) and Rv0227c (0.8) showed minor decrease in expression under hypoxia-induced NRP state in comparison to their actively replicating conditions.

Discussion

Even after multiple control efforts worldwide, TB remains most devastating disease and second largest infectious killer worldwide after human immunodeficiency virus (HIV) [26]. One of the major hurdles in controlling *M. tuberculosis* infection is its ability to switch to a latent or non-replicating persistent form inside human body. Latent TB infection is maintained in the host body for several years, despite acquired host immunity and an extensive treatment regime. Interplay between host and pathogen that escort the infection to a latent stage, and later to reactivation of latent infection remains unearthed completely. There is an alarming need to make an insight into host pathogen interaction and mechanisms underlying latency and reactivation, which would aid in devising better strategies to combat this particular form of infection. Thus, the current scenario demands novel drugs and drug targets, along with novel strategies for identification and validation of the drug targets dedicated and targeted to latent form of TB infection.

In the present study, we report a novel *lacZ* reporter-based strategy for expression analysis and rapid validation of drug targets against latent or persistent TB infection. This is first report of utilizing a reporter-based assay for expression analysis of *M. tuberculosis* genes under hypoxia-induced persistent state.

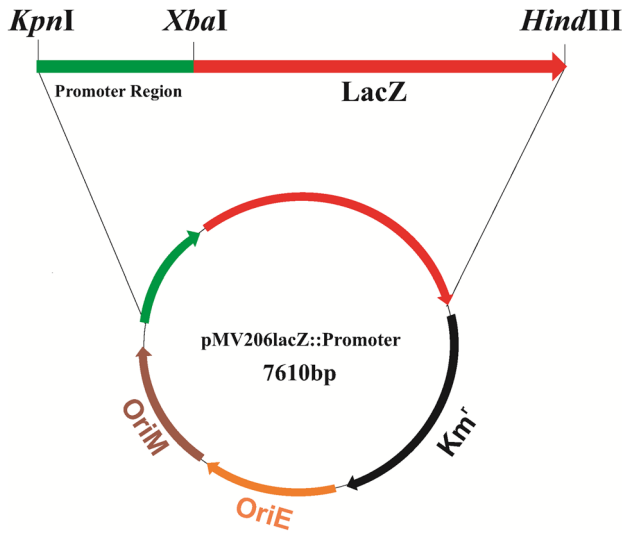

Fig. 1 pMV206::*lacZ* vector pMV206::*lacZ* vector with kanamycin selection marker, containing *oriE* and *oriM* to facilitate its replication in *E. coli* and mycobacterial hosts respectively. MCS allows cloning of upstream promoter region in fusion with *lacZ* reporter gene for expression analysis under different growth/stress conditions

M. smegmatis is a fast growing, non-pathogenic mycobacterial species offering ease of working, as there is no requirement for biosafety level 3 (BSL-3) containment facilities. *M. smegmatis* has a generation time of 2–3 h in comparison to 24 h of *M. tuberculosis* enabling rapid screening of target genes. Hence, to study the expression of genes, a hypoxia-induced NRP model of *M. smegmatis* was employed [8], which has been commonly exploited for study and expression analysis of genes under persistent mycobacterial condition [10, 12].

Most extensively used mycobacterial latency models use hypoxia-induced stress for the development of a NRP state of the bacilli. As expression of β-galactosidase is not affected by low oxygen conditions [6, 29], hence, *lacZ* reporter was chosen for construction of the vector. Other reporter systems such as *gfp* have been utilized as reporter to study mycobacterial–macrophage interactions [7], but there are no reports of its use under hypoxic or latency related condition. Moreover, it also suffers from the problem of quenching [22] and is affected by low oxygen concentrations [4]. *gfp* is also reported to be low in sensitivity as it does not involve enzymic amplification [16]. *lacZ* also has an edge over luminescence-based vectors which require the presence of oxygen for their activity. In a recent study, Carroll et al. [2] have used far-red reporters under hypoxic conditions in mycobacterium, whereas aim of our study was to develop a strategy for expression analysis of genes under hypoxia-induced persistent condition mimicking *M. tuberculosis* latent infection.

A pMV206::*lacZ* vector with *lacZ* reporter system was constructed, which contains an MCS upstream to the reporter gene to facilitate cloning of upstream regulatory sequence of genes for expression analysis. *lacZ* activity of the reporter can be easily monitored using a *p*NPP biochemical assay under hypoxia-induced NRP condition of mycobacteria, and can be correlated to expression level of genes under study.

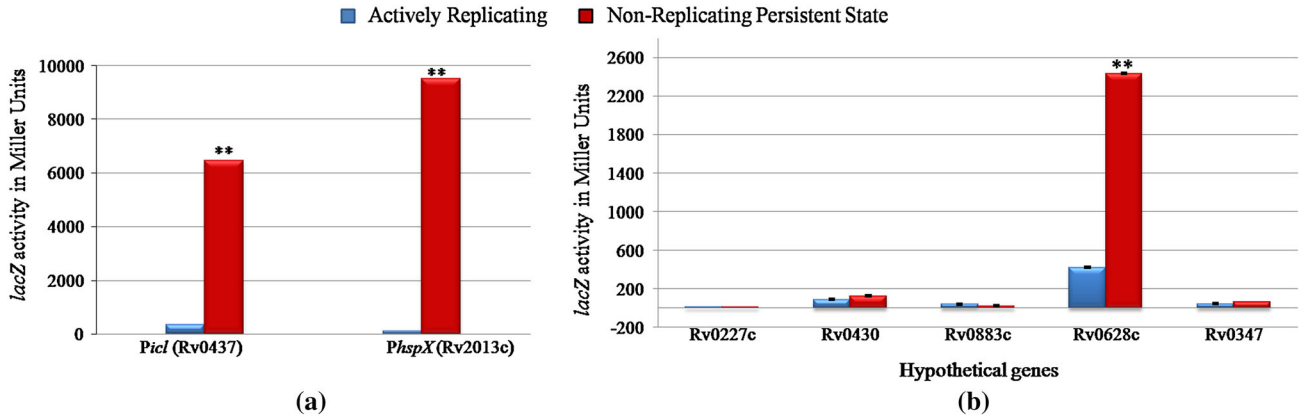

Fig. 2 **a** Validation of β-galactosidase activity under hypoxia induced NRP conditions—*M. smegmatis* strains containing P*hsp60*, P*icl* and P*hspX* constructs were subjected to actively replicating and hypoxia induced NRP conditions and their β-galactosidase activity was determined. P*hspX* and P*icl* showed significant upregulation (91 and 19 fold respectively) under NRP state in comparison to their actively replicating state. Fold change was calculated after normalization of Miller units with respect to P*hsp60* values under actively replicating and NRP states. *Double asterisks* denotes significant ($P \leq 0.05$) statistical difference in expression of actively replicating and NRP states of *icl* and *hspX* promoters. **b** β-galactosidase activity of hypothetical genes—Recombinant *M. smegmatis* strains with upstream regulatory sequence of individual hypothetical genes were subjected to actively replicating and hypoxia induced NRP conditions to determine their expression by *lacZ* activity. Rv0628c showed significant upregulation under NRP condition in comparison to its actively replicating state. *Double asterisks* denotes significant ($P \leq 0.05$) statistical difference in expression of Rv0628c under actively replicating and NRP state. No significant difference was observed for Rv0227c, Rv0430, Rv0883c and Rv0347

Wild-type *M. smegmatis* and the promoterless vector construct showed negligible *lacZ* activity, suggesting that the constructed vector had no background or basal activity of the reporter. *lacZ* activity of the vector was standardized by cloning P*hsp60,* a constitutive promoter from *M. bovis* BCG [5], upstream to the *lacZ* reporter gene and electroporating the construct into *M. smegmatis. M. smegmatis* containing P*hsp60* was subjected to actively replicating and hypoxia-induced NRP conditions. *lacZ* Miller units obtained for P*hsp60* were compared with expression values obtained for wild-type *M. smegmatis*, and *M. smegmatis* containing promoterless vector construct. Significant expression values of P*hsp60* in comparison to the wild-type and promoterless vector construct suggested that the vector could be used for expression analysis under NRP conditions. As the expression levels of constitutively expressing P*hsp60* varied under hypoxic condition in comparison to its aerobic actively replicating state, expression values obtained for P*hsp60* were used for normalization of expression of other genes.

To further validate whether the vector and the reporter strategy is useful for expression analysis under mycobacterial persistence conditions, two genes—*icl* (Rv0467) [13] and *hspX* (Rv2031c) [20], were chosen for expression analysis under actively replicating and NRP states. These genes are established to be up-regulated under *M. tuberculosis* latency and serve as positive control genes for NRP state. β-galactosidase assay of *icl* (Rv0467) and *hspX* (Rv2031c) was done along with P*hsp60* (constitutive) and promoterless vector (negative control). Our study showed elevated levels of *icl* and *hspX* during hypoxia-induced NRP condition. A 91-fold upregulation of *hspX* was recorded, and *icl* also showed 19-fold upregulation under NRP condition.

Icl (iso citrate lyase) is a key gene shown to be involved in *M. tuberculosis* persistent infection. Studies using in vivo and macrophage-based models of latent TB also confirm upregulation and important role for *icl* in mycobacterial latency [18]. There are strong evidences that *hspX* (Rv2031c), a homologue of α-crystalline, has chaperonin activity [8, 12] and is induced during *M. tuberculosis* latency [19]. Thus, upregulation of the promoter of genes already documented to play important role in mycobacterial persistence further validates that the strategy suggested in the report can be exploited for expression analysis of genes, and consecutively can help in identification of novel genes as potential drug targets for mycobacterial latency.

Furthermore, to demonstrate application of the *lacZ*-based strategy, expression analysis of a set of five hypothetical genes was also done. These genes were selected from a bioinformatics-based study of gene expression analysis under multiple stress conditions [15]. Of the five genes studied, Rv0628c showed significant up-regulation under NRP conditions, which is in correlation with earlier reports [24]. Rv0628c is a conserved hypothetical protein, but its definite role in mycobacterial persistence is yet to be assigned. Our study suggests that it could function as a potential drug target for latent form of *M. tuberculosis* infection.

Thus, we have reported a *lacZ* reporter-based method for study of genes under NRP state of *M. tuberculosis* latent infection and have demonstrated its utility by expression analysis of genes. This strategy can be applied for validation of the drug targets deciphered by various dry lab approaches. Hypothetical genes predicted to be novel targets for persistent mycobacterial infection—deduced from bioinformatic, microarray, or mathematical modeling approaches can be successfully validated by utilizing this approach. This strategy can be exploited for faster screening and validation of drug and vaccine targets for their involvement in mycobacterial persistence, thereby reducing the duration of in vitro screening.

There are multiple reports of in vivo exploitation of *lacZ* reporter gene [9, 11] hence this strategy may also be extended for use under in vivo systems mimicking latency or persistent mycobacterial infection. The reported vector may also find its application under similar and variety of stress conditions in different bacteria other than mycobacterial species, albeit with standardization according to the organism and stress condition employed.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no financial/commercial conflicts of interest.

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