A double recombinant *Mycobacterium bovis* BCG strain for screening of primary and rationale-based antimycobacterial compounds.

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Abstract

Conventional antimycobacterial screening involves CFU analysis which poses a great challenge due to slow growth of mycobacteria. Recombinant strains carrying reporter genes under the influence of constitutive promoters allow rapid and wide screening of compounds, but, without revealing their modes of action. Reporter strains using pathway-specific promoters provide a better alternative but allow a limited screening of compounds interfering with only a particular metabolic pathway. This reduces these strains to merely a second-line screen system as they fail to identify even the more potent compounds if they are not inhibiting the pathway of interest. In this study, we have generated a double recombinant *Mycobacterium bovis* BCG strain carrying firefly and *Renilla* luciferase genes as two reporters under the control of a constitutive and an inducible mycobacterial promoter. The presence of dual reporters allows simultaneous expression and analysis of two reporter enzymes within a single system. The expression profile of the firefly luciferase gene, rendered by a constitutive mycobacterial promoter, corroborates with the decline in bacterial growth in response to a wide range of antimycobacterial drugs, while the enhanced expression of *Renilla* luciferase mirrors the selective induction of the reporter gene expression as a result of pathway-specific inhibition. Thus, the double recombinant strain allows the screening of both primary and rationally synthesized antimycobacterial compounds in a single assay. The inhibiting response of drugs was monitored by Dual-Luciferase Reporter Assay which can be easily adapted in high-throughput mode.

Key words: double recombinant, promoter-inducible assay, screen system, *M. bovis* BCG.
Introduction

Despite the existing regimen of drugs tuberculosis remains a leading cause of death worldwide (www.who.int/tb/publications/global_report). The continued emergence of multidrug-resistant strains has made the present line of drugs largely ineffective. Efforts are going on to identify new antimycobacterial compounds against novel targets, and new series of compounds against known targets (1). This requires screening of a large number of compounds using high-throughput screen-systems. Conventional screening involves whole-cell based screening against growing bacterial cells where cell mortality is scored upon treatment with varying concentration of the compounds to be tested (2). This approach is the most widely used as it allows to test a wide range of compounds acting against different biochemical pathways and metabolic processes affecting survival of the microbes. A large number of existing antimicrobial drugs were discovered using this classical approach. However, the targets of these drugs remained either unknown or were identified after several rounds of experimentation. Alternatively, target based approach is used to identify inhibitors against specific biochemical reaction or some important intermolecular interaction (3). This is a straightforward approach with high level of sensitivity and technologically more adaptive from drug development perspective. Being mostly directed towards a well known target it allows rationale-based optimization of lead molecules. However, it employs purely in vitro screening methodologies and, therefore, the lead molecules are not tested for their penetration, efflux and metabolic properties which are key druggable parameters.

In recent years, the whole-cell based screening was performed using genetically modified bioluminescent strains which offered a good alternative to avoid these difficulties (4, 5, 6). These bacterial cells carry luciferase reporter genes fused to constitutive bacterial promoters, which transcriptionally respond to a wide range of drugs (4, 5, 6, 7). Assays based on the reporter gene expression measured drug activities that were parallel to MICs determined by conventional CFU methods (4, 5, 6). Several bioluminescent mycobacterial strains have been...
developed using this strategy which allowed rapid in vitro screening of a large range of antimycobacterial compounds (4, 5, 6, 7), but one of the limitations of this system is that the modes of action of leads remained unknown. The introduction of pathway-specific inducible promoters allowed an enhanced expression of the reporter gene selectively in response to inhibition of a certain biochemical pathway by a line of drugs (8, 9, 10, 11). The selective induction of the reporter gene indicates that a compound is perturbing the pathway of interest at some point, even if the inhibited targets are not known (8, 11). This kind of screening system renders advantage over others as they enable a systematic screening of compounds interfering with a given metabolic pathway. However, a serious limitation of this approach is the inability of the recombinant strains to identify even the more potent compounds if they are not acting through the pathway of interest. Thus, these recombinant strains are reduced to merely a second-line screening system and miss a large number of compounds effective against different molecular targets and biochemical pathways.

To overcome this problem, in this study, we have generated a double recombinant Mycobacterium bovis BCG strain carrying firefly and Renilla luciferase genes as two reporters under the control of a constitutive and an inducible mycobacterial promoter, respectively. The presence of dual reporters allows simultaneous expression and analysis of two individual reporter enzymes within a single system. This eliminates the experimental variability, such as differences in treatment conditions and concentration of drugs, cell lysis efficiency, variation in pipetting and in assay efficiency, and allows more reliable interpretation of the experimental data by minimizing extraneous influences. The reporter gene expression was validated by screening a wide range of known antimycobacterial drugs and other antibiotics. The inhibiting response of drugs was monitored by Promega’s Dual-Luciferase Reporter Assay. The expression profile of the firefly luciferase gene (Fluc), which is under the control of a constitutive mycobacterial promoter, corroborates with the decline in bacterial growth in response to a wide range of antimycobacterial drugs acting through different mechanism of
action, while the enhanced expression of Renilla luciferase (Rluc) mirrors the selective induction of the reporter gene activity as a result of pathway-specific inhibition. *M. bovis* BCG was chosen as surrogate organism due to its high degree of relatedness with *M. tuberculosis*, but relative lack of pathogenicity (4, 6). We describe the generation and evaluation of the double recombinant strain’s response against existing anti-tubercular drugs and, thereby, establishing the authenticity and usefulness of this strain for screening of primary and rationale-based antimycobacterial compounds.

**Materials and methods**

**Bacterial strains, plasmids and cultures**

*Mycobacterium tuberculosis* (H37Rv) and *M. bovis* BCG were obtained from departmental stocks. Other bacteria and plasmids used in the study are described in Table 1. *Escherichia coli*-mycobacteria shuttle plasmid vectors pMV206 and PMV306 were kind gift from William R. Jacobs, Einstein College of Medicine, NY, USA. *E. coli* cultures were grown in LB medium with addition of ampicillin (100µg/ml), kanamycin (25µg/ml) and hygromycin (100µg/ml), as and when required. *M. bovis* BCG cultures were grown in Middlebrook 7H9 medium supplemented with 0.4% glycerol, 0.05% Tween 80 without or with antibiotics as per requirements (hygromycin, 50µg/ml and kanamycin, 25µg/ml). *M. bovis* BCG cultures were plated after serial dilution on Middlebrook 7H10 plates with 0.05% Tween 80 for CFU assay. All antibiotics were obtained from Sigma.

**Determination of minimum inhibitory concentration (MIC)**

MIC was determined as described earlier (11). Briefly, *M. bovis* BCG culture grown to 0.6 OD600 were diluted to 0.05 OD600 with fresh 7H9 medium, and ~ 10^6-7 bacilli were transferred to different tubes containing 5 ml fresh medium with antibiotics. The cultures were allowed to grow for 12 h at 37°C and plated thereafter in duplicates following 10-fold serial dilution for
CFU analysis. Total number of colonies appeared in untreated control was considered as 100% and the MIC was calculated as the concentration of drug at which >90% growth inhibition was observed. Under these conditions, the MIC obtained was 0.50 µg/ml for ethambutol, 1 µg/ml for streptomycin, 1.37 µg/ml for INH, 1 µg/ml for rifampicin, 40 µg/ml for vancomycin, 12.5 µg/ml for polymyxin B sulphate, 50 µg/ml for ethionamide, 35 µg/ml for thiolactomycin, 4 µg/ml for cerulenin and 10 µg/ml for triclosan. Wild type and rBCG strains showed similar MIC for tested drugs.

PCR amplifications, cloning and construction of M. bovis BCG recombinant strains

*M. tuberculosis hsp60*pr (Rv0440), *sigA*pr (Rv2703), *16s rRNA*pr (MTB000019) (12, 13, 14) and *kas* operon (Rv2243-47) *43pr* (11) were PCR amplified from H37Rv genomic DNA using appropriate primers (Table 1) and amplicons were cloned into pTZ57R/T. Firefly and Renilla luciferase genes were amplified from pGL3-Basic and pFN11A vectors, respectively and cloned into pTZ57R/T vector. All plasmid constructs were validated by restriction digestion and DNA sequencing. DNA sequencing was performed using ABI Prism Big Dye Terminator cycle sequencing kit as per the manufacturer’s protocol on ABI 310 DNA sequencer. Firefly and Renilla luciferase genes were relocated to pMV306 and pMV206 vectors respectively at XbaI/EcoRI site. Different promoters, *hsp60*pr, *sigA*pr, *16s rRNA*pr and *43pr* were subsequently subcloned into pMV306 or pMV206 vector to the upstream of Firefly/Renilla luciferase genes using enzyme sites present in primers (Table 1).

Double recombinant strain and reporter gene expression

Various pMV306 plasmid constructs carrying *hsp60*pr, *sigA*pr, *16s rRNA*pr and Flux gene were electroporated into M. bovis BCG using standard protocol. Cells were recovered in 7H9 medium for 16 h at 37°C and then plated on 7H10 with hygromycin to select the transformants. Primary rBCG strain was then transformed with pMV206 vector carrying *kas* operon *43pr* in fusion with Renilla luciferase gene to get double recombinant BCG strain. Recombinants were
grown in 7H9 broth supplemented with hygromycin and kanamycin at 37°C up to 0.5 OD600, cultures were diluted to 0.04–0.05 OD600 with fresh medium with kanamycin and allowed to grow for 4 h at 37°C. Various drugs were added subsequently at different concentrations; ethambutol (0.25µg/ml, 0.50µg/ml, 1.0µg/ml), streptomycin (0.50µg/ml, 1.0µg/ml, 2.0µg/ml), INH (0.55µg/ml, 1.37µg/ml, 2.74µg/ml), rifampicin (0.50, 1.0µg/ml, 2.0µg/ml), vancomycin (14.99µg/ml, 40µg/ml, 80µg/ml), polymyxin B (5.56µg/ml, 12.5µg/ml, 25µg/ml), ethionamide (2µg/ml, 50µg/ml, 100µg/ml), thiolactomycin (20µg/ml, 35µg/ml, 75µg/ml), cerulenin (3µg/ml, 4µg/ml, 8µg/ml) and triclosan (5µg/ml, 10µg/ml, 20µg/ml) and cultures were grown for 12 h. Luciferase assay was performed using Promega’s Dual-Luciferase assay system according to kit’s protocol. Briefly, 80µl culture containing 107-8 bacterial cells was taken and mixed with 20 µl Passive Lysis Buffer (PLB). After 10 minutes incubation on ice, suspension was sonicated (Vibracell VCX-750; Ultrasonics, USA) briefly to lyse the cells. Thereafter, 100µl LAR-II containing luciferase substrate and buffer were added and firefly luciferase activity was measured using Berthold’s luminometer (Germany). Then, 100µl Stop and Glo buffer was added to quench firefly luciferase and Renilla luciferase activity was measured in the same sample. To determine the percent growth inhibition total luminescence in untreated samples was considered as 100%. Experiments were carried out in duplicates for each drug concentration and luciferase activity was calculated for each set individually. The cultures from each time point were also plated to confirm the decline in viability of cells after drug treatment. Three independent experiments were performed and similar trends in results were obtained.

Real Time RT-PCR analyses of M. bovis BCG cultures after antibiotics treatment

Log phase M. bovis BCG: hsp60pr-Fluc culture was split into 5 ml aliquots and treated with specified concentrations of antibiotics (Fig. 3) for 4 h at 37°C. Post-treatment cells were processed for RNA isolation as described earlier (15). The total RNA sample was treated with DNase I (Turbo DNase, Ambion) at 37°C to remove any DNA contamination. The first strand cDNA was synthesized using random hexamers and transcriptor reverse transcriptase (Roche)
as per kit protocol. Quantitative real-time PCR was performed using SYBR green master mix (Roche) as described earlier (16). RNA samples that had not been reverse transcribed were included as control in all the experiments. Expressions of target genes were normalized with the sigA transcript level. The average relative expression levels and standard deviations were determined from the data generated from three independent experiments.

Statistical analysis

Data were collected from three independent experiments and at each point experiments were set up in duplicates. The mean value and standard deviations were plotted for each set of data.

Results and Discussion

Selection of mycobacterial promoters and generation of *M. bovis* BCG primary recombinant strain

Promoters are the main regulatory component of the gene expression which has been correlated with the growth and physiology of the bacteria (12). They impart constitutive expression of genes throughout the growth and selectively induce or repress the expression of genes temporally, spatially and during cellular and environmental stress conditions (13, 17). A prerequisite for selecting constitutive mycobacterial promoters was to identify the promoters that allow the constitutive expression of genes and respond broadly to a range of antibiotics at the level of gene expression. Three mycobacterial promoters, *hsp60*<sub>pr</sub>, *sigA*<sub>pr</sub> and *16srRNA*<sub>pr</sub>, were selected owing to their earlier description of being constitutive promoters (12, 13, 14, 17). These promoters were amplified, cloned upstream of the firefly luciferase ORF and the recombinant constructs were transferred to the *M. bovis* BCG genome via an integrative mycobacterial vector. This allowed a stable Fluc expression as a reporter gene under the control of the chosen promoters in the recombinant strains. All three recombinant *M. bovis* BCG (rBCG) strains showed luciferase activity from early log phase to stationary phase (Fig. 1), but the *hsp60*<sub>pr</sub> rendered a luciferase activity much stronger than *sigA*<sub>pr</sub> and *16srRNA*<sub>pr</sub>. The
luciferase activity was appreciably reduced during the stationary phase in all three strains. This could be either due to the reduced activity of the promoters or to the lower metabolic rate of the stationary phase cells. It may be noted that the luminescence was measured using same number of bacterial cells from different growth stages of the recombinant strains. Earlier, Andreu et al. (18) evaluated three mycobacterial promoters, \textit{hsp60}\textsubscript{pr}, \textit{myc}\textsubscript{tetO}\textsubscript{pr} and \textit{G13}\textsubscript{pr} with three different luminescence reporters, firefly luciferase (Fluc), \textit{Gaussia} luciferase (Gluc) and bacterial luciferases Lux, in \textit{M. tuberculosis} and \textit{M. smegmatis} and found that the highest luminescence was obtained using \textit{hsp60}\textsubscript{pr} in both species. It may be noted that while, \textit{hsp60}\textsubscript{pr} was derived from \textit{M. bovis} BCG (19), \textit{myc}\textsubscript{tetO}\textsubscript{pr} and \textit{G13}\textsubscript{pr} were isolated from \textit{M. smegmatis} (20) and \textit{M. marinum} (21), respectively. Both these promoters were reported to be stronger than \textit{hsp60}\textsubscript{pr} but they observed that the \textit{hsp60}\textsubscript{pr} drove the highest reporter gene expression. In present study, we analyzed three \textit{M. tuberculosis} promoters (\textit{hsp60}\textsubscript{pr}, \textit{sigA}\textsubscript{pr} and \textit{16srRNA}\textsubscript{pr}), which shared nearly complete homology with the surrogate mycobacterial strain, \textit{M. bovis} BCG. The results obtained are similar to those previously described (18) as the \textit{hsp60}\textsubscript{pr} rendered the maximum expression (Fig. 1).

**Antibiotic response of the promoters**

Antibiotic response of the recombinant strains was analyzed upon treatment with a range of antibiotics having different mechanism of actions and the luminescence was measured as a function of gene expression. Decline in the bacterial growth upon antibiotics treatment affects the ongoing gene expressions which in turn reduce the total luminescence in the treated sample as compared to the untreated bacterial cells. All three rBCG strains showed reduction in luminescence upon antibiotics treatment (Fig. 2), but the decline was more pronounced when the reporter was under \textit{hsp60}\textsubscript{pr} transcriptional control. The higher level of reporter gene expression in \textit{M. bovis} BCG: \textit{hsp60}\textsubscript{pr} - Fluc strain allows the screening of antimycobacterial compounds using lower number of bacterial cells as they produce markedly higher luminescence in comparison to rBCG strains carrying \textit{sigA}\textsubscript{pr} and \textit{16srRNA}\textsubscript{pr}. Thus, the further
studies were performed using this as a primary recombinant strain. The \textit{hsp60}_pr-Fluc construct was integrated in single copy at an ectopic site on the \textit{M. bovis} BCG genome. To ensure that the \textit{hsp60}_pr rendered similar level of expression from its native and ectopic sites, we performed real time quantitative RT-PCR of \textit{hsp60} and Fluc genes using RNA samples from \textit{M. bovis} BCG: \textit{hsp60}_pr-Fluc strain upon treatment with different antimycobacterial drugs. A similar decline in the expression of \textit{hsp60} and Fluc was noticed upon treatments with different antibiotics suggesting that the expression derived from the native and the ectopic \textit{hsp60} promoters are similar (Fig. 3). This also ensured that the total luciferase activity is indeed derived from the \textit{hsp60}_pr driven Fluc expression.

**Correlation between luminescence and growth inhibition of the recombinant \textit{M. bovis} BCG: \textit{hsp60}_pr-Fluc strain.**

Conventional antimicrobial screening involves counting of bacterial colonies from dilution plating after antibiotics treatment (22). This takes several days in case of mycobacteria even after using fast growing mycobacterial species. The recombinant strain expressing luciferase constitutively allows fast and real time analysis of diminishing bacterial growth upon antibiotics treatment (5, 6, 7). We noticed an apparent decline in the luminescence after four hours of antibiotics treatments and it gradually increased as the treatment continued. The time required for appreciable decline may vary because of the drug’s mode of action. Drugs, such as rifampicin and streptomycin that interfere with transcription and translation and block further synthesis of luciferase should show an effect after a short incubation period. In contrast, drugs that do not directly interfere with luciferase activity, such as those that block cell wall synthesis, may require longer incubation before loss of luciferase activity is detectable. The RLU assay was performed after twelve hours of antibiotics treatment and at this point the decline in bacterial growth of the recombinant strain, measured in CFU, well corresponded with decline in RLU (Fig. 4). We determined the percent inhibition of the bacterial growth based on the varying level of luminescence and also by counting bacterial colonies after...
treatment with different concentration of antibiotics (IC\textsubscript{50}, MIC(IC\textsubscript{90}), 2xMIC). For experimental analysis $10^{6-7}$ bacterial cells were taken per sample, which gave little higher MIC values but consistently ensured the minimal error in RLU measurement. A similar inhibition profile was obtained through both approaches (Fig. 4), which established the usefulness of \textit{M. bovis} BCG: \textit{hsp60}\textsubscript{pr}\textendash Fluc strain for the screening of a wide range of antimycobacterial compounds in fast and efficient manner.

**Double recombinant \textit{M. bovis} BCG strain and screening of a primary and rationale based antimycobacterial compounds**

\textit{M. bovis} BCG: \textit{hsp60}\textsubscript{pr}\textendash Fluc strain contained the \textit{hsp60}\textsubscript{pr}\textendash Fluc recombinant construct integrated into the genome. The promoter responded to a wide range of antimycobacterial compounds uniformly by reducing the level of reporter gene expression, and therefore allowed the screening of compounds inhibiting mycobacterial growth irrespective of their mode of actions. In earlier studies reporter strains carrying pathway-specific promoters were used for selective screening of rationally synthesized compounds against a given metabolic pathway of bacteria (8, 9, 10, 11). To enhance the screening repertoire of this strain we reasoned that if a promoter, which selectively induces the reporter gene expression sensing the inhibition of a particular biochemical pathway, is combined with a reporter gene, compatible with the Fluc expression and its assay, is introduced into \textit{M. bovis} BCG: \textit{hsp60}\textsubscript{pr}\textendash Fluc strain, the newly generated double recombinant strain would allow the screening of both primary as well as rationally designed pathway specific inhibitors. To enable this we selected the \textit{kas} operon promoter which was reported to be selectively induced in response to antimycobacterial drugs targeting the FAS-II elongation pathway in mycobacteria (11, 23). The organization of \textit{kas} operon genes are highly conserved in mycobacteria (11) and its upstream regulatory regions are almost identical in \textit{M. bovis} BCG and \textit{M. tuberculosis}. The \textit{kas} operon promoter was combined with \textit{Renilla} luciferase gene and the recombinant construct was transferred to \textit{M. bovis} BCG: \textit{hsp60}\textsubscript{pr}\textendash Fluc strain via an extrachromosomal mycobacterial plasmid vector to generate \textit{M. bovis} BCG: \textit{hsp60}\textsubscript{pr}\textendash Fluc 43pr-
Rluc, a double recombinant strain. The double recombinant strain produces Fluc through hsp60pr while Rluc expression is rendered by 43pr (kas operon promoter). Dissimilar enzyme structures and substrate requirements of Firefly and Renilla luciferases make it possible to selectively discriminate between their respective bioluminescent reactions and allow simultaneous assay of the both enzymes. After quantifying the firefly luminescence, this reaction was quenched, and the Renilla luciferase reaction was subsequently initiated in the same tube. Quenching of firefly luciferase luminescence and concomitant activation of Renilla luciferase are accomplished by adding Stop & Glo Reagent (Promega) to the sample tube immediately after quantitation of the firefly luciferase reaction. The assay system was optimized so that the both reporters yield linear assays with utmost sensitivities and no endogenous activity of either reporter in the host cells. Further, we performed only firefly luciferase assay and measured total RLU derived from Fluc activity taking equal number of bacilli from single and double rBCG culture. Nearly same RLU level was obtained in samples from both recombinant strains (data not shown), which ensured that these two enzymes are distinctly dissociated and there is no cross reaction between firefly and Renilla luciferase in our assay. We performed dual luciferase assay using this strain after treatment with different antimycobacterial drugs. The reduced Fluc expression was obtained in the entire treated samples (Fig. 4 & 5) as this expression was rendered by hsp60pr, while Rluc expression was diminished only in samples treated with ethambutol, streptomycin, rifampicin, vancomycin and polymyxin B, which act through pathways other than FAS-II. Rluc expression derived through 43pr which is selectively induced after treatment with FAS-II pathway inhibitors was induced only after in cells (Fig. 5) treated with, isoniazid, ethionamide and thiolactomycin (known FAS-II inhibitors). The induction was apparent as early as three hours after drug treatment and showed a gradual increase up to 24 hours (data not shown). The induction was noticeable nearly at IC50, increased to maximum at the MIC of drug but it did not increase proportionately at concentrations that exceeded far the MIC value. At exceeding concentration of drugs we noticed a rapid and more pronounced induction of luciferase expression (data not
shown), but there was a gradual decline in the expression level with prolonged exposure at higher concentrations as compared with the enhanced expression obtained at MIC level, which could be due to death of more cells at exceeding dose of drugs (Fig. 4 & 5).

Recombinant *M. bovis* BCG and *M. tuberculosis* strains expressing luciferase were used in earlier studies for screening of antimycobacterial compounds (4, 6). These recombinants expressed luciferase exclusively through the *hsp60*<sub>pr</sub>, and based on luciferase profile showed growth inhibition and MIC parallel to conventional CFU based method. The double recombinant strain uses *hsp60*<sub>pr</sub> driven Fluc expression to screen a wide range of compounds which are further evaluated by 43<sub>pr</sub> driven Rluc expression for their inhibitory activity. We noticed a parallel decline in the viability of cells as demonstrated by both luciferase enzymes in response to all those compounds which are working through other than FAS-II pathway (Fig. 5). This allowed a dual evaluation of the inhibitory activities of compounds through two independent mycobacterial promoters and is of definite advantage over earlier described single recombinant strains (4, 5, 6, 7). Moreover, 43<sub>pr</sub> respond to a particular class of compounds inhibiting FAS-II pathway by upregulating the Rluc expression in a single luciferase assay. In present study all our observations were recorded after twelve hours of antibiotics treatment, which ensured the sufficient decline in the luminescence level in response to compounds which are either acting through other than the selected pathway or known to elicit a delayed transcriptional response. Both Fluc and Rluc expressions showed nearly a similar decline in the viability of cells treated with different drugs and more cells died at higher dose of drugs (Fig. 5). Differences in the level of Fluc and Rluc luminescence were noticed because of the strength and response of the 43<sub>pr</sub> and *hsp60*<sub>pr</sub> promoters (Fig. 5). The double recombinant strain carried the *hsp60*<sub>pr</sub>-Fluc construct in pMV306 integrative vector which rendered stable Fluc expression upon integration in the genome. Conversely, 43<sub>pr</sub>-Rluc construct is present in pMV206 extrachromosomal vector, and, therefore, required to be maintained throughout the growth. It is possible that the reduced Rluc expression appeared not due to inhibition of bacterial growth in response to antibiotics treatment but because
of loss of plasmids from a large number of bacterial cells in growing culture. We always grew the bacterial cultures in the presence of kanamycin to ensure that the extrachromosomal plasmids carrying the reporter fusion constructs were maintained throughout the studies.

Susceptibility testing and drug screening based on bioluminescent *M. tuberculosis* strain have been previously described (5, 6), but this requires a biosafety level 3 facility in order to minimize the hazards of dealing with *M. tuberculosis*. As the aim was to create a rapid and hassle-free screen system, we used *M. bovis* BCG, which is likely to be more predictive of anti-*M. tuberculosis* activity than rapidly growing mycobacteria used in other studies (7). The double recombinant *M. bovis* BCG strain expressing two co-assayable luciferase enzymes requires less stringent laboratory practices and facilities. To the best of our knowledge, this is the first demonstration of dual mycobacterial promoters being simultaneously screened in a single screen system. A major advantage of this system is that it allows parallel screening of primary as well as rationally designed antimycobacterial compounds affecting FAS-II elongation pathway. Most anti-tuberculosis whole cell-based drug screening programmes start with a quick initial screening at one fixed concentration, subsequent MIC determination for the best hits, followed by antimycobacterial activity testing in macrophages for those compounds that have been shown to lack eukaryotic cytotoxicity and, finally, in vivo testing in animal models (5, 24, 25). In recent years, the bioluminescent strains have been developed for rapid measurement of antitubercular drug activity in macrophages (5, 26), and for real-time, non-invasive assessment of drug and vaccine efficacy using in vivo imaging of mycobacterial infection (27, 28). These strains mostly used Lux (28) and Fluc (27) as bioluminescent reporters for in vivo imaging. While the former yields autoluminescent mycobacteria, the latter requires the administration of the substrate luciferin. It would be of interest to apply this concept of dual reporter assay in such application. The adaptation of the double recombinant strains for ex vivo studies and in vivo imaging requires a detailed analysis of the activities of pathway-specific promoters upon infection in macrophages and under in vivo conditions.
Earlier, we had used this *M. tuberculosis* kas operon promoter to generate a recombinant *M. aurum* strain (11) which was subsequently utilized for screening antmycobacterial compounds against FAS-II pathway (29, 30). In a separate study (31), the primary recombinant strain *M. bovis* BCG: *hsp60pr-Fluc* was used to test the efficacy of a Beta casein fragment peptide on the clearance of *M. bovis* BCG from THP-1 cells. Based on RLU data we demonstrated that treatment with this peptide enhanced the clearance of rBCG over untreated control cell in a dose dependent manner (31).

In conclusion, we have generated a double recombinant *M. bovis* BCG strain expressing firefly and *Renilla* luciferase, two co-assayable reporter enzymes, under the control of a constitutive and an inducible mycobacterial promoter, respectively. The recombinant strain serves as an assay system and allows parallel screening of primary as well as rationally designed antmycobacterial compounds affecting FAS-II elongation pathway. Moreover, the primary recombinant strain carrying the *hsp60pr-Fluc*, integrated in the genome, can be effectively utilized to create a panel of double recombinant strains wherein different pathway specific promoters combined with Rluc reporter could be introduced through an extrachromosomal plasmid vector. However, one possible limitation of this screen system could be the genetic differences between *M. bovis* BCG and *M. tuberculosis* which may require that all potentially active test compounds be evaluated for activity against *M. tuberculosis* for further evaluation.

Initial screening for activity does not require an accurate MIC, and only a few concentrations of each compound are tested in the preliminary screening. The availability of a rapid assay will allow large-scale screening and will encourage the search for new classes of agents urgently needed to control this disease. With the simplicity of luciferase enzyme assay the system can be easily adapted in high-throughput mode.

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References:


Table 1: Strains, plasmids and primers used in this study.

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<th>Primers, plasmids and strains</th>
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<td>Plasmid vector with Rluc ORF, amp'</td>
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ampr, ampicillin resistant; hygr, hygromycin resistant; kmr, kanamycin resistant
Legends to Figures:

Fig. 1. Bioluminescence (RLU) was measured in rBCG strains expressing Fluc under the control of different mycobacterial promoters. Cultures were grown in 7H9 broth supplemented with hygromycin (50 µg/ml), and nearly equal number of bacilli (10^7-8) from different stages of the growth were taken for RLU assay. rBCG carrying hsp60pr-Fluc showed maximum luciferase activity. The values and error bars represent the mean and standard deviations from three independent experiments.

Fig. 2. Antibiotic response was monitored using bioluminescent rBCG strains. Early log phase cultures of the recombinant strains were treated with different antibiotics and RLU was determined, as described in the text, after twelve hours of the treatment. Decline in RLU was noticed widely in response to all the antibiotics tested in all three recombinant strains.

Fig. 3. Real Time RT-PCR analysis was performed using RNA samples from M. bovis BCG: hsp60pr-Fluc log phase culture treated with antibiotics. The expressions of hsp60 and Fluc were derived from native and ectopic hsp60 promoters, respectively. Note, nearly a similar decline in the expression of hsp60 and Fluc in the treated samples. Expression levels of both genes were normalized with sigA.

Fig. 4. Susceptibility of the rBCG strain (M. bovis BCG: hsp60pr-Fluc) to different antibiotics was analyzed by CFU (-■-) method and the decline in the RLU (-▲--) level. Percent inhibition was calculated, as described in the text, in reference to the untreated sample at the same time point. Similar growth inhibition profile was noticed at three different concentrations of antibiotics using both approaches. The values and error bars represent the mean and standard deviations from three independent experiments.

Fig. 5. Dual luciferase assay was performed using double rBCG strain (M. bovis BCG: hsp60pr-Fluc: 43pr-Rluc). Firefly and Renilla luciferase activities were assayed together in treated samples and fold change in the RLU was determined with respect to basal level expression in untreated control. Note the reduced Fluc level (■) in response to all the treated drugs, while enhanced Rluc level (▲) is seen in response to isoniazid, ethionamide and thiolactomycin, known FAS-II pathway inhibitors. In response to other drugs Rluc too showed diminished activity like Fluc. Data shown are the mean and standard deviation from three independent experiments.