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Double Recombinant *Mycobacterium bovis* BCG Strain for Screening of Primary and Rationale-Based Antimycobacterial Compounds

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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 screening 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, coincides 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 with a dual-luciferase reporter assay which can be easily adapted in high-throughput mode.

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 to identify new antimycobacterial compounds against novel targets and new series of compounds against known targets are ongoing (1). This requires screening of a large number of compounds using high-throughput screening systems. Conventional screening involves whole-cell-based screening against growing bacterial cells where cell mortality is scored upon treatment with various concentrations of the compounds to be tested (2). This approach is the most widely used, as it allows testing of 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 either remained unknown or were identified after several rounds of experimentation. Alternatively, a target-based approach is used to identify inhibitors against a specific biochemical reaction or some important intermolecular interaction (3). This is a straightforward approach with a high level of sensitivity and is technologically more adaptive from a drug development perspective. Being directed mostly toward 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 drug parameters.

In recent years, 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 lead molecules 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 has an 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 that are 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

Received 20 June 2013 **Returned for modification** 18 September 2013
Accepted 8 December 2013
Published ahead of print 16 December 2013
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This article is communication 8578 of CDRI.
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doi:10.1128/AAC.01301-13

expression and analysis of two individual reporter enzymes within a single system. This eliminates the experimental variability, such as differences in treatment conditions, concentrations of drugs, and cell lysis efficiency and 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 with Promega's dual-luciferase reporter assay. The expression profile of the firefly luciferase (Fluc) gene, 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 mechanisms 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 *Mycobacterium tuberculosis* but relative lack of pathogenicity (4, 6). We describe the generation and evaluation of the double recombinant strain's response against existing antitubercular drugs and thereby establish 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*-mycobacterium shuttle plasmid vectors pMV206 and PMV306 were kind gifts from William R. Jacobs, Einstein College of Medicine, New York, NY, USA. *E. coli* cultures were grown in LB medium with addition of ampicillin (100 μg/ml), kanamycin (25 μg/ml), and hygromycin (100 μg/ml) as required. *M. bovis* BCG cultures were grown in Middlebrook 7H9 medium supplemented with 0.4% glycerol and 0.05% Tween 80 without or with antibiotics as per requirements (hygromycin, 50 μg/ml, and kanamycin, 25 μg/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 MIC. MIC was determined as described earlier (11). Briefly, *M. bovis* BCG cultures grown to an optical density at 600 nm (OD_{600}) of 0.6 were diluted to an OD_{600} of 0.05 with fresh 7H9 medium, and ~10^6 to 10^7 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. The total number of colonies that appeared in the untreated control tube was considered 100%, and the MIC was calculated as the concentration of drug at which >90% growth inhibition was observed. Under these conditions, the MICs obtained were 0.50 μg/ml for ethambutol, 1 μg/ml for streptomycin, 1.37 μg/ml for isoniazid (INH), 1 μg/ml for rifampin, 40 μg/ml for vancomycin, 12.5 μg/ml for polymyxin B sulfate, 50 μg/ml for ethionamide, 35 μg/ml for thiolactomycin, 4 μg/ml for cerulenin, and 10 μg/ml for triclosan. Wild-type *M. bovis* BCG and recombinant *M. bovis* BCG (rBCG) strains showed similar MICs for the tested drugs.

PCR amplifications, cloning, and construction of recombinant *M. bovis* BCG strains. *M. tuberculosis hsp60p* (Rv0440), *sigAp* (Rv2703), *16S rRNAp* (MTB000019) (12, 13, 14), and *kas* operon (Rv2243 to -47) *43p* (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 the pGL3-Basic and pFN11A vectors, respectively, and cloned into the pTZ57R/T vector. All plasmid constructs were validated by restriction digestion and DNA sequencing. DNA sequencing was performed using an ABI Prism BigDye Terminator cycle sequencing kit as per the manufacturer's protocol on an ABI 310 DNA sequencer. Firefly and *Renilla* luciferase genes were relocated to pMV306 and pMV206 vectors, respectively, at XbaI/EcoRI sites. Different promoters, i.e., *hsp60p*, *sigAp*, *16S rRNAp*, and *43p*, were subsequently subcloned into the pMV306 or pMV206 vector 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 *hsp60p*, *sigAp*, *16S rRNAp*, and Fluc genes were electroporated into *M. bovis* BCG using standard protocols. Cells were recovered in 7H9 medium for 16 h at 37°C and then plated on 7H10 with hygromycin to select the transformants. The primary rBCG strain was then transformed with the pMV206 vector carrying *kas* operon *43p* in fusion with the *Renilla* luciferase gene to obtain the double recombinant BCG strain. Recombinants were grown in 7H9 broth supple-

TABLE 1 Strains, plasmids, and primers used in this study

Primer, plasmid, or strain	Relevant details[a]	Source
Primers		
43pr1	5′-TAGCGGCCGCACTGAAGCAT-3′	This study
43pr2	5′-GACTCTAGACGTGTCTAAGAG-3′	This study
sigA1	5′-GGTACCGTCGCAGATACGACGCAC-3′	This study
sigA2	5′-TCTAGAATACACCCCTTCGGTCGT-3′	This study
16s1	5′-GGTACCTCGGTGCCGAGATCGAAC-3′	This study
16s2	5′-TCTAGACCCAAACACTCCCTTTGG-3′	This study
60P1	5′-TAGCGGCCGCTAGAGGTGAC-3′	This study
60P2	5′-GCATCTAGATGCGAAGTGATTC-3′	This study
Fluc1	5′-TCTAGATGGAAGACGCCAAAAACAT-3′	This study
Fluc2	5′-GGAATTCTTACACGGCGATCTTTC-3′	This study
Rluc1	5′-TCTAGAATGACTTCGAAAG-3′	This study
Rluc2	5′-GAATTCTTATTGTTCATTTTTGAG-3′	This study
sigART1	5′-GTGGCAGCGACCAAAGCAAG-3′	This study
sigART2	5′-CACTAGCGGACTTCGCCGCT-3′	This study
60RT1	5′-CCAAGACAATTGCGTACGACGA-3′	This study
60RT2	5′-CCACCCCACTTCTTTTCCAGGA-3′	This study
16sRT1	5′-CCTGGCTCAGGACGAACGCT-3′	This study
16sRT2	5′-TCCCGAAGTGCAGGGCAGAT-3′	This study
FlucRT1	5′-CCATTCTATCCGCCTGGAAG-3′	This study
FlucRT2	5′-CGTAAGTGATGTCCACCTCG-3′	This study
Plasmids		
pGL3-Basic	Plasmid vector with *Fluc* ORF, Amp^r	Promega
pFN11A	Plasmid vector with *Rluc* ORF, Amp^r	Promega
pTZ*hsp60p*	Plasmid vector carrying *hsp60p*, Amp^r	This study
pTZ*sigAp*	Plasmid vector carrying *sigAp*, Amp^r	This study
pTZ*16Sp*	Plasmid vector carrying *16S rRNAp*, Amp^r	This study
pTZFluc	Plasmid vector carrying *Fluc* ORF, Amp^r	This study
pTZRluc	Plasmid vector carrying *Rluc* ORF, Amp^r	This study
pTZ*43p*	Plasmid vector carrying *43p*, Amp^r	This study
306h:*Fluc*	Integrative vector carrying *Fluc* ORF, Hyg^r	This study
306h:$hsp60_{pr}$-*Fluc*	Integrative vector carrying *hsp60p* and *Fluc* ORF, Hyg^r	This study
306h:$sigA_{pr}$-*Fluc*	Integrative vector carrying *sigAp* and *Fluc* ORF, Hyg^r	This study
306h:*16S p-Fluc*	Integrative vector carrying *16S rRNAp* and *Fluc* ORF, Hyg^r	This study
206:*43p-Rluc*	Extrachromosomal plasmid vector carrying *43p* and *Rluc* ORF	This study
Bacterial strains		
NEB10	Δ(*lacZ*)*M15 mcrA galU recA1 endA1 nupG rpsL* (Str^r)	NEB
M. bovis BCG	Pasteur strain	Lab stock
BCG *hsp60p-Fluc*	*M. bovis* BCG carrying 306h:*hsp60p-Fluc*, Hyg^r	This study
BCG *sigAp-Fluc*	*M. bovis* BCG carrying 306h:*sigAp-Fluc*, Hyg^r	This study
BCG *16S rRNAp-Fluc*	*M. bovis* BCG carrying 306h:*16S-p-Fluc*, Hyg^r	This study
BCG *hsp60p-Fluc 43p-Rluc*	*M. bovis* BCG carrying 306h:*hsp60p-Fluc*:206-43p-*Rluc*, Hyg^r Km^r	This study

[a] Amp^r, ampicillin resistant; Hyg^r, hygromycin resistant; Km^r, kanamycin resistant.

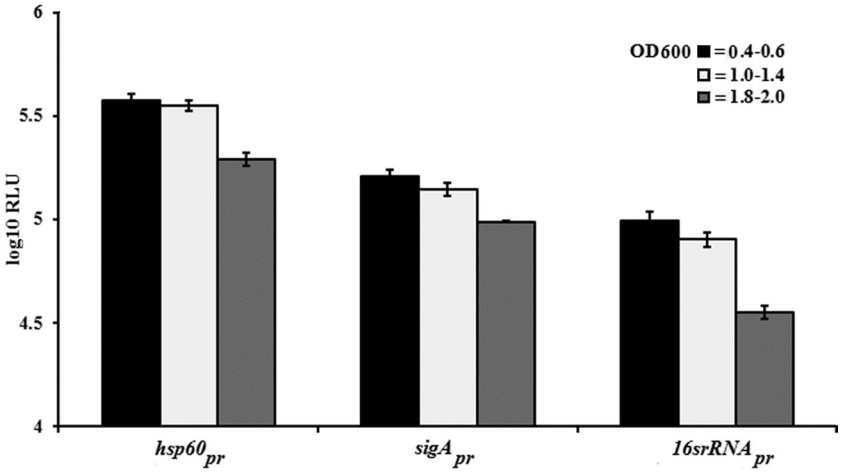

FIG 1 Bioluminescence (RLU) was measured in rBCG strains expressing Fluc under the control of different mycobacterial promoters. Cultures were grown in 7H9 broth supplemented with hygromycin (50 μg/ml), and nearly equal numbers of bacilli (10^7 to 10^8) from different stages of growth were taken for RLU assay. rBCG carrying *hsp60p-Fluc* showed maximum luciferase activity. The values represent the means and standard deviations from three independent experiments.

mented with hygromycin and kanamycin at 37°C to an OD_{600} of 0.5, and cultures were diluted to an OD_{600} of 0.04 to 0.05 with fresh medium with kanamycin and allowed to grow for 4 h at 37°C. Various drugs were subsequently added at different concentrations, i.e., ethambutol (0.25 μg/ml, 0.50 μg/ml, and 1.0 μg/ml), streptomycin (0.50 μg/ml, 1.0 μg/ml, and 2.0 μg/ml), INH (0.55 μg/ml, 1.37 μg/ml, and 2.74 μg/ml), rifampin (0.50, 1.0 μg/ml, and 2.0 μg/ml), vancomycin (14.99 μg/ml, 40 μg/ml, and 80 μg/ml), polymyxin B (5.56 μg/ml, 12.5 μg/ml, and 25 μg/ml), ethionamide (2 μg/ml, 50 μg/ml, and 100 μg/ml), thiolactomycin (20 μg/ml, 35 μg/ml, and 70 μg/ml), cerulenin (3 μg/ml, 4 μg/ml, and 8 μg/ml), and triclosan (5 μg/ml, 10 μg/ml, and 20 μg/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, 80 μl of culture containing 10^7 to 10^8 bacterial cells was taken and mixed with 20 μl passive lysis buffer (PLB). After 10 min of incubation on ice, the suspension was sonicated (Vibracell VCX-750; Ultrasonics, USA) briefly to lyse the cells. Thereafter, 100 μl LAR-II containing luciferase substrate and buffer were added, and firefly luciferase activity was measured using a Berthold luminometer (Germany). Then, 100 μl 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 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 reverse transcription-PCR (RT-PCR) analyses of *M. bovis* BCG cultures after antibiotic treatment. A log-phase *M. bovis* BCG *hsp60p-Fluc* culture was split into 5-ml aliquots and treated with specified concentrations of antibiotics (see Fig. 3) for 4 h at 37°C. Posttreatment 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 the 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 controls in all the experiments. Expression of target genes was 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 values and standard deviations were plotted for each set of data.

RESULTS AND DISCUSSION

Selection of mycobacterial promoters and generation of an *M. bovis* BCG primary recombinant strain. Promoters are the main regulatory components of the gene expression which has been correlated with the growth and physiology of the bacteria (12). They impart constitutive expression of genes throughout growth and selectively induce or repress the expression of genes temporally, spatially, and under 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, *hsp60p*, *sigAp*, and *16S rRNAp*, were selected owing to their earlier description as constitutive promoters (12, 13, 14, 17). These promoters were amplified and cloned upstream of the firefly luciferase open reading frame (ORF), and the recombinant constructs were transferred to the *M. bovis* BCG genome via an integrative mycobacterial vector. This allowed stable expression of the Fluc gene 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 *hsp60p* rendered luciferase activity much stronger than those with *sigAp* and *16S rRNAp*. The luciferase activity was appreciably reduced during the stationary phase in all three strains. This could be due to either the reduced activity of the promoters or 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, *hsp60p*, $_{myc}$*tetOp*, and *G13p*, with three different luminescence reporters, firefly luciferase (Fluc), *Gaussia* luciferase (Gluc), and bacterial luciferase Lux, in *M. tuberculosis* and *Mycobacterium smegmatis* and found that the highest luminescence was obtained using *hsp60p* in both

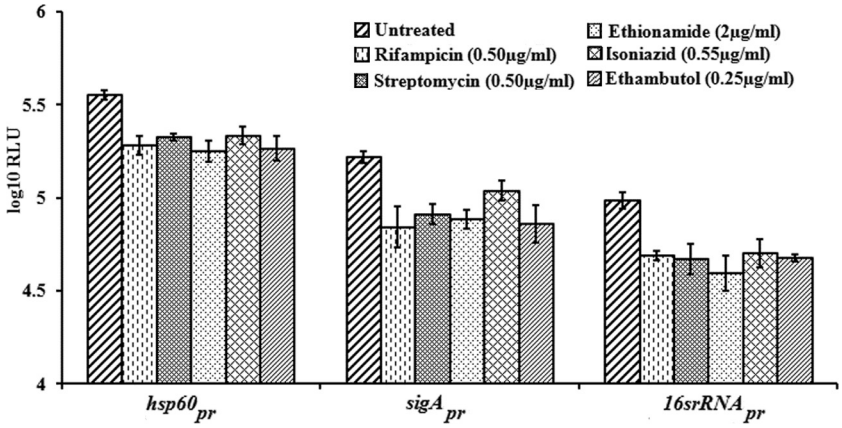

FIG 2 Antibiotic response monitored using bioluminescent rBCG strains. Early-log-phase cultures of the recombinant strains were treated with different antibiotics, and RLU were determined, as described in the text, after 12 h of the treatment. A decline in RLU was seen widely in response to all the antibiotics tested in all three recombinant strains.

species. It may be noted that while *hsp60p* was derived from *M. bovis* BCG (19), $_{myc}$*tetOp* and *G13p* were isolated from *M. smegmatis* (20) and *Mycobacterium marinum* (21), respectively. Both these promoters were reported to be stronger than *hsp60p*, but it was observed that *hsp60p* drove the highest reporter gene expression. In present study, we analyzed three *M. tuberculosis* promoters (*hsp60p*, *sigAp*, and *16S rRNAp*) which shared nearly complete homology with those of the surrogate mycobacterial strain, *M. bovis* BCG. The results obtained are similar to those previously described (18), as *hsp60p* rendered the maximum expression (Fig. 1).

Antibiotic responses of the promoters. The antibiotic responses of the recombinant strains were analyzed upon treatment with a range of antibiotics having different mechanisms of actions, and the luminescence was measured as a function of gene expression. Decline in the bacterial growth upon antibiotic treatment affects the ongoing gene expression, which in turn reduces the total luminescence in the treated sample compared to the untreated bacterial cells. All three rBCG strains showed reductions in luminescence upon antibiotic treatment (Fig. 2), but the decline was more pronounced when the reporter was under *hsp60p* transcriptional control. The higher level of reporter gene expression in the *M. bovis* BCG *hsp60p-Fluc* strain allows the screening of antimycobacterial compounds using lower number of bacterial cells, as they produce markedly higher luminescence than rBCG strains carrying *sigAp* and *16S rRNAp*. Thus, further studies were performed using this as a primary recombinant strain. The *hsp60p-Fluc* construct was integrated in single copy at an ectopic site on the *M. bovis* BCG genome. To ensure that *hsp60p* rendered similar levels of expression from its native and ectopic sites, we performed real-time quantitative RT-PCR of the *hsp60* and *Fluc* genes using RNA samples from the *M. bovis* BCG *hsp60p-Fluc* strain upon treatment with different antimycobacterial drugs. A similar decline in the expression of *hsp60* and *Fluc* was noticed upon treatment with different antibiotics, suggesting that the levels of expression derived from the native and the ectopic *hsp60* promoters are similar (Fig. 3). This also ensured that the total luciferase activity is indeed derived from the *hsp60p*-driven Fluc expression.

Correlation between luminescence and growth inhibition of the recombinant *M. bovis* BCG *hsp60p-Fluc* strain. Conventional antimicrobial screening involves counting of bacterial colonies from dilution plating after antibiotic treatment (22). This takes several days in the case of mycobacteria, even with fast-growing mycobacterial species. The recombinant strain expressing luciferase constitutively allows fast and real-time analysis of diminishing bacterial growth upon antibiotic treatment (5, 6, 7). We noticed an apparent decline in the luminescence after 4 h of antibiotic treatments, and it gradually increased as the treatment continued. The time required for an appreciable decline may vary because of the drug's mode of action. Drugs, such as rifampin 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

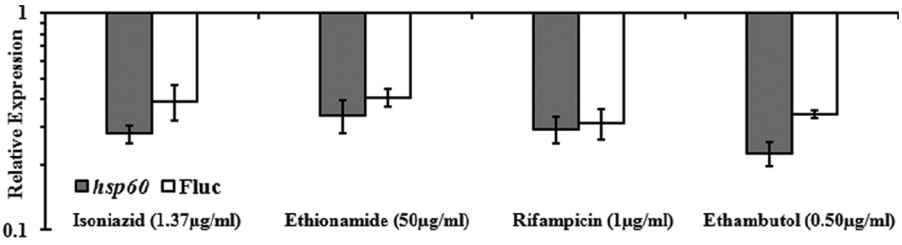

FIG 3 Real-time RT-PCR analysis performed using RNA samples from *M. bovis* BCG *hsp60p-Fluc* log-phase culture treated with antibiotics. The expression of *hsp60* and *Fluc* was derived from native and ectopic *hsp60* promoters, respectively. Note that there was a similar decline in the expression of *hsp60* and *Fluc* in the treated samples. The expression levels of both genes were normalized with *sigA*.

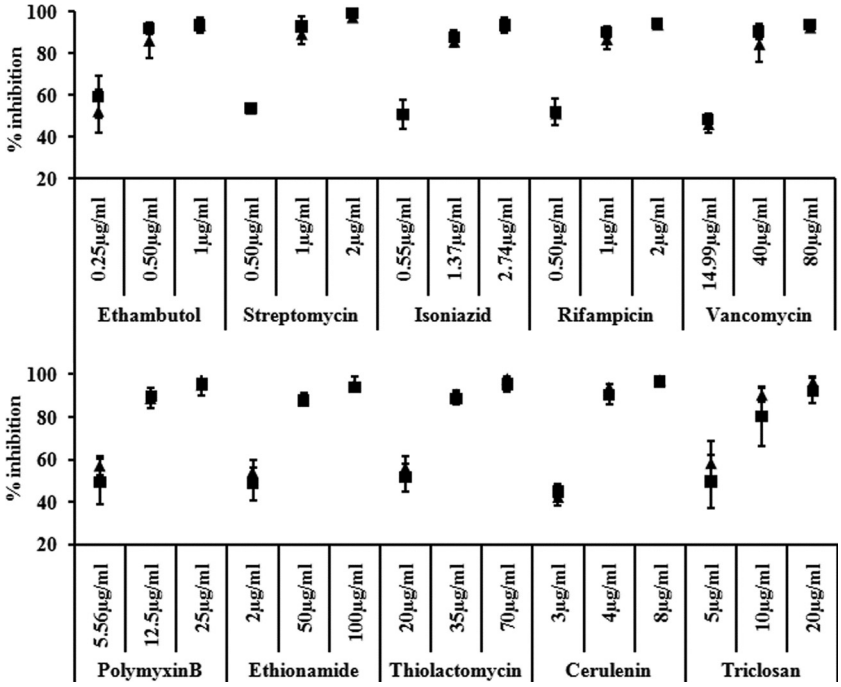

FIG 4 Susceptibility of the rBCG strain (*M. bovis* BCG *hsp60p-Fluc*) to different antibiotics was analyzed by the CFU (■) method and the decline in RLU (▲). Percent inhibition was calculated, as described in the text, in reference to the untreated sample at the same time point. Similar growth inhibition profiles were seen at three different concentrations of antibiotics using both approaches. The values represent the means and standard deviations from three independent experiments.

cell wall synthesis, may require longer incubation before loss of luciferase activity is detectable. The relative light unit (RLU) assay was performed after 12 h of antibiotic treatment, and at this point the decline in bacterial growth of the recombinant strain, measured in CFU, corresponded well with the 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 [50% inhibitory concentration (IC_{50}), MIC($IC_{>90}$), and 2× MIC]. For experimental analysis, $>10^6$ to 10^7 bacterial cells were taken per sample, which gave slightly higher MIC values but consistently ensured minimal error in RLU measurement. Similar inhibition profiles were obtained through both approaches (Fig. 4), which established the usefulness of the *M. bovis* BCG *hsp60p-Fluc* strain for the screening of a wide range of antimycobacterial compounds in a fast and efficient manner.

Double recombinant *M. bovis* BCG strain and screening of primary and rationale-based antimycobacterial compounds. The *M. bovis* BCG *hsp60p-Fluc* strain contained the *hsp60p-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 it therefore allowed the screening of compounds inhibiting mycobacterial growth irrespective of their mode of action. 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 Fluc expression and its assay and is introduced into the *M. bovis* BCG *hsp60p-Fluc* strain, the newly generated double recombinant strain would allow the screening of both primary and rationally designed pathway-specific inhibitors. To enable this, we selected the *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 *kas* operon genes is highly conserved in mycobacteria (11), and its upstream regulatory regions are almost identical in *M. bovis* BCG and *M. tuberculosis*. The *kas* operon promoter was combined with the *Renilla* luciferase gene, and the recombinant construct was transferred to the *M. bovis* BCG *hsp60p-Fluc* strain via an extrachromosomal mycobacterial plasmid vector to generate *M. bovis* BCG *hsp60p-Fluc 43p-Rluc*, a double recombinant strain. The double recombinant strain produces Fluc through *hsp60p*, while Rluc expression is rendered by *43p* (the *kas* operon promoter). The dissimilar enzyme structures and substrate requirements of the 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 were accomplished by adding Stop and Glo reagent (Promega) to the sample tube immediately after quantitation of the firefly luciferase reaction. The assay system was optimized so that both reporters yield linear assays with utmost sensitivities and no endogenous activity of either reporter in the host cells. Further, we performed only the firefly luciferase assay and measured total RLU derived from Fluc activity, taking an equal number of bacilli from single and double

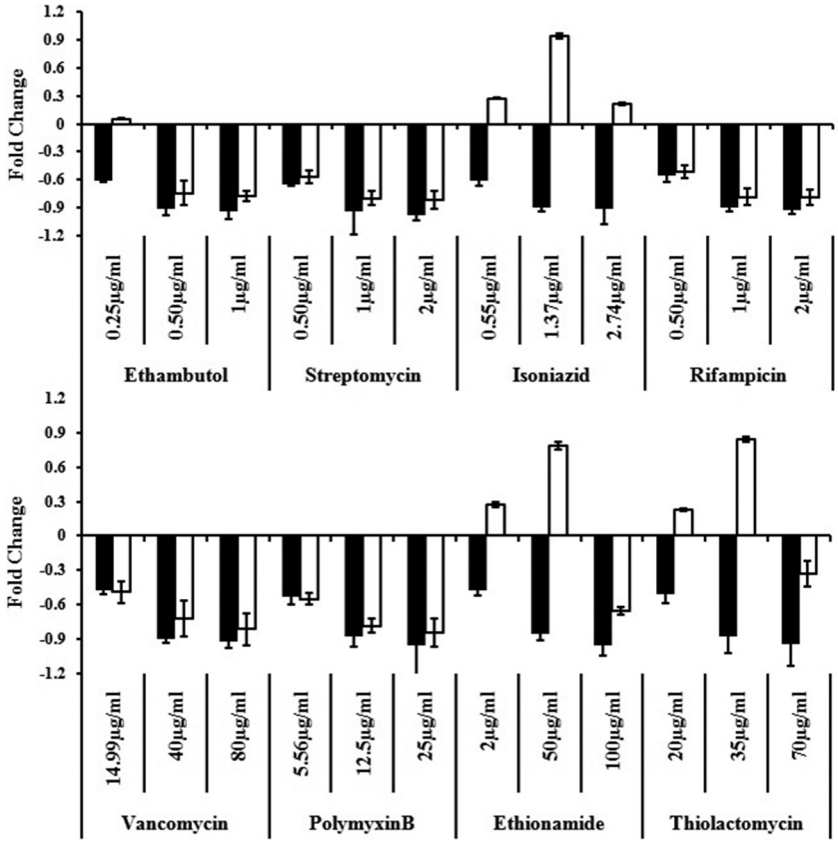

FIG 5 The dual-luciferase assay was performed using the double rBCG strain (*M. bovis* BCG *hsp60p-Fluc 43p-Rluc*). Firefly and *Renilla* luciferase activities were assayed together in treated samples, and the fold change in RLU was determined with respect to basal-level expression in the untreated control. Note the reduced Fluc level (■) in response to all the drug treatments, while an enhanced Rluc level (□) is seen in response to isoniazid, ethionamide, and thiolactomycin, which are known FAS-II pathway inhibitors. In response to other drugs, Rluc showed diminished activity like Fluc. Data the means and standard deviations from three independent experiments.

rBCG cultures. Nearly the same RLU level was obtained in samples from both recombinant strains (data not shown), which ensured that these two enzymes are distinctly dissociated and that there is no cross-reaction between firefly and *Renilla* luciferases in our assay. We performed the dual-luciferase assay using this strain after treatment with different antimycobacterial drugs. Reduced Fluc expression was obtained in the entire treated samples (Fig. 4 and 5) as this expression was rendered by *hsp60p*, while Rluc expression was diminished only in samples treated with ethambutol, streptomycin, rifampin, vancomycin, and polymyxin B, which act through pathways other than FAS-II. Rluc expression derived through *43p*, which is selectively induced after treatment with FAS-II pathway inhibitors, was induced only in cells (Fig. 5) treated with isoniazid, ethionamide, and thiolactomycin (known FAS-II inhibitors). The induction was apparent as early as 3 h after drug treatment and showed a gradual increase up to 24 h (data not shown). The induction was noticeable at nearly the IC_{50} and increased to maximum at the MIC of the drug, but it did not increase proportionately at concentrations that far exceeded the MIC value. At drug concentrations exceeding the MIC, 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 compared with the enhanced expression obtained at the MIC level, which could be due to the death of more cells at drug doses exceeding the MIC (Fig. 4 and 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 *hsp60p* and, based on luciferase profiles, showed growth inhibition and MICs parallel to those found by the conventional CFU-based method. The method with the double recombinant strain uses *hsp60p*-driven Fluc expression to screen a wide range of compounds, which are further evaluated by *43p*-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 work through other than the 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, *43p* responds to a particular class of compounds inhibiting FAS-II pathway by upregulating the Rluc expression in a single luciferase assay. In the present study, all our observations were recorded after 12 h of antibiotic treatment, which ensured a sufficient decline in the luminescence level in response to compounds which either act through other than the selected pathway or are known to elicit a delayed tran-

scriptional response. Both Fluc and Rluc expression showed a nearly similar decline in the viability of cells treated with different drugs, and more cells died at higher doses of drugs (Fig. 5). Differences in the levels of Fluc and Rluc luminescence were noticed because of the strength and response of the *43p* and *hsp60p* promoters (Fig. 5). The double recombinant strain carried the *hsp60p-Fluc* construct in the pMV306 integrative vector, which rendered stable Fluc expression upon integration in the genome. Conversely, the *43p-Rluc* construct is present in the pMV206 extrachromosomal vector and therefore is required to be maintained throughout growth. It is possible that the reduced Rluc expression was not due to inhibition of bacterial growth in response to antibiotic treatment but was because of loss of plasmids from a large number of bacterial cells in the 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* strains 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 convenient screening system, we used *M. bovis* BCG, which is likely to be more predictive of anti-*M. tuberculosis* activity than the rapidly growing mycobacteria used in other studies (7). The double recombinant *M. bovis* BCG strain expressing two coassayable 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 screening system. A major advantage of this system is that it allows parallel screening of primary as well as rationally designed antimycobacterial compounds affecting the FAS-II elongation pathway. Most antituberculosis whole-cell-based drug screening programs start with a quick initial screening at one fixed concentration, subsequent MIC determination for the best hits, then 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, bioluminescent strains have been developed for rapid measurement of antitubercular drug activity in macrophages (5, 26) and for real-time, noninvasive assessment of drug and vaccine efficacy using *in vivo* imaging of mycobacterial infection (27, 28). These strains used mostly 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 use this concept of dual-reporter assay in such an 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 antimycobacterial compounds against the FAS-II pathway (29, 30). In a separate study (31), the primary recombinant strain *M. bovis* BCG *hsp60p-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 that from untreated control cells in a dose-dependent manner (31).

In conclusion, we have generated a double recombinant *M. bovis* BCG strain expressing firefly and *Renilla* luciferases, two coassayable 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 antimycobacterial compounds affecting the FAS-II elongation pathway. Moreover, the primary recombinant strain carrying *hsp60p-Fluc*, integrated in the genome, can be effectively utilized to create a panel of double recombinant strains where different pathway-specific promoters combined with the Rluc reporter could be introduced through an extrachromosomal plasmid vector. However, one possible limitation of this screening 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 the luciferase enzyme assay, the system can be easily adapted in high-throughput mode.

ACKNOWLEDGMENTS

This work was supported by CSIR network project BSC0103. Vandana Singh is a Senior Project Fellow in this network project, and Rajesh Kumar Biswas was the recipient of a CSIR Senior Research Fellowship.

Statistical analysis was performed with the help of Mukesh Srivastava from the Statistics Department of CDRI. We are thankful to Riccardo Manganelli for critical reading of the manuscript and valuable suggestions.

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