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Bioluminescence for Assessing Drug Potency against Nonreplicating *Mycobacterium tuberculosis*

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Targeting dormant *Mycobacterium tuberculosis* represents a challenge to antituberculosis drug discovery programs. We previously reported and validated the use of the streptomycin (STR)-dependent *M. tuberculosis* 18b strain as a tool for assessing drug potency against nonreplicating bacteria both *in vitro* and *in vivo*. In this study, we generated a luminescent 18b strain, named 18b-Lux, by transforming the bacteria with a vector expressing the *luxCDABE* operon from *Photorhabdus luminescens*. Luciferase expression was demonstrated under replicating conditions, and, more importantly, luminescence levels significantly above background were detected following STR removal. The sensitivity of STR-starved 18b-Lux to approved and candidate antituberculosis therapeutic agents was evaluated by means of a luciferase assay in a 96-well format. Results mirrored the data obtained with the standard resazurin reduction microplate assay, and the luminescence readout allowed time course assessments of drug efficacy *in vitro*. Specifically, we proved that bedaquiline, the rifamycins, and sutezolid displayed time-dependent activity against dormant bacteria, while pyrazinamide and SQ109 showed bactericidal effects at the highest concentrations tested. Overall, we established the optimal conditions for an inexpensive, simple, and very sensitive assay with great potential for future applications.

Tuberculosis (TB) represents one of the most important and life-threatening infectious diseases caused by a single etiological agent, *Mycobacterium tuberculosis*. The World Health Organization (WHO) estimated that approximately one-third of the world's population is latently infected with the pathogen, and 1.5 million people died from TB in 2013 (1). The current directly observed therapy, short-course (DOTS), approach includes a four-drug combination regimen (isoniazid [INH], rifampin [RIF], ethambutol [EMB], and pyrazinamide [PZA]) for 2 months, followed by RIF and INH for 4 months. The lengthy therapy and frequent adverse side effects have resulted in poor compliance and the appearance of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains, which have worsened the problem on a global scale. In addition, *M. tuberculosis* can enter a dormant or latent state characterized by reduced growth rate, limited metabolic activity, and phenotypic resistance to most currently available compounds (2, 3), thus making anti-TB treatment even more challenging. Therefore, the research community has been compelled to look for new medications, novel targets, and innovative strategies to fight TB.

The streptomycin (STR)-dependent *M. tuberculosis* strain 18b has been extensively validated in our laboratory as a simple but robust model for testing drug potency against nonreplicating bacilli *in vitro* and *in vivo* (4–6). We demonstrated an absence of growth upon STR removal, an extremely stable STR-dependent phenotype, good agreement between *in vitro* and *in vivo* drug efficacy studies, consistency with previously developed models of nonreplicating persistence, and, finally, suitability for high-throughput screening (HTS).

By using STR-starved 18b (SS18b), we confirmed the activity of several candidate compounds that are currently in development and have been demonstrated to target various essential biological processes, such as DNA, RNA, protein, and ATP synthesis. Moxifloxacin, rifapentine (RFP), pretomanid (PA-824), clofazimine, sutezolid (STZ) (PNU-100480), and bedaquiline (BDQ) (TMC207) were found to be bactericidal against SS18b under nonreplicating conditions, both *in vitro* and *in vivo* (4–6), which supports further evaluation of these candidates for inclusion in novel experimental regimens.

One of the main advantages associated with SS18b resides in the ability to conduct HTS studies in a straightforward manner accessible to virtually all laboratories. We have exploited the resazurin reduction microplate assay (REMA) as an approach that allows rapid evaluation of drug activity through measurement of the reduction of resazurin to resorufin in living bacteria. While this assay has incontrovertible advantages (such as reduced costs and widespread utilization), it represents an endpoint analysis and is not applicable to time course investigations in which the same plates are monitored periodically.

In the past, several reports described the use of luminescent reporters as a means to detect bacterial viability upon drug treatment (7–17). Most of those studies employed firefly luciferase, which requires an exogenously added substrate to detect luminescent bacteria. This issue can be bypassed by using bacterial luciferase, since all of the components are encoded by the *luxCDABE*

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operon and the nucleotide cofactors are endogenously available. The bacterial luciferase system was exploited for *in vitro* drug efficacy studies and for *in vivo* evaluations of vaccine efficacy (7, 8, 17, 18); however, application of the luciferase assay to nonreplicating bacteria has not been reported to date.

In order to provide a tool suitable for time course studies, we present here the development of a bacterial luciferase-based assay using SS18b. We exploited a synthetic *luxCDABE* operon (18) encoding luciferase and all of the components required for the enzymatic reaction, which allows both dose- and time-dependent experiments. This assay compares favorably with and is technically superior to the well-established REMA for assessment of drug activity in nonmultiplying bacteria.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Mycobacterium tuberculosis* strains 18b and 18b-Lux were grown at 37°C, with shaking, in 7H9 broth (Difco) supplemented with 10% albumin-dextrose-catalase (ADC), 0.2% glycerol, 0.05% Tween 80, and 50 μg/ml STR or on solid Middlebrook 7H10 agar (Difco) supplemented with 0.5% glycerol, 10% oleic acid-albumin-dextrose-catalase (OADC), and 50 μg/ml STR. Alternatively, 18b-Lux was grown in 7H9 medium with acetate (0.1% final concentration) supplemented with 10% albumin-NaCl, 0.05% Tween 80, and 50 μg/ml STR. Hygromycin B (HYG) (50 μg/ml) was added to 18b-Lux cultures. Nonreplicating STR-starved 18b-Lux (SS18b-Lux) was generated as follows. 18b-Lux was grown to mid-logarithmic phase in STR- and HYG-containing medium and was washed three times in phosphate-buffered saline (PBS) containing 0.05% Tween 80 (PBST). The final bacterial pellets were resuspended in medium without STR and were frozen in 15% glycerol, in 0.5-milliliter aliquots, at −80°C. When needed, one aliquot was defrosted and inoculated in 7H9 medium without STR. SS18b-Lux cultures were maintained at an optical density at 600 nm (OD_{600}) of 0.2 to 0.5 for 2 weeks (with the addition of fresh medium if necessary), by which time they had stopped replicating.

Growth curves. In order to characterize the growth of *M. tuberculosis* 18b-Lux before and after STR removal, the strain was grown in two different media (7H9 medium with glucose and 7H9 medium with acetate), with or without 50 μg/ml STR. Cultures were diluted to an OD_{600} of ≤0.05 and incubated at 37°C, with shaking. OD_{600} values were recorded at different time points to obtain growth curves. The experiments were carried out with two biological replicates.

Antimicrobials. 4-Aminosalicylic acid (PAS), clofazimine, ethambutol (EMB), isoniazid (INH), moxifloxacin, pyrazinamide (PZA), rifampin (RIF), and streptomycin (STR) were purchased from Sigma-Aldrich and hygromycin B (HYG) from Roche. Experimental drugs were kindly provided by Sanofi-Aventis (rifapentine [RFP]), Tibotec (bedaquiline [BDQ]), V. Makarov (benzothiazinone [BTZ043]), M. Jackson (adamantyl urea [AU1235]), H. Boshoff (SQ109), and C. E. Barry III (sutezolid [STZ]). Drugs for *in vitro* experiments were either dissolved or suspended as follows: AU1235, BTZ043, clofazimine, EMB, moxifloxacin, PA-824, PAS, PZA, RIF, RFP, SQ109, STZ, and BDQ in dimethyl sulfoxide (DMSO) and INH and STR in water.

Construction of *M. tuberculosis* 18b-Lux. Preparation of competent cells and electroporation of *M. tuberculosis* 18b with pEG200 (18) were performed following standard procedures (19). pEG200 carries the *luxCDABE* operon, a cassette conferring hygromycin resistance, the L5 *int-attP* sequences, and the *Escherichia coli* origin of replication. Transformants were selected on 7H10 plates with 50 μg/ml HYG and 50 μg/ml STR. Plates were incubated at 37°C for 4 to 5 weeks. Colonies obtained after electroporation were streaked on 7H10 plates containing 50 μg/ml HYG and 50 μg/ml STR in order to confirm the resistance phenotype. Further validation was obtained by colony PCR using standard procedures, through amplification of the HYG resistance cassette. Positive clones were stored as glycerol stocks at −80°C.

Luciferase assay. 18b-Lux with STR (OD_{600} = 0.0001) or a 2-week-old SS18b-Lux culture (OD_{600} = 0.1) was used in the luminescence assay. Two-fold serial dilutions of each test compound were prepared in white 96-well plates containing the bacilli in a total volume of 100 μl and then were incubated at 37°C. Luminescence was recorded 5 min, 1 day, 4 days, and 6 days after the addition of drugs, by using a Tecan Infinite M200 microplate reader. The same plate was then used for evaluation of the potency of the compounds by means of the resazurin reduction microplate assay (REMA), as detailed below. The experiments were carried out with at least two biological replicates.

Resazurin reduction microplate assay. To determine the *in vitro* efficacy of compounds, 18b-Lux with STR (OD_{600} = 0.0001) or a 2-week-old SS18b-Lux culture (OD_{600} = 0.1) was used in the REMA. Two-fold serial dilutions of each test compound were prepared in white 96-well plates containing the bacilli in a total volume of 100 μl and then were incubated for 6 days at 37°C before the addition of 10 μl of 0.025% resazurin. After overnight incubation, the fluorescence of the resazurin metabolite resorufin was determined (excitation, 560 nm; emission, 590 nm; gain, 70) by using a Tecan Infinite M200 microplate reader. The experiments were carried out with two biological replicates.

Drug susceptibility testing. Drug treatment was performed in the absence of STR. Two independent SS18b-Lux cultures (biological replicates) were exposed to drugs, at the concentrations reported in the text, in 96-well plates for 6 days. Ten-fold serial dilutions of the cultures were plated on 7H10 plates supplemented with glycerol, OADC, STR, and HYG. CFU were counted after 4 to 5 weeks of incubation at 37°C.

Statistical analysis. Data were processed and graphs were constructed with Prism version 5.0 (GraphPad). CFU data were $\log_{10}$ transformed before analysis, expressed as mean $\log_{10}$ CFU ± standard deviation (SD), and compared using Bonferroni's multiple-comparison test in Prism version 5.0 (GraphPad).

RESULTS

Construction of luminescent *M. tuberculosis* 18b. A recent study (18) reported the construction of plasmid pEG200, which carries the *luxCDABE* operon from *Photorhabdus luminescens* cloned downstream of a strong promoter in a mycobacterial integrative vector. The successful expression of luciferase was evaluated in replicating *Mycobacterium smegmatis* and *M. tuberculosis* and was exploited for optimization of a luminescence-based assay for assessing drug potency (18). With the final goal of developing the luciferase assay in nonreplicating bacteria, we transformed *M. tuberculosis* 18b with pEG200 and obtained strain 18b-Lux. Growth was analyzed in 7H9 medium containing either glucose or acetate as the carbon source. Figure 1A presents the results obtained in the presence of STR (i.e., replicating conditions). Bacteria multiplied in both glucose- and acetate-containing media, although they reached the stationary phase earlier in the latter medium. In contrast, removal of STR from the culture medium for STR-starved 18b-Lux (SS18b-Lux) caused growth arrest with similar kinetics with the two different carbon sources (Fig. 1B). SS18b-Lux stopped dividing approximately 12 days after STR withdrawal and the optical density remained stable during the following 10 days, in agreement with our previous data (4).

Evaluation of luciferase expression in 18b-Lux. Luciferase expression was first assessed in the presence of STR. As depicted in Fig. 1A, luminescence levels gradually increased according to bacterial growth and reached a plateau in the late exponential phase, when the optical density was between 0.6 and 1.9 in glucose-containing medium and between 0.5 and 0.9 in acetate-containing medium. This steady-state signal was slightly lower in the presence of acetate, reflecting the growth kinetics under these conditions. Longer incubation in the presence of STR was associated not with

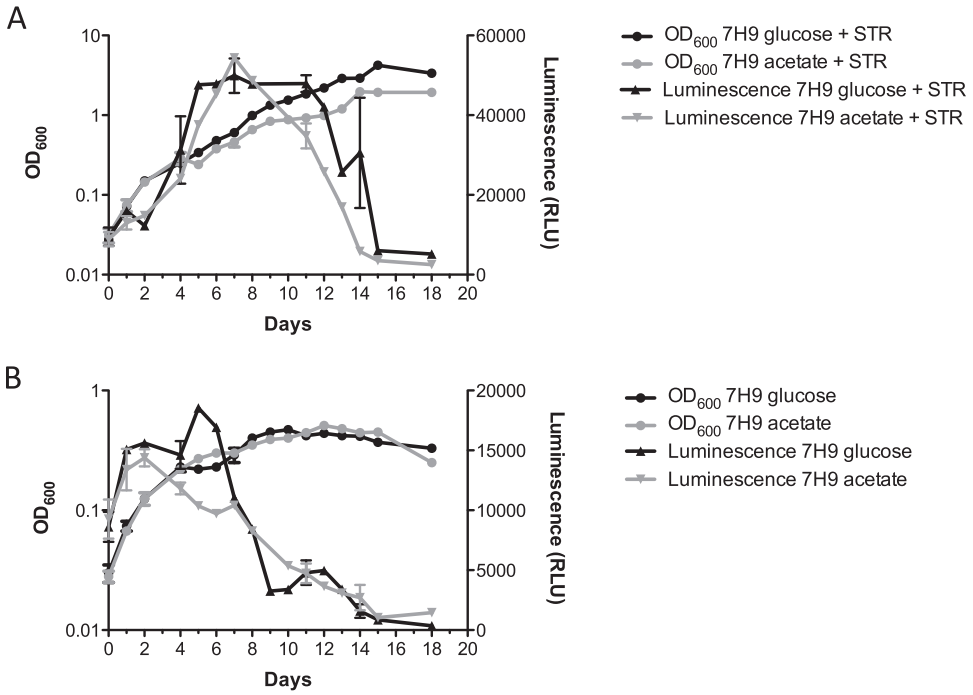

FIG 1 Growth curves for *M. tuberculosis* 18b-Lux and evaluation of luciferase expression. (A) 18b-Lux was grown at 37°C, with shaking, in glucose- or acetate-containing 7H9 medium with 50 μg/ml STR. (B) SS18b-Lux was incubated under the same conditions as in panel A except for the addition of STR. The OD_{600} and luminescence were recorded at different time points and used to compile the graphs. RLU, relative luciferase units. The experiments were carried out with two biological replicates.

a further increase in luminescence but rather with a decline, which coincided with the onset and subsequent establishment of the stationary phase.

Removal of STR caused a reduction in luciferase activity, which was more evident after the first 6 to 8 days, as indicated in Fig. 1B. Despite the lack of multiplication and the translational arrest in STR-starved bacilli, however, detectable basal luciferase activity was maintained after STR withdrawal.

To test the sensitivity of the luciferase reporter under nonreplicating conditions, serial 2-fold dilutions of 2-week-old SS18b-Lux cultures were then monitored (Fig. 2A). Luminescence was constantly above the background levels in both glucose- and acetate-containing media, underlining the high sensitivity of the luciferase readout. We arbitrarily decided to work with bacterial cultures with OD_{600} values of 0.1, which matches the experimental conditions defined previously for the REMA (6).

In order to evaluate the stability of the luminescence signal over time, the expression of luciferase by an SS18b-Lux culture at an OD_{600} of 0.1 was then measured at different time points with different carbon sources (Fig. 2B and C). The signal was maintained at higher levels with acetate (Fig. 2C) than with glucose (Fig. 2B). Thus, the latter condition provided a sufficiently large window for measuring drug activity. Based on these results, we selected an optical density of 0.1 in acetate-containing medium as the reference conditions for future work carried out with SS18b-Lux.

Robustness and reproducibility of luciferase assay with SS18b-Lux. Standard evaluations of drug activity are carried out in 96- or 384-well plates. Therefore, we sought to determine the applicability of the luciferase assay to this format. Before testing a panel of anti-TB compounds in 96-well plates, we measured the robustness and reproducibility of the luciferase reporter by calculating the Z-factor (20). This parameter reflects the dynamic range of the signals and the statistical variations in the data. We incubated SS18b-Lux in the presence or absence of 1 μg/ml RIF and obtained 0.65 and 0.75 as Z-factor values at days 4 and 6 after drug addition, respectively. These values are considered satisfactory (20). The luciferase assay in nonreplicating *M. tuberculosis* is thus robust enough for further applications.

Susceptibility of 18b-Lux to anti-TB compounds. Initially, the potency of two representative anti-TB drugs, namely, INH and moxifloxacin, against growing 18b-Lux and SS18b-Lux was analyzed. Luminescence was measured at different time points after the addition of various concentrations of the compounds (Fig. 3). INH effectively killed replicating bacteria in a time-dependent manner, and we could measure a MIC value of 0.6 μg/ml at day 6 (Fig. 3A). In contrast, no effect was seen against nonmultiplying cells; indeed, luminescence increased, as shown in Fig. 3B. Moxifloxacin was more powerful than INH in the presence of STR (MIC of 0.001 μg/ml at day 6) (Fig. 3C) and showed a bactericidal effect on SS18b-Lux too, as demonstrated by the reduction in the luminescence values (Fig. 3D). These results were in agreement with those of a REMA carried out under the same culture conditions (see Fig. S1 in the supplemental material). Overall, these data confirmed that replicating 18b-Lux is as susceptible as 18b (4) to standard anti-TB compounds and, more importantly, they suggest that luminescence can be used as a readout for evaluation of drug activity in the nonreplicating state.

Comparison of luciferase assay and REMA. To corroborate our findings, we tested 12 approved or experimental anti-TB drugs, with different targets, in the luciferase assay (Fig. 4) and

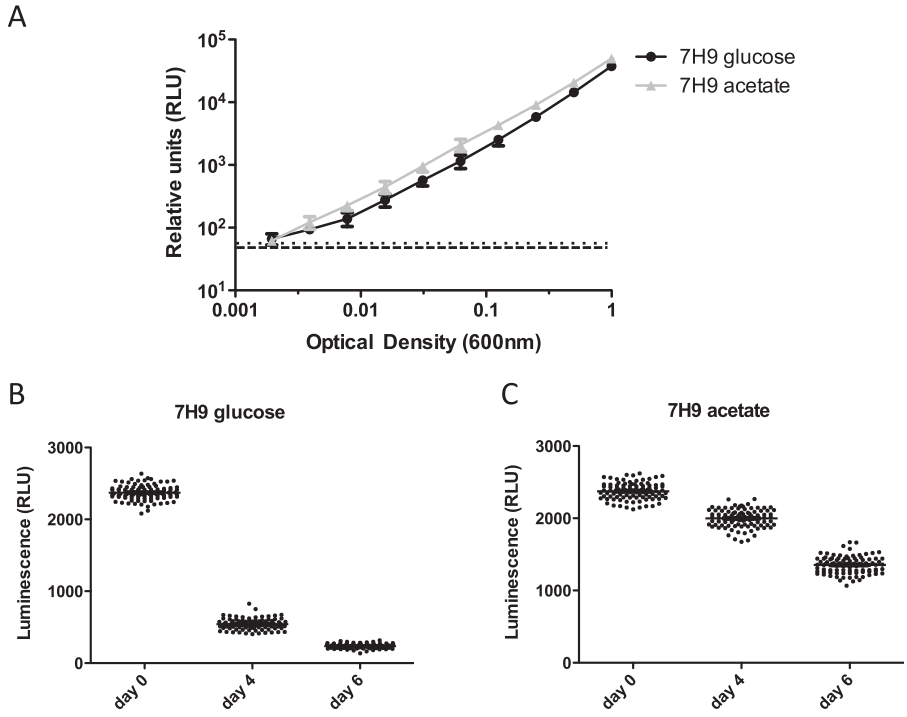

FIG 2 Sensitivity of the luciferase reporter. (A) Limit of detection of luciferase activity in SS18b-Lux. Serial 2-fold dilutions of bacterial cultures with glucose or acetate were used to determine the detection limit for luciferase activity. Dashed line, background luminesce measured for the culture medium (63 ± 1.7 RLU); dotted line, background luminescence measured for SS18b not transformed with the plasmid carrying the *luxCDABE* operon (70.7 ± 7.4 RLU). The experiments were carried out with four technical replicates. (B) Luminescence produced by SS18b-Lux, at an OD_{600} of 0.1, at different time points in glucose-containing medium. The mean values and variability range in a 96-well plate are presented. The experiments were carried out with two biological replicates. (C) Luminescence produced by SS18b-Lux, at an OD_{600} of 0.1, at different time points in acetate-containing medium. The mean values and variability range in a 96-well plate are presented. RLU, relative luciferase units. The experiments were carried out with two biological replicates.

compared the outcomes with the standard REMA results (see Fig. S2 in the supplemental material). The first group of compounds included BTZ043 (21), PAS (22, 23), EMB (24), and AU1235 (25). These molecules, whose targets were found to be in the cell wall biosynthetic process except for PAS, did not display any activity against nonmultiplying cells (Fig. 4). No decrease in the luminescence values was observed during 6-day incubations. Curiously, for three of the compounds (i.e., PAS, EMB, and AU1235), increases in luminescence were noted. These data were fully consistent with those obtained in the REMA (see Fig. S2 in the supplemental material).

The second subset was composed of drugs that showed activity against nonreplicating *M. tuberculosis*, namely, PZA, PA-824 (26), BDQ (27), RFP, SQ109 (28), and STZ (29). The time course evaluations performed with the luciferase assay demonstrated clear time-dependent activity for BDQ, RFP, and STZ (Fig. 4). PZA and SQ109 were effective at the highest concentrations tested (>62.5 μg/ml for PZA and >5 μg/ml for SQ109). Overall, these compounds were potent under nonreplicating conditions, and their efficacy in the luciferase assay mirrored the profiles obtained with the REMA (see Fig. S2 in the supplemental material).

The last group comprised RIF and clofazimine. Both of these drugs caused decreases in the luminescence values immediately after addition to the bacterial cultures (day 0 in Fig. 4). This reduction was more pronounced with clofazimine and took place starting at 0.3 μg/ml, whereas the same finding was noted with concentrations of RIF above 1 μg/ml. We attributed this phenomenon to the dark red color of the drugs, which probably had a quenching effect on the light emitted by the luminescent bacteria. Despite this unexpected outcome, RIF proved to be effective and its activity was time dependent, like the other rifamycin, RFP. The control experiment with the REMA confirmed that both compounds were active (see Fig. S2 in the supplemental material). Taken together, these results validated the newly established luciferase assay and indicated that it compares favorably with and can potentially replace the REMA.

Validation by CFU assay. To independently confirm the results obtained with the luciferase assay, we determined the bactericidal activity of some of the drugs by CFU evaluation. Data are reported in Fig. 5. The no-drug sample confirmed that bacteria were not multiplying, while the killing effect of RIF, used as a positive control, resulted in a 2.6-log-unit decrease with respect to the untreated control ($P < 0.0001$). BTZ043 ($P = 0.8236$), INH ($P = 0.5349$), and AU1235 treatment did not cause any reduction of the viable counts, even at the highest concentration tested for AU1235 ($P = 0.2213$ for 1.25 μg/ml and $P = 0.0713$ for 10 μg/ml). Conversely, clofazimine ($P = 0.0323$) and SQ109 at 10 μg/ml ($P = 0.0323$) displayed statistically significant bactericidal effects, causing 0.48- and 0.4-log-unit decreases, respectively. These data corroborate the aforementioned findings obtained with the luciferase methodology.

DISCUSSION

Our previous studies reported the exploitation of the STR-dependent *M. tuberculosis* 18b strain for assessment of drug potency

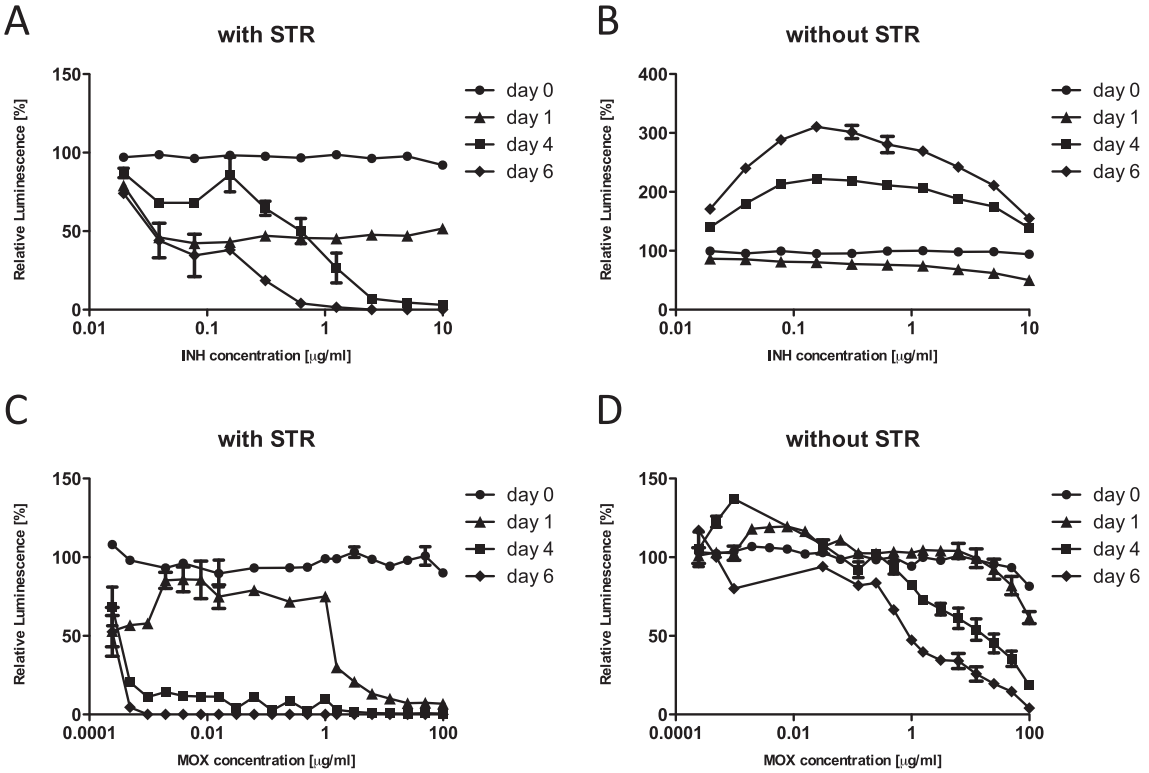

FIG 3 Sensitivity of 18b-Lux, with and without STR, to INH and moxifloxacin. (A) Potency of INH at different time points in acetate-containing medium in the presence of STR. (B) Potency of INH at different time points after STR removal. (C) Potency of moxifloxacin (MOX) at different time points in acetate-containing medium in the presence of STR. (D) Potency of moxifloxacin at different time points after STR removal. Relative luminescence, luminescence values normalized to the untreated control values. Data represent the means and standard deviations of two independent experiments (biological replicates).

against dormant bacteria *in vitro* and *in vivo* (4–6). In this work, we refined our model by validating a simple luciferase-based assay for performing time course evaluations of anti-TB therapy in a 96-well format. We proved that the synthetic *luxCDABE* operon (30) is expressed in *M. tuberculosis* 18b and that the luminescence signal is maintained at sufficiently high levels under nonreplicating conditions. This finding has important implications, as it suggests that, although bacteria do not multiply and *de novo* protein synthesis does not take place any longer, residual (albeit decreasing) metabolic activity is still present and allows luminescence to be generated.

In contrast to the standard REMA, with its endpoint readout, bacterial luminescence measurements allow time course evaluations of drug activity. The same plate can be assessed multiple times, as no addition of exogenous substrate is required. This reduces the costs and the risk of cross-contamination between samples and can potentially shorten the time needed to determine the efficacy of a compound, since bactericidal activity may be detected at earlier time points, in contrast to the REMA, which requires 7 days. In addition, the method can shed light on the mode of action of the drugs by providing information regarding time- and concentration-dependent efficacy. For example, we demonstrated that BDQ, RFP, RIF, and STZ are characterized by time-dependent potency, whereas PZA, PA-824, and SQ109 are associated with dose-dependent responses. Of note, the presence of the plasmid carrying the *luxCDABE* operon did not affect the sensitivity of the strain to standard antibiotics when these were tested under permissive conditions.

By growing the bacteria in media containing different carbon sources (i.e., glucose and acetate), we noticed that the luminescence signal was higher with glucose when bacteria were actively dividing. In addition, the luciferase activity reached the maximum level during the late exponential phase, before a decline was observed at the onset of the stationary phase. This is not surprising, since *luxCDABE* is a metabolic reporter whose activity is dependent on NADH levels. Koga and coworkers already reported a decline in luciferase expression for *Escherichia coli* carrying the P*lac*UV5-*lux* fusion gene during the transition from exponential growth to the stationary phase (31). They correlated this with a decrease in the availability of reduced flavin mononucleotide ($FMNH_2$). The expression kinetics with acetate resembled those observed with glucose, although the luminescence levels were lower. Interestingly, after removal of STR, the signal remained higher with acetate than with glucose, thus prompting us to choose the former carbon source as the reference for our assay. Beside the technical reasons, the choice of acetate may have additional implications, since it may represent more closely the *in vivo* situation, in which the bacteria feed on short-chain fatty acids (32, 33). Moreover, conducting drug screening programs with acetate can avoid false-positive results such as those observed previously in glycerol-containing medium (34).

Comparison of the drug sensitivity results generated here by luminescence with those presented previously (5, 6) revealed extensive overlap, including the cell wall inhibitor signature CWPRED, which was evident in the REMA (6) and confirmed in the luciferase assay. CWPRED was initially described as an in-

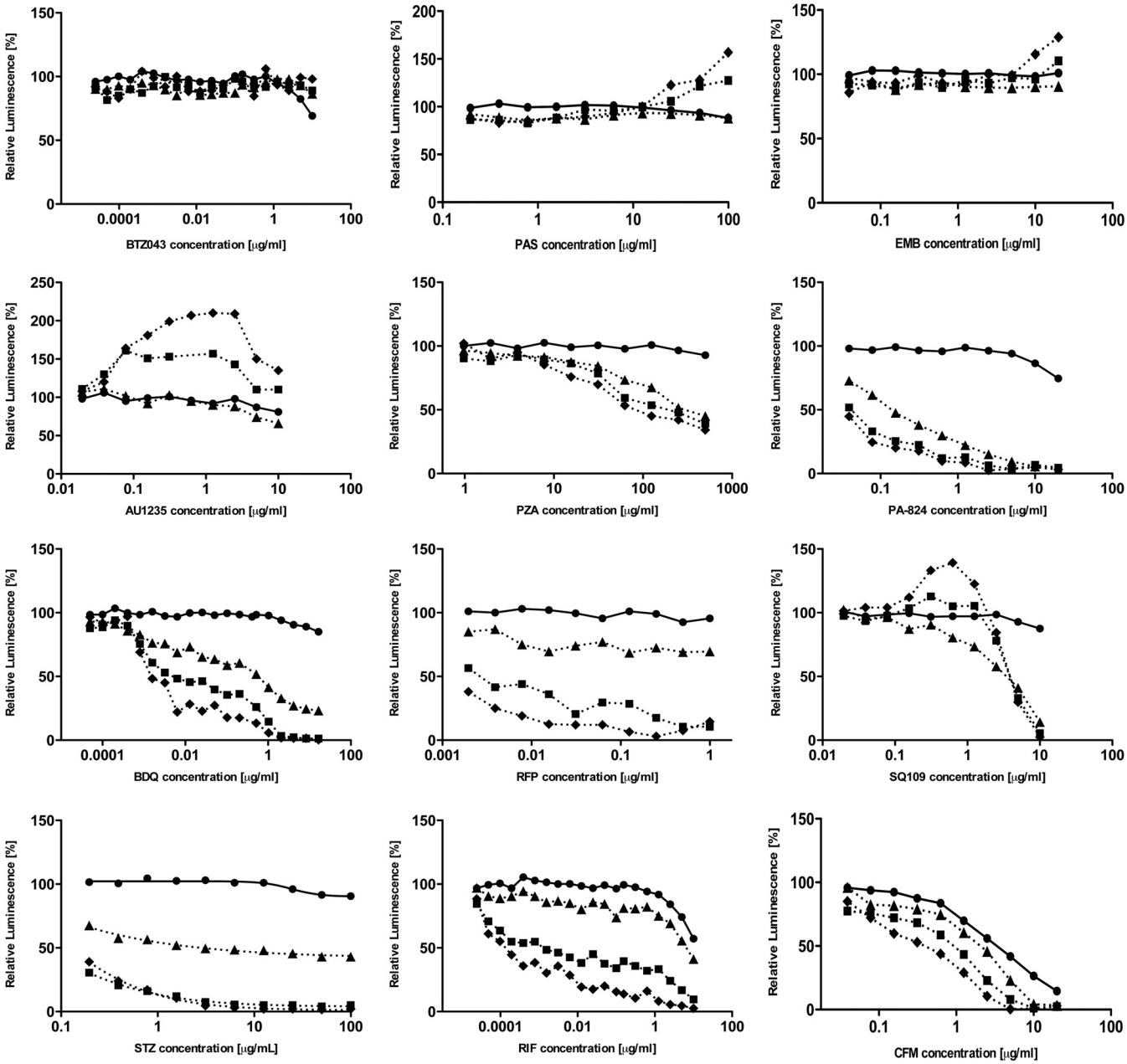

FIG 4 Luciferase assay with SS18b-Lux. Different concentrations of approved and experimental drugs were assessed in the luciferase-based assay with SS18b-Lux. Luminescence was measured at different time points, and data were used to construct killing curves. Relative luminescence, luminescence values normalized to the untreated control values. Data from one representative experiment of three biological replicates are shown. CFM, clofazimine. ●, day 0; ▲, day 1; ■, day 4; ◆, day 6.

crease in fluorescence values in the REMA when cell wall inhibitors were tested (6). Given the specificity for drugs that target the different cell wall layers, CWPRED facilitates target identification. We also noticed that PZA, STZ, RFP, and RIF showed stronger killing effects in the luciferase assay, compared to the REMA, under the same culture conditions (i.e., acetate). We attributed this discrepancy to the greater sensitivity of the luminescence readout, which may be able to detect even subtle differences in the effects caused by the various drug concentrations tested. Interestingly, SQ109, which had previously been regarded as a cell wall inhibitor (6), showed activity against dormant cells in our assay, in agreement with the findings of Li and colleagues, who described effects on menaquinone biosynthesis and on the electron transport chain (35). Importantly, these effects were restricted to SQ109; AU1235, which also contains an adamantyl group and inhibits the same target (MmpL3) (25), displayed no activity. Therefore, the luciferase assay can discriminate between the different modes of action of anti-TB compounds.

We observed that the luminescence signal was quenched immediately after the addition of clofazimine (>0.3 μg/ml) and with high concentrations of RIF (>1 μg/ml). We hypothesize that this phenomenon may be related to the strong red color of these com-

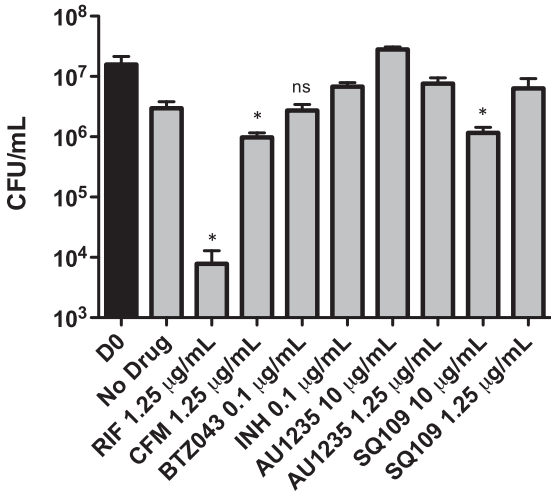

FIG 5 Drug activity evaluated as CFU. SS18b-Lux was incubated for 1 week with the various compounds at the concentrations indicated. An untreated sample (no drug) served as a control. D0, CFU detected at day 0. CFM, clofazimine. Ten-fold serial dilutions were plated on 7H10 plates containing STR, and CFU were enumerated after 4 to 5 weeks of incubation at 37°C. Mean values and standard deviations from two biological replicates are presented. *, $P < 0.05$ (Bonferroni's multiple-comparison test); ns, not significant.

pounds, which possibly masks the readout. However, their efficacy at lower doses could be measured and was not biased by the quenching effect.

In conclusion, we adapted a simple, inexpensive, and sensitive assay, the luciferase assay, to nonreplicating *M. tuberculosis* 18b and demonstrated its robustness and suitability for time course evaluations of drug efficacy. Our assay can be implemented in *in vitro* assessments of novel drug combinations against *M. tuberculosis* and other slowly growing mycobacteria, such as *Mycobacterium ulcerans*. Additional applications are foreseen in immunology experiments, where the fluorescent reporter can be replaced by luminescence (36), and possibly in *in vivo* studies that employ whole-body luminescence as a means of detecting tuberculosis infections and monitoring throughout therapy (9, 17).

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