ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Feb. 1996, p. 400–407 Vol. 40, No. 2
0066-4804/96/$04.00+0

Luciferase In Vivo Expression Technology: Use of Recombinant Mycobacterial Reporter Strains To Evaluate Antimycobacterial Activity in Mice

MARK J. HICKEY, TARAQ M. ARAIN, RIBHI M. SHAWAR, DEBBI J. HUMBLE, MICHAEL H. LANGHORNE, JENNA N. MORGENROTH, AND C. KENDALL STOVER*

Department of Molecular Microbiology, PathoGenesis Corporation, Seattle, Washington 98119

Received 18 August 1995/Returned for modification 23 October 1995/Accepted 21 November 1995

The development of new drugs and vaccines directed against *Mycobacterium tuberculosis* is severely impeded by the slow growth of this organism and the need to work under stringent biosafety conditions. These difficulties pose considerable obstacles when animal studies with *M. tuberculosis* are performed. We investigated whether a novel approach termed luciferase in vivo expression, using an enhanced luciferase-expressing mycobacterial strain, could be used to evaluate antimycobacterial activity in mice. Vectors that expressed firefly luciferase (*lux* gene) at high levels in the bacillus Calmette-Guérin (BCG) strain of *Mycobacterium bovis* were constructed for use in vivo. One recombinant BCG reporter strain (rBCG-*lux*) was selected for high-level expression of the *lux* gene product and for its ability to replicate in mice. Methodology to monitor in vivo growth of the rBCG-*lux* reporter strain in mice by direct assay of luciferase luminescence in organ homogenates was developed. The utility of this approach for assessing the in vivo efficacies of antimycobacterial compounds was evaluated. The activities of standard antimycobacterial drugs were directly apparent in mice infected with the rBCG-*lux* reporter strain by statistically significant reductions in spleen luminescence. In addition, antimycobacterial immunity was also evident in BCG-immunized mice, in which suppression of rBCG-*lux* growth in comparison with that in naive mice was clearly observed. The use of luciferase in vivo expression for the in vivo evaluation of antimycobacterial activity compared favorably with standard CFU determinations in terms of time, labor, expense, and statistical significance but permitted the evaluation of antimycobacterial drugs and immunity in mice in 7 days or less. Thus, the use of this technology can greatly accelerate the process of evaluation of antibiotics and immunogens in animal models for the slowly growing pathogenic mycobacteria.

Mycobacterium tuberculosis infects 10 million people and kills 3 million each year, making it the greatest cause of mortality by a single infectious agent worldwide (22). The surge in cases of tuberculosis associated with AIDS (4) combined with the increase in *M. tuberculosis* strains resistant to front-line antimycobacterial drugs such as rifampin (RIF) and isoniazid (INH) clearly indicate that current options for chemotherapy are inadequate and that new, more effective drugs are needed. Furthermore, the efficacy of the current tuberculosis vaccine is still controversial, and vaccination is not recommended in the United States because of its impact on the surveillance of tuberculosis by use of immunity-based skin tests. Despite the continued use of the *Mycobacterium bovis* BCG vaccine in other countries, it is generally accepted that a more efficacious vaccine is needed. However, efforts to develop new drugs and vaccines for the treatment or prevention of tuberculosis are seriously impeded by difficulties associated with the laboratory study of *M. tuberculosis*. With a doubling time of 18 to 24 h for the organism and the need to work under stringent biosafety level 3 conditions, almost all facets of *M. tuberculosis* research are impeded, including studies with animal models. The capacity of *M. tuberculosis* to survive in an intracellular environment also complicates the evaluation of novel compounds. Antimicrobial compounds that are active against *M. tuberculosis* in vitro may not demonstrate activity against intracellular organisms in vivo if the compound cannot enter the infected host cell. Therefore, it is advantageous to test promising antimycobacterial compounds in vivo as early as possible in a drug evaluation strategy.

Current protocols for the in vivo evaluation of compounds for antimycobacterial activity require CFU determinations with organ homogenates from infected animals. Such experiments generally require incubation of agar plates for 3 to 4 weeks before colonies can be accurately counted. Because these studies are extremely laborious, require multiple serial dilutions, and use large numbers of agar plates, it is difficult to test more than a few compounds in any one experiment. For these reasons, some investigators prefer to test novel agents against intracellular mycobacteria in cultured macrophages (2, 3, 9, 20, 21). While this approach does not require animals, lengthy CFU determinations are still necessary. Furthermore, antimycobacterial activity in infected macrophages does not necessarily indicate activity in vivo, where factors such as drug absorption, distribution, metabolism, and clearance become relevant.

Assay strategies employing reporter genes are attractive alternatives to the cumbersome drug susceptibility assays otherwise employed for the slowly growing mycobacteria. The use of recombinant mycobacteria and reporter mycobacteriophages expressing firefly or bacterial luciferase to measure drug susceptibility in vitro has been described (1, 5, 6, 12, 24). In these reporter gene assays the ability of a compound to inhibit growth of a mycobacterial reporter strain is measured by a decrease in luminescence resulting from a reduction in lucif-

* Corresponding author. Mailing address: PathoGenesis Corporation, 201 Elliott Ave. W., Seattle, WA 98119. Phone: (206) 467-8100. Fax: (206) 282-5065. Electronic mail address: kstover@path.path.com.

erase expression and intracellular ATP levels, both of which are requisites for enzymatic activity. While the application of this technology has the potential to enhance efforts directed towards in vitro drug susceptibility testing and compound screening, improved methodology to test intracellular or in vivo antimycobacterial activity against the slowly growing mycobacteria has not emerged. Here we describe the development of recombinant BCG (rBCG) reporter strains that express the firefly luciferase reporter gene at levels sufficient for direct detection of luminescence in infected mouse organs. The development of these avirulent reporter strains and the luciferase in vivo expression (LIVE) technology permits a more convenient and rapid means of assessing the in vivo potentials of novel antimicrobial compounds or immunogens at an early stage in drug or vaccine development.

MATERIALS AND METHODS

Construction of mycobacterial reporter vectors. Plasmid pMV206 (29) was modified by the addition of a synthetic regulatory region (termed mycobacterial optimal promoter [MOP]) consisting of promoter sequences derived from the BCG *hsp*70 heat shock protein gene and an *Escherichia coli tac* promoter (27a). A PCR-derived fragment containing the strong *E. coli rho*-independent *rrnAB* t1t2 terminator (25) was inserted 5′ to the direction of transcription for the synthetic MOP to yield vector pMH29. A luciferase expression cassette was derived by PCR amplification of a DNA fragment containing the *lux* open reading frame from plasmid pT3/T7*luc* (Clontech, Palo Alto, Calif.). A 5′ PCR primer was used to introduce a unique *Bam*HI site and a ribosomal binding site 5′ to the *lux* coding sequence and to change the seventh codon, encoding isoleucine, from ATA to ATC. The 3′ PCR primer contained a unique *Sal*I site to facilitate directional cloning. Restriction enzyme digestion of the PCR product with *Bam*HI and *Sal*I followed by ligation into pMH29 produced the extrachromosomal expression vector pMH30. The same *lux* PCR product was inserted into pMV261 (29) in front of the BCG *hsp*60 promoter, giving pMV261-*lux*. An integrative shuttle vector designated pMV361-*lux* was generated by cloning the *hsp*60-luciferase expression cassette from pMV261-*lux* into pMV306 (18, 28, 29) as a *Not*I-*Sal*I restriction fragment. All constructions were verified by restriction enzyme mapping, and *lux* gene expression was confirmed by luminescence assay.

rBCG culture and transformation. Conditions for liquid culture of BCG, introduction of purified plasmid DNAs by electroporation, and selection of recombinants have been described previously (28, 29). Vectors pMH30, pMV261-*lux*, and pMV361-*lux* were introduced into BCG substrain Connaught (ATCC 35745) by electroporation. Putative transformant colonies were transferred into 200 μl of Middlebrook 7H9 medium, sonicated to disperse bacterial cells (VibraCell VCX600; Sonics and Materials, Danbury, Conn.), and tested for luciferase expression in luminescence assays.

Mouse infection studies. Female BALB/c mice (B & K, Redmond, Wash.) aged 4 to 6 weeks were used in all experiments. Strains of rBCG-*lux* were grown to an optical density at 540 nm of 0.8 to 1.0 in Middlebrook 7H9 broth supplemented with 10% albumin-dextrose-catalase (Difco, Detroit, Mich.), 0.5% glycerol, and 20 μg of kanamycin per ml. On the day of infection, the luminescence of a culture aliquot was measured, and cultures were sonicated briefly to disperse clumps. A 150-μl volume of culture containing approximately 10^7 rBCG organisms was injected intravenously (i.v.) into the tail vein of each mouse. Animals were sacrificed by cervical dislocation at various times after infection.

For drug protection experiments with mice, therapy was initiated 4 to 24 h after infection with rBCG-*lux*. Dosages were calculated by assuming average animal weights of 20 g and were based on results of previously described studies (13–16, 30). Drug stocks were prepared as follows: INH (2.5 mg/ml), ethambutol (EMB) (12.5 mg/ml), and amikacin (AMK) (4.0 mg/ml) were made in deionized water (dH_2O); RIF (2.0 mg/ml) was dissolved in dimethyl sulfoxide and diluted to its final concentration in dH_2O; and ofloxacin (OFX) (30 mg/ml) was prepared as a suspension in dH_2O from prescribable tablets that contained approximately 0.5 mg of antibiotic per mg (dry weight). KRM-1648.1 (KRM) (PathoGenesis Corp.) was initially solubilized in ethanol and diluted with dH_2O to 2.5, 0.5, and 0.1 mg/ml in a final ethanol concentration of 5%. All drugs except OFX and KRM were obtained from Sigma (St. Louis, Mo.), stored as aliquots at −20°C, and thawed immediately before use. With the exception of amikacin, which was administered subcutaneously, all drugs were administered daily in 200-μl volumes by oral gavage.

For experiments to evaluate immune status, mice were immunized by i.v. injection of 10^5 log-phase BCG Connaught organisms in 7H9 medium 8 weeks prior to challenge with 10^7 rBCG-*lux* organisms. In some experiments, immunosuppression was induced by intraperitoneal injection of 1.5 mg of cyclophosphamide (CYP) (Sigma) in dH_2O twice per week beginning 1 day postinfection (27).

In vitro luminescence assay. Luciferin (R & D Systems, Minneapolis, Minn.) was stored at −20°C as a 1 mM stock solution in 100 mM trisodium citrate, pH 5.1. Aliquots were thawed slowly in the dark and allowed to equilibrate to room temperature before use. For 96-well-format assays, 10- to 50-μl aliquots of liquid bacterial culture were diluted with 100 mM trisodium citrate (pH 5.1) to a final sample volume of 100 μl in microtiter plates (MicroLite #1; Dynatech Inc., Chantilly, Va.). Reactions were initiated by automated injection of 100 μl of luciferin stock in an AutoLumat model 96P luminometer (Wallac Instruments, Gaithersburg, Md.), and luminescence was measured for 15 s without a preset delay. Luminescence was expressed as the number of relative light units (RLU) detected during the measurement period. For tube assays employing an AutoLumat model 953B luminometer (Wallac), 10- to 50-μl aliquots were diluted to 200 μl with citrate buffer in polystyrene tubes (12 by 75 mm), and luminescence was determined under the conditions described above. The 953B luminometer generally detected twofold more RLU than the 96P model with equivalent amounts of culture. All assays were performed in duplicate.

Luminescence assay with organ homogenates. All procedures were performed at room temperature. Organs were aseptically removed and weighed, and 5% (wt/vol) homogenates were prepared in sterile Dulbecco's phosphate-buffered saline (Dulbecco's PBS) (Gibco Laboratories, Grand Island, N.Y.) containing 1% Triton X-100 (TX-100) by using disposable 50-ml tissue grinders (Sage Products Inc., Crystal Lake, Ill.). In preliminary experiments, organ homogenates prepared from uninfected mice were spiked with $\sim 5 \times 10^5$ RLU of log-phase rBCG-*lux* to determine quenching of luminescence. Organs from rBCG-*lux*-infected animals were homogenized as described above and assayed directly for luminescence. For experiments using the 96-well luminometer (model 96P), duplicate 10-μl aliquots of homogenate were diluted with PBS–TX-100 to a final sample volume of 100 μl. In protocols that utilized the tube luminometer (model 953B), duplicate 200-μl aliquots of the homogenate were assayed without dilution. When CFU determinations were performed in parallel, organ homogenates were maintained on ice until culturing procedures were completed.

CFU determinations. Homogenates of mouse spleens were diluted 1:100 in sterile 0.85% sodium chloride. Fifty-microliter aliquots of diluted or undiluted homogenates were inoculated in duplicate on Middlebrook 7H11 agar (Remel, Lenexa, Kans.) by using an Autoplate model 3000 spiral plater (Spiral Biotech, Bethesda, Md.). The plates were incubated for 15 to 28 days at 37°C. Colonies were counted with a Laser Colony Scanner (model 500A) and associated Bacterial Enumeration software (Spiral Biotech).

Nucleotide sequence accession number. The nucleotide sequence of the luciferase expression plasmid pMH30 (see Fig. 1) has been assigned GenBank accession number U40374.

RESULTS

Construction and in vitro characterization of mycobacterial *lux* expression vectors. An extrachromosomal shuttle expression vector termed pMH29 was constructed with the synthetic MOP. This promoter combines elements of an ideal *E. coli* promoter with sequences immediately 5′ to the BCG *hsp*70 transcription start site (27a). Other studies have employed the extrachromosomal pMV261 mycobacterial expression vector with the BCG *hsp*60 promoter (P-*hsp*60) to drive expression of the firefly luciferase *lux* gene or the *Vibrio harveyi luxA* and *luxB* genes for in vitro drug susceptibility testing (1, 5). Both pMH29 and pMV261 are extrachromosomal shuttle vectors which carry the Tn*903* gene encoding kanamycin resistance and replicons for selection and replication in both *E. coli* and mycobacteria. An identical *lux* gene cassette that had been modified at its 5′ end by PCR was inserted 3′ to the BCG *hsp*60 promoter in pMV261 to construct pMV261-*lux* and 3′ to the MOP in pMH29 to generate pMH30 (Fig. 1). An integrative expression vector was also constructed by inserting the P-*hsp*60 *lux* expression cassette into vector pMV306 to yield pMV361-*lux* (29). The three resulting expression vectors pMV261-*lux*, pMV361-*lux*, and pMH30 were all shown to express luciferase in *E. coli* and were subsequently transformed into the BCG Connaught strain. Colonies resulting from transformations with pMV261-*lux* and pMH30 were very slow to develop (6 weeks) in comparison with the single-copy integrative vector pMV361-*lux* and control extrachromosomal or integrative plasmids without the *lux* gene (3 weeks). These disparities suggested that high-level expression of *lux* on multicopy vectors was not well tolerated by the BCG strain and inhibited growth on plates. rBCG transformants containing extrachromosomal plasmids pMH30 and pMV261-*lux* also grew more slowly in liquid culture than rBCG transformants containing

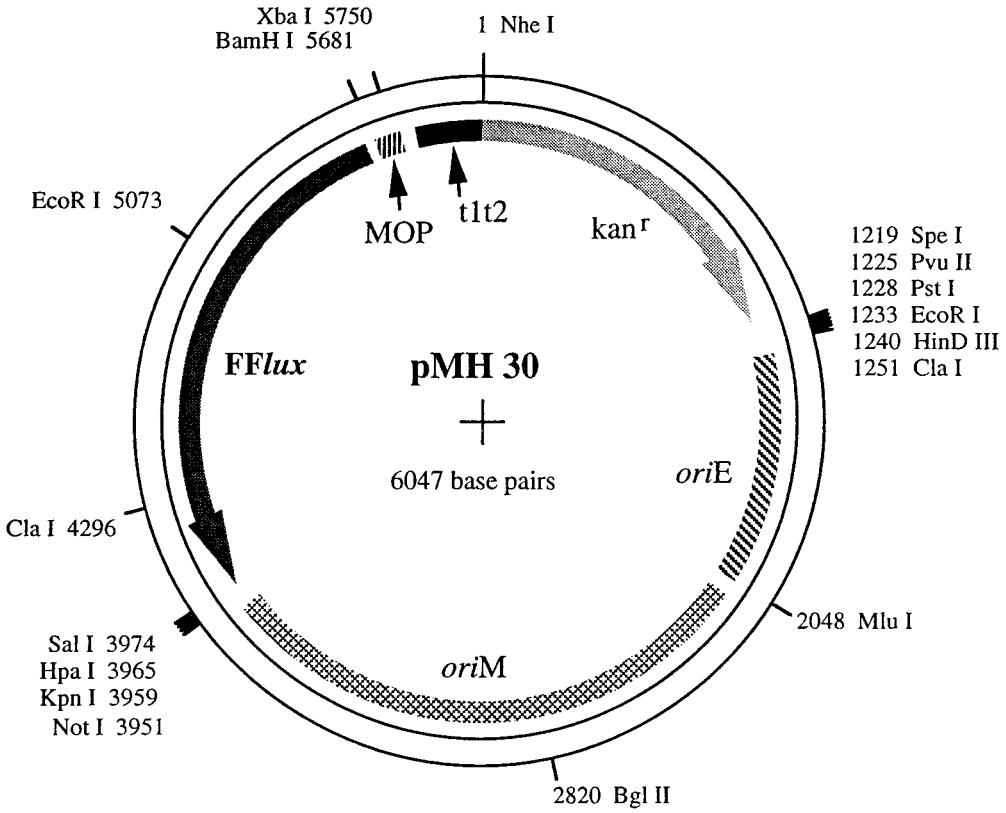

FIG. 1. Extrachromosomal luciferase expression plasmid pMH30. The vector carries the selectable kanamycin resistance marker encoded by the *aph* gene from Tn*903* (kan^r), the ColE1 replicon of the *E. coli* plasmid pUC19 (*ori*E), and a modified mycobacterial plasmid replicon from pAL5000 (*ori*M) constructed as previously described (29). A ribosomal t1t2 terminator is positioned upstream of a synthetic promoter (MOP) combining elements of the *E. coli tac* and BCG *hsp*70 promoters (25). The firefly (FF) luciferase (*lux*) gene cassette was modified by PCR as described in Materials and Methods. Restriction enzyme sites and nucleotide coordinates are indicated.

the single-copy pMV361-*lux* vector. Luciferase assays performed with log-phase liquid cultures revealed that the rBCG:pMH30 transformant consistently expressed a level of luminescence approximately fivefold greater than that of rBCG:pMV261-*lux* transformants and 10- to 30-fold greater than that of rBCG:pMV361-*lux*. The expression of luciferase from all three vectors was unchanged after passage for approximately 20 generations without kanamycin selection, indicating that the extrachromosomal and integrating vectors were equally stable (data not shown).

rBCG-*lux* luminescence in organ homogenates. Direct inhibitory effects on the luciferase enzyme or interference and quenching of luminescence due to color or opacity have not been observed with pure compounds evaluated for antimycobacterial activity at relevant concentrations with rBCG-*lux* reporter strains in bioluminescent culture-based assays (data not shown). However, rBCG-*lux* bioluminescence assays evaluating antimycobacterial activity in natural product extracts indicated that some colored solutions could interfere with the detection of bioluminescence at very high concentrations (data not shown). Therefore, we first investigated luciferase assay conditions in murine liver and spleen homogenates spiked with rBCG:pMH30 to determine if a complex organ homogenate suspension would interfere with luciferase enzymatic activity or the ability to detect luminescence. In contrast to results of a previous study with an *M. tuberculosis* reporter strain (5), it was not necessary to lyse the rBCG-*lux* bacteria. Standard phosphate buffer (pH 7.4) with the addition of 1% TX-100 (26) was found to be optimal for detection of luminescence in rBCG-*lux*-spiked organ homogenates and was therefore used in all subsequent experiments. Interference with luminescence detection due to the opacity of the organ homogenate was dependent upon the luminometer used and was found to be minimized by a 1:10 dilution of the organ homogenate. Luminescence in organ homogenates spiked with rBCG:pMH30 was remarkably stable even after 4 h on ice or at room temperature.

BALB/c mice were infected i.v. with rBCG:pMH30 to determine whether luminescence from rBCG-*lux* could be detected in infected-organ homogenates by using the assay conditions determined in spiking experiments. Twenty-four hours after infection, spleens, livers, and lungs were harvested and homogenized in PBS or PBS–TX-100. Again, luminescence was readily detected in spleen and liver homogenates without procedures to lyse or concentrate the rBCG-*lux* reporter bacteria. Luminescence was undetectable in lung tissues, indicating the inability of rBCG-*lux* to survive in this site 24 h after i.v. infection. The use of TX-100 in spleen and liver homogenates substantially enhanced (ca. fivefold) rBCG-*lux* luminescence, suggesting that the lysis of infected cells by the detergent enhanced the entry of the luciferin into the bacteria or improved the detection of luminescence from the reporter strain (26). Thus, luciferase activity could be assayed directly in infected mouse spleens and livers, suggesting that the method could be used as an index of rBCG-*lux* growth if the strain could proliferate in vivo for a sufficient period of time.

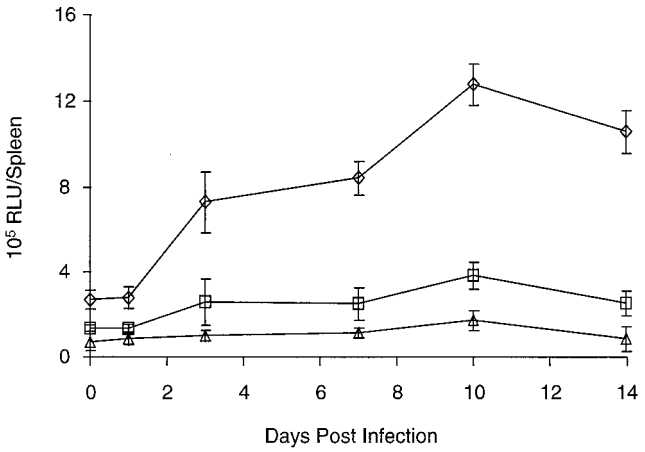

FIG. 2. Comparison of in vivo growths of rBCG-*lux* reporter strains. BALB/c mice were injected with rBCG-*lux* strains containing extrachromosomal vector pMH30 (◇) or pMV261-*lux* (□) or the integrative single-copy vector pMV361-*lux* (△). The growth of each strain in infected spleens was assessed over a 2-week time course by measuring luminescence RLU. Mean values determined from duplicate assays with groups of five mice are plotted. Error bars indicate standard deviations from the means.

Growth of rBCG-*lux* reporter strains in mice. We compared the three rBCG-*lux* reporter strains for their abilities to replicate in BALB/c mice following i.v. infection. In vivo growth curves based on RLU from organ homogenate luminescence assays correlated with growth curves based on CFU (Fig. 2). As expected, the rBCG:pMH30 strain exhibited substantially greater luminescence in vivo than either rBCG:pMV261-*lux* or rBCG:pMV361-*lux*. Despite their markedly different levels of *lux* expression, all three strains grew comparably over 2 weeks in vivo as measured by CFU in spleens (data not shown). This result contrasted markedly with results of in vitro culture growth rate determinations, in which rBCG constructs containing extrachromosomal *lux* expression vectors pMH30 and pMV261-*lux* grew more slowly. Maximal rBCG-*lux* spleen RLU and CFU levels were detected approximately 10 days postinfection, after which greater mouse-to-mouse variation and a gradual reduction in spleen luminescence and CFU were observed. Liver homogenates were also assayed for reporter strain growth and luminescence following i.v. rBCG-*lux* infection. Liver luminescence far exceeded spleen luminescence 24 h after infection but dropped precipitously to insignificant levels thereafter (data not shown). These data indicated a limited time frame of 10 to 14 days for proliferation of rBCG-*lux* strains in immunocompetent mice and determined the selection of the spleen as a target organ for further investigation of the LIVE method. On the basis of its capacity to proliferate in mice and its superior luciferase expression, rBCG:pMH30 was selected for all subsequent experiments. rBCG:pMH30 was reisolated from mouse spleens 14 days after infection and plated on nonselective medium. All colonies tested were kanamycin resistant and expressed a level of luciferase activity per bacterium that was comparable to that of the original inoculum (data not shown). These findings demonstrated that this vector was stable in vivo and indicated that the reduction in apparent growth determined by luminescence was due to a reduction in CFU and not to loss of the vector.

Evaluation of antimicrobial activity by using an rBCG-*lux* mycobacterial reporter strain. As in previous in vitro studies with cultured *M. tuberculosis* and *Mycobacterium avium*, the expression of luciferase in rBCG did not appreciably affect the drug susceptibility profiles in comparison with those of nonrecombinant BCG or *M. tuberculosis* (reference 6 and data not shown). Therefore, mice were infected with rBCG-*lux* and treated daily over a 7-day period with three antimycobacterial compounds to determine whether LIVE technology could be used to test compound activity in vivo. Luminescence in the spleens of INH- and EMB-treated animals was reduced in comparison with that in untreated control groups within 7 days of treatment initiation (Table 1). Groups treated with the potent experimental antibiotic KRM exhibited a dose-dependent reduction in luminescence after a single oral dose, indicating very rapid in vivo activity for this semisynthetic ansamycin (23). Treatment with KRM was terminated for all groups after the administration of the third dose, since spleen luminescence in the KRM-treated groups had already reached undetectable levels. Recrudescence in organ luminescence was not observed 7 days after the termination of treatment. The observed reductions in luminescence for all drug-treated groups was statistically significant ($P < 0.05$) and suggested that in vivo antimycobacterial activity could be directly determined by the reduction of rBCG-*lux* luminescence in spleens.

To further investigate the utility of LIVE for in vivo drug evaluation, five drugs with different mechanisms of action were selected for treatment of rBCG-*lux*-infected animals: two cell wall-active drugs (INH and EMB), a transcriptional inhibitor (RIF), a DNA gyrase inhibitor (OFX), and an aminoglycoside translational inhibitor (AMK). CFU determinations were made in parallel with RLU determinations to compare the data obtained by each method. Again, a statistically significant reduction in rBCG-*lux* spleen luminescence in INH- and EMB-treated mice was evident within 7 days of treatment initiation, with a 1-log-unit reduction observed by day 10 (Fig. 3 and

TABLE 1. In vivo antimicrobial activities of INH, EMB, and KRM against rBCG-*lux* determined by luminescence assay of spleen homogenates

Treatment	Day	Mean RLU	SD (%)[a]	% Reduction[b]	P[c]
Control	0	76,377	18.1		
	1	444,430	23.2		
	3	905,473	29.4		
	7	1,651,260	20.2		
INH	1	396,453	9.4	10.8	0.613
	3	824,110	19.6	9.0	0.596
	7	311,337	32.6	81.1	0.026
EMB	1	398,663	27.0	10.3	0.664
	3	977,557	8.6	(8.0)	0.694
	7	756,733	15.3	54.2	0.040
KRM[d] (25 mg/kg)	1	71,347	24.6	83.9	0.017
	3	5,943	85.6	99.3	0.029
	7	0	0.0	100.0	0.013
KRM[d] (5 mg/kg)	1	138,260	20.7	68.9	0.021
	3	9,123	42.6	99.0	0.028
	7	0	0.0	100.0	0.013
KRM[d] (1 mg/kg)	1	277,253	44.8	37.6	0.323
	3	67,120	49.2	92.6	0.039
	7	0	0.0	100.0	0.013

[a] Standard deviation as a percentage of the mean RLU.
[b] Percent reduction from control RLU on the same day; the number in parentheses indicates a value higher than the control value.
[c] Statistical significance from a paired, two-tailed t test.
[d] Administration of KRM was terminated after day 3.

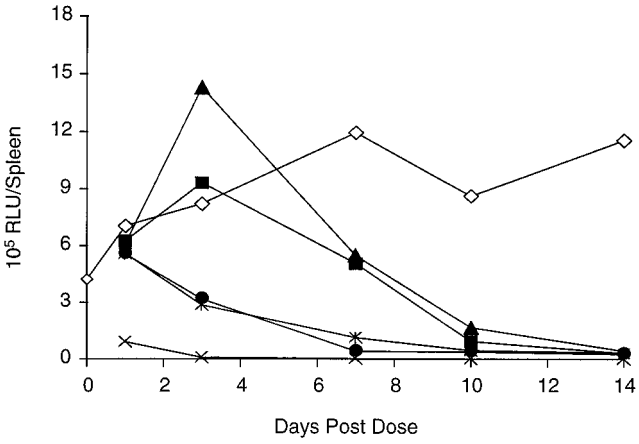

FIG. 3. In vivo activity of standard antimycobacterial drugs evaluated by rBCG-*lux* luminescence. BALB/c mice were infected with rBCG-*lux* carrying plasmid pMH30. Each drug was administered daily at the dosages indicated in Materials and Methods beginning 24 h after infection and was continued for 10 days. INH (■), EMB (▲), RIF (×), and OFX (∗) were administered by oral gavage; AMK (●) was given by subcutaneous injection. ◇, control. The growth of rBCG-*lux* in each group was evaluated by luminescence assays of mouse spleen homogenates at 1 day postinfection (day 0) and 1, 3, 7, 10, and 14 days after initiation of drug treatment. Mean RLU per spleen were determined from duplicate assays with groups of three mice. Mean RLU, standard deviations from the mean, percent reductions in comparison with the control group, and statistical significances are shown in Table 2.

Table 2). Treatment with OFX or AMK resulted in more rapid decreases in luminescence that were evident by day 3, with statistically significant reductions of greater than 1 log unit by day 7. With RIF, a reduction of almost 1 log unit was observed after a single oral dose. A comparison of luminescence and CFU data for control and INH-treated mice revealed that the assessment of growth and antimycobacterial activity by LIVE was equivalent to evaluation by standard CFU methodology (Fig. 4). However, in AMK-treated animals, reductions in luminescence at day 3 were greater in comparison with results for control mice than was observed with CFU determinations, suggesting that translational inhibition and effects on *lux* expression were evident before the reporter strain was killed (data not shown). With EMB, significant increases in luminescence were observed in comparison with results for control mice at day 3, but this increase was not observed by CFU determination (data not shown). This result with EMB has been reproducible in other experiments and may be due to a drug-related effect on mycobacterial cell wall permeability to the luciferin substrate. Although RLU and CFU data at early time points did not correlate for AMK and EMB, concomitant reductions in RLU and CFU were observed with all tested drugs by day 7.

rBCG-*lux* use in immunosuppressed mouse models. Because the growth of BCG is limited in immunocompetent mice, we investigated rBCG-*lux* growth and the effect of drug treatment in immunosuppressed mice. Normal BALB/c mice and mice immunosuppressed with CYP were treated daily with INH to determine the relative effect of this standard drug on rBCG-*lux* growth. After 7 days of comparable growth in the spleens of control mice, the rBCG-*lux* strain proliferated in the immunosuppressed animals to levels approximately sevenfold greater than those observed in immunocompetent mice by day 14 (Table 3). INH treatment resulted in statistically significant reductions of rBCG-*lux* luminescence in comparison to that for untreated control mice in both immunocompetent and immunosuppressed mouse groups by day 7. However, the degree of INH activity, expressed as the percent reduction in RLU, was considerably greater in immunocompetent mice than in immunosuppressed mice. Continuing INH administration diminished luminescence to nearly background levels in immunocompetent mice by day 14, while INH treatment for the same period resulted in no further reduction of luminescence in CYP-treated mice (Table 3). These data indicate that the dynamic range of the rBCG-*lux* infection model in immunosuppressed mice is considerably greater than that in immunocompetent mice, but compound testing in this model may provide a more stringent test for antimycobacterial compounds in the absence of a murine immune response.

Evaluation of antimicrobial immunity by using an rBCG-*lux* mycobacterial reporter strain. We also investigated whether antimycobacterial immunity could be evaluated by LIVE. BALB/c mice which had been immunized 8 weeks previously with BCG (substrain Connaught) and naive sibling mice were challenged with rBCG:pMH30. Growth of the reporter strain in the BCG-immunized and naive mouse groups was monitored over a 10-day period by measuring spleen luminescence. rBCG-*lux* luminescence was significantly suppressed ($P < 0.0003$) at all time points in BCG-immunized mice, while nor-

TABLE 2. In vivo activities of standard antimycobacterial drugs evaluated by rBCG-*lux* luminescence

Treatment	Day	Mean RLU	SD (%)[a]	% Reduction[b]	P[c]
Control	0	420,820	23.7		
	1	703,320	18.3		
	3	821,530	30.0		
	7	1,194,320	13.8		
	10	862,683	20.2		
	14	1,155,647	48.0		
INH	1	625,640	31.0	11.0	0.331
	3	931,460	7.7	(13.4)	0.435
	7	507,103	17.7	57.5	0.005
	10	93,623	67.9	89.1	0.020
	14	28,897	66.3	97.5	0.073
EMB	1	612,330	6.5	12.9	0.292
	3	1,430,870	61.1	(74.2)	0.431
	7	548,107	46.6	54.1	0.007
	10	166,977	46.5	80.6	0.040
	14	42,377	87.1	96.3	0.067
RIF	1	88,900	11.0	87.4	0.016
	3	8,513	12.6	99.0	0.029
	7	2,240	114.7	99.8	0.006
	10	960	173.2	99.9	0.014
	14	0	NA[d]	100.0	0.069
OFX	1	557,620	12.0	20.7	0.323
	3	285,773	16.4	65.2	0.044
	7	111,597	46.3	90.7	0.004
	10	45,340	24.8	94.7	0.016
	14	32,487	66.5	97.2	0.077
AMK	1	562,190	17.0	20.1	0.288
	3	324,760	34.6	60.5	0.126
	7	47,163	65.9	96.1	0.005
	10	45,347	40.9	94.7	0.012
	14	32,360	59.4	97.2	0.075

[a] Standard deviation as a percentage of the mean RLU.
[b] Percent reduction from control RLU on the same day; numbers in parentheses indicate values higher than the control values.
[c] Statistical significance from a paired, two-tailed t test.
[d] NA, not applicable.

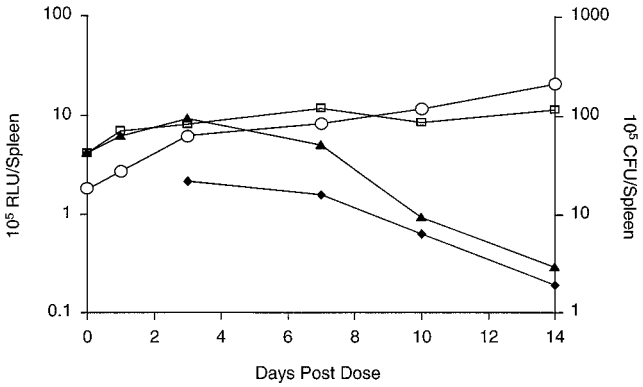

FIG. 4. Comparison of CFU and RLU in rBCG-*lux*-infected mice. rBCG-*lux* RLU from untreated control mice (□) and INH-treated mice (▲) in the experiment whose results are depicted in Fig. 3 are compared with CFU (○ and ◆, respectively) obtained from the same spleen homogenates to correlate luminescence (RLU) and CFU.

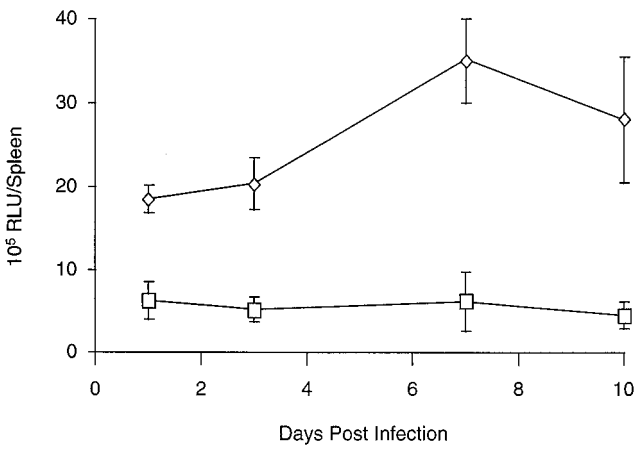

FIG. 5. Antimycobacterial immunity evaluated by rBCG-*lux* infection. BALB/c mice were immunized with BCG substrain Connaught. After 8 weeks, these immunized mice (□) and control mice (◇) were challenged with rBCG:pMH30. Reporter strain growth was determined by duplicate luminescence assays (expressed in RLU) on spleens from naive and immunized mice (five mice per time point) at 1, 3, 7, and 10 days postinfection. Bars indicate standard deviations from the means.

mal growth and rBCG-*lux* luminescence were observed in the control group (Fig. 5). These data suggested a utility for LIVE in the evaluation of antimycobacterial immunity.

DISCUSSION

M. tuberculosis and *M. bovis* are closely related members of the tuberculosis complex, and because of their genetic identity, it has been suggested that they be considered strains of the same species (8, 11). Although it grows as slowly as *M. tuberculosis*, the avirulent *M. bovis* BCG strain offers distinct advantages over the faster-growing mycobacteria in the study of the pathogenic tubercle bacilli. With the exception of susceptibility to pyrazinamide, BCG's drug susceptibility profile is very similar to that of fully susceptible *M. tuberculosis* bacilli. Furthermore, studies with BCG can proceed under less-stringent laboratory conditions, and BCG will replicate and persist longer in mice than the rapidly growing avirulent mycobacteria (e.g., *Mycobacterium smegmatis*). Thus, we chose recombinant BCG as a candidate challenge strain for a first test of the LIVE strategy in an infected-mouse model.

TABLE 3. Comparison of growth of rBCG-*lux* and activity of INH in immunocompetent and immunosuppressed mice

Treatment	Day	Mean RLU	SD (%)[a]	% Reduction[b]	P[c]
None	1	33,208	33.2		
	3	56,611	41.1		
	7	69,726	12.6		
	14	102,977	14.0		
INH	3	61,857	11.5	(9.3)	0.781
	7	7,537	32.0	89.2	0.006
	14	287	116.8	99.7	0.006
CYP	1	48,916	15.9		
	3	87,202	23.0		
	7	110,455	2.1		
	14	690,679	27.5		
INH + CYP	3	85,209	13.6	2.3	0.732
	7	46,827	13.1	57.6	0.005
	14	53,634	5.8	92.2	0.029

[a] Standard deviation as a percentage of the mean RLU.
[b] Percent reduction from control RLU on the same day; the number in parentheses indicates a value higher than the control value.
[c] Statistical significance from a paired, two-tailed *t* test.

Experience with the expression of foreign antigen genes in rBCG for recombinant vaccine studies has indicated that no single expression vector is optimal for the expression of every gene and that high-level expression of some foreign proteins is deleterious to the recombinant host strain (28, 29). We therefore tested several BCG Connaught transformants carrying integrative and extrachromosomal mycobacterial expression vectors, with the goal of selecting a vector that expressed levels of a reporter gene sufficient for its detection in organ homogenates following infection and growth in vivo. The construction of the pMH30 vector, which stably expressed luciferase at high levels, and the selection of an rBCG:pMH30 transformant that was capable of replicating in mice were crucial to the function of the LIVE strategy developed in this study. Although high-level expression of *lux* in rBCG appeared to adversely affect its growth in culture, a similar attenuating affect was not observed in mice during the 2-week time frame tested, and it should be noted that the rBCG-*lux* reporter grew exponentially beyond 14 days in immunosuppressed mice (Table 3 and data not shown). The pMH30 vector and *lux* expression are very stable even in the absence of antibiotic selection, as we have never been able to isolate BCG which had lost the vector after growth in rBCG-*lux*-infected mice. These findings are in agreement with those of previous studies in which foreign antigens were expressed at high levels in rBCG from extrachromosomal vectors based on the pAL5000 replicon (28, 29). In addition, it was possible to perform luminescence assays directly on organ homogenates without having to purify or concentrate the bacteria and without having to use an inordinately high inoculum of rBCG-*lux*. It also proved to be unnecessary to lyse the rBCG-*lux* cells, allowing intracellular ATP levels to fuel the conversion of luciferin to oxyluciferin. Equally important was the finding that the luminescence of the reporter strain was remarkably stable both on ice and at room temperature, allowing the processing of organ homogenates en masse and ensuring that organ homogenate *lux* assays were reproducible. These findings combine to make the use of LIVE practical as well as feasible.

Reporter genes other than the firefly luciferase gene were

considered for use in in vivo reporter strains. Genes encoding enzymes with chemiluminescence activity are particularly desirable because bioluminescence is more easily detected in a turbid organ homogenate and the dynamic range of bioluminescence detection is generally much wider than is usually achievable with colorimetric assays. The bacterial *luxAB* luciferase genes of *V. harveyi* have also been expressed in the mycobacteria, and it is known that their aliphatic aldehyde substrates will readily enter mycobacteria without lysis (1). However, a recent report indicates that expression of *Vibrio* luciferase may lead to oxidative stress in the host bacteria (10). Furthermore, the use of firefly luciferase has an added advantage over the use of bacterial luciferases in that it requires endogenous mycobacterial ATP for chemiluminescence activity, a more direct measure of bacterial viability than reduced flavin mononucleotide, which is required for bacterial luciferase luminescence. With the exception of the bacterial luciferase from *Xenorhabdus luminescens*, firefly and bacterial luciferases are substantially less active at physiological temperatures (19). This was not a problem in these studies because firefly *lux* activity is restored upon cooling, and all assays were performed at room temperature (data not shown). Another candidate reporter gene which has been stably expressed at high levels in mycobacteria and for which sensitive bioluminescence assays now exist is that for *E. coli* β-galactosidase (29). However, because this enzyme does not require endogenous ATP and may have significant activity in mycobacteria even after cell death, reductions in its activity due to drug effects on mycobacteria are apparently slower than those observed with the eukaryotic firefly luciferase (data not shown). Another recently identified gene, encoding the green fluorescent protein, could offer potential advantages as a reporter in mycobacteria, since the green fluorescent protein requires only the appropriate-wavelength light for excitation fluorescence in the absence of an exogenous substrate (17). However, it remains to be determined whether background fluorescence in tissues will permit sensitive detection of green fluorescent protein expression in organs without laborious microscopy analysis.

Although LIVE is ultimately not as sensitive as a CFU assay, which can theoretically detect a single viable organism in a spleen, as few as 200 organisms can be detected in a luminescence assay (roughly 2,000 CFU per spleen) by using rBCG-*lux* with the pMH30 construct (data not shown). However, the use of reporter strains for in vivo antimycobacterial drug evaluation has a number of distinct advantages. RLU data are obtained directly, without the need for laborious dilution and plating procedures and long incubations, and are not subject to the problems of plate contamination. With CFU determinations, organ homogenates must be serially diluted to obtain colony numbers on a plate in a countable range (30 to 300 CFU per plate), and colony counting with mycobacteria is less precise than it is with other bacteria because of mycobacterial clumping. Using LIVE, we have observed the variation in RLU between duplicate samples to average 6 to 8% (data not shown). Therefore, smaller statistically significant reductions in mycobacterial counts due to drug treatment are more easily observed in short-term studies with fewer animals. This was confirmed in independent experiments with INH and EMB treatment, in which we observed statistically significant reductions in spleen RLU, in comparison with those in untreated mice, within 7 days of treatment initiation, with as few as three mice per time point (Tables 1 to 3). Therefore, the precision of the LIVE approach may allow novel drugs and vaccines to be tested more rapidly for initial efficacy in animals. Furthermore, short-term LIVE experiments testing new drugs require substantially smaller quantities of experimental compounds. Accordingly, it is possible to evaluate candidate compounds in vivo at an early stage of drug development.

While advantageous as an avirulent reporter strain, BCG shows limited growth in mice in comparison with *M. tuberculosis*, especially in lung tissues. We nevertheless routinely observed appreciable rBCG-*lux* growth over a 10- to 14-day period in immunocompetent BALB/c mice, which was sufficient to allow short-term drug and vaccine testing. Experiments employing immunocompromised mice showed that rBCG-*lux* can grow logarithmically for at least 21 days (data not shown). The use of immunocompromised mice could extend the use of the rBCG-*lux* to longer-term experiments in which it is desirable to separate the host immune response from the activity of an experimental drug (7). Experiments with rBCG-*lux* infection in CYP-treated mice suggest the potential for an immunosuppressed LIVE model which mimics the treatment situation in mycobacterium-infected AIDS patients, in whom the antimicrobial activity of a drug is not augmented by an immune response.

Whereas the use of rBCG-*lux* and LIVE provides a convenient initial analysis of in vivo antimycobacterial activity, further in vivo testing of the most promising compounds and immunogens against virulent *M. tuberculosis* is warranted. The same vectors developed in this study for rBCG-*lux* also function in *M. tuberculosis* strains. Initial experiments with recombinant *M. tuberculosis lux* reporter strains indicate increased growth and persistence in mice in comparison with rBCG-*lux*, particularly in lung tissues, even at very early times following infection (1a). These initial data suggest an important difference in the establishment of bacteria in the lung early in the infectious process and also suggest a potential utility for LIVE in the evaluation of tissue tropism and pathogenic mechanisms for *M. tuberculosis*. Initial results with *M. tuberculosis lux* also indicate that the application of LIVE technology can be extended to use with virulent *M. tuberculosis* in longer-term studies to more critically evaluate new antimycobacterial compounds for potential clinical effects. However, the effect of *lux* hyperexpression on *M. tuberculosis* in vivo growth, pathogenicity, and drug susceptibility must be compared with standard CFU determinations for any *M. tuberculosis* strain selected for LIVE before data generated by this methodology are accepted as a real indicator of a significant clinical effect for a new drug. At this time the application of LIVE technology is most appropriate for initial short-term studies in what can be characterized as an in vivo screen to indicate potential for in vivo activity of new lead compounds with promising in vitro activity.

The LIVE technology developed in this study is most appropriate for use with slowly growing pathogens for which simple animal models with disease or death end points do not exist, and for which procedures to measure the pathogen burden are necessary. We have developed recombinant mycobacterial reporter strains that express firefly luciferase at levels suitable for the direct detection of reporter strain luminescence in infected-organ tissues and the methodology for direct chemiluminescence assay on reporter strain-infected organs. Using a variety of standard antimycobacterial compounds with different mechanisms of action, we have demonstrated the potential for LIVE technology to simplify the evaluation of mycobacterial viability in vivo and to accelerate the in vivo analysis of new anti-infective drugs and immunogens against the slowly growing mycobacteria. LIVE technology approaches should also facilitate the study of these difficult pathogens in their natural environment and may also have broader application to in vivo studies of regulatory mechanisms which are essential for pathogenesis and in vivo survival.

ACKNOWLEDGMENTS

We are grateful to Michael Cynamon and Lynn Rose for their assistance in planning these studies and to Lynn Rose, David Sherman, and Jeff Ried for critical and constructive comments on the manuscript. We are also thankful for the excellent technical assistance of Geri Orta, Devinder Singh, and Chris Nywall.

REFERENCES

1. **Andrew, P. W., and I. S. Roberts.** 1993. Construction of a bioluminescent mycobacterium and its use for assay of antimycobacterial agents. J. Clin. Microbiol. **31:**2251–2254.
1a. **Arain, T.** Unpublished results.
2. **Bermudez, L. E., and L. S. Young.** 1988. Activities of amikacin, roxithromycin, and azithromycin alone or in combination with tumor necrosis factor against *Mycobacterium avium* complex. Antimicrob. Agents Chemother. **32:** 1149–1153.
3. **Carlone, N. A., G. Acocella, A. M. Cuffini, and M. Forno-Pizzoglio.** 1985. Killing of macrophage-ingested mycobacteria by rifampicin, pyrazinamide, and pyrazinoic acid alone and in combination. Am. Rev. Respir. Dis. **132:** 1274–1277.
4. **Collins, F. M.** 1989. Mycobacterial disease, immunosuppression, and acquired immunodeficiency syndrome. Clin. Microbiol. Rev. **2:**360–377.
5. **Cooksey, R. C., J. T. Crawford, W. R. Jacobs, Jr., and T. M. Shinnick.** 1993. A rapid method for screening antimicrobial agents for activities against a strain of *Mycobacterium tuberculosis* expressing firefly luciferase. Antimicrob. Agents Chemother. **37:**1348–1352.
6. **Cooksey, R. C., G. P. Morlock, M. Beggs, and J. T. Crawford.** 1994. Bioluminescence method to evaluate antimicrobial agents against *Mycobacterium avium*. Antimicrob. Agents Chemother. **39:**754–756.
7. **Cooper, A. M., D. K. Dalton, T. A. Stewart, J. P. Griffin, D. G. Russell, and I. M. Orme.** 1993. Disseminated tuberculosis in interferon gamma gene-disrupted mice. J. Exp. Med. **178:**2243–2247.
8. **Frothingham, R., H. G. Hills, and K. H. Wilson.** 1994. Extensive DNA sequence conservation throughout the *Mycobacterium tuberculosis* complex. J. Clin. Microbiol. **32:**1639–1643.
9. **Gangadharam, P. R., and M. D. Reddy.** 1994. Contributions of animal and macrophage models to the understanding of host parasite interaction of *Mycobacterium avium* complex (MAC) disease. Res. Microbiol. **145:**214–224.
10. **Gonzales-Flecha, B., and B. Demple.** 1994. Intracellular generation of superoxide as a by-product of *Vibrio harveyi* luciferase expressed in *Escherichia coli*. J. Bacteriol. **176:**2293–2299.
11. **Imeada, T.** 1985. Deoxyribonucleic acid relatedness among strains of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium bovis* BCG, *Mycobacterium microti*, and *Mycobacterium africanum*. Int. J. Syst. Bacteriol. **35:** 147–150.
12. **Jacobs, W. R., Jr., R. G. Barletta, R. Udani, J. Chan, G. Kalkut, G. Sosne, T. Kieser, G. J. Sarkis, G. F. Hatfull, and B. R. Bloom.** 1993. Rapid assessment of drug susceptibilities of *Mycobacterium tuberculosis* by means of luciferase reporter phages. Science **260:**819–822.
13. **Ji, B., C. Truffot-Pernot, M. C. Raviglione, R. J. O'Brien, P. Olliaro, G. Roscigno, and J. Grosset.** 1993. Effectiveness of rifampin, rifabutin, and rifapentine for preventative therapy of tuberculosis in mice. Am. Rev. Respir. Dis. **148:**1541–1546.
14. **Klemens, S. P., M. S. DeStefano, and M. H. Cynamon.** 1993. Therapy of multidrug-resistant tuberculosis: lessons from studies with mice. Antimicrob. Agents Chemother. **37:**2344–2347.
15. **Klemens, S. P., M. A. Grossi, and M. H. Cynamon.** 1994. Activity of KRM-1648, a new benzoxazinorifamycin, against *Mycobacterium tuberculosis* in a murine model. Antimicrob. Agents Chemother. **38:**2245–2248.
16. **Klemens, S. P., C. A. Sharpe, M. C. Rogge, and M. H. Cynamon.** 1994. Activity of levofloxacin in a murine model of tuberculosis. Antimicrob. Agents Chemother. **38:**1476–1479.
17. **Kremer, L., A. Baulard, J. Estaquier, O. Poulain-Godefroy, and C. Locht.** 1995. Green fluorescent protein as a new expression marker in mycobacteria. Mol. Microbiol. **17:**913–922.
18. **Lee, M. H., L. Pascopella, W. R. Jacobs, Jr., and G. F. Hatfull.** 1991. Site-specific integration of mycobacteriophage L5: integration-proficient vectors for *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, and bacille Calmette-Guerin. Proc. Natl. Acad. Sci. USA **88:**3111–3115.
19. **Meighen, E.** 1991. Molecular biology of bacterial luminescence. Microbiol. Rev. **55:**123–142.
20. **Mor, N., and L. Heifets.** 1993. MICs and MBCs of clarithromycin against *Mycobacterium avium* within human macrophages. Antimicrob. Agents Chemother. **37:**111–114.
21. **Rastogi, N., V. Labrousse, K. S. Goh, and J. P. de Sousa.** 1991. Antimycobacterial spectrum of sparfloxacin and its activities alone and in association with other drugs against *Mycobacterium avium* complex growing extracellularly and intracellularly in murine and human macrophages. Antimicrob. Agents Chemother. **35:**2473–2480.
22. **Raviglione, M. C., D. E. J. Snider, and A. Kochi.** 1995. Global epidemiology of tuberculosis—morbidity and mortality of a worldwide epidemic. JAMA **273:**220–226.
23. **Saito, H., H. Tomioka, K. Sato, M. Emori, T. Yamane, K. Yamashita, K. Hosoe, and T. Hidaka.** 1991. In vitro antimicrobial activities of newly synthesized benzoxazinorifamycins. Antimicrob. Agents Chemother. **35:**542–547.
24. **Sarkis, G. J., W. R. Jacobs, Jr., and G. F. Hatfull.** 1995. L5 luciferase reporter mycobacteriophages: a sensitive tool for the detection and assay of live mycobacteria. Mol. Microbiol. **15:**1055–1067.
25. **Sarmientos, P., J. E. Sylvester, S. Contente, and M. Cashel.** 1983. Differential stringent control of the tandem *E. coli* ribosomal RNA promoters from the *rrnA* operon expressed *in vivo* in multicopy plasmids. Cell **32:**1337–1346.
26. **Simpson, W. J., and J. R. M. Hammond.** 1991. The effect of detergents on firefly luciferase reactions. J. Biolumin. Chemilumin. **6:**97–106.
27. **Smee, D. F., R. A. Burger, J. Coombs, J. H. Huffman, and R. W. Sidwell.** 1991. Progressive murine cytomegalovirus disease after termination of ganciclovir therapy in mice immunosuppressed by cyclophosamide treatment. J. Infect. Dis. **164:**958–961.
27a. **Stover, C. K.** Unpublished results.
28. **Stover, C. K., G. P. Bansal, M. S. Hanson, J. E. Burlein, S. R. Palaszynski, J. F. Young, S. Koenig, D. B. Young, A. Sadziene, and A. G. Barbour.** 1992. Protective immunity elicited by recombinant BCG expressing OspA lipoprotein: a candidate lyme disease vaccine. J. Exp. Med. **178:**197–209.
29. **Stover, C. K., V. F. de la Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R. G. Barletta, W. R. Jacobs, and B. R. Bloom.** 1991. New use of BCG for recombinant vaccines. Nature (London) **351:**456–460.
30. **Truffot-Pernot, C., B. Ji, and J. Grosset.** 1991. Activities of pefloxacin and ofloxacin against mycobacteria: *in vitro* and mouse experiments. Tubercle **72:** 57–64.