ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1993, p. 1348–1352
0066-4804/93/061348-05$02.00/0

Vol. 37, No. 6

A Rapid Method for Screening Antimicrobial Agents for Activities against a Strain of *Mycobacterium tuberculosis* Expressing Firefly Luciferase

ROBERT C. COOKSEY,[1]* JACK T. CRAWFORD,[1] WILLIAM R. JACOBS, JR.,[2] AND THOMAS M. SHINNICK[1]

Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333,[1] and Howard Hughes Medical Institute, Albert Einstein College of Medicine, Bronx, New York 10461[2]

Received 17 February 1993/Accepted 14 April 1993

We developed a rapid method to screen the efficacy of antimicrobial agents against *Mycobacterium tuberculosis*. A restriction fragment carrying a promoterless firefly luciferase gene was cloned into a 4,488-bp shuttle vector, pMV261, and luciferase was expressed under the control of a mycobacterial heat shock promoter. The resulting plasmid, pLUC10, was introduced by electroporation into the avirulent strain *M. tuberculosis* $H_{37}Ra$. Luciferase assays of sonic lysates of Triton X-100-treated cells of *M. tuberculosis* $H_{37}Ra$(pLUC10) yielded bioluminescence in excess of 1,000 relative light units/$\sim 10^9$ tubercle bacilli, compared with 0.0025 for the same number of parental cells. A 48-h microdilution antimicrobial agent-screening assay using this strain was developed.

The number of reported cases of tuberculosis in the United States has shown a steady increase since 1988 (4). In 1991, 26,283 new cases, representing an increase of approximately 18% from 1985, were reported to the Centers for Disease Control (4). Outbreaks of tuberculosis caused by multidrug-resistant strains of *Mycobacterium tuberculosis*, especially among persons with human immunodeficiency virus infection, have also been reported (3, 10). These trends underscore the need for improved methods for detection, identification, and antimicrobial susceptibility testing of clinical isolates of this species, as well as for improved methods for identifying new antituberculosis drugs.

One potentially useful approach to determining drug activity against mycobacteria is based on the use of a firefly luciferase reporter system, which has already proved useful in studying the metabolic activity and control of gene expression in other organisms (1, 13). Luciferase, in the presence of ATP, Mg^{2+}, and O_2, oxidizes luciferin through an intermediate compound, luciferyl-AMP, to oxyluciferin, AMP, PP_i, CO_2, and light. A commercially available luciferase assay system (Promega Corp., Madison, Wis.) employs an alternative pathway involving luciferyl-coenzyme A to prevent self-inhibition of the enzyme and produce light emissions of longer duration. These emissions are proportional to the amount of luciferase in a sample and are measured with luminescence spectrophotometers or scintillation counters. When introduced into mycobacteria, the luciferase reporter gene may permit rapid assessment of growth or inhibition in the presence of antimicrobial agents. We constructed a plasmid containing a firefly luciferase gene under the transcriptional control of the promoter for *hsp60*, the gene for a mycobacterial heat shock protein, and transformed it into an avirulent strain of *M. tuberculosis*. We evaluated the production of light by the strain in the presence or absence of various antituberculosis drugs to develop a rapid screening test for in vitro evaluation of drugs potentially useful against *M. tuberculosis*.

MATERIALS AND METHODS

Strains and plasmids. Plasmid pLUC10 contains the mycobacterial origin of replication, the *Escherichia coli* origin of replication, and the kanamycin resistance gene from pMV261 (11), as well as the promoter and ribosome-binding site for the *M. tuberculosis* 65-kDa antigen from pTB12 (9) and the luciferase gene from pT3/T7-LUC (Clontech Laboratories, Palo Alto, Calif.). To construct pLUC10, pTB12 was first cleaved with *Bal*I; *Hin*dIII linkers were added and then cleaved with *Hin*dIII and self-ligated to generate pTB29. The *hsp60* promoter was then isolated as a 600-bp *Kpn*I-*Hin*dIII fragment from pTB29 and inserted into *Kpn*I-*Hin*dIII-cleaved pMV261 to generate pTB30. The 1.9-kbp *Hin*dIII fragment from pT3/T7-LUC was then cloned into *Hin*dIII-digested pTB30. Finally, a clone carrying the luciferase gene in the proper orientation relative to the *hsp60* promoter was designated pLUC10 (Fig. 1). This plasmid (~6,400 bp) was propagated in *E. coli* XL1. *M. tuberculosis* $H_{37}Ra$ (ATCC 25177) was obtained from the Centers for Disease Control Mycobacteriology Laboratory stock collection. This strain is avirulent, immunogenic, and susceptible to all of the antimicrobial agents used in this study as evaluated by the standard method of proportion (7).

Plasmid preparation and electroporation. Plasmid DNA was isolated from *E. coli* XL1(pLUC10) by using an extraction column (Qiagen-tip 100) and the manufacturer's protocol (Qiagen Inc., Chatsworth, Calif.). *M. tuberculosis* $H_{37}Ra$ was cultivated in 25 ml of Middlebrook 7H9 broth with 10% Middlebrook ADC enrichment (complete Middlebrook 7H9 broth; GIBCO Laboratories, Madison, Wis.) for 21 days at 37°C before harvesting by centrifugation for 20 min at ~3,400 × *g* and 4°C. Cells were washed twice in cold 10% glycerol, resuspended in 50 μl of the same solution, and combined with 5 μg of plasmid in 5 μl of reagent grade water. The number of bacteria was estimated to be ~25 × 10^8 by

* Corresponding author.

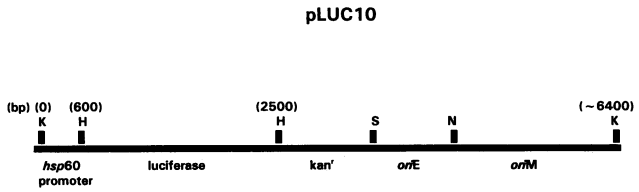

FIG. 1. Map of plasmid pLUC10 linearized at the unique *Kpn*I site. H, *Hin*dIII; S, *Spe*I; N, *Not*I; K, *Kpn*I.

the formula of David (5), which assumes that the wet weight of 10^8 tubercle bacilli is 1 mg. Electroporation was performed with a BTX Electro Cell Manipulator 600 (Biotechnologies and Experimental Research, Inc., San Diego, Calif.) equipped with a disposable cuvette chamber (0.2-cm gap). Instrument settings were 2.5 kV and 129 Ω. Electroporated cells were incubated for 16 h at 37°C in 5 ml of complete Middlebrook 7H9 broth before plating on Dubos Oleic Acid Agar (Difco Laboratories, Detroit, Mich.) containing 10 μg of kanamycin per ml. Colonies appeared after 25 to 30 days of incubation at 37°C. Species identification was confirmed by high-performance liquid chromatography (HPLC) (2). Plasmids were isolated from the transformants by the method of Kado and Liu (6), by using cells cultivated for 14 days in 50 ml of complete Middlebrook 7H9 broth and treated with 1 mg of D-cycloserine per ml for 16 h before harvesting.

Luciferase assays. Ten milliliters of stationary-phase (21-day) culture in Middlebrook complete 7H9 broth (supplemented with 0.05% Tween 80 and 10 μg of kanamycin per ml) was centrifuged (12,000 × *g*, 10 min, 4°C), and 10 mg of the pellet was resuspended in 1 ml of the same broth to give a concentration of ~10^9 tubercle bacilli. Tenfold dilutions of these suspensions were made in broth, and 1 ml of each was centrifuged (12,000 × *g*, 4°C, 10 min) in 1.5-ml conical polypropylene tubes. The pellets were washed in phosphate-buffered saline (pH 7.2) and resuspended in 100 μl of a detergent lysis buffer (1% Triton X-100, 25 mM Tris [pH 7.8 with H_3PO_4], 10 mM trans-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid (CDTA), 10 mM dithiothreitol, 50% glycerol) supplied in a commercial luciferase assay system (Promega Corp.). These suspensions were sonicated for 5 min (5-s cycles at a 60% pulse) with a 431A cuphorn attachment on a W-380 cell disruptor (Heat Systems-Ultrasonics, Farmingdale, N.Y.), cooled to 0°C with a circulating solution of 50% methanol, and then dispensed into 96-well microdilution plates (flat bottom, white; Microlite 2; Dynatech Laboratories, Inc., Chantilly, Va.). Direct freeze-thaw microdilution plate lysis was performed in a similar manner, except that 100 μl of the unwashed cell dilutions, followed by 100 μl of Promega lysis buffer, was placed directly into microdilution plates, which were incubated at −70°C for 30 min, and then thawed at room temperature. Lysates were tested for luciferase activity by adding 50 μl of Promega Luciferase Assay Substrate (470 μM luciferin, 270 μM coenzyme A [lithium salt], 530 μM ATP) and immediately measuring bioluminescence in an ML 1000 Luminometer (Dynatech) at 20°C by using the minimum (2-s) instrument delay. The mean value of 15 cycles in relative light units (RLU) was recorded since no increase in bioluminescence was detectable in additional cycles; however, the correlation of RLU to photons emitted by the luciferase reaction is nonlinear.

Susceptibility testing. Microdilution susceptibility testing was performed in clear 96-well polystyrene microdilution plates by the method of Wallace et al. (12), with twofold dilutions of an antimicrobial agent in complete Middlebrook 7H9 broth containing 0.05% Tween 80. Rifabutin was obtained from Adria Pharmaceuticals, Inc., Columbus, Ohio; all other antimicrobial agents were obtained from Sigma Chemical Company, St. Louis, Mo. The drugs (50 μl) were dispensed into the plates, followed by the inocula (50 μl), which were prepared from stationary-phase cultures diluted 10^{-2} or 10^{-4} in fresh broth, yielding approximately 10^5 or 10^3 bacilli per 50 μl. Plates were sealed in plastic bags and incubated at 37°C for 21 days. Luciferase susceptibility tests were performed on replicate samples at three different times with only the smaller inoculum (10^3 bacilli) in Microlite 2 plates (Dynatech). Luciferase assays were performed by the freeze-thaw lysis method as described above, at 2, 7, and 14 days of incubation. Macrodilution MIC testing (8) was performed by using twofold drug dilutions in 5 ml of complete Middlebrook 7H9 broth with 0.05% Tween 80. Cultures were inoculated with 10^3 bacilli. The MIC was defined as the lowest drug concentration for which no visible growth was observed after 21 days.

RESULTS

A map of plasmid pLUC10 linearized at its unique *Kpn*I site is shown in Fig. 1. The 1,900-bp *Hin*dIII fragment containing the firefly luciferase gene and the *hsp60* promoter was inserted into shuttle vector pMV261 (Fig. 1). Approximately 2,500 kanamycin-resistant colonies were obtained 21 days after plating of ~25 × 10^8 cells of *M. tuberculosis* $H_{37}Ra$ electroporated with 5 μg of plasmid pLUC10. Four typical colonies were selected for further evaluation. Cultures of each displayed the same mycolic acid pattern as the

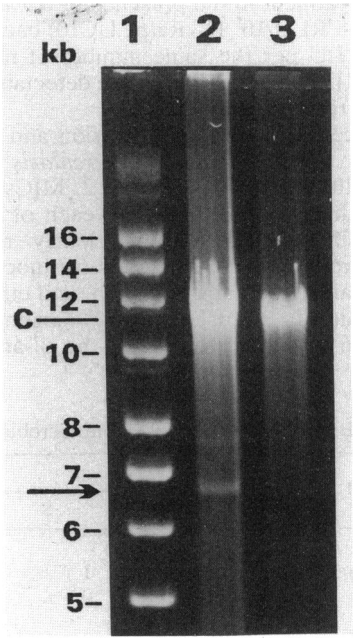

FIG. 2. Plasmid profile of *M. tuberculosis* $H_{37}Ra$(pLUC10). Miniplasmid preparations (6) were electrophoresed in a 1% agarose–Tris–borate–EDTA horizontal gel for 6 h at 80 V. Size standards (supercoiled DNA, lane 1) are shown in approximate kilobase pairs (kb) on the left. Lanes: 2, $H_{37}Ra$(pLUC10); 3, $H_{37}Ra$. The arrow indicates plasmid pLUC10. C, chromosome.

TABLE 1. Bioluminescence produced by *M. tuberculosis* $H_{37}Ra$(pLUC10)

Lysis method	Bioluminescence (RLU) produced by the following no. of bacilli[a]							
	10^9	10^8	10^7	10^6	10^5	10^4	10^3	10^2
Sonication	>1,000[b]	120	9.3	0.94	0.10	0.013	0.002	0.002
Freeze-thaw	14.5	2.3	0.8	0.12	0.07	0.005	0.000	0.000

[a] Numbers of bacilli were estimated with the formula 10^9 tubercle bacilli = ~10 mg (5). The bioluminescence of parent strain $H_{37}Ra$ was ≤0.0025 RLU with both lysis methods, regardless of the estimated number of bacilli.
[b] Maximum instrument reading.

parent strain by HPLC analyses. Each was shown to contain an extrachromosomal DNA band equal in size to plasmid pLUC10 (Fig. 2).

After 14 days of culture in complete Middlebrook 7H9 broth containing 10 μg of kanamycin per ml, cells from 10 ml (~10^9 bacilli) were harvested, washed in cold phosphate-buffered saline, suspended in 100 μl of lysis buffer, and sonicated. When these lysates were tested for luciferase activity, >1,000 RLU/10^9 bacilli was found in each of the four samples. These values were ≥4 × 10^5-fold greater than those for the naive recipient strain (0.0025 RLU/10^9 bacilli). Typical results for one strain are shown in Table 1. Luciferase activity in sonicated samples was proportional to cell concentration in samples containing as few as 10^4 bacilli but was not distinguishable from parental background values (0.0020 RLU/10^4 bacilli) below this cell concentration (Table 1). Lysates were prepared from duplicate samples of the cell dilutions stored for 14 days at −70°C, and luciferase activity varied by no more than ±5% from the initial values for any of the dilutions. Variation in RLU among the 15 cycles of luminometer readings was ≤5% for all of the samples tested. Nonspecific luminescence (i.e., in controls containing no bacilli) ranged from 0 to 5 × 10^{-4} RLU.

Lysis of cells in microdilution plate wells by −70°C freeze-thaw treatment in the presence of 0.5% Triton X-100 resulted in 14.5 RLU/10^9 $H_{37}Ra$(pLUC10) bacilli, compared with 0.0025 RLU per the same number of naive recipient bacilli (Table 1). Light emissions were detectable with as few as 10^4 bacilli treated in this manner.

Results of conventional macrodilution and microdilution susceptibility testing of *M. tuberculosis* $H_{37}Ra$ and $H_{37}Ra$(pLUC10) are shown in Table 2. MICs varied by no more than 1 drug concentration for each of the six drugs tested by both methods. As previously reported (12), however, microdilution MICs varied by inoculum size for three drugs (kanamycin, streptomycin, and ethambutol). As expected, strain $H_{37}Ra$(pLUC10) was kanamycin resistant (MIC, 32 to 64 μg/ml) because of the 3′-aminoglycoside phosphotransferase production encoded on plasmid pLUC10.

Luciferase susceptibility testing of nine drugs was performed by the freeze-thaw lysis method (Table 3). Bioluminescence readings after 2, 7, and 14 days were <20 × 10^{-4} RLU for the lowest concentration tested (0.06 μg/ml) for streptomycin, isoniazid, rifabutin, and rifampin. For the remaining five compounds, bioluminescence readings sharply decreased at higher drug concentrations that closely correlated with MICs determined by the conventional susceptibility testing methods, indicating that the luciferase method can be used to measure the inhibitory activity of these compounds.

DISCUSSION

The shuttle vector used in this study, pMV261, was constructed by Stover et al. (11) to facilitate the expression of foreign antigens in *M. bovis* BCG. We used this vector to express a firefly luciferase reporter gene in *M. tuberculosis* $H_{37}Ra$. Bioluminescence by this strain, which contains plasmid pLUC10, was at least 4 × 10^5-fold greater than that of the naive parent strain when ~10^9 cells of each, lysed by sonication, were tested. Luciferase activity was also measured when cells of *M. tuberculosis* $H_{37}Ra$ were lysed directly in microdilution plates by detergent and freeze-thaw treatments; however, less luciferase activity was detected when cells were treated in this manner. The freeze-thaw method nonetheless enabled our development of a direct microdilution plate bioluminescence method for measurement of antimicrobial agent activity against *M. tuberculosis* $H_{37}Ra$(pLUC10). With the luciferase assay, drug activities that were parallel to MICs determined by conventional methods were demonstrated within 48 h. The time required for demonstration of activity may vary as a result of the drug's mode of action. Drugs, such as rifampin, that shut off transcription and immediately block further synthesis of luciferase should show an effect after a short incubation

TABLE 2. Comparison of antimicrobial susceptibility test results for *M. tuberculosis* $H_{37}Ra$ and $H_{37}Ra$(pLUC10)

Strain and method	MIC[a] (μg/ml) of:							
	K	S	INH	CS	RBT	EMB	RIF	CIP
$H_{37}Ra$								
Macrodilution	4	1	≤0.06	4	NT	2	≤0.03	NT
Microdilution	2 (4)	0.5	≤0.06	4	1	1 (2)	≤0.03	0.12
$H_{37}Ra$(pLUC10)								
Macrodilution	64	0.5	≤0.06	4	NT	2	≤0.03	NT
Microdilution	32 (64)	0.25 (0.5)	≤0.06	4	0.5	1 (2)	≤0.03	0.12

[a] MICs in parentheses were obtained with 10^5 bacilli as the inoculum compared with the standard 10^3 bacilli if MICs were not the same for both inocula. Abbreviations: K, kanamycin; S, streptomycin; INH, isoniazid; CS, D-cycloserine; RBT, rifabutin; EMB, ethambutol; RIF, rifampin; CIP, ciprofloxacin; NT, not tested.

TABLE 3. Bioluminescence of *M. tuberculosis* $H_{37}Ra$(pLUC10) in the presence of antituberculosis drugs

Antimicrobial agent and time (days)	Bioluminescence (RLU, 10^{-4})[a] at drug concn (μg/ml) of:											
	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	≥128
Kanamycin												
2	NT	80	84	78	77	65	75	52	8	1	1	1
7	NT	180	221	156	99	150	102	81	32	0	0	NT
14	NT	1,213	656	1,960	2,039	2,153	871	1,282	3,099	3,530	16	NT
Streptomycin												
2	NT	4	1	1	0	1	1	0	1	1	2	NT
7	NT	4	0	0	1	0	2	0	0	0	0	NT
14	NT	8	6	3	4	3	1	1	7	3	0	NT
Isoniazid												
2	6	0	0	3	2	4	1	3	1	2	NT	NT
7	4	4	3	0	1	2	0	0	0	0	NT	NT
14	7	1	1	2	0	2	7	0	1	2	NT	NT
Cycloserine												
2	NT	NT	NT	NT	NT	32	17	5	3	5	9	2
7	NT	NT	NT	NT	NT	133	33	31	7	6	6	3
14	NT	NT	NT	NT	NT	583	20	6	0	4	7	4
Rifabutin												
2	7	7	2	1	0	1	7	0	1	NT	NT	NT
7	6	4	6	0	2	2	1	1	0	NT	NT	NT
14	9	2	0	2	0	3	13	0	11	NT	NT	NT
Ethambutol												
2	NT	NT	60	21	17	0	0	1	0	0	0	0
7	NT	NT	117	121	38	4	2	0	1	1	0	0
14	NT	NT	1,194	24	19	0	4	1	0	0	3	0
Ethionamide												
2	NT	NT	44	9	1	0	0	1	0	1	0	0
7	NT	NT	100	30	2	1	1	2	0	0	0	0
14	NT	NT	285	8	1	0	0	0	0	1	2	0
Rifampin												
2	0	1	1	1	0	2	0	0	0	NT	NT	NT
7	2	1	0	1	0	1	0	0	0	NT	NT	NT
14	1	1	3	0	0	0	1	0	0	NT	NT	NT
Ciprofloxacin												
2	101	17	0	1	2	0	1	0	0	NT	NT	NT
7	144	39	2	2	1	2	0	1	0	NT	NT	NT
14	485	89	1	3	0	1	0	6	4	NT	NT	NT

[a] Control values (no drug present) 85 × 10^{-4} (2 days), 222 × 10^{-4} (7 days), and 3,055 × 10^{-4} (14 days) RLU. NT, not tested.

period. In contrast, drugs that do not directly interfere with luciferase activity, such as those that block cell wall synthesis, may require longer incubation before loss of luciferase activity is detectable. Although avirulent strain *M. tuberculosis* $H_{37}Ra$ was used in the present study, the luciferase plasmid has been introduced into virulent strains of *M. tuberculosis* as well. Use of strains resistant to a single drug will allow assessment of cross-resistance, for example, screening of rifampin derivatives against a rifampin-resistant strain.

Most of the drugs currently prescribed for treatment of tuberculosis have been used for many years. The standard treatment regimen consists of isoniazid, rifampin, pyrazinamide, and ethambutol. Various second-line drugs may be added to the regimen if studies on the initial isolates indicate resistance. The increasing problem of drug resistance has heightened interest in development of new antituberculosis agents. Isolates of *M. tuberculosis* resistant to six or more drugs have been encountered, leaving physicians with few choices of active drugs and no drugs with the efficacy of isoniazid or rifampin.

Development of new drugs has been hampered by the lack of a simple method for screening of compounds for activity against *M. tuberculosis*. Routine methods using solid media or radiometric procedures are satisfactory for testing of small numbers of highly promising agents but are not suitable for large-scale screening of compounds, many of which are available only in very small quantities. The luciferase assay holds great promise in this regard, since the assay uses very small drug quantities. Initial screening for activity does not require an accurate MIC, and only a few concentrations of each compound need to be 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.

ACKNOWLEDGMENTS

We thank the following employees of the Respiratory Diseases Branch, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, CDC: from the Mycobacteriology Laboratory, Ray Butler and James Kilburn for performance of HPLC analyses and Charlotte Hale-Smith and Glenn Morlock for plasmid analyses and luminometer assays; from the Hansen's Disease Laboratory, Morris Sammons for plasmid analyses; and Robert Good, Branch Chief, for assistance in preparation of the manuscript. We also thank Kendall Stover of MedImmune, Inc., Gaithersburg, Md., for supplying plasmid pMV261.

REFERENCES

1. **Alam, J., and J. L. Cook.** 1990. Reporter genes: application to the study of mammalian gene transcription. Anal. Biochem. **188:**245–254.
2. **Butler, W. R., and J. O. Kilburn.** 1988. Identification of major slowly growing pathogenic mycobacteria and *Mycobacterium gordonae* by high-performance liquid chromatography of their mycolic acids. J. Clin. Microbiol. **26:**50–53.
3. **Centers for Disease Control.** 1991. Nosocomial transmission of multidrug-resistant tuberculosis among HIV-infected persons—Florida and New York, 1988–1991. Morbid. Mortal. Weekly Rep. **40:**585–591.
4. **Centers for Disease Control.** 1992. Tuberculosis morbidity—United States: final data, 1991. Morbid. Mortal. Weekly Rep. **41:**240.
5. **David, H. L.** 1976. Laboratory diagnostic procedures, p. 159–162. *In* Bacteriology of the mycobacterioses. U.S. Department of Health, Education, and Welfare, Atlanta, Ga.
6. **Kado, C. I., and S.-T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
7. **Kent, P. T., and G. P. Kubica.** 1985. Public health mycobacteriology: a guide for the level III laboratory. U.S. Department of Health and Human Services publication no. 86-8230, p. 159–184. U.S. Department of Health and Human Services, Washington, D.C.
8. **Sahm, D. F., and J. A. Washington.** 1991. Antibacterial susceptibility tests: dilution methods, p. 1105–1116. *In* A. Balows, W. J. Hausler, Jr., K. L. Herrmann, H. D. Isenberg, and H. J. Shadomy (ed.), Manual of clinical microbiology, 5th ed. American Society for Microbiology, Washington, D.C.
9. **Shinnick, T. M.** 1987. The 65-kilodalton antigen of *Mycobacterium tuberculosis*. J. Bacteriol. **169:**1080–1088.
10. **Snider, D. E., Jr., G. M. Cauthen, L. S. Farer, G. D. Kelly, J. O. Kilburn, R. C. Good, and S. W. Dooley.** 1991. Drug-resistant tuberculosis. Am. Rev. Respir. Dis. **144:**732.
11. **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, Jr., and B. R. Bloom.** 1991. New use of BCG for recombinant vaccines. Nature (London) **351:**456–460.
12. **Wallace, R. J., Jr., D. R. Nash, L. C. Steele, and V. Steingrube.** 1986. Susceptibility testing of slowly growing mycobacteria by a microdilution MIC method with 7H9 broth. J. Clin. Microbiol. **24:**976–981.
13. **Williams, T. M., J. E. Burlein, S. Ogden, L. J. Kricka, and J. A. Kant.** 1989. Advantages of firefly luciferase as a reporter gene: application to the interleukin-2 gene promoter. Anal. Biochem. **176:**28–32.