

## Construction of a Bioluminescent Mycobacterium and Its Use for Assay of Antimycobacterial Agents

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Received 1 March 1993/Returned for modification 17 April 1993/Accepted 20 May 1993

To show, as a model system, that mycobacteria can express heterologous luciferase genes and that bioluminescence can be a rapid method of measuring antimycobacterial activity, a bioluminescent form of *Mycobacterium smegmatis* was made by transformation with a *Mycobacterium-Escherichia coli* shuttle vector containing the *luxAB* genes from *Vibrio harveyi*. The antimycobacterial effects of antibiotics and biocides could be assayed in real time by using bioluminescent *M. smegmatis*.

Diseases caused by pathogenic mycobacteria are a major cause of morbidity and mortality in humans and animals. *Mycobacterium tuberculosis* alone accounts for over 3 million deaths annually (12). Mycobacterial diseases are an increasing public health problem worldwide. Cases of disease caused by *M. tuberculosis* and by nontuberculous mycobacteria are increasing in association with the increase in the numbers of immunocompromised individuals (4, 19). A part of any program for the control of mycobacterial diseases is the use of chemotherapeutic drugs and biocides to resolve infection and to prevent cross-infection.

There is a need to identify new drugs for use in the chemotherapy of mycobacterial diseases. Chemotherapy for the control of nontuberculous mycobacteria is inadequate (1). Current regimens for the treatment of disease caused by *M. tuberculosis*, although effective, still require extended periods of time and can be associated with toxicity (3). In addition, concern has been expressed about the increasing emergence of multi-drug-resistant *M. tuberculosis* (8, 20).

Biocides are widely used for ridding heat-labile surgical instruments, such as bronchoscopes, of mycobacteria (6). Routine monitoring of the efficacies of these procedures should be done. Furthermore, mycobacteria are one of the panel of microorganisms used during the evaluation of new biocides (2).

A drawback in assaying antimycobacterial activity is the slow growth rate of pathogenic mycobacteria, which means that methods that are reliant on measuring the growth of bacteria are time-consuming. A method that could measure antimycobacterial activity faster and that could be amenable to automation would be an obvious improvement.

Bioluminescence, which is generated by a luciferase enzyme, has been shown before to be a sensitive, real-time measure of cell viability and can be used to assay the activities of antibacterial agents as they occur, i.e., in real time (17). We investigated whether mycobacteria can express a bioluminescent phenotype and if the use of bioluminescent mycobacteria could quicken assays of drugs and biocides. As a model, we chose to do this by constructing a recombinant *Mycobacterium smegmatis* isolate containing the *luxA* and *luxB* genes from *Vibrio harveyi* (13).

### MATERIALS AND METHODS

**Bacteria.** *Escherichia coli* TG1 (15) was maintained on Luria agar, and for use, it was grown in Luria broth. Transformed *E. coli* isolates were selected on Luria agar containing 15 µg of kanamycin ml<sup>-1</sup>.

*M. smegmatis* mc<sup>2</sup>155 (16) was a gift from W. R. Jacobs, Albert Einstein College of Medicine, New York, N.Y. It was maintained on Lowenstein-Jensen medium and was grown in Middlebrook 7H9 broth (Difco) containing albumin-dextrose complex enrichment (Difco) and 0.05% Tween 80. Transformed *M. smegmatis* isolates were selected on Middlebrook 7H11 agar (Difco) containing oleic-albumin-dextrose complex supplement (Difco) and 15 µg of kanamycin ml<sup>-1</sup>.

**Plasmids.** The *E. coli-Mycobacterium* shuttle vector pMV261 (18) was obtained from C. K. Stover, MedImmune Inc., Gaithersburg, Md. The vector was propagated in *E. coli* TG1 grown in Luria broth containing 15 µg of kanamycin ml<sup>-1</sup>.

The genes for the two structural subunits of *V. harveyi* luciferase were contained in the plasmid pSB226 (10), which was obtained as a gift from G. S. A. B. Stewart, University of Nottingham, Nottingham, United Kingdom. This plasmid was propagated in *E. coli* TG1 grown in Luria broth containing 100 µg of ampicillin ml<sup>-1</sup>.

**Manipulation of DNA.** Large-scale preparations of plasmids from *E. coli* were made by the lithium chloride-polyethylene glycol precipitation method described previously (15).

Plasmid DNA was introduced into transformation-competent *E. coli* as described previously (15). *M. smegmatis* was transformed by the electroporation method of Snapper and coworkers (16). Transformants were first selected by growth on kanamycin. Bioluminescent transformants were identified by visual identification after exposure of colonies to vapor of the luciferase substrate decyl aldehyde (Aldrich). Identification was confirmed by assay of bioluminescence.

**Assay of bioluminescence.** Bioluminescence was measured in a Lab-Line ATP photometer. The photometer was calibrated with [1-<sup>14</sup>C] hexadecane as described by Hastings and Weber (9). Bacteria were grown to the mid-log phase in the appropriate broth. Bioluminescence was measured for 6 s at 10 s after the addition of 10 µl of 1% (vol/vol) decyl aldehyde in ethanol to 400 µl of bacterial culture.

**Assay of antimycobacterial activity.** Solutions of antibiotics and biocides were prepared in distilled water immediately before use. Bioluminescent *M. smegmatis* was grown to the

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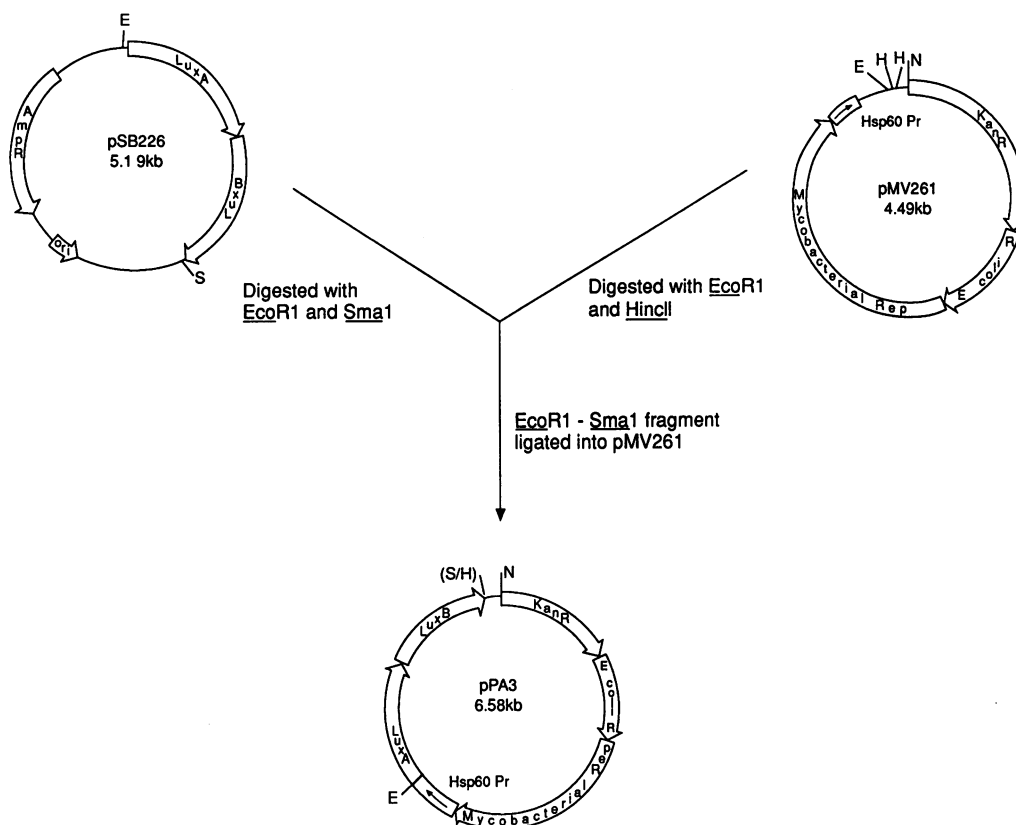


FIG. 1. Construction of the shuttle vector pPA3 containing the luciferase genes *luxA* and *luxB*. Plasmid pSB226 contains the *luxA* and *luxB* genes from *V. harveyi* (18). Plasmid pMV261 (15) has a Tn903-derived gene conferring kanamycin resistance (Kan<sup>r</sup>), an *E. coli* origin of replication (E coli R), a mycobacterial origin of replication (Mycobacterial Rep), and the 5' regulatory region of the *M. bovis* BCG *hsp60* gene (Hsp60Pr). Restriction endonuclease sites are abbreviated as follows: E, *Eco*R1; H, *Hinc*II; S, *Sma*I; N, *Nhe*I.

mid-log phase in 7H9 broth containing ADC enrichment (Difco), 0.05% Tween 80, and 15  $\mu\text{g}$  of kanamycin  $\text{ml}^{-1}$ . For assay of biocides, samples of the culture were mixed with an equal volume of double-strength biocide solution. For assay of antibiotics, 9.8 ml of culture was mixed with 0.2 ml of antibiotic solution (7). Control samples were diluted in water. The samples were incubated at 37°C for 10 min with biocides and 24 h with antibiotics before a 0.5-ml sample was removed. Samples with biocides were mixed with 0.5 ml of appropriate neutralizer (14): 2% (vol/vol) Tween 80 for phenol and 2.5% (vol/vol) lenth broth for hititane. Some workers (5) have used 1% (wt/vol) fresh sodium bisulfite to neutralize glutaraldehyde when working with mycobacteria. However, we found this to be toxic to *M. smegmatis*. Therefore, in experiments with glutaraldehyde, samples were diluted in water only at the end of the assay. Samples containing antibiotics were not neutralized. After neutralization, when appropriate, a portion of the sample was taken for measurement of bioluminescence and 50  $\mu\text{l}$  was serially diluted in water (11) before plating onto 7H11 agar containing OADC supplement and 15  $\mu\text{g}$  of kanamycin  $\text{ml}^{-1}$ .

## RESULTS

**Construction of a bioluminescent *M. smegmatis*.** The *Mycobacterium-E. coli* shuttle plasmid pMV261 (18) was chosen as a vector. It contains the Tn903-derived *aph* gene which confers kanamycin resistance in mycobacteria, the promoter from the gene for the mycobacterial heat shock protein

Hsp60, and a transcription terminator (18). The *luxA* and *luxB* genes were isolated on a 2.2-kb DNA fragment after digestion of pSB226 with *Eco*R1 and *Sma*I (Fig. 1). The *Eco*R1-*Sma*I fragment was then ligated with pMV261 that had been digested with *Eco*R1 and *Hinc*II (Fig. 1), and the DNA was used to transform *E. coli* TG1. Plasmid DNAs were extracted from 12 bioluminescent transformants and were analyzed by restriction enzyme digestion and agarose electrophoresis. One plasmid, pPA3, which consisted of pMV261 containing the *luxA* and *luxB* genes downstream of the mycobacterial promoter was then used to transform *M. smegmatis* mc<sup>2</sup>155.

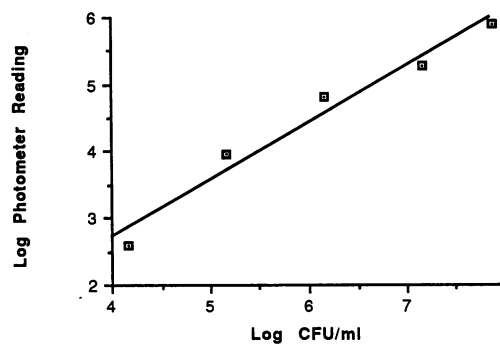


FIG. 2. Relationship between luminescence and number of *M. smegmatis* (pPA3). Results are means of three experiments.

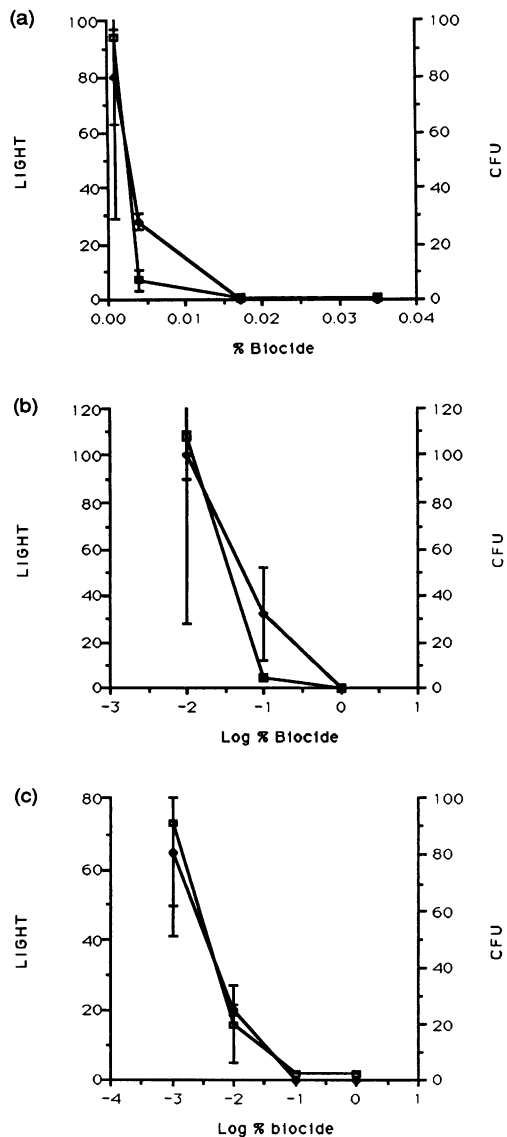


FIG. 3. Effects of biocides on the viability and luminescence of bioluminescent *M. smegmatis*. *M. smegmatis* was incubated for 10 min with hhibitane (a), phenol (b), or glutaraldehyde (c) before dilution in neutralizer and subsequent assay for luminescence and viability. Luminescence (□) and viability (◆) are given as percentages of those measured in the absence of biocide. Data are the means, with standard deviations, of three experiments.

Bioluminescent *M. smegmatis* transformants were identified by visual inspection of colonies in the dark. Colonies were luminescent only after the addition of the substrate decyl aldehyde to the lids of the petri dishes. Bioluminescence was confirmed by measurement in an ATP luminometer. The specific luminescences of transformants and controls were determined to be 134 and 0.2 quanta  $\cdot$  s $^{-1}$   $\cdot$  cell $^{-1}$ , respectively.

As shown in Fig. 2, there was a linear relationship between the extent of luminescence and the viability of *M. smegmatis*. These data suggest that bioluminescence could be suitable as a measure of viability in mycobacteria.

**Use of luminescence to measure killing of *M. smegmatis*.** The suitability of bioluminescence for assaying the antimicrobial

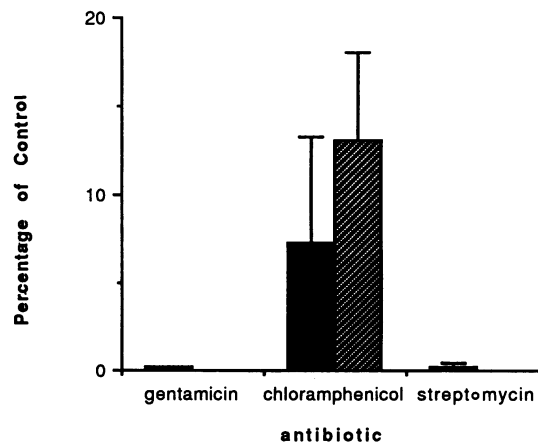


FIG. 4. Effects of antimicrobial drugs on the viability and luminescence of bioluminescent *M. smegmatis*. *M. smegmatis* was incubated for 24 h with 2.5  $\mu$ g of gentamicin ml $^{-1}$ , 25  $\mu$ g streptomycin ml $^{-1}$ , or 25  $\mu$ g chloramphenicol ml $^{-1}$  before assaying for luminescence (filled column) and viability (hatched column). Luminescence and viability are given as percentages of those measured in the absence of antibiotic. Data are the means, with standard deviations, of three experiments.

effects on mycobacteria was assessed with biocides and antibiotics and with *M. smegmatis*.

Figure 3 shows the effects of phenol, hhibitane, and glutaraldehyde on the luminescence and viable count on the luminescent *M. smegmatis*. In each case, the decrease in CFU with time paralleled the decrease in luminescence with increasing biocide concentration.

A similar picture was seen with the chemotherapeutic agents (Fig. 4). Gentamicin at 2.5  $\mu$ g ml $^{-1}$  and 25  $\mu$ g of streptomycin ml $^{-1}$  caused a total loss of viability over 24 h. There was a concomitant absence of luminescence. With 25  $\mu$ g of chloramphenicol ml $^{-1}$ , there was an 87% decrease in cell viability over 24 h. This was accompanied by a decrease in bioluminescence of 93%.

## DISCUSSION

The data presented here show that mycobacteria can express luciferase genes and that the product of this expression can be used to assay antimycobacterial agents rapidly. The bioluminescence of the recombinant *M. smegmatis* appears to be a true reflection of the viability of that culture, and as such, it is suitable for use in assaying the antimycobacterial activities of drugs and biocides. The crucial difference between this method and methods that are reliant on mycobacterial growth is that results with bioluminescence are obtained in real time, whereas 2 days must elapse before viability can be measured. The conclusion of the present study is not that a bioluminescent *M. smegmatis* isolate is the ideal tool for assessment of antimicrobial activity against all species of mycobacteria. Clearly, individual mycobacterial species will differ in their susceptibilities. What the data do suggest is that efforts to make bioluminescent forms of slow-growing mycobacteria will be worthwhile since the time savings in evaluation of the antimycobacterial activities of biocides and drugs will be great.

## ACKNOWLEDGMENTS

P. W. A. was in receipt of a Science Research Fellowship from The Nuffield Foundation.

We thank G. S. A. B. Stewart, University of Nottingham, for helpful discussions.

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