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

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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.

**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⁻¹.

*M. smegmatis* mc²155 (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⁻¹.

**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⁻¹.

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⁻¹.

**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 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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mid-log phase in 7H9 broth containing ADC enrichment (Difco), 0.05% Tween 80, and 15 μg of kanamycin 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) lethen broth for hibitane. 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 μl was serially diluted in water (11) before plating onto 7H11 agar containing OADC supplement and 15 μg of kanamycin 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 EcoRI and SmaI (Fig. 1). The EcoRI-SmaI fragment was then ligated with pMV261 that had been digested with EcoRI and HincII (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 mc²155.

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 (Kanr), 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, EcoRI; H, HincII; S, SmaI; N, NheI.

FIG. 2. Relationship between luminescence and number of M. smegmatis (pPA3). Results are means of three experiments.
Bioluminescent M. smegmatis transformants were identified by visual inspection of colonies in the dark. Colonies were luminous 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·s⁻¹·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 effects on mycobacteria was assessed with biocides and antibiotics with M. smegmatis.

Figure 3 shows the effects of phenol, hibitane, 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 µg ml⁻¹ and 25 µg of streptomycin ml⁻¹ caused a total loss of viability over 24 h. There was a concomitant absence of luminescence. With 25 µg 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.

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