ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Mar. 1995, p. 754–756 Vol. 39, No. 3
0066-4804/95/$04.00+0


# Bioluminescence Method To Evaluate Antimicrobial Agents against *Mycobacterium avium*

ROBERT C. COOKSEY,[1]* GLENN P. MORLOCK,[1] MARJORIE BEGGS,[2] AND JACK T. CRAWFORD[1]

*Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia,[1] and Medical Research Service, McClellan Veterans Hospital, Little Rock, Arkansas[2]*

Received 28 September 1994/Returned for modification 14 November 1994/Accepted 12 December 1994

**Plasmid pLUC10, carrying the firefly luciferase gene, was transformed by electroporation into *Mycobacterium avium* A5. Bioluminescence production by strain A5(pLUC10), as measured in a microdilution plate luminometer, was ~1 relative light unit per $2 \times 10^6$ viable bacilli, whereas it was 0.0005 relative light unit for an equal number of parental cells. The susceptibility of strain A5(pLUC10) to eight concentrations of each of eight antimicrobial agents was evaluated by the luciferase microplate assay in parallel with a conventional broth macrodilution method with antimicrobial agents. Decreases in bioluminescence to levels that were ≤10% of those of drug-free controls were observed in microplate wells containing inhibitory concentrations of drugs in as few as 3 days. The close correlation of these inhibitory concentrations with the MICs determined by a conventional broth macrodilution method suggests that the luciferase microplate method may offer a convenient and reliable means of evaluating the in vitro activities of antimicrobial agents against the *M. avium* complex.**

*Mycobacterium avium* complex (MAC) organisms are ubiquitous in the environment and are more frequently associated with human disease than are other nontuberculous mycobacteria (4). Disseminated MAC infections are now recognized as substantial contributors to both morbidity and mortality in AIDS patients and may become an inevitable complication in AIDS patients who do not succumb to other opportunistic infections (3, 7). Recommended treatment regimens include ethambutol and a newer macrolide (azithromycin or clarithromycin) used in conjunction with clofazimine, rifabutin, ciprofloxacin, or amikacin (4, 6). However, successful treatment of established MAC infections in immunocompromised patients is difficult.

Considering the need for improved therapies for disseminated MAC infection, novel methods for in vitro screening of antimicrobial drugs against MAC may be warranted. We previously described the expression of firefly luciferase in the *Mycobacterium tuberculosis* type strain H37Ra after insertion of the luciferase plasmid pLUC10 and development of a luciferase assay for in vitro screening of antituberculosis drugs (1). More recently, we have streamlined the screening procedure by eliminating the need for cell lysis prior to the addition of the enzyme substrate luciferin (our unpublished data).

As a component of our continuing development of the luciferase microplate screen for antimycobacterial drug efficacies, we have introduced the plasmid carrying the firefly luciferase gene, plasmid pLUC10, into an isolate of *M. avium* (designated strain A5) from an AIDS patient. This strain was used to evaluate the usefulness of the luciferase assay for screening antimicrobial agents in vitro against MAC organisms.

**Strains and plasmid.** *M. avium* A5 was obtained from Tobin Hellyer. The strain was isolated from the blood specimen of an AIDS patient in the United Kingdom (2). The strain lacks plasmids, and testing at the Centers for Disease Control and Prevention showed the strain to be serovar 4. Colonies of A5 grown on Dubos oleic acid agar (Difco Laboratories, Detroit, Mich.) were uniformly opaque and smooth edged. The construction, propagation, and isolation of the firefly luciferase shuttle plasmid pLUC10 were described previously (1). Transformation of plasmid pLUC10 into A5 was performed as follows. Recipient cells were grown for 3 to 5 days in Middlebrook 7H9 broth containing 10% Middlebrook ADC enrichment (complete Middlebrook 7H9 broth; GIBCO Laboratories, Madison, Wis.) in a sidearm flask, and the cell density was determined with a Klett model 800-3 colorimeter with a green filter (Klett Manufacturing Co. Inc., New York, N.Y.). When the Klett value reached approximately 100 the cells were collected by centrifugation and were washed three times in 0.5 volume of 10% glycerol. Plasmid DNA (200 ng) was mixed with 60 μl of cells, and the mixture was electroporated in a sterile cuvette (1-mm gap) at 12 kV/cm for 5 ms in a BTX Electro Cell Manipulator 600 (Biotechnologies and Experimental Research, Inc., San Diego, Calif.). Cells were washed from the cuvette with 0.9 ml of medium and were allowed to incubate for 2 h at 37°C before plating on medium containing 50 μg of kanamycin per ml. Plasmids were isolated from the transformants after cultivation for 7 days in complete Middlebrook 7H9 broth containing 50 μg of kanamycin per ml by the method of Kado and Liu (5) by using previously described modifications and were resolved by electrophoresis (1).

**Luciferase assays.** Cultures of A5(pLUC10) were diluted 10-fold in complete Middlebrook 7H9 broth, and 100-μl samples were placed into wells of a 96-well microdilution plate (flat-bottom, white; Microlite 2; Dynatech Laboratories, Inc., Chantilly, Va.). The subsequent addition of 50 μl of luciferase substrate, luciferin, to each well and measurement of bioluminescence in a microplate luminometer (ML 1000; Dynatech Laboratories) were performed as described previously (1).

**Susceptibility testing.** Broth macrodilution susceptibility testing was performed as described previously with drug dilutions in 5 ml of complete Middlebrook 7H9 broth containing 0.05% Tween 80 (1). The inoculum size was ~$10^6$ bacilli per 0.1 ml, and MICs were interpreted as the lowest concentrations of drugs that prevented visible growth after incubation for 7

* Corresponding author. Phone: (404) 639-1280. Fax: (404) 639-1287.

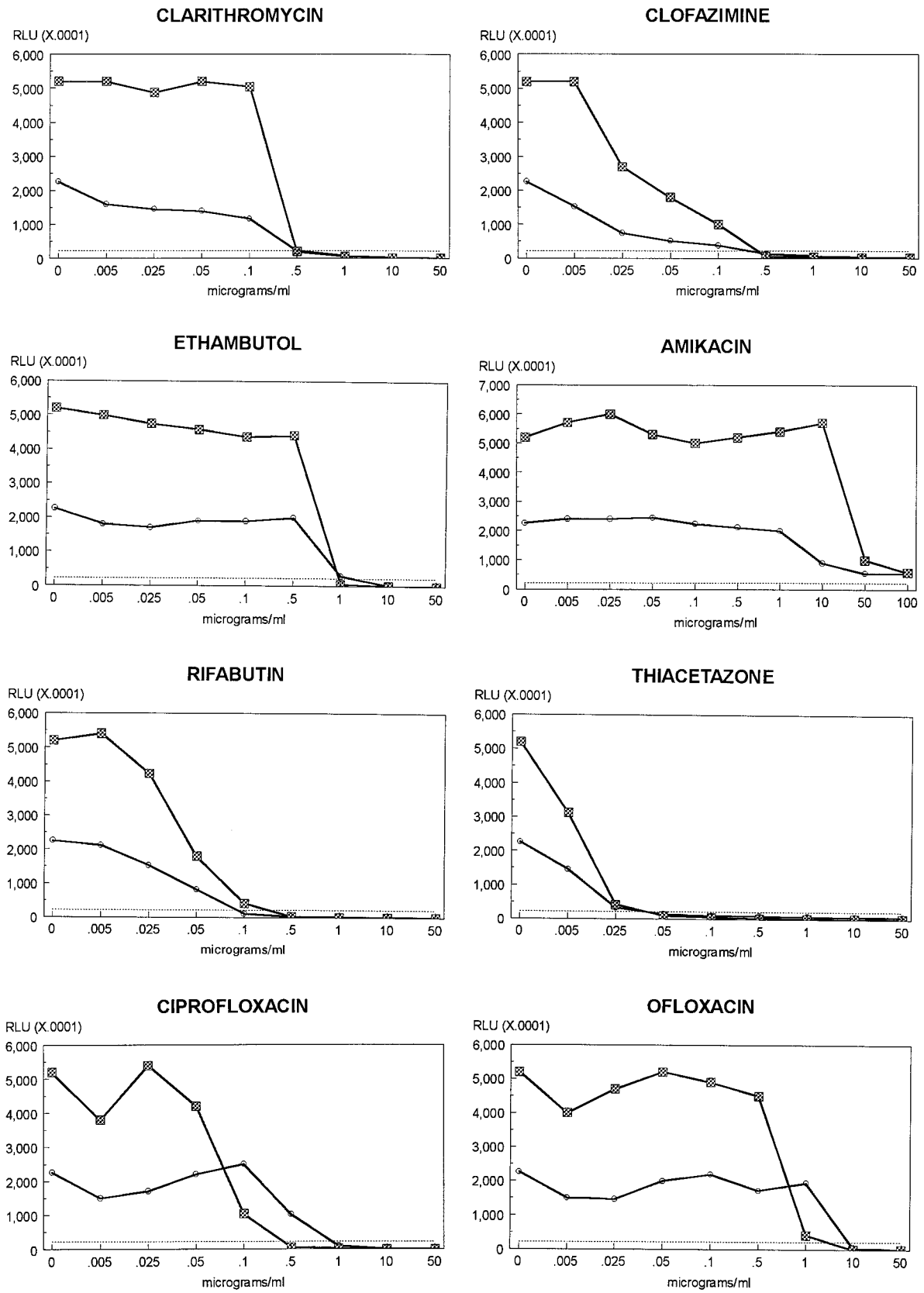


FIG. 1. Bioluminescence of *M. avium* A5(pLUC10) after incubation for 3 days (○) or 7 days (▨) in microdilution plate broth cultures containing antimicrobial agents. The mean bioluminescence of the inoculum (· · ·) sample (10 μl from a total well volume of 200 μl) was $220 \times 10^{-4}$ RLUs (standard deviation, $12 \times 10^{-4}$ RLUs). The mean bioluminescence of 16 drug-free controls included in each drug plate was $2,260 \times 10^{-4}$ RLUs (standard deviation, $203 \times 10^{-4}$ RLUs) after 3 days and $5,200 \times 10^{-4}$ RLUs (standard deviation, $167 \times 10^{-4}$ RLUs) after 7 days.

days and again after 14 days. Luciferase microdilution plate susceptibility assays were performed in duplicate by incubating $10^5$ bacilli plus antimicrobial agent dilutions (0.2-ml total volume per well) in complete Middlebrook 7H9 broth with 0.05% Tween 80. A 10-μl sample was removed from each well immediately after inoculation (time zero) and after incubation for 3 and 7 days and was assayed for luciferase activity. Rifabutin was obtained from Adria-SP Inc., Albuquerque, N.M., clarithromycin was obtained from Abbott Laboratories, Abbott Park, Ill., ofloxacin was obtained from Ortho Pharmaceutical Corporation, Raritan, N.J., and ciprofloxacin was obtained from Miles Inc., Kankakee, Ill. All other antimicrobial agents were obtained from Sigma Chemical Company, St. Louis, Mo.

Colonies of strain A5 presumed to contain plasmid pLUC10 were observed on agar plates after 10 to 14 days of incubation. The efficiency of transformation was $\sim 10^4$ colonies per 200 ng of DNA. Four colonies were examined for bioluminescence and for the presence of an extrachromosomal DNA band equal in size to that of plasmid pLUC10. The mean bioluminescence for the four clones was 0.5017 relative light unit (RLU; range, 0.4960 to 0.5221 RLU; standard deviation, 0.0138 RLU). A plasmid with electrophoretic mobility equal to that of plasmid pLUC10 (~6.4 kbp) was present in all four isolates. There was a linear relationship between the number of bacilli and bioluminescence in a range of 10-fold dilutions yielding from $2 \times 10^7$ bacilli (10 RLUs) to $2 \times 10^4$ bacilli (0.01 RLU). The bioluminescence of $2 \times 10^7$ bacilli of the A5 parent strain was 0.0005 RLU, which is approximately equal to background instrument readings when substrate and uninoculated broth are present.

The effects on bioluminescence of culturing *M. avium* A5(pLUC10) in the presence of the eight antimicrobial agents are shown in Fig. 1. The bioluminescences of 16 drug-free control wells averaged 0.2260 RLU (standard deviation, 0.0203 RLU) after 3 days and 0.5200 (standard deviation, 0.0167 RLU) after 7 days. The bioluminescence in wells containing concentrations of drugs that were inhibitory to growth was ≤10% that in drug-free control wells (≤0.0226 RLU after 3 days and ≤0.0520 RLU after 7 days). Inhibitory drug concentrations also prevented increases in the bioluminescences of the inocula (time zero readings), which averaged 0.0220 RLU (standard deviation, 0.0012 RLU; Fig. 1). MICs determined by the broth macrodilution method were as follows: rifabutin, 0.1 μg/ml; thiacetazone, 0.05 μg/ml; ciprofloxacin, 0.5 μg/ml; ofloxacin, 0.5 μg/ml; clarithromycin, 0.5 μg/ml; clofazimine, 0.5 μg/ml; ethambutol, 5 μg/ml; and amikacin, >100 μg/ml. These MICs correlated closely with the minimum drug concentrations found to be inhibitory by the luciferase assay.

The transformation efficiency of plasmid pLUC10 into *M. avium* A5 on the basis of the numbers of kanamycin-resistant colonies that grew after electroporation was at least equal to that which we previously reported for *M. tuberculosis* H37Ra (1). The expression of firefly luciferase in strain A5(pLUC10), however, was approximately fivefold greater than that in H37Ra(pLUC10) when equal numbers of intact bacilli were measured for their bioluminescences (data not shown). This superior bioluminescence enabled detection of dramatic decreases in bioluminescence at inhibitory drug concentrations in as few as 3 days and optimally after 7 days. The primary advantage of the luciferase microplate method for screening the activities of antimicrobial agents against MAC organisms is not the short turnaround times for results but rather the convenience of performing assays reliably in 96-well microdilution plates, which require less drug, broth, and inoculum. Luciferase testing in *M. avium* is further facilitated by the absence of cell clumping during microdilution plate broth culture, a factor that contributes to well-to-well variations in the bioluminescence of *M. tuberculosis*. Establishing breakpoints in bioluminescence that reliably correlate with MICs, however, will require repeated testing of additional MAC strains possessing luciferase reporter genes and further standardization of the system.

The colonial morphology of strain A5 was not altered by the presence of plasmid pLUC10 or by exposure to antimicrobial agents. When A5(pLUC10) was subcultured from microdilution plate wells containing subinhibitory concentrations of the eight antimicrobial agents onto Dubos oleic acid agar plates, only smooth, opaque colonies identical to those of the parent strain appeared. MIC interpretations by the standard broth macrodilution susceptibility assay were the same for both A5 and A5(pLUC10) with the exception of amikacin. The MIC of amikacin was 5 μg/ml for the parent strain, whereas it was >100 μg/ml for A5(pLUC10). It is not known whether the kanamycin resistance gene *aph* from transposon Tn*903*, used to construct plasmid pLUC10, conferred resistance to amikacin. Amikacin has not been reported, however, to be an efficient substrate for the 3′-aminoglycoside phosphotransferase encoded by this gene. Development of improved luciferase expression vectors in the mycobacteria, for example, plasmids capable of stable integration into the host genome, those without resistance genes such as the kanamycin resistance gene on pLUC10, or both, is under way.

## REFERENCES


1. **Cooksey, R., 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.
2. **Hellyer, T. J., I. N. Brown, J. W. Dale, and C. S. F. Easmon.** 1991. Plasmid analysis of *Mycobacterium avium-intracellulare* (MAI) isolated in the United Kingdom from patients with and without AIDS. J. Med. Microbiol. **34:**225–231.
3. **Horsburgh, C. R., Jr.** 1991. *Mycobacterium avium* complex infection in the acquired immunodeficiency syndrome. N. Engl. J. Med. **324:**1332–1338.
4. **Inderlied, C. B., C. A. Kemper, and L. E. M. Bermudez.** 1993. The *Mycobacterium avium* complex. Clin. Microbiol. Rev. **6:**266–310.
5. **Kado, C. I., and S.-T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145:**1365–1373.
6. **Masur, H., and The Public Health Service Task Force on Prophylaxis and Therapy for *Mycobacterium avium* Complex.** 1993. Recommendations on prophylaxis and therapy for disseminated *Mycobacterium avium* complex disease in patients infected with the human immunodeficiency virus. N. Engl. J. Med. **329:**898–904.
7. **Nightingale, S. D., L. T. Byrd, P. M. Southern, J. D. Jockusch, S. X. Cal, and B. A. Wynne.** 1992. Incidence of *Mycobacterium avium-intracellulare* complex bacteremia in human immunodeficiency virus-patients. J. Infect. Dis. **165:** 1082–1085.