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LETTER TO THE EDITOR

Rapid Whole-Cell Assay of Antitubercular Drugs Using Second-Generation Fluoromycobacteriophages

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Tuberculosis (TB) is a major human health concern and kills about 3,500 people each day (1). The emergence of *Mycobacterium tuberculosis*-resistant strains has become a serious public health problem worldwide, complicating treatment and control of the disease. There is a need for new and efficient antitubercular drugs that shorten the time of treatment and frequency of administration, have less toxicity, and require less patient surveillance (2–5). A novel, rapid, and sensitive assay to be used in activity testing and screening of new compounds could be part of the solution to the TB health challenges.

We previously described the development of fluoromycobacteriophages as a new class of reporter phages that provide a simple means of indicating the metabolic state of *M. tuberculosis* cells and thus their response to antibiotics (6) (7). Mycobacterial cells can

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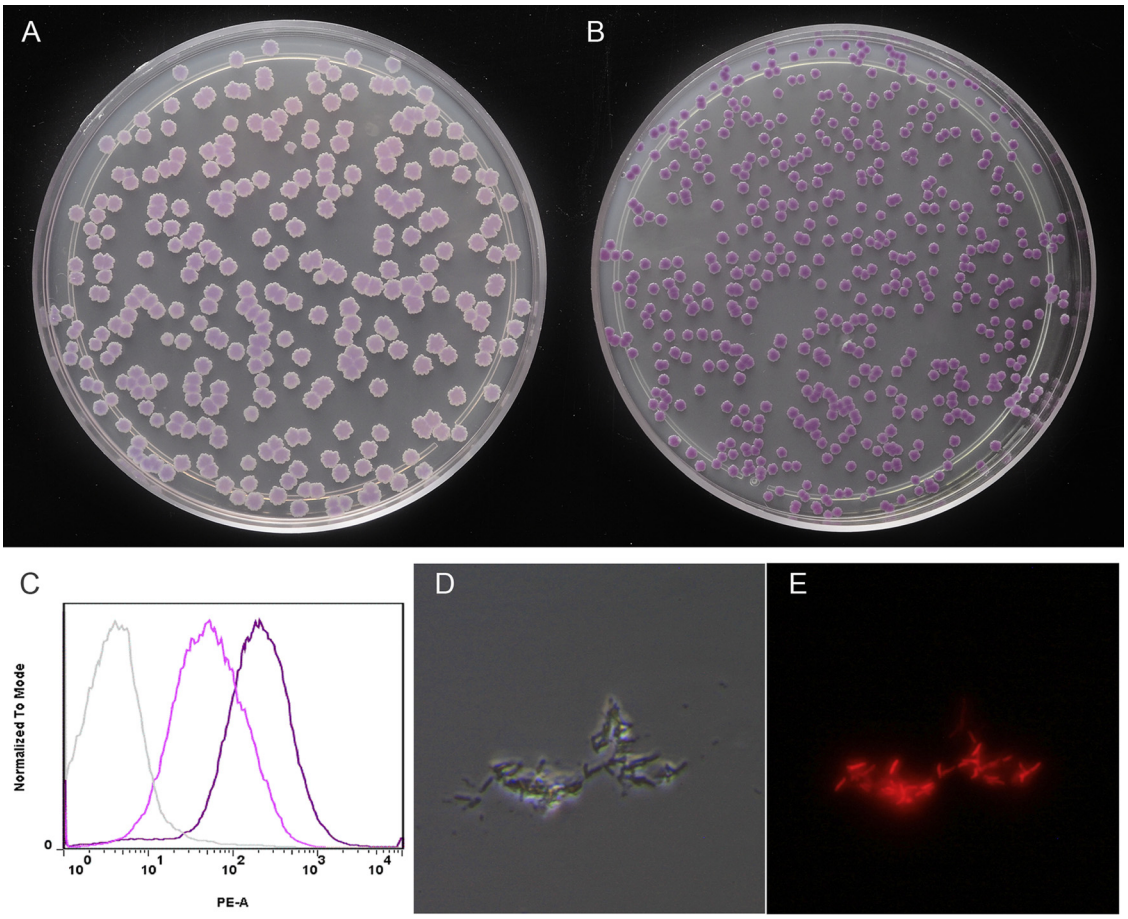

FIG 1 Construction of new *mCherry*$_{bomb}$ϕ. (A and B) *M. smegmatis* mc^{2}155 colonies after transformation with pJL37::*hsp60*$_{prom}$-*mCherry*$_{bomb}$ (A) or pJL37::*hsp60*$_{prom}$-*gp9RBS-mCherry*$_{bomb}$ (B). (C) Flow cytometry analysis of *M. smegmatis* mc^{2}155 control (gray) transformed as described for panel A (pink) or panel B (purple). The intensity of fluorescence is plotted against the number of events normalized to the mode. PE-A, phycoerythrin A. (D and E) Infection of *M. tuberculosis* mc^{2}6230 with the new *mCherry*$_{bomb}$ϕ for 5 h at 37°C. Phase-contrast images (D) and fluorescence micrograph images (E) are shown. Magnification, ×1,000.

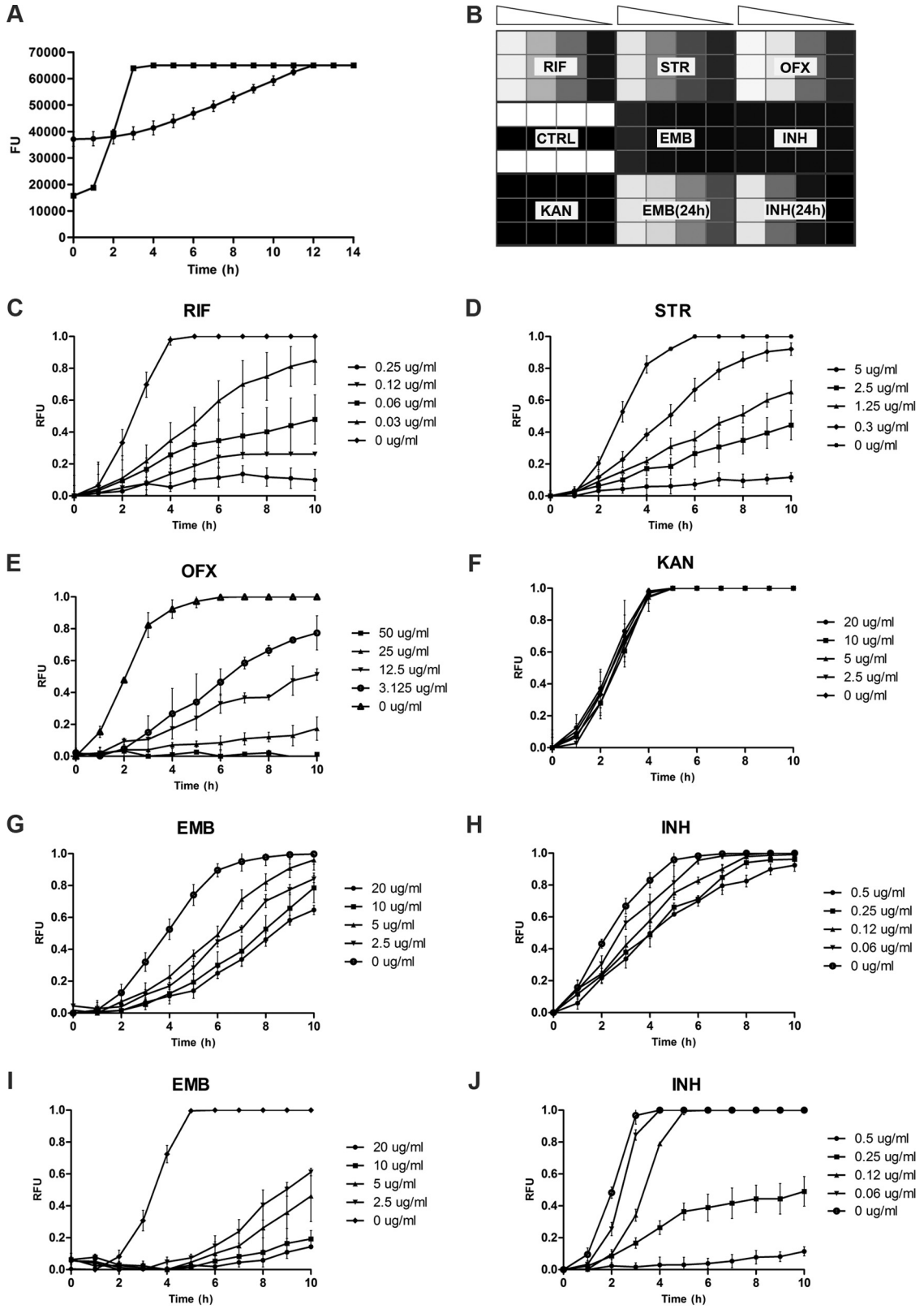

FIG 2 Monitoring of fluorescence after infection of *M. tuberculosis* with fluoromycobacteriophages using a fluorimeter. (A) Fluorescence units (FU) versus time after infection of *M. tuberculosis* mc^{2}6230 with phAE87::*hsp60-EGFP* (circles) or *mCherry*$_{bomb}$ϕ (squares). (B) Schematic representation of the multiwell plate setup and output fluorescence reading for activity testing of drugs. Black represents maximal FU and white absence of signal. Different shades of gray are used for intermediate values. Control (CTRL) wells without drug are shown. EMB and EMB(24), EMB without and with 24 h of preincubation; INH and INH(24), INH without and with 24 h of preincubation. (C to J) Relative fluorescence units (RFU) versus time after infection of *M. tuberculosis* mc^{2}6230 Kan^r with second-generation fluoromycobacteriophages in the presence of increasing concentrations of the following antibiotics: rifampin (RIF) (C); streptomycin (STR) (D); ofloxacin (OFX) (E); kanamycin (KAN) (F); ethambutol (EMB) (G and I); isoniazid (INH) (H and J). Antibiotics were added simultaneously with *mCherry*$_{bomb}$ϕ (C to H) or preincubated with cells for 24 h before infection (I and J).

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easily be detected by fluorescence microscopy or flow cytometry after infection with the reporter phage.

To construct second-generation fluoromycobacteriophages with greater sensitivity and shorter time to *M. tuberculosis* detection, we used a modified $mCherry_{bomb}$ version with codon usage optimized for mycobacteria under the control of the *hsp60* promoter and with the predicted ribosome binding site (RBS) of gp9 of mycobacteriophage TM4, the major capsid protein (8, 9). Panels A to C of Fig. 1 show a comparison between mycobacterial cells transformed with the expression plasmid before and after substitution of the RBS associated with the *hsp60* promoter. The original vector in shuttle phasmid phAE159 (10) was replaced with pYUB854 carrying the *hsp60*prom-*gp9*RBS-$mCherry_{bomb}$ cassette. Phage particles were obtained as previously described (6), and the newly constructed phage was called $mCherry_{bomb}\Phi$.

After infection of *M. tuberculosis* $mc^2$6230 (Δ*RD1* Δ*panCD*) (11), bright red cells were observed by fluorescence microscopy after only 5 h (Fig. 1D and E), showing a notable reduction of the time required to detect the signal compared to previous phAE87::*hsp60-EGFP* (enhanced green fluorescent protein) (6) and other fluorescent reporter phages (12). We then evaluated the detection of *M. tuberculosis*-infected cells by monitoring the expression kinetics of $mCherry_{bomb}$ using an automated multiwell plate reader fluorimeter. After infection with $mCherry_{bomb}\Phi$, the maximal readout was reached at 4 to 6 h versus 10 h with the previous GFP phage (6). Moreover, the signal/background ratio (total number of fluorescence units/number of fluorescence units of cells plus phage at time zero) was higher for the new reporter phage, allowing better discrimination between noninfected and infected cells (Fig. 2A).

We further measured the fluorescent signal after infection in the presence of 2-fold dilutions of the drugs most commonly used for TB treatment. A schematic representation of the 96-well plate setup and the output signal for this assay is presented in Fig. 2B. To avoid background differences among $mCherry_{bomb}\phi$ stocks, we calculated relative fluorescence units (RFU) as follows:

$$\text{RFU} = \frac{\text{mean fluorescence units} - \text{background}}{\text{maximal fluorescence reading units} - \text{background}}$$

A decrease in the fluorescent signal was observed for increasing concentrations of the tested compounds (Fig. 2), allowing MIC determinations. With this assay, we were able to corroborate the antibiotic resistance phenotype of the *M. tuberculosis* $mc^2$6230 kanamycin-resistant (Kan^r) derivative strain used in this work (Fig. 2F).

As we previously reported (6), when the target of the tested drug is gene expression (transcription or translation), phage and antibiotics could effectively be added simultaneously (Fig. 2C to F). When the antibiotic had a different target (e.g., cell wall synthesis), a preincubation of 24 h was necessary prior to addition of $mCherry_{bomb}\Phi$ (Fig. 2G to J). Based on this requirement, in testing new compounds, a preliminary mechanism of action of the drug can be inferred.

In comparison with other whole-cell assays for activity testing of compounds (13–18), results using fluoromycobacteriophages can be obtained in hours in contrast to days; no addition of substrate is needed (13, 14, 19), and, since mycobacterial cultures can be maintained constantly growing, they are readily available when needed. All these features make $mCherry_{bomb}\Phi$ in combination with automated fluorimetric detection a convenient tool for activity testing of new antitubercular drugs and a potential rapid drug susceptibility testing (DST) assay of *M. tuberculosis* clinical isolates.

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