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ϕ^2GFP10, a High-Intensity Fluorophage, Enables Detection and Rapid Drug Susceptibility Testing of *Mycobacterium tuberculosis* Directly from Sputum Samples

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The difficulty of diagnosing active tuberculosis (TB) and lack of rapid drug susceptibility testing (DST) at the point of care remain critical obstacles to TB control. This report describes a high-intensity mycobacterium-specific-fluorophage (ϕ^2GFP10) that for the first time allows direct visualization of *Mycobacterium tuberculosis* in clinical sputum samples. Engineered features distinguishing ϕ^2GFP10 from previous reporter phages include an improved vector backbone with increased cloning capacity and superior expression of fluorescent reporter genes through use of an efficient phage promoter. ϕ^2GFP10 produces a 100-fold increase in fluorescence per cell compared to existing reporter phages. DST for isoniazid and oxofloxacin, carried out in cultured samples, was complete within 36 h. Use of ϕ^2GFP10 detected *M. tuberculosis* in clinical sputum samples collected from TB patients. DST for rifampin and kanamycin from sputum samples yielded results after 12 h of incubation with ϕ^2GFP10. Fluorophage ϕ^2GFP10 has potential for clinical development as a rapid, sensitive, and inexpensive point-of-care diagnostic tool for *M. tuberculosis* infection and for rapid DST.

There is an urgent need for improved assays for both diagnosis of tuberculosis (TB) and drug susceptibility testing (DST) that are accurate, rapid, and inexpensive (25). The most important advances would be assays that could be applied at the point of care in the developing world. Recent advances in nucleic acid amplification approaches ("genotypic assays"), especially the Xpert MTB/RIF assay, are important contributions to the rapid initial diagnosis of pulmonary TB. However, Xpert MTB/RIF detects only rifampin resistance (Rif^r) and relies on the fact that almost all clinical Rif^r strains identified to date harbor one of three specific point mutations (2, 7, 8, 21, 28, 44). Other clinical drug resistance phenotypes have more varied genetic underpinnings (e.g., hundreds of different mutations can lead to isoniazid resistance [39]) and therefore may prove refractory to diagnosis via sequence-specific approaches. Both multidrug-resistant tuberculosis (MDR-TB) and extensively drug-resistant tuberculosis (XDR-TB) strains are endemic in South Africa and contribute significantly to mortality among hospitalized patients (12, 20). Since both MDR-TB and XDR-TB strains are rifampin resistant, Xpert MTB/RIF cannot distinguish between them, potentially leading to inappropriate therapy for weeks or even months until DST results become available. The usefulness of the Xpert MTB/RIF test is also limited by the cost, which is still too high for the test to be affordable in resource-poor settings (24). Therefore, there is still a need for an inexpensive and rapid point-of-care test for TB diagnosis.

Phenotypic assays recognize the organismal response of bacteria to antibiotics without limitation to any particular antibiotic, allele, or mechanism. Unlike nucleic acid tests, phenotypic assays are not dependent upon the foreknowledge of a specified candidate sequence leading to drug resistance. DST by culture remains the gold standard for phenotypic assays, but a solid-medium culture identification of *Mycobacterium tuberculosis* takes 4 to 8 weeks. Newer approaches, such as microscopic-observation drug susceptibility (MODS) testing, have shortened the time needed for phenotypic assays to between 1 and 2 weeks (29). The high-intensity fluorophage described here may potentially shorten the time to diagnosis and complete DST to 1 to 2 days.

Fluorophages are a type of "reporter phage" that inject their DNA specifically into mycobacteria (24). Fluorescence is produced by expression of a fluorescent reporter gene (11) cloned into the phage. Each metabolically active mycobacterial cell that is infected with the reporter phage transcribes and translates the gene for fluorescent protein. The reporter signal is not affected by antibiotic in a genetically resistant strain, but in a sensitive strain it is greatly attenuated (35). Since the proof-of-principle demonstration in 1993 (23), mycobacterial reporter phages have remained a potentially elegant solution to the problem of TB diagnosis and DST. In laboratory cultures, including cultures derived from clinical isolates, reporter phages detect mycobacterial cells and allow assays of drug susceptibility in appreciably less time than culture alone (3, 4, 23, 32, 35, 36). However, existing reporter phages are unable to identify mycobacteria directly in clinical specimens. To date, the key limitations of reporter phages have been (a) poorly fluorescent reporter signals, requiring prolonged exposure time to distinguish fluorescent mycobacteria from back-

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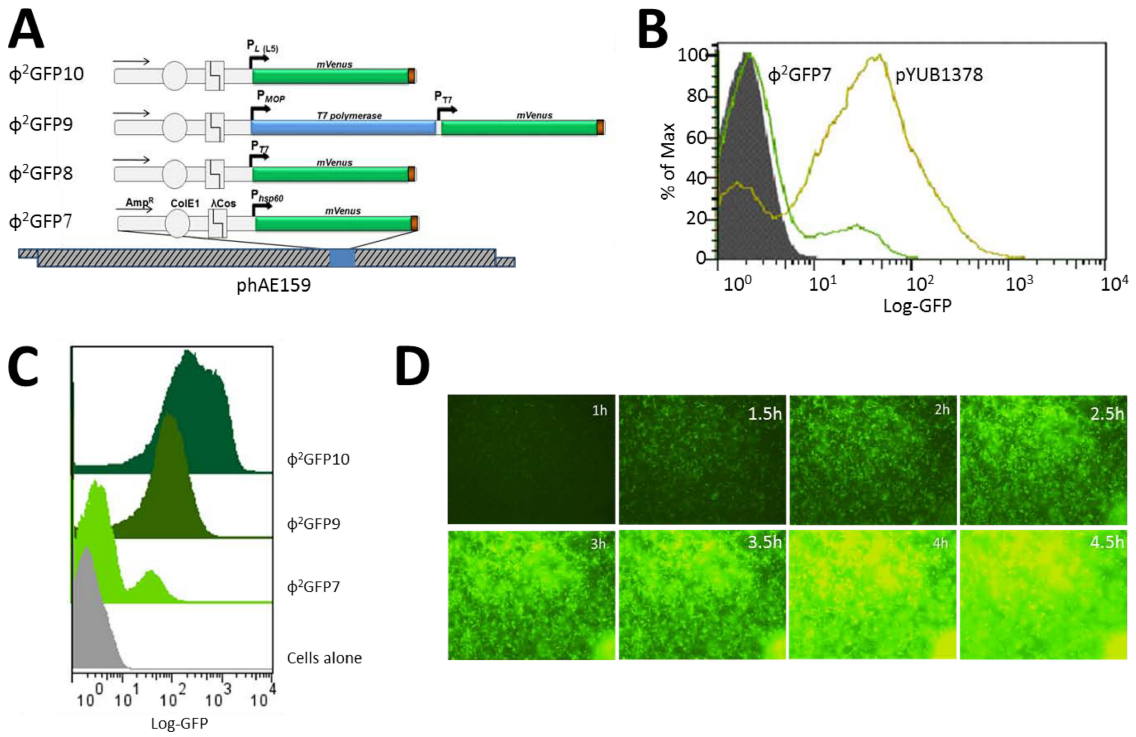

FIG. 1. Generation of high-intensity fluorophage. (A) Schematic representation of fluorophages constructed in the present study. (B) Comparison of fluorescence obtained from *M. smegmatis* infected with fluorophage ϕ^2GFP7 (dark green) and *M. smegmatis* expressing an *hsp60*-mVenus cassette from episomal plasmid pYUB1378 (light green). (C) Flow cytometric comparison of fluorescent signal obtained after infection of *M. smegmatis* mc^{2}155 with various fluorophages. The MFI values for cells alone and cells infected with ϕ^2GFP7, ϕ^2GFP9, and ϕ^2GFP10 were 2.03, 4.03, 10.4, and 168, respectively. (D) Time-lapse microscopy of mVenus expression in *M. smegmatis* mc^{2}155 after infection with fluorophage ϕ^2GFP10. Images were taken every 30 min using an exposure time of 30 ms; the fluorescent detector was saturated after 4.5 h.

ground autofluorescence, and (b) the inability of fluorophages to produce detectable signal in clinical sputum samples.

We have been able to overcome the above-described limitations by engineering a fluorophage with a substantially more powerful promoter, an improved vector backbone, and a more intense fluorescence reporter gene. This high-intensity fluorophage (ϕ^2GFP10) allows—for the first time—direct microscopic visualization of phage-encoded fluorescence of *M. tuberculosis* cells in sputum samples. ϕ^2GFP10 has potential both as a rapid and inexpensive point-of-care diagnostic tool and as a tool for DST of *M. tuberculosis* in clinical sputum samples.

MATERIALS AND METHODS

Generation of high-intensity fluorophage. Vector pYUB1378 was generated by cloning the mVenus gene downstream of the *hsp60* promoter in pMV261 (42). Vector pYUB1229 was generated by introducing a lambda-cos-site and a unique PacI site by the use of BspH1 in pEXP1-DEST (Invitrogen). pYUB1229 was used as a backbone for construction of all the plasmids. An *hsp60-mVenus* cassette was cloned from pYUB1378 into pYUB1229 at BglII-NdeI sites to generate pYUB1167. A T7-*mVenus* cassette was generated by replacing the *hsp60* promoter with a T7 phage promoter by the use of BglII and NdeI sites, and the plasmid was designated pYUB1361. T7 polymerase gene (14, 45) was cloned upstream of the T7-*mVenus* cassette in pYUB1361 at the BglII site to generate pYUB1383. The expression of T7 polymerase in pYUB1383 is driven by a synthetic mycobacterial optimal promoter (MOP) consisting of promoter sequences derived from the BCG *hsp70* heat shock protein gene and an *Escherichia coli tac* promoter (22). An L5-*mVenus* cassette was generated by cloning an L5 promoter (9) between the BglII and NdeI sites in plasmid pYUB1361, and the plasmid was designated pYUB1391. A vector map illustrating all the features and unique restriction sites for the plasmids described above is shown in Fig. S1 in the supplemental material. Plasmids pYUB1167, pYUB1361, pYUB1383, and pYUB1391 were digested with PacI and ligated to PacI-digested phAE159. The ligation mix was packaged into lambda phage heads by the use of an *in vitro* packaging extract (MaxPlax; Epicentre [19]) to generate shuttle phasmids. A 500-ng volume of each shuttle phasmid DNA was electroporated into 300 μl of *M. smegmatis* mc^{2}155 (40). The electroporation mix was recovered for 1 h at 37°C after addition of 1 ml of 7H9 media (Middlebrook 7H9 broth supplemented with 10% oleic acid-albumin-dextrose-catalase [OADC], 0.5% glycerol, and 50 μg/ml pantothenate). A 300-μl volume of recovered sample was mixed with 3 ml of top agar and was poured over 7H10 plates (5, 23). Plates were incubated at 30°C for 3 days to obtain reporter phages. Plasmids pYUB1167, pYUB1361, pYUB1383, and pYUB1391 correspond to fluorophages ϕ^2GFP7, ϕ^2GFP8, ϕ^2GFP9, and ϕ^2GFP10, respectively (Fig. 1A). Individual plaques were picked and propagated at 30°C to obtain high-titer fluorophages (5).

Infection of mycobacterial strains with fluorophages. *M. tuberculosis* mc^{2}6230 is a fully drug-sensitive Δ*RD1* Δ*panCD* mutant certified for use in the BSL2 facility of Albert Einstein College of Medicine (38). This was used as a surrogate strain for the virulent *M. tuberculosis* H-37Rv. Drug-resistant mutants of *M. tuberculosis* mc^{2}6230 were isolated by selecting cells on 7H10 plates in the presence of various drugs at 5 times the MIC value (18, 27). The *M. tuberculosis* mc^{2}7201, mc^{2}7202, and mc^{2}7203 strains are resistant to rifampin, kanamycin, or both rifampin and kanamycin, respectively. For identification or to perform DST using fluorophage, all the mycobacterial strains were grown to an optical density at 600 nm (OD_{600}) of 0.8 to 1.0 in 7H9 media containing 0.05% Tween 80. The cells were washed twice with MP buffer (50 mM Tris [pH 7.6], 150

mM NaCl, 10 mM $MgCl_2$, and 2 mM $CaCl_2$) to remove detergent and resuspended in 7H9 media to an OD_{600} of 1.0. Washed cells (5 μl) were divided into aliquots and added to a 96-well plate containing 50 μl of 7H9 media. A 200-μl volume of fluorophage was added to each well to obtain a multiplicity of infection (MOI) of 100, and the plates were incubated for 12 h at 37°C. Following this, each sample was split into two parts. One part of the sample was acquired using flow cytometry (FACSCalibur, BD Biosciences, CA), and the other part was centrifuged at 15,000 × *g* for 10 min. Excess phage and media were removed from centrifuged samples, and the pellet was resuspended in 10 μl of 7H9 media. A 5-μl volume of sample was spotted on a glass slide and visualized on a fluorescence microscope (Nikon Ti) using a differential interference contrast (DIC) filter for bright-field analysis and a fluorescein isothiocyanate (FITC) filter for green fluorescence. Flow cytometry data were analyzed using the FlowJo software package (version 7.6.1; Tree Star Inc., Ashland, Oregon). Fluorescent bacilli after incubation with phage indicate the presence of mycobacteria in that analysis. The significance of a difference between any two populations was determined by the Kolmogorov-Smirnov test.

To determine relative fluorescence and the percentage of cells infected as a function of MOI, *M. tuberculosis* mc^{2}6230 cells were infected with ϕ^2GFP10 at the appropriate phage dilution to obtain an MOI of 100, 10, 1, 0.1, 0.01, 0.001, and 0.0001. After infection, samples were acquired using FACSCalibur and data were analyzed using the FlowJo software, version 7.6.1.

For DST, 5 μl of washed cells was divided into aliquots and added to a 96-well plate containing 50 μl of 7H9 media. Antibiotics were added into individual wells to obtain the following concentrations: kanamycin at 5 μg/ml, rifampin at 2 μg/ml, isoniazid at 0.4 μg/ml, and ofloxacin at10 μg/ml. A 10-μl volume of MP buffer was added in a control well. At this stage, 200 μl of ϕ^2GFP10 (MOI = 100) was added for time (t) = 0, or the sample was preincubated at 37°C, as noted for the respective experiments, before addition of ϕ^2GFP10. After phage addition, samples were incubated for 12 h at 37°C. The assay was terminated by mixing with an equal volume of 4% paraformaldehyde. At the end of the experiment, samples were acquired using FACSCalibur or analyzed on a fluorescence microscope as described above.

***M. tuberculosis* detection and DST in sputum samples.** Anonymous, deidentified sputum samples were collected from smear-positive patients with TB in Durban, South Africa, prior to initiation of treatment. Samples were split into two halves, and one part was treated by a standard N-acetyl-L-cysteine-NaOH (NALC) method (10) for DST using the agar proportion method and the other aliquot for reporter phage. The optimal ϕ^2GFP10 signal required using 0.625% NaOH for sputum processing instead of the current standard clinical recommendation of 2% (26, 41). Samples were resuspended in 500 μl of 7H9 media. A 50-μl volume of sample was used for Ziehl-Neelsen staining, and 100 μl of sample was incubated with 500 μl of phage (titer, 10^9/ml) in the presence or absence of antibiotic. After incubation for 12 h at 37°C, samples were fixed in 5% formaldehyde for 3 h, centrifuged, resuspended in ~25 μl of leftover volume after removal of the supernatant, and analyzed by fluorescence microscopy as described above.

RESULTS

Development of high-intensity fluorophage. To address the main shortfall of the existing fluorophages for detection of *M. tuberculosis*, which is poor signal-to-background ratio, two complementary strategies were employed. First, a new mycobacteriophage TM4 vector backbone (phAE159) was used to generate fluorophages that can deliver up to 10 kb of recombinant DNA into mycobacterial cells. Second, the promoters originating from bacteriophages were used to enhance the expression of reporter genes. Initially, the monomeric fluorescent protein mVenus, which is brighter than the existing fluorescent proteins (30), was cloned under the control of *hsp60* promoter (42) to generate ϕ^2GFP7 (Fig. 1A). Unfortunately, the fluorescence intensity obtained from ϕ^2GFP7 was similar to that of the previous reporter phage phAE87::*hsp60*-EGFP (33) and was at least 10-fold lower than that of the cells harboring the pYUB1378 multicopy plasmid (Fig. 1B).

The enhanced cloning capacity of phAE159 was then used to search for stronger endogenous promoters in the *M. tuberculosis* genome which would improve reporter gene expression from mycobacteriophages. A random library of *M. tuberculosis* H-37Rv genomic DNA was generated by cloning approximately 2-kb fragments upstream of the reporter gene in phAE159 vector. This approach revealed no fragment that significantly enhanced the expression of the reporter gene compared to the P_{hsp60} promoter. The use of G13 promoter from *Mycobacterium marinum*, isolated from a promoter trap library and 40 times stronger than the mycobacterial *hsp60* promoter on an episomal plasmid (6), also did not increase the mVenus expression signal to a level appreciably higher than that of the *hsp60* promoter in phAE159 mycobacteriophage (data not shown). Apparently, a strong promoter in the context of an episome is not necessarily strong in the context of phage. We hypothesized that phage promoters might be better suited for high expression of reporter genes from mycobacteriophages, since phages have evolved to express large quantities of their structural and lytic proteins in short periods of time. We first tested the late promoter of *E. coli* T7 phage, which has been extensively used on episomal plasmids to overexpress recombinant proteins in *E. coli* and mycobacteria (17, 43, 45). Fluorophage ϕ^2GFP8 was generated by cloning the T7 phage promoter upstream of mVenus (Fig. 1A). The T7 promoter functions only with its cognate RNA polymerase (43, 45); therefore, no fluorescence signal was observed when ϕ^2GFP8 infected *M. smegmatis* strain (mc^{2}155) lacking T7 polymerase. In contrast, infection of *M. smegmatis* strain expressing T7 polymerase (mc^{2}4517) with ϕ^2GFP8 resulted in a high level of fluorescence. Fluorescent cells were detected as early as 2 h after infection (movie S1 in the supplemental material). The population of fluorescent cells increased with time, and more than 90% of the cells were fluorescent after 10 h of infection with ϕ^2GFP8 (movie S1 in the supplemental material). Following the above-described results, the T7 polymerase gene, driven by the MOP promoter (22), was cloned upstream of T7 promoter in ϕ^2GFP8 to generate ϕ^2GFP9. ϕ^2GFP9 overcame the requirement of a strain to endogenously express T7 polymerase, and a strong fluorescent signal was obtained when ϕ^2GFP9 infected the wild-type *M. smegmatis* strain (mc^{2}155) (Fig. 1C). Based on these results, another reporter phage, ϕ^2GFP10, was generated using P_{Left} lytic promoter of mycobacteriophage L5 (31). This phage promoter requires only the mycobacterial host RNA polymerase. The comparison of these two phage promoters to the *hsp60* promoter shows that the fluorescence obtained from ϕ^2GFP10 was almost 100-fold greater than that obtained from the cells infected with ϕ^2GFP7 and was also higher than that obtained from the cells infected with ϕ^2GFP9 under similar conditions (Fig. 1C). Fluorescent cells were observed after 1 h of infection with ϕ^2GFP10, and the fluorescence signal increased over time. More than 90% of the cells were fluorescent after 4.5 h and resulted in the saturation of the fluorescence detector (Fig. 1D).

Comparison of fluorophages in *M. tuberculosis*. Attenuated *M. tuberculosis* strain mc^{2}6230, a drug-sensitive Δ*RD1* Δ*panCD* mutant of *M. tuberculosis* H-37Rv (38), was infected with the reporter phages described in the previous section. Individual bacilli

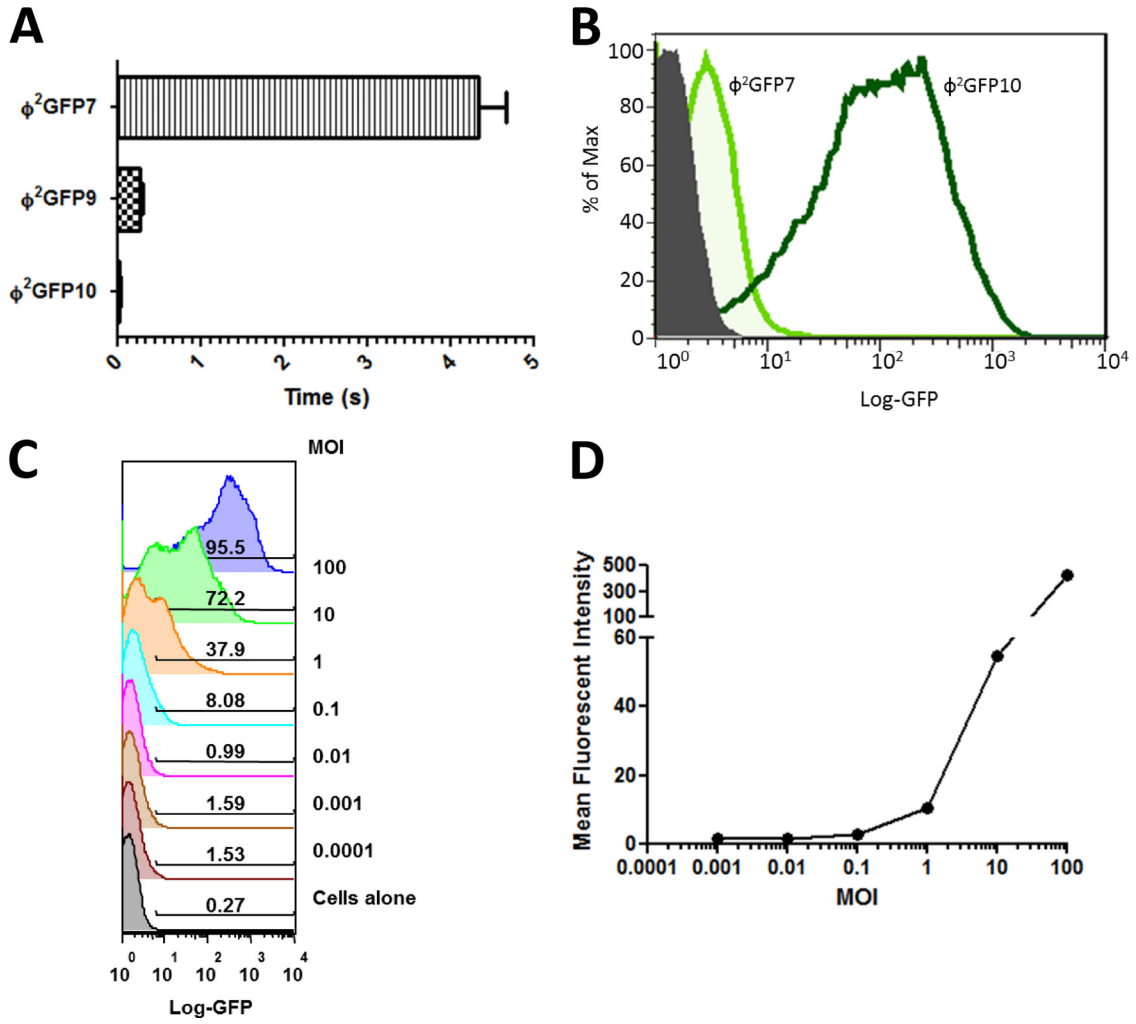

FIG. 2. Comparison of fluorophages in *M. tuberculosis*. (A) Time required to capture and visualize fluorescent cells by a Nikon Ti microscope after infection of *M. tuberculosis* mc^{2}6230 for 12 h with the indicated fluorophages. The error bars represent the variation in exposure time between biological replicates, and the exposure time for a sample is the mean of time required to capture images for a sample at 5 different fields. (B) Comparison of fluorescent intensities obtained after 12 h of infection of *M. tuberculosis* mc^{2}6230 with ϕ^2GFP7 and ϕ^2GFP10 determined by the use of flow cytometric analysis. The MFI values were 2.05 and 287.9, respectively, for cells infected with ϕ^2GFP7 and ϕ^2GFP10. The MFI value for cells alone was 1.37. (C) Efficiency of detection of *M. tuberculosis* mc^{2}6230 by the use of fluorophage ϕ^2GFP10 at various MOI. *M. tuberculosis* cells were infected with ϕ^2GFP10 at different MOI, ranging from 0.0001 to 100. Percent fluorescent population was determined at each MOI using flow cytometry. (D) MFI of *M. tuberculosis* cells infected with ϕ^2GFP10 was plotted at different MOI. The increase in MFI at higher MOI indicates the infection of *M. tuberculosis* cells by more than one ϕ^2GFP10.

were fluorescent in all cases but displayed a wide range of fluorescence intensity. *M. tuberculosis* cells infected with ϕ^2GFP10 required an average exposure time of 15 to 20 ms to record an image, and ϕ^2GFP9 required an average time of 100 to 300 ms. Both ϕ^2GFP9- and ϕ^2GFP10-infected cells could be visualized easily through the microscope eyepiece. In contrast, mc^{2}6230 cells infected with ϕ^2GFP7 were not identifiable with optics alone and required 4 to 5 s of exposure to capture and visualize the fluorescent cells (Fig. 2A). Flow cytometry data paralleled microscopy results and showed that mc^{2}6230 cells infected with ϕ^2GFP10 fluoresced at approximately a 100-fold-greater intensity than mc^{2}6230 cells infected with ϕ^2GFP7 (Fig. 2B). The fluorescence intensity did not increase significantly after prolonged incubation of ϕ^2GFP7 with mc^{2}6230. Significant differences in the fluorescence intensities of *M. tuberculosis* cells were also observed after 48 h of infection with ϕ^2GFP7 compared to 12 h of infection with ϕ^2GFP10 (see Fig. S2 in the supplemental material). Overall, the P_{Left} promoter of L5 mycobacteriophage in ϕ^2GFP10 drives the maximum expression of the reporter in *M. tuberculosis*.

Next, we determined the relationship of the multiplicity of infection (MOI) of ϕ^2GFP10 to the reporter signal obtained per cell. The proportion of fluorescent cells in a given sample increased with increasing MOI of ϕ^2GFP10 (Fig. 2C). At MOIs of 1, 10, and 100, approximately 38%, 72%, and 95.5% of the cells, respectively, were fluorescent (Fig. 2C). The mean fluorescent intensity (MFI) signal per cell also increased as a function of MOI (Fig. 2D). The increase in fluorescence at higher MOI implies that a single mycobacterial cell can be incited to express product from more than one reporter phage. *M. smegmatis* or *M. tuberculosis* cells infected with other fluorophages (ϕ^2GFP7, ϕ^2GFP9) also resulted in increased MFI with increased MOI (data not shown),

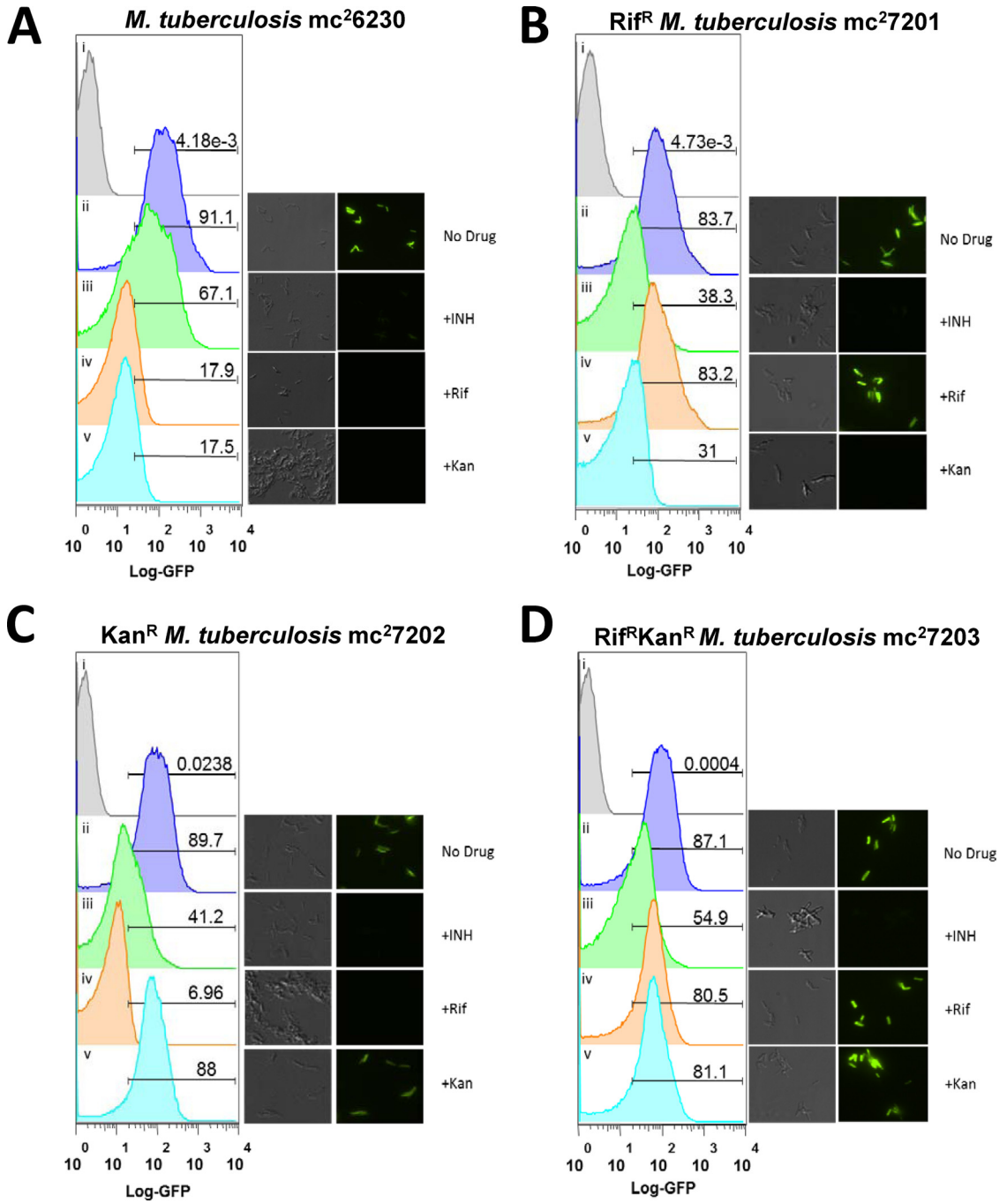

FIG. 3. DST for *M. tuberculosis* strains using fluorophage ϕ^2GFP10. (A) Drug-sensitive *M. tuberculosis* mc^{2}6230. (B) Rifampin-resistant *M. tuberculosis* mc^{2}7201. (C) Kanamycin-resistant *M. tuberculosis* mc^{2}7202. (D) Rifampin- and kanamycin-resistant *M. tuberculosis* mc^{2}7203. Strains were incubated with ϕ^2GFP10 and the indicated antibiotics simultaneously for 12 h and examined by flow cytometry (left panels) and microscopy (right panels). i, cells alone on the fluorescence-activated cell sorter (FACS) plot; ii, cells incubated with ϕ^2GFP10; iii, cells incubated with ϕ^2GFP10 in the presence of isoniazid (INH); iv, cells incubated with ϕ^2GFP10 in the presence of rifampin (Rif); v, cells incubated with ϕ^2GFP10 in the presence of kanamycin (Kan). The number indicated in each FACS plot represents percent fluorescent population. Failure to fluoresce in the presence of antibiotic indicates sensitivity, and a fluorescence signal in the presence of a specific antibiotic indicates resistance to that antibiotic.

suggesting that the new vector backbone supports multiple infections. Based on the results, an MOI of 100 was used in subsequent experiments.

Phenotypic drug susceptibility testing using ϕ^2GFP10 fluorophage. In the absence of drug treatment, approximately 90% of *M. tuberculosis* cells were positive for fluorescence after incubation with ϕ^2GFP10 by both flow cytometry and microscopy (Fig. 3A). When rifampin or kanamycin was added to cells along with ϕ^2GFP10 (time $t = 0$), the population of fluorescent *M. tuberculosis* cells diminished by approximately 85%, as analyzed by flow

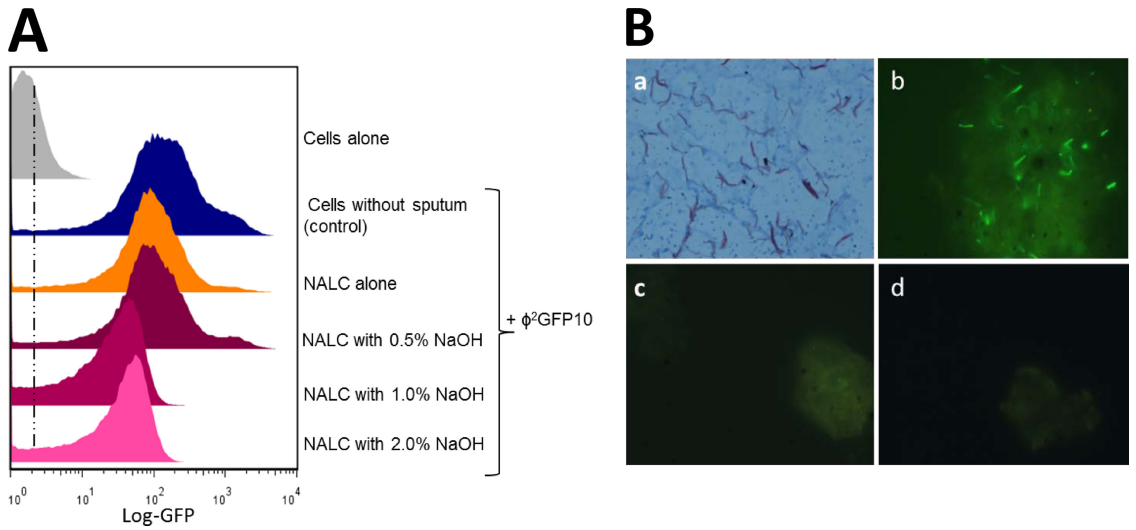

FIG. 4. Detection of *M. tuberculosis* in sputum samples with ϕ^2GFP10. (A) Laboratory-cultured *M. tuberculosis* mc^{2}6230 cells were spiked into sputum samples to a final concentration of 10^4 cells per ml. The sample was decontaminated with NALC alone, or NALC and various NaOH concentrations, and incubated with ϕ^2GFP10 for 12 h at 37°C before analysis by flow cytometry. (B) Diagnosis and DST of *M. tuberculosis* in primary clinical samples from patients with active TB. Sputum samples were collected from patients with TB and analyzed for the presence of *M. tuberculosis* by the use of (a) Ziehl-Neelsen staining and (b) ϕ^2GFP10. The DST for (c) rifampin and (d) kanamycin was performed on the same sample by the use of ϕ^2GFP10. The DST results, available after 12 h of incubation with ϕ^2GFP10, showed that the patient was infected with a drug-sensitive *M. tuberculosis* strain.

cytometry (Fig. 3A). Longer preincubation with these drugs ($t = 3$ h and $t = 6$ h) did not significantly alter the results, indicating that drug and phage can be added simultaneously at the time of assay for DST (see Fig. S3A in the supplemental material). In contrast, inhibition of fluorescence in the presence of isoniazid was sensitive to the time of addition of drug and phage. When ϕ^2GFP10 was added simultaneously (time $t = 0$) or after $t = 3$ h and 6 h of isoniazid addition, fluorescent cells were detected by both flow cytometry and microscopy, although the MFI was lower than that of the untreated control (Fig. 3A; see also Fig. S3A in the supplemental material). Isoniazid pretreatment for 18 h prior to phage addition increased the difference between treated and nontreated samples. Ofloxacin behaved similarly to isoniazid, in that an 18-h pretreatment with antibiotic increased the signal differences in DST (see Fig. S3B in the supplemental material). Isoniazid and ofloxacin disrupt mycolic acid metabolism and DNA replication, respectively. Since ϕ^2GFP10 analysis assesses the transcription and translation ability of the cells, we reason that the inhibition of transcription and translation are later and secondary effects of these drugs. All four drugs displayed similar kinetics of killing, as measured by CFU, and in all cases a drop in CFU was observed only after 48 h of antibiotic treatment (data not shown). These results suggest that ϕ^2GFP10 detects inhibition of essential metabolic processes prior to the inhibition becoming irreversible. Thus, ϕ^2GFP10 gives a rapid but absolutely predictive DST.

The fluorescence patterns of rifampin-resistant strain mc^{2}7201 after infection with ϕ^2GFP10 were similar in the presence and absence of rifampin. Rifampin resistance did not change the result of treatment with kanamycin (Fig. 3B). Conversely, for kanamycin-resistant *M. tuberculosis*, mc^{2}7202 fluorescence decreased in the presence of rifampin but not in the presence of kanamycin (Fig. 3C). Fluorescence results consistently agreed with CFU plating results (data not shown). *M. tuberculosis* mc^{2}7203 is doubly resistant to kanamycin and rifampin, and its fluorescence intensity did not change in the presence of either antibiotic (Fig. 3D). These results show that ϕ^2GFP10 distinguishes *M. tuberculosis* strains that are resistant to a single drug from those strains that are resistant to multiple drugs.

Detection of *M. tuberculosis* cells in spiked sputum samples with ϕ^2GFP10. Previous generations of reporter phage gave no detectable signal from mycobacteria in sputum (either reconstruction or clinical specimens). The high signal per cell encouraged us to proceed with reconstruction experiments in which defined laboratory-grown log-phase mycobacteria were spiked into human sputum. Deidentified clinical sputa from patients without mycobacterial disease were spiked with 10^4 *M. tuberculosis* cells per ml, processed with NALC and NaOH, and incubated for 12 h with ϕ^2GFP10. A fluorescent signal was readily observed if and only if *M. tuberculosis* cells were present. Processing with NALC alone, or with NALC plus 0.5% NaOH, did not decrease the reporter signal (Fig. 4A). However, fluorescence intensity/cell decreased ~5-fold when samples were processed with a higher concentration of NaOH (1% to 2%) prior to phage incubation. These data show that with ϕ^2GFP10, 90% of *M. tuberculosis* cells were detected in the spiked sputum samples. Outgrowth of nonmycobacterial species was not observed during the assay period in any of the 16 sputum samples from individual patients or in 4 independent sputum sample pools (each consisting of sputum from ~20 patients). Therefore, treatment with 0.5% NaOH was sufficient to eliminate interference of nonmycobacterial species in the ϕ^2GFP10-based assay.

Detection of *M. tuberculosis* cells in clinical samples. Considering the differences in metabolism, as reflected in the transcriptome of laboratory-grown *M. tuberculosis* compared to the expression thought to occur in patient sputum samples (16), it was not certain that ϕ^2GFP10 would be able to detect bacilli in clinical specimens. To test this, sputum samples from patients with smear-positive TB were obtained prior to the initiation of treat-

ment in Durban, South Africa. These were processed using the procedure detailed above for the spiking experiments and incubated with ϕ^2GFP10 for 12 h at 37°C. Fluorescent bacilli were readily detected by fluorescence microscopy. Figure 4B shows the result from a representative patient. DST of the clinical *M. tuberculosis* was determined for kanamycin and rifampin in parallel (Fig. 4B). Results obtained after 12 h of incubation with ϕ^2GFP10 indicated that the strain was sensitive to both of the drugs. These results were in 100% agreement with the results of DST determined using the agar proportion method (34), which were available only after 3 weeks. We have successfully performed DST for kanamycin and rifampin by the use of ϕ^2GFP10 on 7 sputum samples so far where agar proportionation was performed on parallel aliquots from same clinical specimens. There was 100% agreement with the DST compared with the results obtained using the agar proportionation method. All seven samples were sensitive to both the drugs. Notably, all samples subjected to DST were multibacillary.

DISCUSSION

This paper describes the construction and performance characteristics of a high-intensity fluorophage, ϕ^2GFP10. In cultured *M. tuberculosis* where direct comparison was possible, ϕ^2GFP10 yielded 100-fold more per-cell signal than any previously described reporter phage (33). ϕ^2GFP10 also allowed the visualization of individual *M. tuberculosis* bacilli expressing a phage-delivered reporter gene in clinical sputum samples. The phenotypic DST using ϕ^2GFP10 was easy to perform and required minimal processing of sputum samples. ϕ^2GFP10-based assay has the potential to be a low-cost assay for TB diagnosis and DST for detecting MDR and XDR strains of *M. tuberculosis* in resource-poor settings with minimal infrastructure.

We speculate that the phage promoter works so well in ϕ^2GFP10 because phages have been selected during evolution to achieve the maximum level of gene expression in the shortest amount of time. For example, mycobacteriophage D29 yields 120 new phages from a single infected cell of *M. tuberculosis* in 3 h, whereas the doubling time of *M. tuberculosis* itself is approximately 24 h (15). The new reporter phage vector, phAE159, is also deleted for the TM4-gp49 gene, which encodes a product that may inhibit bacteriophage superinfection (37). Deletion of TM4-gp49 might be responsible for the enhanced per-cell signal at higher MOI (Fig. 2C). Unlike all previous reporter phages, ϕ^2GFP10 illuminates *M. tuberculosis* directly from clinical sputum samples without prior culture. Until now, the immediate assay of clinical samples has not been possible with any previous reporter phage because the background fluorescence was too high in comparison with the weaker signal from the reporter phage.

This study was the first time a reporter phage approach has worked in sputum, either in the form of reconstructions or—most importantly—directly from clinical samples. Thus, development of ϕ^2GFP10 appears to eliminate a long-existing obstacle to the use of reporter phage assays in clinical settings for the detection and DST of *M. tuberculosis*. Previous generations of reporter phages worked only with cultured samples (3, 4, 36), extending the time required for assay and diminishing their advantage compared to other approaches. The results presented here with a small sample size are sufficient to demonstrate a proof of principle that (i) ϕ^2GFP10 can identify *M. tuberculosis* in sputum and that (ii) rapid DST can be performed directly from sputum by the use of ϕ^2GFP10. We are in the process of extending the study to a larger group of samples. A large-scale clinical study is required to compare the efficacy of the fluorophage ϕ^2GFP10 system with other methods for the detection and DST of *M. tuberculosis*.

More bacilli were visible in a typical field of view by Ziehl-Neelsen staining than by ϕ^2GFP10-based analysis when parallel aliquots from the same sample were compared (Fig. 4B, panels a and b). These results do not mean that acid-fast staining is more sensitive than ϕ^2GFP10 analysis, because Ziehl-Neelsen staining involves drying the sample, which reduces the three-dimensionality of the sample to a single plane that is entirely in focus. Visualization of cells using ϕ^2GFP10 was performed in wet preparations, and not all cells were in one plane (as can be seen in Fig. 4B, panel b). Because for any field of view there were many fluorescent bacilli outside the plane of focus, no direct comparison for sensitivity determinations could be performed. However, given the phenotypic nature of the test, the fluorophage detects only live and metabolically active cells, whereas Ziehl-Neelsen acid staining detects active, quiescent, and dead cells. The presence of dead bacilli in the sputum sample of patients undergoing treatment is expected to contribute to the differences in sensitivity between the two techniques. It has been proposed that the determination of the ratio of live to dead bacilli in clinical samples by the use of fluorophage in combination with acid-fast staining could be developed as a tool to assay for early bactericidal activity (24).

Two results seen with ϕ^2GFP10 imply the existence of distinct metabolic subpopulations within log-phase cultures of *M. tuberculosis*. (i) When isoniazid and phage were added together at time $t = 0$, the average fluorescence measured at 12 h decreased by 64%, and yet a subpopulation of cells retained maximal fluorescence (Fig. 3A). (ii) In experiments performed to determine the optimal MOI of infection, the whole population shifted to a higher MFI with increasing MOI, and yet the population spread of the dynamic range of fluorescence intensity per cell, i.e., the variance around the MFI, did not change with increasing MOI (Fig. 2C; compare MOI 10 and 100). According to the persister model, a metabolically less active subpopulation survives transient antibiotic exposure (1). An alternative hypothesis is that a rapidly metabolizing subpopulation survives by some other means, for example, by inducing a drug efflux pump (13). Further work is required to determine if persisters are enriched in one or the other metabolic subpopulation identified by ϕ^2GFP10 and to validate alternative mechanisms for persistence in *M. tuberculosis*.

Rapid DST of TB samples by the use of fluorphages has the potential to become a low-cost diagnostic method in resource-poor settings. The fluorescence microscope and appropriate filters needed for DST with ϕ^2GFP10 are already included in the recommended diagnostic equipment for laboratories in resource-poor settings (46). The necessary reagents for fluorophage growth and amplification are inexpensive, safe, and universally available. The cost associated with TB diagnosis and DST using three drugs is approximately $2, which is less than the cost of any other diagnostic test available, at least any test that can also assess antibiotic susceptibilities (24, 36). The assay is adaptable to a 96-well plate format, such that multiple samples can be processed in parallel.

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