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Conditionally Replicating Luciferase Reporter Phages: Improved Sensitivity for Rapid Detection and Assessment of Drug Susceptibility of *Mycobacterium tuberculosis*

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TM4 is a lytic mycobacteriophage which infects mycobacteria of clinical importance. A luciferase reporter phage, phAE40, has been constructed from TM4 and was previously shown to be useful for the rapid detection and drug susceptibility testing of *Mycobacterium tuberculosis*. However, the lytic nature of the phage results in a loss of detectable light output and limits the sensitivity of detection. We describe several strategies aimed at improving the luciferase activity generated by TM4 luciferase phages, including (i) varying the position of the luciferase gene in the phage genome, (ii) isolating host-range mutants of the phage, and (iii) introducing temperature-sensitive mutations in the phage such that it will not replicate at the infecting temperature. Several new phages generated by these methods show increased intensity of luciferase production compared to the first-generation reporter phage phAE40, and one phage, phAE88, also demonstrates an enhanced duration of luciferase activity. This has allowed the detection of as few as 120 BCG cells and the determination of drug susceptibilities of *M. tuberculosis* in as little as 1 day.

Tuberculosis (TB) is one of the most neglected global health problems and remains endemic in most parts of the world. The causative agent, *Mycobacterium tuberculosis*, is the single leading infectious cause of mortality worldwide, with 10 million infections and 3 million deaths estimated to occur each year (16). In many countries, tuberculosis is the most common opportunistic disease associated with human immunodeficiency virus infection (4). Furthermore, the increase in multidrug-resistant strains of *M. tuberculosis* is alarming. When undetected, multidrug-resistant TB has resulted in high mortality rates (23) and produced several hospital outbreaks (20). Since the best prevention of tuberculosis is detection and cure of infectious cases (24), current efforts are focused on improving the rapidity of identification of *M. tuberculosis*, allowing prompt initiation of appropriate therapy.

Recently, the luciferase reporter mycobacteriophage technique has been described as an efficient system to decrease the time required for diagnosis and drug susceptibility testing of *M. tuberculosis* and other mycobacteria (10, 15, 18). The first luciferase reporter phage (LRP), phAE40 (10), was constructed from the mycobacteriophage TM4, a lytic phage able to infect mycobacteria of clinical importance, including *M. tuberculosis* (22). However, the lytic nature of the phage produces a rapid mycobacterial cell lysis, resulting in a loss of detectable light output and limiting the sensitivity of detection to 10^4 mycobacterial cells (10). In contrast, a second reporter phage, phGS18, made from the temperate phage L5, forms lysogens after infection of *M. smegmatis*, resulting in accumulation of the luciferase protein, sustained increases in light output, and up to a 1,000-fold-improved limit of detection (18). However, L5 fails to efficiently infect and lysogenize *M. tuberculosis*, limiting its clinical utility. D29, a relative of L5, does infect *M. tuberculosis* but, like TM4, is lytic, and the LRPs derived from D29 have similar sensitivity characteristics (15).

In order to improve the luciferase activity from TM4 lytic phages, several independent strategies were investigated. In this study, we show (i) that the position of the luciferase gene within the phage genome affects the luciferase activity, (ii) that host-range mutants of TM4 that plaque more efficiently reduce luciferase activity, and (iii) that conditionally replicating mutants which plaque less efficiently produce more luciferase signal. Our results suggest that the latter phages can improve the performance of the luciferase assay and will provide a more sensitive tool to allow the detection and the determination of antibiotic susceptibilities of *M. tuberculosis* with fewer numbers of organisms.

MATERIALS AND METHODS

Bacteriophages, bacterial strains, and plasmids. The phages, bacterial strains, and plasmids used in this study are summarized in Table 1. The novel phages generated in this work are described below in some detail. The ethambutol-resistant strain of BCG designated mc^{2}2511 was isolated on Middlebrook 7H10 media (Difco, Detroit, Mich.) containing 5 μg of ethambutol (Sigma Chemical, St. Louis, Mo.)/ml. Cosmid pYUB556 was constructed by replacing the ampicillin resistance cassette of pYUB216 (10) with a *Nhe*I-*Spe*I fragment carrying the kanamycin resistance gene (*aph*) from pMV261 (21).

Cultivation of mycobacterial strains. *M. smegmatis* mc^{2}155 (19) was grown in Middlebrook 7H9 broth (Difco) with 0.4% Casamino Acids (Difco), 0.2% dextrose, and 0.05% Tween-80. BCG, *M. tuberculosis* Erdman, and clinical strains of *M. tuberculosis* (obtained from our clinical mycobacteriology laboratory) were grown in Middlebrook 7H9 broth with 10% ADS (0.5% bovine albumin fraction V [Sigma], 0.2% glucose, 140 mM NaCl), 0.5% glycerol, and 0.025% Tween-80. The drug-resistant BCG cells were grown in the latter medium containing either rifampin (50 μg/ml), streptomycin (250 μg/ml), isoniazid (INH) (5 μg/ml), or ethambutol (5 μg/ml) for mc^{2}768, mc^{2}767, mc^{2}765, or mc^{2}2511, respectively.

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TABLE 1. Bacterial strains, plasmids, and mycobacteriophages used in this study

Reagents	Description[a]	Reference or source
Mycobacterium (-a)		
M. smegmatis mc^{2}155	Phage-propagating strain	19
M. bovis var. BCG Pasteur		ATCC 35734
M. tuberculosis Erdman		ATCC 35801
M. bovis BCG mc^{2}765	INH resistant	10
M. bovis BCG mc^{2}767	Streptomycin resistant	10
M. bovis BCG mc^{2}768	Rifampin resistant	10
M. bovis BCG mc^{2}2511	Ethambutol resistant	This work
M. tuberculosis clinical strains	Unique clinical isolates (n = 6)	This work
Plasmid		
pYUB216	Cosmid with Amp^r, *E. coli* replicon, and *FFlux* under BCG hsp60 promoter	10
pYUB556	pYUB216::Kan^r, Amp^s	This work
pMV261	Mycobacterial cloning vector, Kan^r	21
pYUB328	Excisable cosmid cloning vector, Amp^r	1
Shuttle phasmid(s)		
phAE80, phAE81, phAE82	Unique TM4 shuttle phasmids with pYUB328	This work
phAE101	ts TM4 shuttle phasmid with pYUB328, fails to replicate at 37°C	This work
LRP(s)		
phAE40	Original TM4-based LRP, plaques BCG	10
phAE83, phAE84	phAE80 with either orientation of pYUB556	This work
phAE85	phAE81 with pYUB556	This work
phAE86	phAE82 with pYUB556	This work
phAE88	ts mutant of phAE101, fails to replicate at 37°C	This work
phAE115	Mutant of phAE85, plaques BCG as efficiently as *M. smegmatis*	This work
phAE117 to phAE122	Unique ts mutants of phAE85, fail to replicate at 37°C	This work
phAE123 to phAE125	Unique ts mutants of phAE115, fail to replicate at 37°C	This work
phAE130	Revertant of phAE88, replicates at 37°C	This work

[a] ts, temperature sensitive; *FFlux*, firefly luciferase gene.

Antibiotics were obtained from Sigma Chemical and were dissolved in water or ethanol (rifampin).

Preparation of high-titer phage lysates and phage DNA. High-titer phage lysates were prepared by infecting *M. smegmatis* mc^{2}155 cells on tryptic soy agar (Difco) plates as described previously (5, 10), either at 30 or 37°C. Lysates were harvested after the addition of 5 ml of phage buffer (10 mM Tris-HCl [pH 7.5], 10 mM $MgSO_4$, 70 mM NaCl, 1 mM $CaCl_2$) for 10 h at 4°C. Upon collection, lysates were filtered (0.45-μm-pore-size cellulose acetate membrane), titered, and stored at 4°C. Titration was performed by plating serial 10-fold dilutions of phage onto lawns of mc^{2}155 and counting plaques at 48 h, as previously described (5). Phage and shuttle phasmid DNAs were prepared by a standard proteinase K digestion phenol-chloroform extraction protocol (9).

Construction of TM4 shuttle phasmids by using pYUB328. TM4 shuttle phasmids, functioning as plasmids in *Escherichia coli* and as phages in mycobacteria, were constructed as described previously (9–11) except that the excisable cosmid pYUB328 was used as the cloning vector (1). Tm4 phage DNA was self-ligated to form concatemers and partially digested with *Sau*3AI to generate fragments 40- to 50-kb long. Two arms of pYUB328 were prepared by digestion with *Xba*I, alkaline phosphatase treatment, and *Bam*HI digestion; each arm contains a λ *cos* site and has one end compatible with the *Sau*3AI-digested phage DNA. Phage DNA was then ligated to the pYUB328 arms at a 1:10 molar ratio (insert to arms), and the ligation mixture was transduced into λ-sensitive *E. coli* HB101 with the GigaPack Gold in vitro packaging kit (Stratagene, La Jolla, Calif.). Cosmid DNA was extracted from a pool of 50,000 ampicillin-resistant colonies, representing a library of random pYUB328 insertions into the TM4 genome. Those insertions into nonessential regions of the phage genome are expected to result in viable recombinant phages. These were obtained by transforming the pooled cosmid DNA into *M. smegmatis* by electroporation (19) and selecting the mycobacteriophage plaques resulting after 24 h of incubation at 37°C. The presence of pYUB328 DNA in the genome of these phasmids was confirmed by direct plaque blotting analysis using the ECL direct nucleic acid labelling and detection systems (Amersham, Arlington Heights, Ill.); 98% of the plaques hybridized to a pYUB328 probe. Three such plaques were isolated as phAE80, phAE81, and phAE82; a fourth shuttle phasmid, phAE101, was derived from wild-type TM4 that had been previously mutagenized (see below).

Construction of TM4 LRPs from shuttle phasmids. In order to construct LRPs, the pYUB328 in the TM4 shuttle phasmids had to be replaced by a luciferase-bearing cosmid, pYUB556. DNA from each of the shuttle phasmids phAE80, phAE81, phAE82, and phAE101 was self-ligated to form concatemers and digested by *Not*I to excise pYUB328. The fragments were then ligated to the *Not*I-linearized pYUB556, the ligation was in vitro packaged, and the resultant recombinant cosmids were transduced into *E. coli* HB101 in the manner described for the generation of shuttle phasmids, except that kanamycin selection was used. The cosmid DNA was extracted and electroporated into *M. smegmatis* as before, and resulting plaques were checked by direct hybridization with a probe from a 1,378-bp *Bam*HI-*Eco*RV fragment of the *FFlux* gene; 100% of the plaques tested hybridized to the probe. The orientation of the insert was determined by restriction analysis for 10 independent colonies of each shuttle phasmid. Both orientations of the luciferase cassette pYUB556 were obtained only from phAE80, yielding phAE83 and phAE84. The LRPs derived from phAE81 (i.e., phAE85), phAE82 (i.e., phAE86), and phAE101 (i.e., phAE88) were present in only one orientation (see Fig. 1). High-titer phage lysates were prepared from individual plaques of each LRP.

Restriction analysis of recombinant shuttle phasmids. Recombinant phasmid DNA was analyzed by restriction analysis to localize the insertion site of the cosmid pYUB556 within the LRPs. After *Not*I digestion to remove pYUB556, linear phage DNA, which was either concatemerized or unligated, was cut with *Nde*I, allowing identification of a terminal *Nde*I fragment containing each of the cosmid insertions (Fig. 1). The presence of deletions in the recombinant phage genome was ascertained by comparing the *Nde*I digest of wild-type TM4 DNA (13.6 kb) with the same digest of each of the recombinant phage DNAs, containing the additional 5.1 kb of pYUB556 insertion.

Host-range phage mutants of TM4 LRPs. While all the LRPs derived as described above form plaques on *M. smegmatis* mc^{2}155, it is notable that they do so inefficiently on BCG, as is also found with their parent phage TM4 (10). However, TM4-derived phages repropagated from single plaques on BCG (occurring at a frequency of less than 10^{-8}) (9) are able to efficiently plaque on BCG, while retaining their plaquing on *M. smegmatis*. One such phage, phAE115 derived from phAE85, is able to plaque BCG even after a further round of phage propagation in *M. smegmatis*.

Hydroxylamine mutagenesis to generate conditionally replicating TM4 phages. In order to isolate TM4 conditionally replicating mutants which will plaque less efficiently on *M. smegmatis* at 37°C, but will plaque at 30°C, we mutagenized phage lysates using hydroxylamine (NH_2OH) in a standard protocol (3, 14) with minor modifications suitable for phage. Thus, 0.2 ml of phage lysates in phage buffer (at 10^{11} PFU/ml) was added to 0.4 ml of 0.5 M KH_2PO_4 (pH 6.0) and 5 mM EDTA, 0.8 ml of freshly prepared 1 M NH_2OH (pH 6.0), 20 μl of 1 M $MgSO_4$, and 0.6 ml of sterile water. Aliquots of each reaction were taken for titering after 24, 48 and 72 h of mutagenesis at 37°C to determine at which point only 0.1% of input phage survives, providing an intensity of mu-

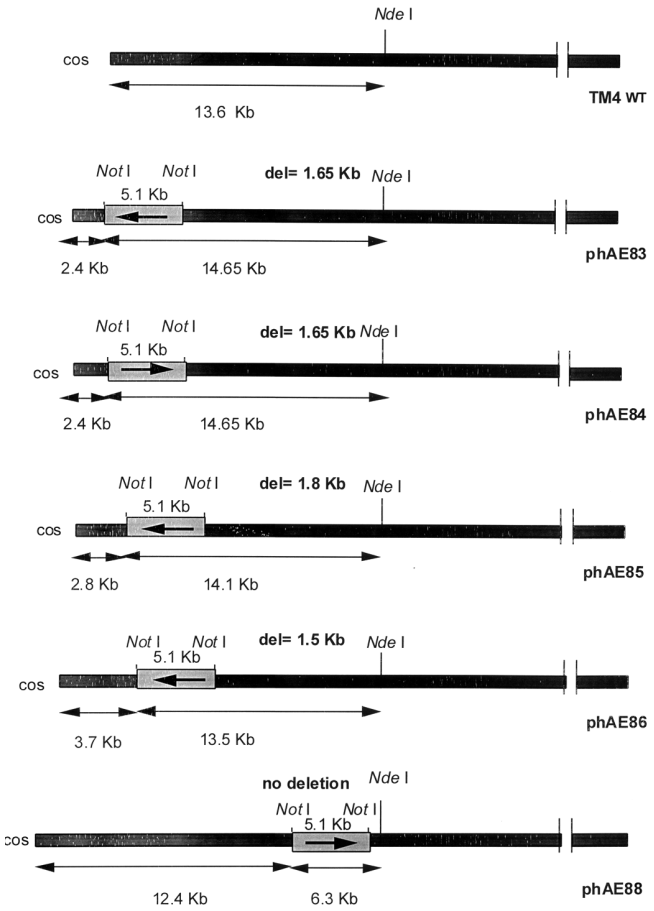

FIG. 1. Restriction map of TM4 wild type (WT) and five independent LRPs based on digests with *Not*I and *Nde*I. The luciferase cassettes are all located at one end of the TM4 genome between its *cos* site and an *Nde*I site. The direction of promoter transcription for the luciferase gene is indicated by an arrow. The estimated length of the deletion in the phage genome for each construct is noted, assuming the wild-type *Nde*I fragment is 13.6 kb and the cosmid pYUB556 is 5.1 kb.

tagenesis required to observe temperature-sensitive mutations. At each time point, the hydroxylamine reaction was stopped by being diluted 1:100 in cold LBSE medium (Luria broth medium containing 1 M NaCl–1 mM EDTA), and the solution was incubated for 1 h on ice, diluted two times in phage buffer, and stored at 4°C. Over 3,000 single plaques from sufficiently mutagenized stocks of phAE85 or phAE115 plated on *M. smegmatis* at 30°C were picked and transferred onto a *M. smegmatis* lawn subsequently grown at 37 and 42°C. Nine phages, designated phAE117 through phAE125, yielded no or only pinpoint plaques at 37°C and were repropagated and titered at both 30 and 37°C, with a 10^5- to 10^6 fold-lower titer at the latter temperature. To create phAE101, the shuttle phasmid precursor of phAE88, a second round of mutagenesis was performed on a previously mutagenized small-plaque mutant of wild-type TM4, resulting in a phage with no visible plaques at 37°C and a reversion frequency (ratio of titers at 37 and 30°C) of 10^{-8}.

Luciferase assays. BCG or *M. tuberculosis* cells were grown to stationary phase (BCG, optical density at 600 nm of 1.8 to 2.0; *M. tuberculosis*, turbidity of >2 McFarland units [Remel, Lenexa, Kans.]), washed twice, and resuspended (after 1:50 dilution) to approximately 10^7 cells/ml in Middlebrook 7H9 broth (Difco) with 10% ADS without Tween. The cells were incubated standing for 24 h at 37°C to optimize their infectibility by phage (10). Cells (1-ml samples) were infected with the LRPs at a multiplicity of infection of 10^3. At regular intervals from 1 to 24 h after phage infection, luciferase synthesis was monitored by assaying 100-μl aliquots (containing 10^6 CFU of mycobacteria) with 100 μl of 0.22 mM D-luciferin (Sigma) in 0.067 M sodium citrate (pH 4.5) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, Calif.). Determinations at all time points were performed in duplicate and graphed as average values ± standard errors. Experiments with virulent *M. tuberculosis* Erdman were performed in a Biosafety Level 3 facility by using a LUMAC 2010 luminometer (Perstorp Analytical, Silver Spring, Md.) within a biocontainment hood.

For assays of the sensitivity of detection, 10-fold dilutions of washed BCG in media without Tween were incubated at 37°C overnight and then infected with 10^{10} PFU of the LRP being tested. After 3 to 24 h of LRP infection at 37°C, 100-μl aliquots were removed for luciferase determinations. The titer of BCG was confirmed by plating serial dilutions of cells onto Middlebrook 7H10 plates (Difco) supplemented with oleic acid-albumin-dextrose-catalase (OADC) (BBL, Cockeysville, Md.).

Determination of drug susceptibility by using LRP phAE88. Light production from drug-sensitive and drug-resistant BCG, *M. tuberculosis* Erdman, or two clinical isolates of *M. tuberculosis* was determined after antibiotic treatment and infection with phAE88. The mycobacteria were washed twice, resuspended to a concentration of approximately 10^7 cells/ml, and incubated standing at 37°C for 24 h in the presence or absence of drug. Drug-sensitive BCG cells were incubated with rifampin (2 μg/ml), streptomycin (2 μg/ml), ethambutol (2.5 μg/ml), or INH (0.1 μg/ml). *M. tuberculosis* strains were incubated with INH (0.1 or 0.5 μg/ml), rifampin (2 μg/ml), ethambutol (5 μg/ml), or streptomycin (6 μg/ml). Drug-resistant BCG cells were treated with the antibiotic to which they are resistant (5 μg/ml). Phage infection was performed by addition of phAE88 lysates at a multiplicity of infection of 10^3, followed by incubation at 37°C and determination of luciferase activity at 2, 4, 6, and 8 h postinfection.

RESULTS

Construction and analysis of novel TM4 LRPs. In order to isolate a library of cosmid insertions into nonessential regions of the phage genome, we employed a shuttle phasmid technique as previously described (9–11), except that the cosmid cloning vector we used (pYUB328) was excisable from the phage genome by a *Not*I restriction digest. Since *Not*I does not cut anywhere in the phage genome, such a strategy inserts unique cloning sites within the phage DNA in a region which does not affect the viability of the phage.

From the restriction analysis of the initial phasmid library, we isolated three unique constructs, phAE80, phAE81, and phAE82, differing by the location of the cosmid in the phage genome. We converted these three shuttle phasmids into reporter phages by replacing the cosmid pYUB328 with pYUB556 containing the luciferase cassette. We sought to isolate LRPs with opposite orientations of their luciferase cassettes. Notably, we were able to obtain both orientations of pYUB556 only from the phAE80 construct, creating phAE83 and phAE84. The other unique LRPs were named phAE85 (from phAE81) and phAE86 (from phAE82).

Despite their unique insertion sites (Fig. 1), all the luciferase cassettes in the recombinants were located at one end of the TM4 genome, between its "left" terminus and a *Nde*I site. This clustering suggests that a nonessential region exists here. Further, by comparing the length of the terminal *Nde*I fragment containing the cosmids to that from wild-type TM4, we infer that a deletion occurred in the phage genome of each construct: approximately 1.65 kb for phAE83 and phAE84, 1.8 kb for phAE85, and 1.5 kb for phAE86.

Luciferase activity of novel TM4 LRPs. The luciferase activity of the new LRPs after infection of BCG and *M. tuberculosis* was compared with that of phAE40 as a reference. The kinetics of light production after infection with the reporter phages shows a rapid increase in luciferase production over the first 3 h after infection with each of the phages (Fig. 2A). The highest activity was consistently produced by phAE85, which produced approximately 3 to 10 times more light than phAE40 from BCG (Fig. 2A and C). The other constructs produced luciferase activity in a range of values intermediate between phAE85 and phAE40 (Fig. 2A). phAE83 and phAE84 (with opposite orientations of the luciferase cassette) had approximately the same peak activity, suggesting that the orientation of the luciferase cassette in these constructs does not affect expression of the luciferase gene. By 6 h of infection, a rapid decrease in light production had occurred with all the phages, consistent with a mycobacterial cell lysis.

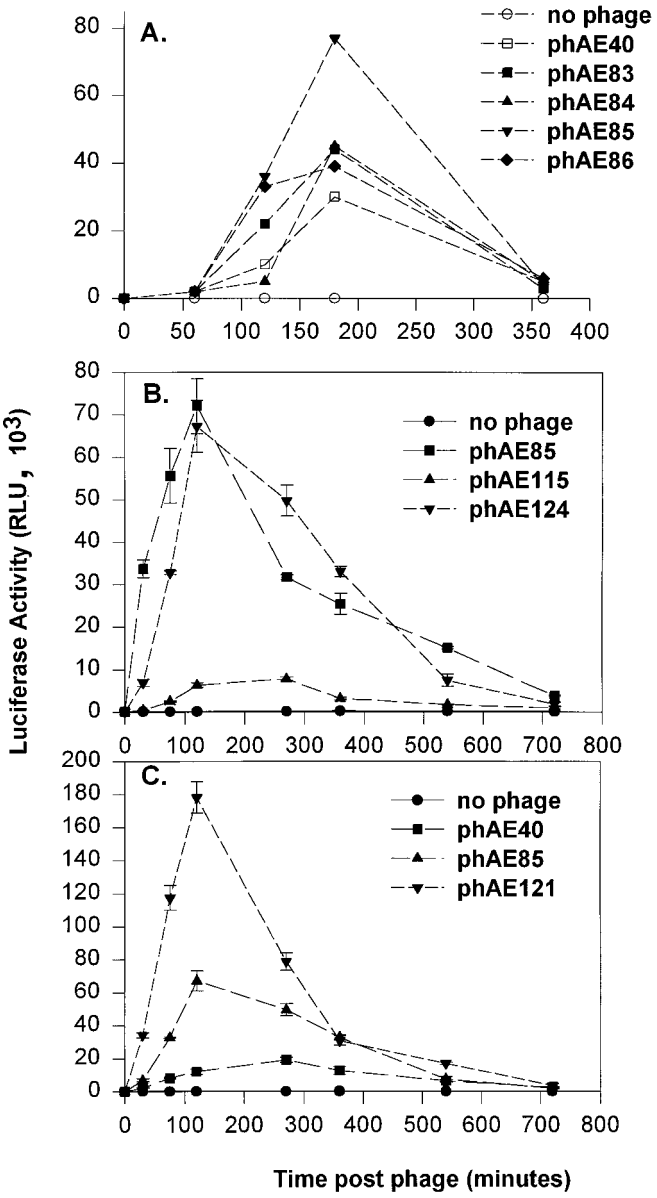

FIG. 2. Kinetics of luciferase activity after infection of BCG with TM4 LRPs. (A) Effects of different luciferase cassette insertion sites and orientation. (B) Effects of host-range and temperature-sensitive mutants of phAE85. (C) Effects of temperature-sensitive mutants of phAE85.

Luciferase activity of host-range mutants of novel LRPs. Since phAE85 was chosen for further studies, we noted that it did not form plaques on BCG, thus suggesting its life cycle was somehow arrested in this mycobacterium. As with other TM4 phages, mutants which do plaque on BCG could be selected (9, 10), and one such isolate, phAE115, was tested for its expression of luciferase activity after infection of BCG. As seen in Fig. 2B, the peak of light production after phAE115 infection of BCG is markedly reduced compared to that of phAE85. Thus, it seems that the nonplaquing phage phenotype of phAE85 is beneficial for eliciting optimal luciferase production from LRP-infected BCG.

Isolation and luciferase testing of conditionally replicating LRPs. Given that a nonplaquing phage results in more light than its plaque-producing relative, we reasoned that other mutations could be introduced into the phage genome which would further attenuate its virulence, allowing more sustained luciferase production and perhaps leading to a greater sensitivity of detection. With the conditionally replicating phage methodology, we planned to obtain a phage that could deliver a desired DNA sequence (the reporter gene and its promoter) without damaging the host cell, thereby mimicking a temperate phage such as L5. We therefore mutagenized phages phAE85 and phAE115 and selected for conditionally replicating mutants, those that can be propagated on *M. smegmatis* at a permissive temperature (30°C) but fail to form plaques at the temperature of the luciferase assay (37°C). Eight conditionally replicating phages were selected for luciferase kinetics studies in BCG based on their differential plaquing at 30 and 37°C; each phage had a plaque titer at least 10^5 times lower at the nonpermissive temperature of 37°C. The luciferase signal derived from representative sets of these phages (phAE121 derived from phAE85 and phAE124 derived from phAE115) after infection of BCG is shown in Fig. 2B and C. In each instance, the conditionally replicating mutant expresses higher peak luciferase activity at the nonpermissive temperature than its parent, but the kinetics of luciferase expression are no different than those from the respective nonmutagenized parental phage.

Construction of a lytic LRP with a thermosensitive mutation that extends the duration of luciferase activity. The decline of the luciferase signal from the conditionally replicating phages suggests that despite not plaquing, these phages are still somehow damaging their host. In order to try to attenuate these phages further, we elected to mutagenize the wild-type phage TM4 rather than the shuttle phasmids, to do so more intensely (using two cycles), and only thereafter to introduce the luciferase cassette. This approach overcomes the potential constraints on the sites of mutagenesis imposed by the deletions in the shuttle phasmids phAE80 to phAE82. These deleted genes may normally serve a redundant function and now become essential to maintain the viability of the phage once their companion genes have been mutagenized.

A phage mutant, PH101, obtained by this approach replicates at the permissive temperature (30°C) but fails to plaque at the nonpermissive temperature (37°C), with a reversion frequency of less than 10^{-8}. Using the shuttle phasmid technology, as previously applied to TM4 wild type in this study, we isolated one construct from a library of pYUB328 cosmid insertions in PH101. The resulting TM4 temperature-sensitive shuttle phasmid phAE101 was converted to the corresponding LRP by replacing the cosmid pYUB328 with pYUB556, creating phAE88. No difference in the temperature sensitivity of phAE88 was found compared to the original phage mutant PH101.

By endonuclease restriction analysis, we determined the location of the luciferase cassette in the phAE88 construct (Fig. 1). Although it was situated in the same end of the phage genome as the other constructs, it was further from the *cos* site (Fig. 1), suggesting a second nonessential region exists in the TM4 genome. Moreover, the restriction analysis shows that no deletion occurred in the phAE88 genome and consequently that the TM4 phage head is capable of accepting approximately 5.1 kb of additional DNA (the size of pYUB556).

Luciferase production after infection with the conditionally replicating LRP phAE88. The luciferase activity after infection of tuberculous mycobacteria with phAE88 was evaluated and compared to that from phAE40 and phAE85 (Fig. 3). While the expected rapid decline in light production occurred with both phAE40 and phAE85, the temperature-sensitive mutant phAE88 demonstrated increased light emission from BCG,

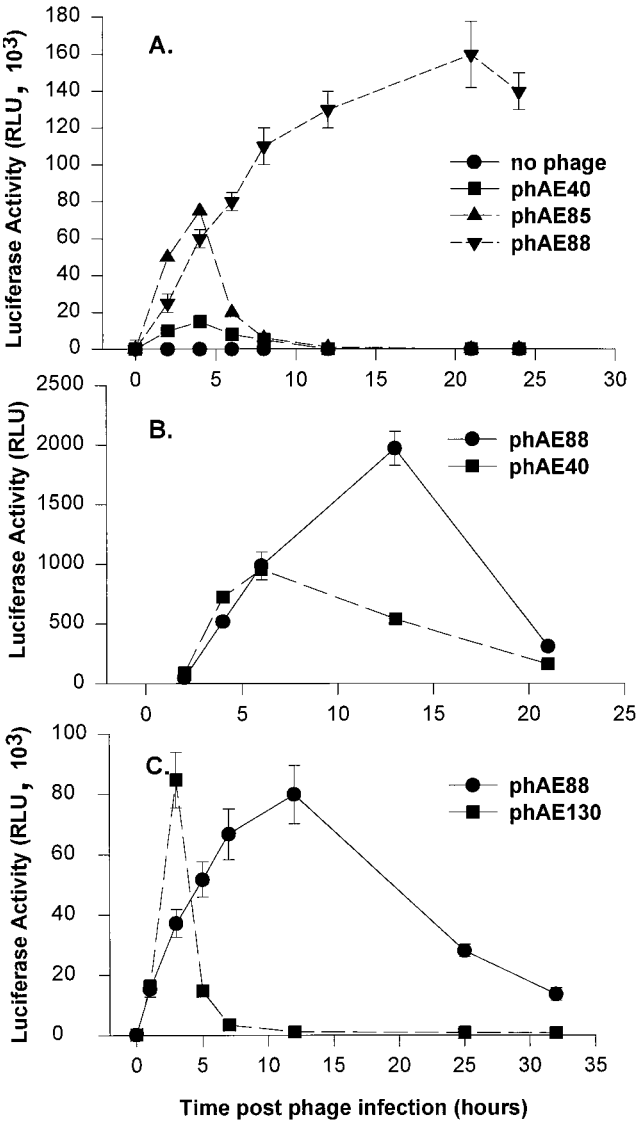

FIG. 3. Kinetics of luciferase activity after infection with the conditionally replicating LRP phAE88. (A) Infection of BCG with indicated phages. (B) Infection of *M. tuberculosis* Erdman with indicated phages. (C) Infection of BCG with indicated phages.

which peaked at 12 to 21 h (Fig. 3A and B) and decreased to the basal level by 36 h after infection. In experiments with phAE88 infection of *M. tuberculosis* Erdman, the duration of light output is similarly extended and increased relative to phAE40 (Fig. 3B). In addition, five clinical isolates of *M. tuberculosis* produce a mean of 9 times (range, 3 to 15) more light than the non-temperature-sensitive phage phAE86. Finally, a spontaneous revertant of phAE88 designated phAE130, which regains its ability to plaque at 37°C, concurrently shows non-extended luciferase expression (Fig. 3C). These results provide strong evidence that the temperature-sensitive mutation in the phage genome, which inhibits the ability of phage to plaque, can also extend and expand the production of luciferase reporter protein after infection of tuberculous mycobacteria.

Increased sensitivity of detection of mycobacteria with conditionally replicating TM4 LRPs. In order to determine the sensitivity of mycobacterial cell detection with phAE85 and phAE88 compared to phAE40, we infected serial dilutions of BCG cells with each of these phages. The cell concentrations were determined by plating mycobacteria prior to infection, and the experiment was repeated twice. Figure 4 shows the luciferase activity derived from 1.2×10^5 to 1.2×10^1 cells. The lytic phage phAE85 detects as few as 1.2×10^4 mycobacteria 4 h after infection, producing a higher signal than phAE40 under the same conditions. With the conditionally replicating phage phAE88, a very clear signal is obtained from only 1,200 cells after 8 h of infection. Furthermore, extending the time for light output to 12 h allows the detection of as few as 120 BCG cells. Clearly, the new generation of phages is capable of detecting a very low number of cells a short time after infection.

Rapid determination of drug susceptibility by using the conditionally replicating LRP phAE88. Differences in light production between drug-sensitive and drug-resistant mycobacteria were determined after 24-h antibiotic treatment and subsequent infection with phAE88. The earliest useful time point, 2 h post phage infection, is shown in most of the panels in Fig. 5, while somewhat greater differences in light production between drug-treated and control cells were evident by repeating the test at 6 to 8 h after phAE88 infection (data not shown). Figure 5A shows results obtained with drug-sensitive BCG cells incubated in the presence or absence of the main antituberculous antibiotics at clinically relevant concentrations. The samples containing no drug showed a rapid increase in their light emission. In contrast, minimal luciferase activity was detected in the samples pretreated with each of the different drugs. Similar results were obtained with an Erdman strain and clinical strains of *M. tuberculosis* (Fig. 5B), one of which was confirmed to be INH resistant by standard methods. The final four panels in Fig. 5 (Fig. 5C to F, respectively) represent the results obtained with the streptomycin-resistant mc^{2}767, the rifampin-resistant mc^{2}768, the INH-resistant mc^{2}765, and the ethambutol-resistant mc^{2}2511 treated with the

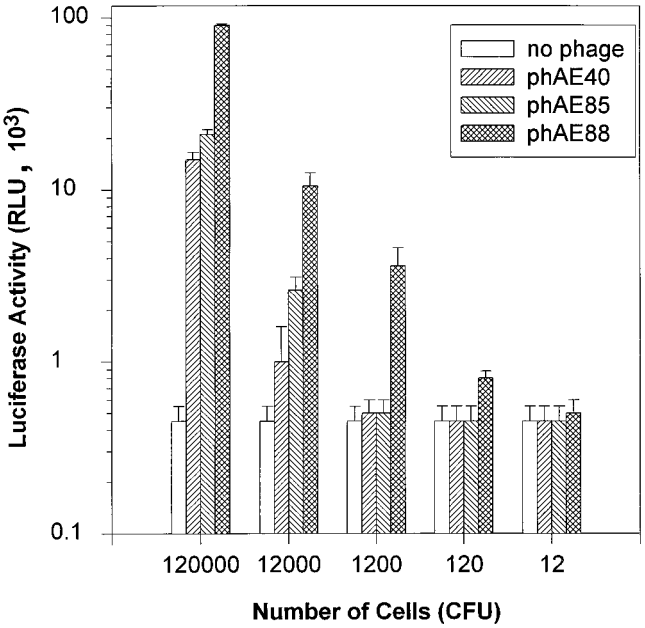

FIG. 4. Sensitivity of detection of mycobacteria with TM4 LRPs. Tenfold serial dilutions of BCG were infected with phAE40, phAE85, or phAE88 at time zero, and peak light output, obtained at 4 h for phAE40 and phAE85 and 12 h for phAE88, was plotted. All readings were performed in duplicate and data are average values ± standard errors.

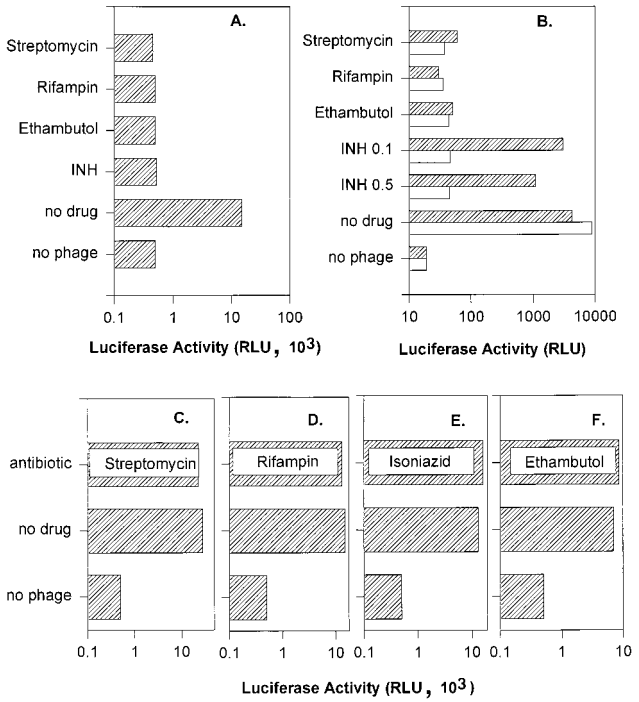

FIG. 5. Determination of drug susceptibility with phAE88. Mycobacteria were incubated for 24 h in the presence or absence of drug and then infected with phage phAE88 for 2 h (for BCG) or 4 h (for *M. tuberculosis*). Background luciferase activity was determined after addition of "no phage" to the mycobacteria. (A) BCG cells tested after incubation with rifampin (2 μg/ml), streptomycin (2 μg/ml), ethambutol (2.5 μg/ml), or INH (0.1 μg/ml). (B) Two clinical strains of *M. tuberculosis* (one fully drug susceptible [open bars] and one INH resistant [shaded bars]) treated with INH (0.1 or 0.5 μg/ml), rifampin (2 μg/ml), streptomycin (6 μg/ml), or ethambutol (5 μg/ml). (C) Streptomycin-resistant BCG mc^2767 treated with or without streptomycin (5 μg/ml). (D) Rifampin-resistant BCG mc^2768 treated with or without rifampin (5 μg/ml). (E) INH-resistant BCG mc^2765 treated with or without INH (5 μg/ml). (F) Ethambutol-resistant BCG mc^2511 treated with or without ethambutol (5 μg/ml).

antibiotic to which they are resistant (5 μg/ml). The luciferase activity from these drug-resistant strains is unaffected by the addition of drug. These results suggest that the drug susceptibility profile of tuberculous mycobacterial strains can be clearly defined 2 h after infection with phAE88. A longer time of infection would permit further confirmation of the result or the ability to work with a lower inoculum.

DISCUSSION

This work describes a new set of improved TM4 LRPs.

We used the excisable cosmid pYUB328 as a cloning vector in the construction of shuttle phasmids, which has allowed the rapid generation, manipulation, and analysis of reporter phages and led to the following observations about the genetic organization of phage TM4. (i) There are at least two nonessential regions within the "left" arm of the TM4 phage genome, one between 2.4 and 3.7 kb from the phage *cos* site for phAE80, phAE81, and phAE82 and one 12.4 kb from the *cos* site for phAE101. (ii) At three of the four sites, only one orientation of the luciferase cassette is tolerated by the phage, as no viable phage results from the luciferase cassette in the opposite direction, presumably due to the polar effects of the powerful hsp60 promoter. (iii) As phAE88 can incorporate the entire luciferase cassette without undergoing any deletions, the TM4 head can package at least 5.1 kb of additional DNA, which is available for cloning. This may be useful in using a temperature-sensitive recombinant phage as a vehicle for delivery of transposons (2), i.e., as a tool for genetic manipulation of mycobacteria.

While the independently generated shuttle phasmid constructs (phAE80, phAE81, and phAE82) map to a small region, there are differences in their expression of luciferase activity upon infection of mycobacteria. In particular, phAE85 expresses significantly more luciferase output at its peak than the first LRP, phAE40. The explanation for this may lie in the phage sequences adjacent to the luciferase cassette, which can contain strong *cis*-acting native phage promoters that dominate reporter gene transcriptional activity and modulate its expression, as seen with the D29 LRPs (15). Alternatively, adjacent phage RNA may stabilize the luciferase transcripts. Thus, the precise location and orientation of the luciferase cassette in the phage genome can significantly modify the luciferase activity which is obtained.

Beyond optimizing the interaction of the phage with the inserted luciferase cassette, we sought to increase the luciferase signal even further by obviating any deleterious effects of lytic phage infection on the host mycobacteria. We used hydroxylamine mutagenesis to generate temperature-sensitive mutations in genes required for the lytic growth of TM4. In phage L1, closely related to L5 and containing a genome size similar to TM4, at least 28 genes (of the total of approximately 88 in L5 [7]) are required for lytic growth (3). These include the "late" genes encoding the lytic machinery of the phage, as well as the "early" genes required to facilitate phage DNA synthesis and replication. Mutations in both types of genes may improve luciferase production from reporter phages upon infection of mycobacteria. Host cell lysis, which occurs "late" (by 3 h [preliminary data]) in the lytic cycle of TM4, disperses the luciferase and ATP required for optimal light generation. Interrupting this process, as has been done in the lysis-deficient protein R and S mutants of lambda phage, can increase the yield of heterologous expressed proteins (14). On the other hand, phage "early gene" functions may encode a system to turn off host protein synthesis, which was demonstrated to occur in the lytic cycle of phage L5 (7). It is conceivable that this process, which down-regulates expression of native mycobacterial stress proteins in L5, would also decrease production of the heterologous *FFlux* driven by a mycobacterial heat shock promoter (hsp60). Mutations in either the lytic machinery or the host protein synthetic shutdown apparatus could clearly enhance luciferase production from reporter phages, assuming these phages were still able to infect the host.

A set of conditionally replicating TM4 phages (phAE117 to phAE125) was therefore isolated, each of which demonstrated improved peak luciferase output relative to its non-temperature-sensitive parent but surprisingly similar kinetics. This suggests that residual phage functions were still deleterious to the mycobacterial host or that revertants (fully lytic phages) arose rapidly and lysed the luciferase-producing mycobacteria. Indeed, the more sustained luciferase activity observed with the more highly mutagenized phAE88 relative to each of the earlier temperature-sensitive LRPs (Fig. 3) shows that multiple mutations may be required to allow an extended duration of luciferase activity. Alternatively, the lower reversion frequency of phAE88 relative to the other temperature-sensitive LRPs, minimizing a second round of lytic infections by revertants such as phAE130, may be sufficient to explain the delayed mycobacterial lysis observed.

Why does the luciferase activity derived from phAE88 eventually fall off? First, as indicated above, there may still be intact phage-expressed genes which prove to be ultimately deleterious to the host. It is unlikely that the luciferase is itself toxic, as

stable transformants of mycobacteria expressing luciferase are quite viable (10). Second, the temperature-sensitive function may simply be delayed in onset rather than fully eliminated at the nonpermissive temperature. Third, revertants of the conditionally replicating phage which are fully lytic may arise, damaging the mycobacterial host in a second wave of lytic infections. Finally, while the kinetics of light output with phAE88 (Fig. 3) resemble those of a temperate phage (18) in the first day after infection, the light signal is rapidly reduced thereafter. One prominent difference between phAE88 and a true temperate phage (phGS18 [18]) lies in the integration of the latter in the mycobacterial chromosome. While integrated phage (prophage) is transmitted to all progeny after cell division, the temperature-sensitive phAE88 may be lost during cell replication. In fact, the decline of luciferase activity with phAE88 corresponds to the time by which cell division is expected to occur in BCG.

Further work in our laboratory will focus on understanding the mechanisms of sustained luciferase production and defining the specific genes mutated in the conditionally replicating phages, using a complementation approach. Once identified, mutations in these genes can be combined and stabilized by engineering deletions or amber suppressor mutations. These will allow the phage to be propagated in a unique *trans*-complementing or suppressor tRNA-expressing mycobacterial strain, while ensuring it does not produce a lytic infection in a target mycobacterial strain. Finally, the forthcoming genome sequence of phage TM4 (6) should facilitate a rational dissection of phage genetics to allow the generation of an ideal reporter gene delivery system.

In this work, we have shown that the sustained, high levels of luciferase activity that result from phAE88 infection of BCG allow the detection of as few as 120 BCG cells after a 12-h infection. This is the first time such a sensitivity of detection has been obtained in a slow-growing mycobacterium with the LRP technique. With improvements in clinical sample processing allowing detection directly from sputum samples (preliminary data), this sensitivity of detection should allow *M. tuberculosis* in most clinical samples (8) to be amenable to LRP protocols.

It had been previously shown that luciferase reporter mycobacteriophages were capable of distinguishing drug-resistant from drug-susceptible *M. tuberculosis* organisms in a 48-h assay with phAE40 (10) and in less time by using the temperate reporter phages with the rapidly growing *M. smegmatis* (18). In this study, we used phAE88 to determine the drug susceptibility profile of various BCG strains. Sensitive and resistant strains were discriminated at clinically relevant concentrations of each antimicrobial agent. We showed for the first time that the susceptibility to ethambutol could be defined in a slow-growing mycobacterium and that a 24-h incubation in the presence of each drug, with 2 h of phage infection, sufficed to define the susceptibility profile. With certain antibiotics (rifampin and streptomycin), the time to susceptibility results was even further reduced, as the drugs could be added simultaneously with the phage (data not shown). It is presumed that the efficacy of these latter agents relates to their direct effects on host processes (transcription and translation) required by the phage to generate its reporter protein (12, 13) and explains their rapid onset of action.

The advantages of the high sensitivity of detection of phAE88 and its ability to rapidly detect drug resistance in mycobacteria extend beyond earlier detection of fewer mycobacteria. For a given inoculum, a higher signal would allow the determination of fractional drug resistance from a mixed population of mycobacteria. Given the close correlation between light output and cell number shown here (Fig. 4) and previously (10), and assuming equal efficiencies of phage infection between drug-susceptible and -resistant strains, the percentage of residual light activity after antibiotic therapy should reflect the percentage of the population which is resistant to the drug. The most sensitive reporter phages should be best able to identify low levels of resistance in a population. Finally, the sustained expression of luciferase activity opens the door to photographic detection modalities presently being developed in our laboratory which integrate light output over time, thus increasing the sensitivity even further. In conjunction with refinements in the specificity of the phage for *M. tuberculosis* utilizing a selective antibiotic (17), these improvements in sensitivity will allow this technology to be compared to current technologies in head-to-head clinical trials.

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