RESEARCH LETTER

Application of BRED technology to construct recombinant D29 reporter phage expressing EGFP

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Abstract

Bacteriophage Recombineering of Electroporated DNA (BRED) has been described for construction of gene deletion and point mutations in mycobacteriophages. Using BRED, we inserted a P_{hsp60}-*egfp* cassette (1143 bp) into the mycobacteriophage D29 genome to construct a new reporter phage, which was used for detection of mycobacterial cells. The cassette was successfully inserted and recombinant mycobacteriophage purified. DNA sequencing of the cassette did not show any mutations even after several phage generations. *Mycobacterium smegmatis* mc^2155 cells were infected with D29::P_{hsp60}-*egfp* (MOI of 10) and evaluated for EGFP expression by microscopy. Fluorescence was observed at around 2 h after infection, but dissipated in later times because of cell lysis. We attempted to construct a lysis-defective mutant by deleting the *lysA* gene, although we were unable to purify the mutant to homogeneity even with complementation. These observations demonstrate the ability of BRED to insert *c.* 1 kbp-sized DNA segments into mycobacteriophage genomes as a strategy for constructing new diagnostic reporter phages.

Introduction

Recombinant bacteriophages are useful tools for studying gene function and construction of phage reporters for detecting viable *Mycobacterium tuberculosis* in clinical specimens. The insertion of gene cassettes into mycobacteriophages was first accomplished with shuttle phasmids based on the lytic mycobacteriophage TM4 (Jacobs *et al.*, 1993). Shuttle phasmids have a combination of phage and plasmid features, can be genetically manipulated *in vitro*, amplified in *Escherichia coli* as plasmids, and used for infecting Mycobacteria where they can be propagated as bacteriophages. Since then, a variety of luciferase phage reporters have been constructed for detection of active and nonreplicating *M. tuberculosis* (Sarkis *et al.*, 1995; Pearson *et al.*, 1996; Carrière *et al.*, 1997; Dusthackeer *et al.*, 2008).

Based on the same technology, a new group of reporter phages (phAE87::P_{hsp60}-*egfp* and phAE87::P_{hsp60}-ZsYellow) expressing fluorescent proteins were constructed (Piuri *et al.*, 2009). These fluoromycobacteriophages allowed the detection of viable mycobacteria in fluorescent microcopy and by flow cytometry. The phage expressing the EGFP (enhanced green fluorescent protein) was further evaluated to detect drug resistance to isoniazid, rifampicin and streptomycin in a total of 155 strains of *M. tuberculosis* showing a low cost per sample and rapid results if compared with standard phenotypic assays; sensitivity for detecting isoniazid and rifampicin resistance was similar to that determined using a resazurin assay, but slightly lower for streptomycin resistance (Rondón *et al.*, 2011).

The expression rate of GFP by fluorophages was recently increased 100-fold by Jain *et al.* (2012), using a more efficient promoter and a new reporter phage vector. This high-level expression of GFP overcame the influence of background fluorescence in the assay and permitted the detection of *M. tuberculosis* in sputum in preliminary results. The performance of this fluoromycobacteriophage in a high-burden tuberculosis setting remains to be fully evaluated.

Genetic manipulation to obtain gene replacement, point mutations, *in vivo* cloning, and unmarked deletions in mycobacteria became less complex with the advent of a recombineering system by Van Kessel & Hatfull (2007). Analysis of bacteriophage Che9c genome revealed genes encoding an exonuclease (gp60) and a recombinase (gp61), and homologs to RecE and RecT from *Escherichia coli* Rac prophage, respectively. These genes were used for constructing pJV62 containing only gp61 to generate target point mutations with ssDNA as substrate and pJV53 containing both genes for dsDNA recombineering.

Recombineering technology has also been applied for generating mutations, deletions, gene replacement, and insertions in mycobacteriophages with high efficiency. Using Bacteriophage Recombineering of Electroporated DNA (BRED), *M. smegmatis* mc^2155 with recombineering functions is simultaneously electroporated with phage genomic DNA and a targeting substrate (Van Kessel & Hatfull, 2008; Van Kessel *et al.*, 2008; Marinelli *et al.*, 2012). Phage particles can then be recovered by plating the electroporated cells. In general, 1–15% of the screened plaques contain the expected mutant, which can be identified by PCR. These primary plaques are mixed, containing nonrecombinant mycobacteriophages as well as the mutant. Several rounds of plating and PCR are needed to isolate recombinants (Marinelli *et al.*, 2008).

There are many advantages in using BRED; there is no need for constructing complex cloning systems, no requirement for a selectable marker, mutations can be made in any part of a mycobacteriophage genome, the recombineering process takes place *in vivo*, and detection of phage recombinants is rapidly accomplished by PCR. Although BRED is faster and less complex than alternative cloning systems, insertion or deletion of large DNA segments is anticipated to be less efficient. This has to be considered for constructing reporter phages containing gene cassettes that are usually larger than 400 bp.

As every phage reporter constructed to date took advantage of shuttle phasmid technology, here we attempted to use the BRED approach for constructing a recombinant D29 mycobacteriophage expressing EGFP under the control of the *hsp60* promoter and applying it to the detection of mycobacteria.

Materials and methods

Electrocompetent cells

Mycobacterium smegmatis mc^2155 cells containing plasmid pJV53 were grown for 48 h at 37 °C in middlebrook 7H9 supplemented with 10% ADC (albumin, dextrose, catalase) (Becton Dickinson GmbH, Heidelberg, GE), 0.05% Tween 80, and kanamycin at 30 μg mL^{-1} (Sigma-Aldrich, St. Louis, MO). A volume of 500–1000 μL was transferred from the culture to 100 mL of 7H9 broth with 1 mM $CaCl_2$, 30 μg mL^{-1} kanamycin, and 0.2% succinic acid (Sigma-Aldrich) to obtain a culture with an optical density ($OD_{600\,nm}$) of 0.02. The culture was incubated at 37 °C, 250 rpm until it reached an OD_{600} of 0.4. Acetamide (Sigma-Aldrich) was added to a final concentration of 0.2%, and the culture was further incubated for 3 h, at 37 °C, 250 rpm. The culture was maintained on ice for 30 min and washed four times with ice cold 10% glycerol (Sigma-Aldrich). The bacterial pellet was suspended in 2 mL of ice cold 10% glycerol. Aliquots were kept on −80 °C until the electroporation or up to 6 months (Piuri *et al.*, 2009).

Table 1. Oligonucleotides used for D29 phage recombinants construction and detection

Oligonucleotide	Sequence
100 bp 82/71	5′-CGTGTTAGTTCAGGAGTTCCTCGATGTCGGGTGGCCAGCACCAGATCATGTGCAGGTTCTTGTAGACGAAGACGCGAATTGGCT TGGGGTTCATGCGATC-3′
D29-p1	5′-GCGGTTCCTTACTGCGTGGGCTCCGCTGGCTACGGAGAACGCACGCGCGTCGTGTTAGTTCAGGAGTTCCTCGAT-3′
D29-p2	5′-TTGAGTCGGACCCGACGCTTCAGCGTCAAAGACAACTACCTGGATGACTGGATCGCATGAACCCCAAGCCAATTC-3′
D29-p3	5′-CGTGTTAGTTCAGGAGTTCCTCGATGTCGGGTGGCCAGCACCAGATCATTTACTTGTACAGCTCGTCCATGCC-3′
D29-p4	5′-GATCGCATGAACCCCAAGCCAATTCGCGTCTTCGTCTACAAGAACCTGCACTCTAGAGGTGACCACAACGAC-3′
100 bp LysA	5′-CATGACGCTCATAGTCACACGCGACCACGCGCAGTGGGTCCACGACATGAACCCCGAGTACCTACAGGCGTACATCGCCAG GAATGGAGCCCTATGAG- 3′
LysA-1	5′-GCAGGTTCGAGTCCTGCTCTCGCGACTTGACAGCCACCACGAAAGGAACCCATGACGCTCATAGTCACACGCGA-3′
LysA-2	5′-TGCCGGGGACGAGAGTGCCGACGTAGTAGAGCGTTTCACGGATCTTGGGGCTCATAGGGCTCCATTCCTGGCGAT-3
D1	5′-TACGAAGGTATCGGCGAGCCATC-3′
D2	5′-GCTAGTGAGCGGCATTGCGG-3′
D3	5′-TCGTTGTGGTCACCTCTAGAGTG-3′
D4	5′-GTTCGAGTCCTGCTCTCGCGA-3′
D5	5′-ACGAGAGTGCCGACGTAGTAGA-3′
D6	5′-GCAGTGGGTCCACGACATGAAC-3′

Construction of a 200-bp recombineering substrate

A 100-base oligonucleotide (Integrated DNA Technologies, San Diego, CA) named 100 bp 82/71 (Table 1), with 50-base upstream and downstream homology to the D29 genome segment to be deleted, was amplified by PCR. Two 75-base primers, D29-p1and D29-p2 (Integrated DNA Technologies), with 50-base overlaps on each end of the 100-mer, were used to generate a 200-bp recombineering substrate with 100-bp homology on either side of the D29 DNA segment selected for being deleted (Fig. 1).

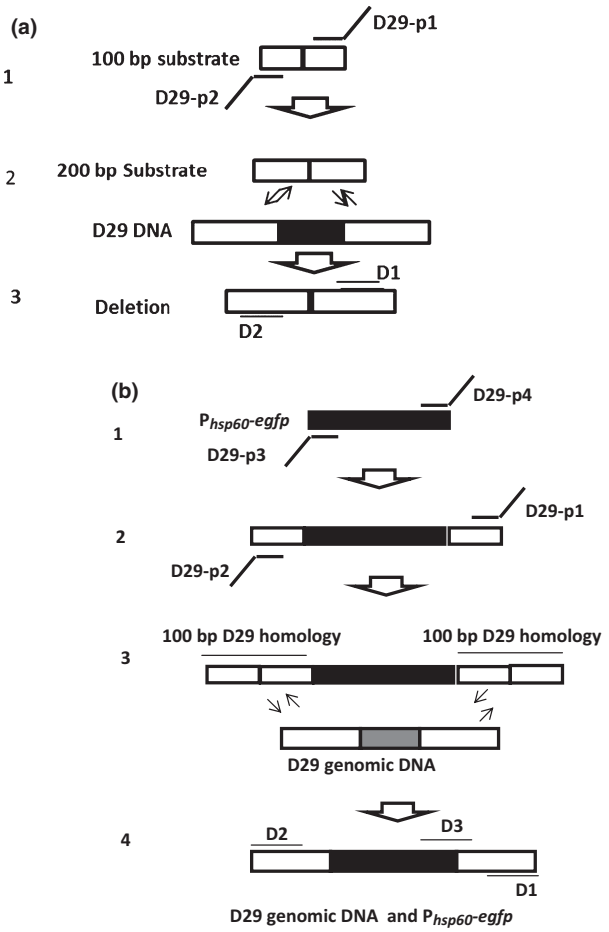

Fig. 1. Deletion of the 472 bp and insertion/replacement of the P_{hsp60}-*egfp* into the phage D29 genomic DNA. (a) 1. Amplification of the 100-bp oligonucleotide to generate a 200-bp substrate with primers D29-p2 and D29-p1; 2. Recombineering involving D29 phage genomic DNA and the 200-bp substrate; 3. Detection of deletion with flanking primers D1 and D2. (b) 1. Addition of 50-bp sequence homolog to D29 genomic DNA targeting sequencing on either side of P_{hsp60}-*egfp* with primers D29-p3 or D29-p4; 2. Second PCR to obtain 100 bp of homology to either side of the targeting sequence; 3. Recombination between P_{hsp60}-*egfp* and the targeting sequence; 4. Detection of the targeting sequence with primers D1/D2 or D1/D3.

The PCR was performed on a 2720 thermal cycler (Applied Biosystems, Foster City, CA) in a 0.2-mL tube containing 1 U of platinum Taq, 0.8 μL of dNTPs at 25 mM each, 4 μL of 2 mM $MgCl_2$, 1 μL of each primer at 25 pmol L^{-1}, 10 μL of 10× buffer (600 mM Tris-SO_4, 180 mM $(NH_4)_2SO_4$), 4 μL of DMSO, and PCR-graded water to a total reaction volume of 100 μL. PCR cycling parameters were set to 2 min at 95 °C followed by 30 cycles at 95 °C for 30 s, 56 °C for 30 s, and 68 °C for 1 min, and a final 10-min step at 68 °C. The PCR products were purified from agarose gel with QIAquick Gel Extraction Kit (Qiagen, Hilden, GE) following manufacturer's protocol.

Construction of the P_{hsp60}-*egfp* recombineering substrate

The construction of recombineering substrate was performed in two independent PCR. Firstly, the cassette P_{hsp60}-*egfp* was amplified from plasmid pYL37-*egfp*. A 50-bp homology flanking the target D29 DNA segment was added upstream and downstream of the cassette (Fig. 1b). Primers D29-p3 and D29-p4 (Table 1) with 50-base (IDT) overlaps on each end of the cassette were used in this first step, as previously described (Marinelli *et al.*, 2008).

The PCR was performed on a 2720 thermal cycler (Applied Biosystems) in a reaction mixture containing 1 U of PFU DNA polymerase, 0.8 μl of dNTPs at 25 mM each, 1 μL of each primer at 25 pmol L^{-1}, 10 μL of 10× buffer (750 mM Tris-HCl (pH 9.0); 500 mM KCl; 200 mM, $(NH4)_2SO_4$), 4 μL of DMSO, 10 ng of plasmid DNA and PCR-graded water to a total reaction volume of 100 μL. The cycling conditions were set to 1 cycle at 95 °C for 5 min, 30 cycles (95 °C for 1 min, 56 °C for 30 s, 72 °C for 1.5 min), and a final cycle at 72 °C for 10 min. The PCR product were gel purified using QIAquick Gel Extraction Kit.

A second PCR was carried out to add more 50 bp on each end of the cassette and obtain a final recombineering substrate with 100-bp homology on either side of the target sequence. The PCR assay was performed on a 2720 thermal cycler (Applied Biosystems) with primers D29-p1and D29-p2 (Table 1) in a reaction containing 0.2 μL (1 U) of Platinum Taq high-fidelity DNA polymerase (Invitrogen Corporation, Carlsbad, CA), 0.8 μL of dNTPs at 25 mM each, 4 μL of 2 mM $MgCl_2$, 1 μL of each primer at 25 pmol L^{-1}, 10 μL of 10× buffer (600 mM Tris-SO_4, 180 mM $(NH4)_2SO_4$), 4 μL of DMSO, 10 ng of the first PCR product, and PCR-graded water to a total reaction volume of 100 μL. Cycling parameters were set to 1 cycle at 95 °C for 2 min, 30 cycles (95 °C for 1 min, 57 °C for 30 s, 68 °C for 1.5 min), and a final cycle at 68 °C for 10 min. The PCR product was gel purified using QIAquick Gel Extraction Kit.

Electroporation of recombineering substrates

Aliquots (100 μL) of electrocompetent *M. smegmatis* mc^2155 carrying pJV53 were mixed with 100 ng of phage DNA and recombineering substrate at concentrations varying over the range 50–700 ng. The solution was maintained on ice for 15 min, loaded into 2-cm cuvettes, and immediately electroporated using Gene Pulser Xcell Electroporation Total System (Bio-Rad, Hercules, CA) using a pulse of 2500 V, 25 μF, and 1000 Ω (Goude & Parish, 2008). Electroporated cells were kept in 7H9 supplemented with 10% ADC and 1 mM $CaCl_2$ at 37 °C/250 rpm/1 h, gently mixed with 4 mL of molten 7H9 soft agar (10% ADC, 1 mM $CaCl_2$, 0,75% agar), and plated onto 7H10 with 500 μL of *M. smegmatis* mc^2 155. After 16-h incubation at 37 °C, each primary plaque was transferred to 100 μL of phage buffer (10 mM Tris-HCl, pH 7.5; 10 mM MgSO4; 68.5 mM NaCl; 1 mM $CaCl_2$).

Detection of 472-bp deletion

Recombinant phages from primary plaques were detected in 1 μL of phages by PCR using PFU DNA polymerase. Primers D1 and D2 (Integrated DNA Technologies) (Table 1) flanking the deleted region were used to amplify a 1002-bp or a 530-bp segment of wild-type and recombinant phage, respectively. The concentrations of each PCR component were the same as described above. The PCR was set to 1 cycle at 95 °C for 5 min, 30 cycles (95 °C for 1 min, 59 °C for 30 s, 72 °C for 1 min), and a final step at 72 °C for 10 min.

Primary plaques containing recombinant and wild-type phages were transferred to 100 μL phage buffer and serially diluted from 10^{-1} to 10^{-6}. Dilutions were incubated with 500 μL of *M. smegmatis* mc^2155 at 37 °C/15 min, mixed with 5 mL of molten 7H9 soft agar and replated onto 7H10 supplemented with 10% ADC and 1 mM $CaCl_2$. New plaques were screened by PCR for mutant detection as described above. This process was performed until only pure recombinant phages were detected in plaques.

To confirm the deletion, DNA sequencing of the recombinant phages was carried out on ABI 3730 DNA Analyzer using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem) with the same set of primers (D1 and D2) previously used for detecting phage mutants. The data obtained were analyzed with BioEdit software v7.0.9 (Ibis Bioscience, Carlsbad, CA).

Detection of the P_{hsp60}-*egfp* insertion/replacement

Primary plaques were first screened by PCR using primers D1 and D3 (Table 1). The PCR was performed on a 2720 thermal Cycler (Applied Biosystems) in a reaction containing 0.2 μL of Platinum Taq high-fidelity DNA polymerase (Invitrogen Corporation), 0.8 μL of dNTPs at 25 mM each, 4 μL of 2 mM $MgCl_2$, 0.1 μL of each primer at 25 pmol L^{-1}, 2.5 μL of 10× buffer (600 mM Tris-SO_4, 180 mM $(NH4)_2SO_4$), 1 μL of DMSO, 1 μL of phage, and PCR-graded water to a total reaction volume of 25 μL. Cycling parameters were set to 1 cycle at 95 °C for 2 min, 30 cycles (95 °C for 1 min, 58 °C for 1 min), and a final cycle at 68 °C for 10 min.

A second PCR was carried out with primers D1 and D2 (Table 1) flanking the recombineering site to confirm the presence of samples containing only recombinant phages. The PCR was performed in a 25 μL reaction containing 1 U of PFU DNA polymerase, 0.4 μL of dNTPs at 25 mM each, 0.1 μL of each primer at 25 pmol L^{-1}, 2.5 μL of 10× buffer (750 mM Tris-HCl (pH 9.0); 500 mM KCl; 200 mM, $(NH4)_2SO_4$), and 1 μL of sample. The cycling conditions were set to 1 cycle at 95 °C for 5 min, 30 cycles (95 °C for 1 min, 59 °C for 30 s, 72 °C for 2 min), and a final cycle at 72 °C for 10 min.

Deletion of lysA

A 200-bp substrate containing 100-bp homology on either side of the *lysA* gene targeting sequence was constructed as previously described using the oligonucleotide 100-bp LysA and primers LysA1 and LysA2 (Integrated DNA Technologies) (Table 1).

Mutant phages were detected by PCR (Marinelli *et al.*, 2008) using platinum Taq (Invitrogen Corporation) with primers D4 and D6, which fully hybridizes only with recombinant phages. The amplification was performed under the following cycling conditions: 2 min at 95 °C, 30 cycles (30 s at 95 °C, 30 s at 59 °C), and a 10 min at 68 °C.

A PCR with PFU DNA polymerase was also performed with primers D4 and D7. This set of primer amplifies the targeting DNA segment of both recombinant and wild-type phage D29, and it was used to confirm the presence of only phage recombinants after several steps of phage purification. The following cycling parameters were performed: 5 min at 95 °C, 35 cycles (30 s at 95 °C, 30 s at 59 °C, and 45 s at 72 °C), and 10 min at 72 °C.

Evaluation of EGFP expression

Three aliquots (500 μL) of *M. smegmatis* mc^2155 were infected with recombinant D29 (MOI of 10) and incubated at 30, 37, and 42 °C. A volume of 100 μL was recovered every 30 min from each aliquot in a total period of 6 h, washed three times with PBS buffer, and fixed with 2% p-formaldehyde. Mycobacteria were concentrated on a

0.22-μm filter and immediately evaluated by fluorescence microscopy (Axiostar Plus, Carl Zeiss, Gottingen, GE) with a 100× objective and oil immersion, using the REL. 4.6 software. Filters 42002-HQ 479/30X, HQ 520/40 m, and Q495LP (Chroma Technology Corporation, Bellows Falls, VT) were used for detection of EGFP. No software was used for enhancing image quality.

Results and discussion

Construction of a D29:: P_{hsp60}-*egfp* reporter phage

Mycobacteriophage D29 is a lytic phage related to the temperate phage L5 (Ford *et al.*, 1998). It carries two lysogeny-related genes partially deleted in its right arm (Ford *et al.*, 1998). Additionally, a nonessential region near the cohesive end in the right arm of D29 genome was previously described using phasmid-based technology (Pearson *et al.*, 1996).

To verify that the region encompassing gene *82/71* (Sarkis *et al.*, 1995; Ford *et al.*, 1998) was nonessential for lytic growth of D29, a segment of 472 bp was deleted (D29 genome coordinates 45.387–45.859). This deletion was accomplished in a single co-electroporation of 100 ng D29 genomic DNA and targeting substrate (Table 1). In a total of 100 plaques screened, over 20% contained the deletion mutant, as identified by PCR of the flanking sequences (Fig. 2). Recombinant bacteriophages were plaque purified and shown to grow with normal plaque morphology and production of phage particles (data not shown).

The region encompassing the 472-bp sequence was than replaced by a P_{hsp60}-*egfp* cassette (Fig. 3). Differently from the previous segment deletion, we started co-electroporating 200 ng of D29 genomic DNA and targeting DNA substrate at concentrations varying from 100 to 700 ng. No plaques containing phage recombinants were found using flanking PCR or mismatched PCR with concentrations of targeting substrate below 600 ng. In a total of 600 primary plaques screened, we found only around 1% of individual plaques containing phage recombinants.

Several steps of serial dilutions followed by recovery and replating mixed populations were needed to obtain pure phage recombinants, indicating that they represented only a minor portion of particles in the primary plaques. This relatively poor recovery of recombinants may be due to the size of the insert, because the previous deletion of the same region with a 200-bp substrate generated a high proportion of mutant phages. DNA sequencing confirmed insertion of the P_{hsp60}-*egfp* cassette into D29 genome, and mutations were not found even after production of several phage stocks. Although mutant detection required multiple rounds of PCR, it was not more laborious than using shuttle phasmid technology for the construction. Although the number of phage recombinants containing

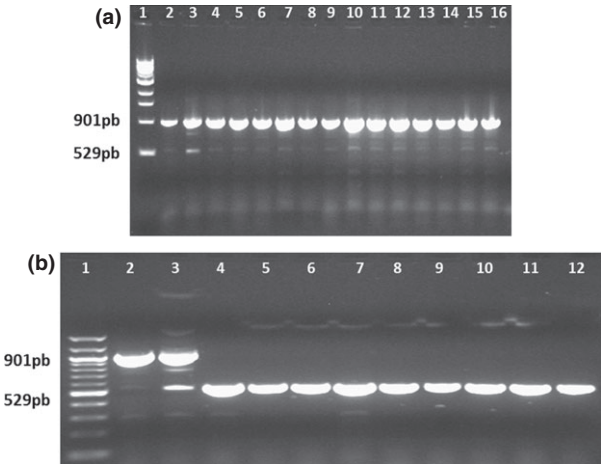

Fig. 2. Gel electrophoresis of PCR products using flanking primers D1 and D2 for detection of plaques containing phage D29 with a deletion in the right arm. (a) 1. 1-kb ladder, 2–16. Primary plaques containing a mixed population of wild-type D29 phages (901 bp) and recombinant D29 phages (529 bp). (b) 1. 100-bp ladder, 2. Negative control, DNA band amplified from phage D29 genomic DNA, 3. Positive control, DNA bands generated from a primary mixed plaque, 4–12. Production of a single 529-pb DNA band from pure plaques of D29 phage recombinants.

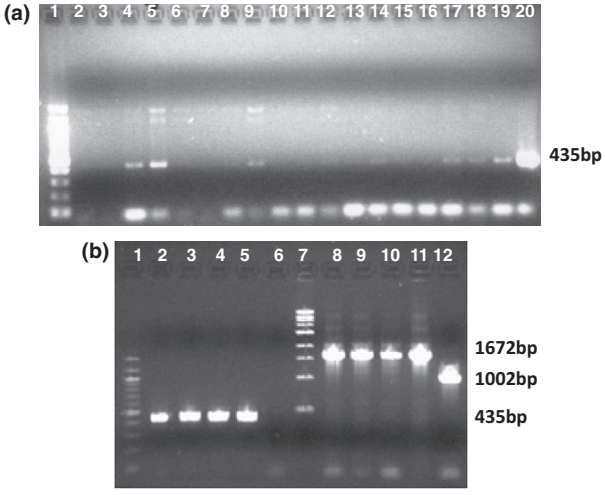

Fig. 3. Gel electrophoresis of PCR products to detect phage D29:: P_{hsp60}-*egfp*. (a) PCR from primary plaques with primers D1 and D3, specific for phage D29::P_{hsp60}-*egfp*. 1. 100-bp ladder, 2. D29 phage genomic DNA used as negative control, 3. 3–20, primary plaques containing recombinant phages (435 bp). (b) 1. 100-bp ladder, 2–5. PCR with primers D1 and D3 from purified phage D29:: P_{hsp60}-*egfp*, 6. D29 phage genomic DNA, 7. 1-kb ladder, 7–11. PCR with primers (D1 and D2) encompassing the $P_{hsp60\text{-}egfp}$ insertion (1672 bp), 12. Phage D29 genomic DNA.

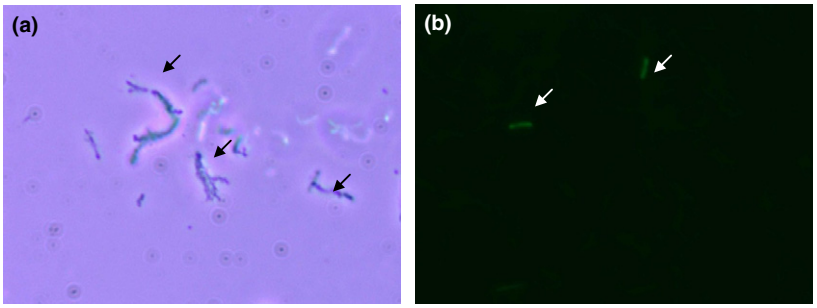

Fig. 4. *Mycobacterium smegmatis* mc^{2}155 infected with phage D29::P_{hsp60}-*egfp* after 2-h infection at 37 °C (1000X). (a) Bright field micrograph image showing rapid killing of mycobacteria (black arrows), (b) Fluorescence micrograph image presenting glowing cells.

the P_{hsp60}-*egfp* cassette was lower than when compared to the cloning of DNA segments under 400 bp, there was no need of carrying a serial of recombineerings to insert the whole segment in parts, what significantly reduced the time and cost of the procedure.

Detection of *M. smegmatis* using the D29::P_{hsp60}-*egfp* reporter phage

To evaluate if EGFP is expressed from the D29:: P_{hsp60}-*egfp* phage at levels visible by fluorescent microscopy, *M. smegmatis* mc^{2}155 was grown to mid-log phase and infected with mycobacteriophage D29::P_{hsp60}-*egfp* at a multiplicity of infection of 10. Samples collected every 30 min during 6 h, washed in PBS buffer, and concentrated in a 0.22 μm membrane were immediately analyzed for detecting fluorescent cells. Fluorescence was not visualized in the first 1 h 30 min of infection. Instead, a growing number of lysed cells were observed in the course of infection. Fluorescent cells were detected in the 2-h infection at 37 °C (Fig. 4b), but phage D29::P_{hsp60}-*egfp* had lysed the majority of cells at this time as observed on Fig. 4a. Infection time over 3 h reduced the living cells to a number difficult to visualize under microscopy. Infections carried out at 30 and 42 °C did not provide fluorescence cells.

Attempts to construct a lysis-defective mutant of D29

We reasoned that we could extend the period of fluorescence and increase its intensity by constructing a lysis defect mutant of the fluorophage. One approach is to delete the *lysA* gene encoding the endolysin, which is known to be required for lysis (Payne *et al.*, 2009). Using BRED to construct this deletion, a *lysA* mutant was detected by PCR in 5% of the recovered primary plaques, showing recombination was successful. Attempts to purify the mutant phage away from the wild-type helper phage particles in the mixed primary plaque were unsuccessful, consistent with lysin A being an essential function for plaque formation. We thus constructed a complementing plasmid encoding the Corndog lysin A, which has been shown previously to be tolerated by *M. smegmatis* and to complement a lysin A mutant of mycobacteriophage Giles (Marinelli *et al.*, 2008). However, we were not able to purify the *lysA* mutant by complementation even after screening over 1600 plaques.

With some reporter phages already developed, the next main steps into improving phenotypic assays based on bacteriophage technology are to reduce the time needed to detect *M. tuberculosis* and increase the sensibility of phage-based assays. The lytic phage machinery may be valuable for construction of recombinant phages presenting high mRNA transcription rate, which can improve mRNA transcription of reporter genes. The advent of BRED technology makes possible the construction of phages containing reporter genes virtually in any portion of a bacteriophage genome under the control of any promoter. Using BRED technology, it was possible to insert the whole P_{hsp60}-*egfp* cassette into the genome of mycobacteriophage D29. This approach may be useful to construct novel reporter phages and improve phasmids expressing reporter proteins already developed.

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Competing interests

The authors declare no competing interests.

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