Mycobacteriophage-repressor-mediated immunity as a selectable genetic marker: Adephagia and BPs repressor selection

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Mycobacteriophages provide an abundance of systems for use in mycobacterial genetics, including manipulation of Mycobacterium tuberculosis. Because of the dearth of antibiotic resistance cassettes and biosafety concerns in constructing recombinant virulent M. tuberculosis strains, we developed the use of mycobacteriophage-encoded repressor genes that can be selected in the presence of lytic versions of their cognate phages. The phage Adephagia repressor gene (43) was identified through its ability to confer immunity to Adephagia superinfection, together with the mapping of mutations in gene 43 that confer a clear-phage phenotype. Plasmid transformants containing either Adephagia 43 or the previously identified BPs repressor 33 can be readily selected following electroporation using engineered lytic derivatives of Adephagia and BPs, respectively. Selection is as efficient as antibiotic selection, can be used with either single-copy integration vectors or with extrachromosomal vectors, and works similarly in both Mycobacterium smegmatis and M. tuberculosis.

INTRODUCTION

Mycobacteriophages have played central roles in the development of genetic systems for Mycobacterium tuberculosis (Hatfull, 2014). They were instrumental in mycobacterial transfection and transformation (Jacobs et al., 1987) and use of antibiotic-resistant selectable markers (Snapper et al., 1988), and are of broad utility for delivery of transposons (Bardarov et al., 1997), reporter genes (Jacobs et al., 1993; Piuri et al., 2009) and allelic exchange substrates (Bardarov et al., 2002). Mycobacteriophage genetic components have also found widespread use in the development of integration-proficient vectors (Hatfull, 2012; Huff et al., 2010; Lee et al., 1991; Morris et al., 2008; Pham et al., 2007) and mycobacterial recombineering systems (van Kessel & Hatfull, 2007, 2008a, b; van Kessel et al., 2008).

Our understanding of mycobacteriophage genetic diversity is greatly advanced by the availability of a large collection of completely sequenced mycobacteriophage genomes emerging from the Science Education Alliance Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES) program (Jordan et al., 2014; Pope et al., 2015). All of these phages were isolated on Mycobacterium smegmatis mc²155, with the exception of DS6A which was isolated in the 1960s and which only infects Mycobacterium tuberculosis complex hosts (Bowman, 1969). They can be grouped into clusters of related genomes (Clusters A–V, and seven singletons that have no close relatives: http://phagesdb.org), several of which can be further divided into subclusters by their degrees of nucleotide sequence relatedness and gene content (Hatfull et al., 2006, 2012, 2013; Pope et al., 2011b, 2015). In general, there is a close correlation between cluster type and the ability to infect M. tuberculosis, as well as M. smegmatis (Jacobs-Sera et al., 2012). For example, all of the phages within each of the five subclusters in Cluster K efficiently infect M. tuberculosis mc²7000, as do many of the phages within Subclusters A2 and A3 (Jacobs-Sera et al., 2012). Phages in Cluster G do not efficiently infect M. tuberculosis (Sampson et al., 2009), but expanded host range mutants can be readily identified that efficiently infect both M. smegmatis and M. tuberculosis, as a result of single amino acid substitutions in a putative tail fibre (Jacobs-Sera et al., 2012).

Many mycobacteriophages form turbid plaques and are evidently temperate, whereas others form clear or hazy plaques. Most Cluster A phages are temperate, although some appear to have lost the ability to lysogenize due to loss or inactivation of the repressor gene (Ford et al., 1998; Pope et al., 2011b). Most Cluster K phages are also temperate, although the well-studied TM4 (Subcluster K2) has lost both its repressor and integration functions (Pope et al., 2011a). The Cluster G phages are temperate but form lysogens at low frequency and use an unusual integration-dependent immunity system for lysogenic establishment.

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and maintenance (Broussard & Hatfull, 2013; Broussard et al., 2013).

In temperate phages, lysogenic maintenance and superinfection immunity are typically mediated by a repressor that downregulates lytic gene expression. In phage lambda and its relatives, the cl repressor and cro are divergently transcribed from an intergenic region containing two promoters and a tripartite operator (Ptashne, 1987). This gene organization is conserved in unrelated phages, but is not universal. For example, in mycobacteriophage L5, the repressor gene is located within the right arm and is leftwards transcribed like all of the other right arm genes (Donnelly-Wu et al., 1993; Hatfull & Sarkis, 1993). However, divergent transcription from an intergenic region containing putative early lytic promoters is present in at least some mycobacteriophages, including Giles (Dedrick et al., 2013) and those like BPs in Cluster G (Broussard et al., 2013). In most mycobacteriophages immunity regulation has yet to be well defined.

We demonstrated previously that the repressor gene encoded by phage L5 can be used as a selectable marker in M. smegmatis, using lytic derivatives of L5 as the selectable agents (Donnelly-Wu et al., 1993; Fullner & Hatfull, 1997). However, L5 only efficiently infects slow-growing bacteria under particular conditions (Fullner & Hatfull, 1997), and there is a clear need for additional selectable markers for M. tuberculosis. However, divergent transcription from an intergenic region containing putative early lytic promoters is present in at least some mycobacteriophages, including Giles (Dedrick et al., 2013) and those like BPs in Cluster G (Broussard et al., 2013). In most mycobacteriophages immunity regulation has yet to be well defined.

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Here we mapped the repressor gene of the Cluster K phage Adephagia, constructed a lytic derivative of Adephagia that can neither lysogenize nor integrate into the host chromosome, and characterized the potential for immunity selection as a selectable marker in both fast- and slow-growing mycobacteria. We demonstrated that both the Adephagia repressor and the repressor of BPs can be used in conjunction with single copy integration vectors and multicopy extrachromosomal vectors as selectable markers in both M. smegmatis and M. tuberculosis.

### METHODS

**Bacterial strains and media.** M. smegmatis mc²155 and M. tuberculosis mc²7000 were grown as described previously (Jacobs-Sera et al., 2012). M. smegmatis mc²155 cultures were grown in 7H9 medium supplemented with carbenicillin (50 μg ml⁻¹), cycloheximide (10 μg ml⁻¹), 10% ADC and 1 mM CaCl₂. M. tuberculosis mc²7000 cultures were grown in 7H9 media supplemented with carbenicillin (50 μg ml⁻¹), cycloheximide (10 μg ml⁻¹), pantothenic acid (100 μg ml⁻¹), 10% OADC and 1 mM CaCl₂. Kanamycin at 20 μg ml⁻¹ was added to media as needed. Adephagia lysogens [mc²155(Adephagia)] were isolated by picking colonies from a turbid Adephagia spot on a lawn of M. smegmatis mc²155. Colonies were streaked twice on agar plates to remove extraneous phage particles and lysogens were confirmed by patching onto mc²155 to observe spontaneous phage induction. Phage propagation was as described previously (Sarkis & Hatfull, 1998).

**Plasmid constructions.** Plasmid pZP13 (Table 1) contains Adephagia gene 43 and ~500 bp flanking DNA (coordinates 34 350–35 226) inserted into the KpnI and XbaI sites of the L5 integration-proficient vector pMH94 (Lee et al., 1991). Plasmid pZP23 was constructed as follows: first, Adephagia 43 was PCR amplified from pZP13 using primers pZP13 A43.fwd (AAAAAAGTTAAA-CATGCCTGAGGACACCGAC; pZP13 A43.rev (TTTTTTCTCGAGTCAAAAGACTCGGAATGTCAGGCCTCAC; Sbf I), and the hsp60 promoter was amplified from plasmid pmCherrybomb. SbfI and HpaI sites were added to the 5’ end of the sequence and an SbfI site added to the 5’ end. The promoter and Adephagia 43 DNA fragments were then digested with HpaI and ligated, reamplified by PCR using flanking primers, and inserted into the SbfI site of pMH94. Plasmid pZP15 was constructed by PCR-amplifying Adephagia gene 43 and the ~500 bp flanking region of pZP13 and inserting it into Nhel and KpnI sites of vector pIL37(-) hsp60. Construction of plasmid pGWB48 was

### Table 1. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Features</th>
<th>Description</th>
<th>Marker</th>
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<td>pZP13</td>
<td>Adephagia 43, L5 attP, int</td>
<td>L5 integrating vector expressing Adephagia repressor from its native promoter</td>
<td>Adephagia gp43, KAN</td>
<td>This study</td>
</tr>
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<td>Adephagia 43, oriM</td>
<td>Replicative vector expressing Adephagia repressor from its native promoter</td>
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<td>This study</td>
</tr>
<tr>
<td>pZP23</td>
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<td>L5 integrating vector expressing Adephagia repressor from the hsp60 promoter</td>
<td>Adephagia gp43, KAN</td>
<td>This study</td>
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<tr>
<td>pZP32</td>
<td>P&lt;sub&gt;hsp60&lt;/sub&gt;-Adephagia 43, L5 attP, int</td>
<td>L5 integrating vector expressing Adephagia repressor under hsp60 promoter</td>
<td>Adephagia gp43</td>
<td>This study</td>
</tr>
<tr>
<td>pGWB48</td>
<td>IR&lt;sup&gt;rep-cro&lt;/sup&gt;-BPs gp33&lt;sup&gt;103&lt;/sup&gt;, L5 attP, int</td>
<td>L5 integrating vector expressing BPs prophage repressor under native promoter</td>
<td>BPs 33, KAN</td>
<td>Broussard et al. (2013)</td>
</tr>
<tr>
<td>pGWB159</td>
<td>IR&lt;sup&gt;rep-cro&lt;/sup&gt;-BPs gp33&lt;sup&gt;103&lt;/sup&gt;, L5 attP, int</td>
<td>L5 integrating vector expressing BPs prophage repressor under native promoter</td>
<td>BPs 33</td>
<td>This study</td>
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<td>pMH94</td>
<td>L5 attP, int</td>
<td>L5 integrating vector</td>
<td>KAN</td>
<td>Lee et al. (1991)</td>
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described previously (Broussard et al., 2013). Briefly, a segment of BPs (coordinates 29 014–29 522) was amplified and blunt-end cloned into pMH94, which was digested with XbaI and blunt-ended. To construct plasmids pZP32 and pGWB159, the kanamycin cassette of pZP23 and pGWB48, respectively, was removed by digestion with HindIII, followed by plasmid religation.

Isolation and characterization of Adephagia clear-plaque mutants. Four independent Adephagia lysates were prepared and plated on M. smegmatis, and spontaneous clear plaques were identified at a frequency of ~10^{-4}. Seven clear-plaque candidates were picked and three rounds of plaque purification performed. Four candidates (mtt4–1) were chosen and high titre lysates prepared. Adephagia region 34 350–35 226 containing gene 43 and 500 bp flanking sequences was PCR-amplified from the lysates of Adephagia WT and clear-plaque mutants 1–4 and sequenced (GeneWiz, NJ, USA).

Superinfection immunity assays. Phage lysates were serially diluted in phage buffer and 3 μl of each dilution was spotted onto lawns seeded with 500 μl of mc2155, mc2155(Adephagia), mc2155pZP13, mc2155pZP15 or mc2155pZP23. Plates were incubated at 37 °C for 24–48 h. For M. tuberculosis immunity assays, 1 ml aliquots of M. tuberculosis mc27000, mc27000pMH94, mc27000pZP13, mc27000pZP15 and mc27000pZP23 cultures were used and plates incubated for 6 days at 37 °C.

Lysogeny assays. M. smegmatis mc2155 was grown to late-exponential phase (without Tween 80) and diluted to OD660 ~ 1.0 in TH9 medium. Six ten-fold serial dilutions were made and 100 μl of each dilution was plated a onto plate seeded with 10^8 p.f.u. and a plate with no phage. Plates were incubated at 37 °C for 4 days and colonies counted. The ratio of colonies on phage-containing plates relative to plates without phage was used to determined lysogeny frequencies. To test strains for phage release, individual colonies were streaked onto solid media, a top agar layer containing 0.5 ml late-exponential phase M. smegmatis mc2155 was added, and the plates incubated for 2 days at 37 °C.

BRED mutagenesis. The BRED technique for phage engineering has been described previously (Marinelli et al., 2008). For Adephagia, a Δ43 mutant was created by co-electroporating 100 ng of a 200 bp deletion substrate (containing 100 bp homology to either side of gene 43) and 50 ng of Adephagia DNA into 100 μl induced electro-competent mc2155:pJV53 recombineering cells (van Kessel & Hatfull, 2007). Reactions were recovered for 1 h at 37 °C in 1 ml TH9/ADC/CaCl2 and plated as top agar lawns with ~ 300 μl mc2155 cells and incubated overnight at 37 °C. Twenty plaques were picked in 50 μl of phage buffer and screened for a 43 deletion by PCR with primers annealing 600 bp upstream and downstream of gene 43. Plaques containing the desired deletion were serially diluted and replated on mc2155, and individual plaques picked and PCR-verified for the 43 deletion. The PCR product was purified (QIAquick PCR-Purification, Qiagen) and sequenced (GeneWiz, NJ, USA). To generate Δ43A41, DNA from Δ43 was prepared and BRED mutagenesis repeated with a deletion substrate targeting 41. For BPsΔ33, a 33 deletion was described previously (Broussard et al., 2013), and a host-range mutant that infects M. tuberculosis was isolated and the mutation mapped to gene 22 that introduces the substitution L462R.

Phage selection of transformants. For transformation of mc2155, a total of 100 ng of plasmid (pZP23, pGWB48 or pMH94) was electroporated into 100 μl of mc2155 at 2.5 kV, 25 μΩ, 25 μl, 900 μl of ice-cold TH9/ADC/CaCl2 medium was added and cells were recovered for 2 h at 37 °C with shaking at 250 r.p.m. For M. tuberculosis mc27000 transformation, 100 ng of plasmid DNA was electroporated into 200 μl of electrocompetent cells, 800 μl of room temperature TH9/OADC/CaCl2 was added and cells were incubated standing for 24–48 h at 37 °C. Following recovery, 0.1 ml of cells was spread on solid medium onto which phage particles (~10^9 p.f.u.) had been spread with glass beads (3 mm, Fisher Scientific). For kanamycin selection, antibiotic at 20 μg ml^{-1} was added to bottom agar media. Plates were incubated at 37 °C for 4 days (mc155) or 4 weeks (mc7000), after which colonies were counted. Individual colonies were patched on selection plates prepared as above.

PCR. PCRs were performed using Cloned Pfu DNA Polymerase (Agilent Technologies) according to manufacturer protocol specifications, except that DMSO was added at 5%. Colony PCR was conducted using a SapphireAmp Fast PCR Master Mix (Takara Bio).

RESULTS

Adephagia gp43 confers superinfection immunity

Prior characterization of the immunity specificities of Cluster K phages showed that phages within Subclusters K1 and K3 are homoimmune (Pope et al., 2011a); at the time of analysis, the sole Subcluster K2 phage, TM4, was shown to be neither temperate nor subject to immunity by K1 and K3 phages (Pope et al., 2011a). The Subcluster K1 and K3 phages all contain an integration cassette located close to the centre of their genomes (Fig. 1a), which is lost as part of a large deletion at the centre of the TM4 genome (Pope et al., 2011a). However, comparative genomic analysis did not provide strong clues as to the location of the repressor genes in Cluster K phages, which could plausibly be located close to the integration cassette – as in Giles and BPs (Broussard et al., 2013; Dedrick et al., 2013) – or closer to the right genome end as in L5 and other Cluster A phages (Brown et al., 1997; Donnelly-Wu et al., 1993; Pope et al., 2011b).

Further bioinformatic inspection reveals that Cluster K phages have two leftwards-transcribed genes (e.g. Adephagia 42 and 43) located immediately to the right of the integration cassette (Fig. 1a). HHpred analysis (So¨ ding et al., 2005) of Adephagia gp43 and its relatives shows strong similarity to helix-turn-helix DNA-binding motifs including transcriptional repressors, suggesting it as a strong candidate for the immunity repressor of the Cluster K phages; no putative function for Adephagia gp42 and its relatives was identified. Adephagia gene 43 and the adjacent gene 44 are divergently transcribed from the intervening region, similar to the canonical lambda organization of cl and cro; HHpred predicts that Adephagia gp44 is also a DNA-binding protein, although its functional equivalency to lambda Cro is unclear. We also note that the deletion at the centre of the TM4 genome removes most of the homologue of Adephagia 43, consistent with its clear-plaque phenotype, and supportive of the assignment of 43 as the repressor gene.

To test the ability of Adephagia 43 to confer immunity to superinfection, a 500 bp fragment containing gene 43 and its flanking regions was cloned into both a multicopy extrachromosomal pAL5000-derived vector

http://mic.sgmjournals.org
(a) Adephagia

(b) Giles

TM4

Angelica

Adephagia

Fionnbarth

mc²155

mc²155 (Adephagia)

mc²155pZP13

mc²155pZP15

mc²155pZP23
Fig. 1. Adephagia gene 43 confers superinfection immunity. (a) Map of the Adephagia genome. Rightwards- and leftwards-transcribed genes are shown as coloured boxes above and below the genome, respectively. Gene numbers are shown within the gene boxes, and the family designation is shown above the box with the number of family members in parentheses; families correspond to groups of related proteins, as described elsewhere (Cresawn et al., 2011; Pope et al., 2015). Genes are coloured according to their family designation. The lower part shows details of the central portion of the genome containing the integrase and repressor genes. (b) Immunity patterns of repressor-expressing strains. Phages Giles (control), TM4 (Subcluster K2), Angelica, Adephagia (Subcluster K1) and Fionnbharth (Subcluster K4) were serially diluted and spotted onto lawns of M. smegmatis mc\(^{2}\)155 (control), an Adephagia lysogen, and repressor-expressing strains mc\(^{2}\)155pZP13, mc\(^{2}\)155pZP19 and mc\(^{2}\)155pZP23, where gene 43 is expressed by its native promoter on an integrating plasmid, by its native promoter on an extrachromosomal plasmid, and by the hsp60 promoter on an integrating plasmid, respectively.

Clear-plaque mutants map to Adephagia gene 43

Because the phage repressor is not the only gene with the potential to confer an immune phenotype, we also isolated and mapped mutants that confer a clear-plaque phenotype. Clear-plaque mutants were readily identified in Adephagia lysates, and four independent mutants (mt 1–mt 4) were isolated and purified (Fig. 2a). We also determined lysogenization frequencies by counting survivors recovered by plating a culture of M. smegmatis mc\(^{2}\)155 on phage-seeded medium. WT Adephagia forms lysogens at 39% (but with variation within three experiments of 15–43%), and mt 1, mt 2 and mt 4 lysozyme at less than 1% (Fig. 2a). Mutant mt3 – whose plaques retain some turbidity – has a lysogenization frequency of 22%, reflecting a defect in efficient lysogenic establishment (Fig. 2a). We also tested the ability of these mutants to infect an Adephagia lysogen, and observed that all are subject to Adephagia immunity (Fig. 2b).

To map the mutations, we PCR-amplified Adephagia 43 and its flanking regions from mt 1–mt 4 (Fig. 2c). Mutants mt 2, mt 3 and mt 4 all generated a PCR product similarly sized to that from WT Adephagia, although mt 1 gave a substantially smaller product (Fig. 2c). Sequencing the mt 1 PCR product showed the presence of a 519 bp deletion that removes all of the 43 ORF as well as 44 bp of gene 42 and 47 bp of the 43–44 intergenic region (Fig. 2d). Sequencing of mt 2, mt 3 and mt 4 identified single base changes in the 43 ORF in all three mutants (Fig. 2d). mt 4 has an arginine to glycine change just downstream of the putative helix–turn–helix motif (R56G), and mt 2 has a tyrosine-to-cysteine substitution at residue 72 (Y72C). mt 3 has a lysine-to-arginine substitution within the predicted helix–turn–helix region (K23R), which presumably compromises the DNA-binding properties of gp43 and enables lysogenization albeit with a reduction in the efficiency of lysogenic establishment (Fig. 2d). The demonstration that gp43 both confers immunity and is mutated in clear-plaque mutants provides strong experimental support to the bioinformatic prediction that gp43 functions as the phage repressor.

BRED engineering of Adephagia

Although the simple explanation for these clear-plaque mutants is that gene 43 is required for lysogenic maintenance, it is plausible that additional unmapped changes elsewhere in the genome contribute to the phenotype. We therefore used Bacteriophage Engineering of Electro-porated DNA (BRED) (Marinelli et al., 2008) to construct a defined deletion mutant that inactivates gene 43, leaving only the three 5′- and 3′-outermost codons intact (Fig. 3a). Adephagia Δ43 forms clear plaques and lysogenizes at less than 0.1% frequency (Fig. 3b), confirming that gp43 is the phage repressor. When strains containing plasmids pZP13, pZP15 or pZP23 were plated onto medium seeded with Adephagia Δ43 particles, they were immune to phage killing, and equivalent numbers of colonies were recovered as on non-phage-seeded medium (Fig. 3c). However, the survivors were lysogenic for Adephagia Δ43, as illustrated by phage release (Fig. 3d) and PCR amplification of the attL attachment junction (Fig. 3e).

To inactivate the integration functions of Adephagia we introduced an additional mutation deleting the integrase gene 41 in combination with the Δ43 mutation, removing all except for the three 5′- and 3′-most codons of gene 41 (Δ41Δ43; Fig. 3a). Adephagia Δ41Δ43 formed clear plaques as expected (Fig. 3b), and repressor-containing strains plated with equal efficiencies on Δ41Δ43-seeded plates as on non-seeded plates (Fig. 3c). However, the surviving
colonies recovered on Δ41Δ43 did not carry an Adephagia Δ41Δ43 prophage, did not release phage (Fig. 3d) and did not contain attL DNA (Fig. 3e).

**Adephagia-repressor-based selection in *M. smegmatis***

Because the Adephagia repressor confers strong immunity to superinfection it offers the potential for use as a selectable marker for mycobacterial genetics, compatible with use of antibiotic resistance cassettes. Perhaps the most demanding configuration for the selection would be for recovery of transformants following electroporation, because it requires both survival of transformed cells, efficient killing of non-transformants cells, and – at least for many applications – avoidance of lysogeny of the transformants. We thus investigated direct selection of plasmid transformants following electroporation of *M. smegmatis*. 

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**Fig. 2.** Isolation of Adephagia clear-plaque mutants. (a) Purified lysates of Adephagia clear plaque mutants (mt 1–mt 4) were serially diluted and spotted on a lawn of *M. smegmatis* mc²155, and show a clear-plaque phenotype relative to WT Adephagia. To the right are shown the lysogeny frequencies expressed as the proportion of survivors following plating of a culture of *M. smegmatis* mc²155 on phage-seeded plates relative to non-seeded plates. (b) Adephagia clear-plaque mutants (mt 1–mt 4) do not infect an Adephagia lysogen compared with Giles control. (c) A 900 bp region including gene 43 and flanking regions was PCR-amplified from lysates of Adephagia and clear-plaque mutants 1–4, mt 1 showed a 500 bp deletion while mt 2, mt 3 and mt 4 generated PCR products of similar size to WT Adephagia. (d) Locations of mutations giving rise to a clear-plaque phenotype. The deletion of gene 43 and surrounding sequence in mt 1 is underlined in red with dotted lines showing the end points of the deletion. The amino acid sequence of gp43 is shown below the genome map with black arrows indicating the amino acid substitutions in mt 2, mt 3 and mt 4. The black line above the sequence indicates the position of the predicted helix–turn–helix motif.
Fig. 3. Construction of defined Δ43 and Δ41Δ43 mutants of Adephagia. (a) A schematic of Adephagia engineered deletions to generate phages ΔΔ43 and ΔΔ43Δ41 is shown. The three 5′- and 3′-outermost codons of each deleted gene are left intact to minimize polarity. (b) Lysates of Adephagia, Δ43 and Δ43Δ41 were serially diluted and plated onto lawns of M. smegmatis mc²155, and both mutants exhibit clear-plaque phenotypes and have undetectable levels of lysogeny, as reflected in the proportion of M. smegmatis survivors when plated on phage-seeded plates. (c) Repressor-expressing strains (pZP13, pZP15 and pZP23) are immune to killing by Δ43 and Δ41Δ43 phages and grow on phage-seeded plates at similar frequencies as non-seeded plates, whereas WT M. smegmatis is efficiently killed by both phages. Strains were grown to OD₆₀₀ of 0.8–0.9, serially diluted and plated to give approximately 400 colonies per plate. Phage-seeded plates contained 10⁹ p.f.u. per plate. (d) Five colonies from each repressor-expressing strain recovered on phage-seeded plates in (c) were tested for lysogeny and phage release by growing patches on cells with a top agar lawn containing M. smegmatis as an indicator strain. Left panels show growth of patches in the absence of the overlay and right panels show the patches with the overlay. All five pZP13 and pZP15 colonies together with three of the five pZP23 colonies recovered on Δ43 were lysogenic and showed spontaneous phage release. In contrast, none of the colonies recovered on Δ41Δ43 were lysogenic, regardless of the repressor-plasmid present. The no phage (top row) control patches were from the M. smegmatis mc²155pZP13 culture plated in the absence of phage. The two patches on the bottom row are an Adephagia lysogen and WT M. smegmatis mc²155 as indicated. (e) Cultures of the mc²155pZP13 colonies in (d) were grown and the attP (top panel) and attB/attL (bottom panel) regions were PCR amplified. For attB/attL a three-primer reaction was used containing a forward primer that amplifies both attB and attL, and two reverse primers, one specific to attB and the other specific for attL.
Plasmids pZP15, pZP23 and pZP32 (a derivative of pZP23 in which the kanamycin resistance cassette was removed) and vector pMH94 control DNA were electroporated into \textit{M. smegmatis} mc^\textsubscript{2}155 and equivalent portions plated on either solid media containing kanamycin or Adephagia \Delta41Δ43 (Fig. 4a). The total number of cells plated was approximately 10^7 c.f.u. and phage selection used approximately 10^8 p.f.u. phage. In both the vector DNA and no DNA controls, efficient killing by Adephagia \Delta41Δ43 was observed, with a small number of survivors (Fig. 4b). Upon retesting these survivors, few grew on phage-seeded plates, indicating that they were phenotypic escapees from the selection, not phage-resistant mutants (although at least one likely spontaneous phage-resistant mutant was recovered; Fig. 4).

Plasmid pZP15, pZP23 and pZP32 transformants were readily recovered on either phage or antibiotic-containing media (Fig. 4a), and for pZP15 and pZP23 the numbers of phage-selected transformants was as high as or higher than with kanamycin selection (Fig. 4a); phage-selected pZP32 transformants were recovered at a similar frequency as pZP15 and pZP23 transformants. Fifty colonies from each of the phage-selected plates were patched onto phage-seeded and kanamycin media, and for pZP15 and pZP23, all phage-selected colonies were also kanamycin-resistant, indicating they were true transformants (Fig. 4b). Transformants were also shown to be phage-free (Fig. 4c, d) and contain plasmid DNA integrated at the L5 attachment site (Fig. 4e).

**BP\textregistered s-repressor-based selection in \textit{M. smegmatis}**

Mycobacteriophage BP\textregistered s is a temperate phage that was previously shown to use an unusual regulatory system referred to as integration-dependent immunity (Broussard et al., 2013). In this and related phages the \textit{attP} site is located within the repressor ORF (33), resulting in loss of the 3’ end of the repressor gene following integration (Fig. 5a). Because the repressor 3’ end encodes an ssrA-like tag that promotes degradation and loss of function, only the prophage form (gp33\textsuperscript{103}) and not the virally encoded form of repressor (gp33\textsubscript{316}) confers immunity to superinfection. BP\textregistered s and related phages grouped in Cluster G do not efficiently infect \textit{M. tuberculosi}s, but mutants can be readily isolated in which substitutions in gp22 enable efficient \textit{M. tuberculosi}s infection (Jacobs-Sera et al., 2012). These properties suggest that the BP\textregistered s immunity system might be effective as an additional selectable system that is compatible with the Adephagia system described above, as the two phages do not share immune specificity.

To evaluate BP\textregistered s selection, we used plasmids pGWB48 and pGWB159, both of which encode the active form of the BP\textregistered s repressor (gp33\textsuperscript{103}) under its native upstream expression signals in an L5 integration-proficient vector; pGWB48 also contains a kanamycin resistance gene, but pGWB159 does not (Table 1). For selection we used a derivative of BP\textregistered s in which the repressor gene is deleted and which – because the phage attachment site is located within the repressor gene (Fig. 5a) – is also defective in integration; as shown previously this leads to the inability to form stable lysogens even in the presence of gp33\textsuperscript{103} (Broussard et al., 2013). The phage used also carries a mutation in BP\textregistered s gene 22 (gp22 L462R) that enables efficient infection of \textit{M. tuberculosi}s as well as \textit{M. smegmatis} and also adsorbs more efficiently to \textit{M. smegmatis} (Jacobs-Sera et al., 2012).

Plasmid pGWB48 transformants were readily recovered following electroporation of \textit{M. smegmatis} at least as frequently as for kanamycin selection, and the phage efficiently killed non-transformed cells (Fig. 5b). Interestingly, killing was more efficient with the gp22 L462R extended host range mutant of BP\textregistered s than with its isogenic parent (data not shown). All of the pGWB48 transformants regrown on phage-seeded plates were also kanamycin-resistant (Fig. 5c). We also showed that the transformants were phage-free and did not release BP\textregistered s phage particles (Fig. 5d).

**Repressor-based selection in \textit{M. tuberculosi}s**

One advantage of developing repressor-based selection using Adephagia and BP\textregistered s is that these phages are among the relatively small subset of phages that either infect \textit{M. tuberculosi}s efficiently (e.g. Adephagia) or for which mutant derivatives that do so can be readily isolated (e.g. BP\textregistered s) (Jacobs-Sera et al., 2012; Pope et al., 2011a). As shown in Fig. 6(a), Adephagia and its Cluster K relatives Angelica (Subcluster K1) and Fionnbharth (Subcluster K4) all efficiently infect \textit{M. tuberculosi}s, and plasmids pZP13, pZP15 and pZP23 confer immunity to superinfection by Adephagia and Angelica, but not Fionnbharth, as seen in \textit{M. smegmatis} (Fig. 1). Adephagia plaques are seen at high titre on mc^\textsubscript{2}7000pZP13 reflecting either incomplete immunity or repressor-insensitive mutants as also seen in \textit{M. smegmatis} (Fig. 1).

Using Adephagia \Delta43\Delta41, \textit{M. tuberculosi}s transformants could be readily selected following electroporation of pZP15, pZP23 and pZP32 (Fig. 6b), with similar numbers of pZP15 and pZP23 transformants selected with phage as with kanamycin. Killing of non-transformed cells with Adephagia \Delta43\Delta41 was efficient, and the primary difference between transformation of \textit{M. tuberculosi}s and \textit{M. smegmatis} was the inability to select pZP13 transformants (Fig. 6b), presumably due to inadequate repressor expression. We also examined BP\textregistered s repressor selection in \textit{M. tuberculosi}s, and observed efficient phage selection of pGWB48 transformants and efficient killing of non-transformed cells (Fig. 6c). Thus, both the Adephagia and BP\textregistered s repressor systems are effective selectable markers for use in both \textit{M. smegmatis} and \textit{M. tuberculosi}s, including the ability to directly select for transformants following electroporation.
Fig. 4. Adephagia repressor selection for M. smegmatis transformants. (a) DNA (100 ng) of Adephagia-repressor-expressing plasmids pZP13, pZP15, pZP23, pZP32, vector pMH94 or no DNA controls were transformed into M. smegmatis electro-competent cells and recovered in 1 ml 7H9/ADC/CaCl$_2$ for 2 h with shaking at 37 °C. Cells (0.1 ml) were spread on plates seeded with AdephagiaΔ43Δ41 at 10$^9$ p.f.u. per plate or on kanamycin (20 μg ml$^{-1}$) plates. (b) Fifty colonies from Adephagia Δ43Δ41-seeded plates from (a) were patched onto phage-seeded or kanamycin plates as indicated. (+) is a pZP23 colony from a kanamycin plate used as control for a true transformant; (−) is an M. smegmatis mc$^2$155 colony. (c) Twelve pZP23 transformants from Adephagia Δ43Δ41-seeded plates were isolated, restreaked and tested for phage release in the absence (left) or presence (right) of an overlay of top agar containing M. smegmatis mc$^2$155 as an indicator strain. None of the colonies released phage. Patches from an Adephagia lysogen (+) or mc$^2$155 (−) are shown as controls. (d) Five of the colonies (1–5) in (c) were tested for integration of Adephagia Δ43Δ41 at its chromosomal attachment site. The top and bottom panels show PCR amplification of attP and attB/attL as described for Fig. 3(e). (e) PCR analysis showing plasmid integration at the L5 attB site. Amplification of L5 attB and attL are shown in the top and bottom panels, respectively. Lanes 1–5 are the same five pZP23 transformants analysed in (d), lanes 6–10 are pZP32 transformants, and lanes 11–13 are mc$^2$155pMH94, pMH94 DNA and mc$^2$155, respectively.
DISCUSSION

We have described here two new systems for repressor-mediated genetic selection as markers for use in mycobacterial genetics. Although selection using the L5 repressor (gp71) in *M. smegmatis* was reported previously (Donnelly-Wu et al., 1993), this was prior to the development of tools for facile manipulation of mycobacteriophages (Marinelli et al., 2008) and was not readily applicable to *M. tuberculosis*. The mapping of the Adephagia repressor and demonstration of two new repressor selectable systems illustrates the advantage of a large collection of genetically diverse mycobacteriophages that provide a variety of immunity systems. Repressor selectable genes are likely to be useful for a variety of applications including plasmid transformation and construction of chromosomal mutations by allelic exchange.

A potential advantage of exploiting phages from Subclusters A2 and A3, Cluster K and Cluster G is that they (or readily isolated mutant derivatives) efficiently infect both *M. smegmatis* and *M. tuberculosis*. Additional selectable

![Diagram of repressor systems](image_url)

**Fig. 5.** BPs repressor selection for *M. smegmatis* transformants. (a) A schematic of the BPs immunity region is shown indicating the integrase (32) and repressor (33) genes. The P<sub>R</sub> and P<sub>rep</sub> promoters and the directions of transcription are indicated. The attP site located within the BPs repressor gene (33) is marked by a black box. A part of the BPs Δ33 genome is represented below, showing deletion of gene 33 and consequent loss of attP. For selection, a derivative of this phage was used that also has a mutation in gene 22 (gp22 L462R) that confers the ability of BPs to efficiently infect *M. tuberculosis* as well as *M. smegmatis*. (b) DNA (100 ng) of BPs-repressor-expressing plasmids pGWB48 and pGWB159 was electroporated into *M. smegmatis* electrocompetent cells and recovered in 1 ml 7H9/ADC/CaCl<sub>2</sub> for 2 h with shaking at 37 °C. A no DNA transformation control was included. Cells (0.1 ml) were spread on plates seeded with BPs Δ33 22L462R at 10<sup>9</sup> p.f.u./plate or on kanamycin (20 μg ml<sup>-1</sup>) plates. (c) Fifty colonies from BPs Δ33 22L462R-seeded plates from (b) were patched onto phage-seeded or kanamycin plates, as described in Fig. 4. (+) is a pGWB48 colony from a kanamycin plate used as control for a true transformant; (−) is an *M. smegmatis* mc<sup>2</sup>155 colony. (d) Phage release assays showing that phage-selected pGWB48 transformants do not carry a BPs prophage; bottom panel shows a BPs lysogen. Right part, with an *M. smegmatis* mc<sup>2</sup>155 overlay; left part, without overlay.
Fig. 6. Repressor-mediated immunity and repressor selection in *M. tuberculosis*. (a) Immunity patterns of *M. tuberculosis* mc²7000-repressor-expressing strains. Phages TM4 (Subcluster K2), Angelica, Adephagia (Subcluster K1) and Fionnbharth (Subcluster K4) were serially diluted and spotted onto lawns of *M. tuberculosis* mc²7000 (control), mc²7000pMH94 vector control, and repressor-expressing mc²7000pZP13, mc²7000pZP15 and mc²7000pZP23 strains. (b) Adephagia repressor selection in *M. tuberculosis* mc²7000 is shown. DNA (100 ng) of Adephagia-repressor-expressing plasmids pZP13, pZP15, pZP23, pZP32 or vector control pMH94 was transformed into *M. tuberculosis* mc²7000 electrocompetent cells and recovered in 1 ml of 7H9/ADC/CaCl₂ for 24 h with shaking at 37°C. Cells (0.1 ml) were spread on plates seeded with Adephagia Δ43Δ41 at 10⁹ p.f.u. per plate or on kanamycin (20 μg ml⁻¹) plates. A no DNA transformation control was included. (c) BPs repressor selection in *M. tuberculosis* mc²7000. The transformation procedure followed was similar to that in (b) but using plasmid pGWB48 and BPs Δ33 22 L462R for selection.
markers are likely to be useful in \textit{M. smegmatis} where relatively few antibiotic resistance cassettes are available, but especially in \textit{M. tuberculosis}, where few useful markers are available for use in drug-resistant strains, or where introduction of additional antibiotic-resistant genes is undesirable for biosafety reasons. These selectable schemes are likely to be useful in other mycobacterial strains that these phages infect, including \textit{Mycobacterium ulcerans} (Rybniker et al., 2006).

Characterization of the Adephagia immunity system should be helpful in developing similar selectable markers that can also be used in both \textit{M. smegmatis} and \textit{M. tuberculosis}, but which are compatible with the ones described here. Cluster K phages form at least six subclusters (K1–K6; Pope et al., 2011a; http://phagesdb.org) and we predict that there is at least one additional – and likely more – different immune specificities based on bioinformatic analyses. One of these could be provided by Fionnbarth, which we show here is heteroimmune with Adephagia (Figs 1 and 6). Other candidates from Cluster K are phages Validus and Milly, which have amino acid deviations in the putative recognition helix of the predicted helix–turn–helix DNA-binding domain, and are all insensitive to the Adephagia repressor encoded in pZP23 (data not shown). Phages in Subcluster A2 and A3 could be similarly used, and a sizeable repertoire of compatible systems can be envisaged.

The phage-mediated selections are simple and both BPs and Adephagia can be easily propagated on \textit{M. smegmatis} to high titre (\(>10^{10}\) p.f.u/ml); phage lysates can also be stored cold for extended periods with minimal loss of titre. Selection can be accomplished by either mixing cells and phage particles followed by plating onto solid media, or spreading phage particles directly onto solid medium following by plating of cells. Selection works well using either extrachromosomal or integration-proficient vector backbones, and the repressor genes are small (\(\sim400\) bp). For many applications, the use of the non-integrating phage derivatives yielding prophage-free transformants is desirable, and with integration vectors they are expected to be stably maintained without ongoing selection. When using extrachromosomal vectors, the absence of ongoing selection may lead to loss of the plasmid due to unstable plasmid inheritance. An alternative configuration for the selection could be to use the Adephagia \(\Lambda^{+}\) phage derivative, such that the transformants also carry the prophage (Fig. 3). This should provide strong selection for plasmid maintenance, because plasmid loss will result in prophage derepression, lytic growth and cell death. This could be particularly useful in the construction of recombinant vaccine strains, where high levels of antigen expression from extrachromosomal plasmids are desirable, coupled with plasmid maintenance in an antibiotic resistance cassette free genetic background. The phage-based selection system outlined here – together with future mycobacteriophage-derived selection schemes – provides facile systems for manipulation of mycobacteria when the use of antibiotic markers is undesirable.

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