AEM Accepts, published online ahead of print on 12 July 2013
Appl. Environ. Microbiol. doi:10.1128/AEM.01016-13

Generation of affinity-tagged fluoromycobacteriophages by mixed assembly of phage capsids

Mariana Piuri[1,2] Liliana Rondón[2] Estefanía Urdaniz[2] and Graham F. Hatfull[1]*

[1]Department of Biological Sciences
University of Pittsburgh
Pittsburgh, PA 15241
(412) 624 6975
gfh@pitt.edu

[2]Departamento de Química Biológica
Facultad de Ciencias Exactas y Naturales
Universidad de Buenos Aires, IQUIBICEN- CONICET, Buenos Aires, Argentina

*Corresponding author

47 **Abstract**

48 Addition of affinity tags to bacteriophage particles facilitates a variety of applications including
49 vaccine construction and diagnosis of bacterial infections. Addition of tags to phage capsids is
50 desirable as modification of the tails can lead to poor adsorption and loss of infectivity. Although
51 tags can readily be included as fusions to head decoration proteins, many phages do not have
52 decoration proteins as virion components. The addition of a small (10 aa) Strep II tag (STAG) to
53 the mycobacteriophage TM4 capsid subunit, gp9, was not tolerated as a genetically
54 homogenous recombinant phage, but could be incorporated into the head by growth of wild-type
55 phage on a host expressing the capsid-STAG fusion. Particles with capsids composed of wild-
56 type and STAG-tagged subunit mixtures could be grown to high titer, show good infectivity, and
57 can be used to isolate phage-bacterial complexes. Preparation of a STAG-labeled
58 fluoromycobacteriophage enabled capture of bacterial complexes and identification of infected
59 bacteria by fluorescence.

60

61

62

63 **INTRODUCTION**

64

65 Mycobacteriophages – viruses that infect mycobacterial hosts including *Mycobacterium*
66 *tuberculosis* – represent powerful toolboxes for mycobacterial genetics and for clinical
67 applications to control human tuberculosis (1-3). Over 220 mycobacteriophage genomes have
68 been sequenced, revealing them to be highly diverse and organizationally mosaic (4, 5). These
69 have been exploited for the development of genetic tools such as for delivery of transposons
70 and allelic change substrates (6, 7), for the construction of integration-proficient plasmid vectors
71 (8-12), non-antibiotic selectable markers (13), and expression systems (14, 15). They have also
72 been proposed for efficient delivery of reporter genes to facilitate simple and rapid determination
73 of drug susceptibilities of *M. tuberculosis* in clinical specimens (15-19).

74

75 TM4 is perhaps one of the best-studied mycobacteriophages. It was isolated in 1984 (20) and
76 used to construct the first shuttle phasmids (21). It has a genome 52,797 bp in length, contains
77 89 protein-coding genes and no tRNA genes (22, 23), and is one of several phages with
78 nucleotide sequence similarity that constitute Cluster K (22). All of these Cluster K phages infect
79 both *Mycobacterium smegmatis* mc^2155 and *M. tuberculosis* H37Rv, and TM4 infects some
80 strains of *M. avium*, as well as *M. ulcerans* (22, 24). TM4 is not temperate and does not form
81 stable lysogens in any known host. However, its Cluster K relatives are temperate, containing
82 integrase genes and forming lysogens in *M. smegmatis* and *M. tuberculosis* (22). Comparative
83 genomic analysis suggests that TM4 is a recent virulent derivative of a temperate parent.
84 Temperature-sensitive non-replicating TM4 mutants have been isolated for use as phage
85 delivery vehicles (7) and the mutations have been mapped (22).

86

87 Morphologically, TM4 is siphoviral and contains an isometric icosahedral capsid joined to a long
88 flexible non-contractile tail (23). The 305 amino acid capsid subunit is encoded by gene *9*, is

covalently crosslinked in mature particles (23), and is predicted to fold similarly to phage HK97 (Fig. 1). The gene is approximately 100 residues shorter than HK97 gp5 because it lacks the N-terminal 104 aa delta region that provides an essential assembly function in HK97 (25). The upstream gene [*8*] of TM4 encodes a putative scaffold protein but genome and virion protein analysis suggests that there are no decoration proteins (22, 23) such as Hoc and Soc that are components of the phage T4 capsid (26). Hoc and Soc are dispensable for T4 assembly, but can be modified such as to display foreign peptides on the capsid surface, with the potential for vaccine construction and antigen delivery (27). As there are no equivalent proteins in TM4, any modification to the capsid surface would likely require modifications of the capsid subunit itself.

Here we describe an approach for addition of a short affinity tag to TM4 particles. This enables the easy purification of phage particles, and the capture of phage-bacterium complexes with potential to simplify diagnostic applications. The approach should be generally applicable to other bacteriophage systems.

MATERIALS AND METHODS

Bacterial strains and reagents. *M. smegmatis* mc^2155 has been described previously (28) and was grown at 37°C in Middlebrook 7H9 broth (Difco), containing ADC (2 g l^{-1} Dextrose, 5 g l^{-1} Albumin, 0.85 g l^{-1} NaCl) and 0.05% v/v Tween 80 or TH10 (Difco) containing ADC. Middlebrook Top Agar (MBTA) was prepared using 4.7 g l^{-1} Middlebrook 7H9, 7 g l^{-1} Bacto Agar. Tween was omitted when cultures were used for phage infection. When appropriate, kanamycin 25 μg ml^{-1} was used. *E. coli* strains were grown in L-Broth. Phage buffer contained 10 mM Tris HCl pH 7.5, 68.5 NaCl, 10 mM $MgSO_4$, 1 mM $CaCl_2$.

BRED experiments. Insertion of a STAGII addition to the C-terminus of gp9 of phAE87::*hsp60-EGFP* (abbreviated gfpϕ) (18) was done using BRED as already described (29). Briefly, a 230 bp target substrate with 100 bp of homology to each side of the 3´of gp9 of TM4 was designed using a 100 nt oligo (5´-acaagacgccggtcgccgtcgtggcaccggctgccAGCGCTTGGAGCCACCCGCAGTTCGAAAAAtagtgcgc tatcgccacgccttgacgggggcggtt-3´) containing the coding sequence for STAGII plus two amino acids used as a linker (shown in capital letters) and two 85-mer external primers, one forward (5´-cgccctgcggctcaaggcgcggttcgcctacgtgctgggtgtgagcgcgaccgctcagggcgccaacaagac gccggtcgccgtc-3´) and one reverse (5´-taccgggtccagttgtcgtcgccctcgacgagggcggccagcag ggtgccctcacgcaccccgataaccgcccccgtcaaggcgt-3´), with 20-base overlaps on either side of the 100-mer. Combination PCR was carried out by mixing 10 ng of the 100-mer and 25 pmoles of the 85-mers in a 100 µl PCR reaction using Cloned Pfu DNA polymerase (Agilent Technologies, Santa Clara, USA). The resulting PCR product, a 230 bp targeting substrate, was extracted from the gel using the QIAquick Gel Extraction Kit (Qiagen, USA) and reconstituted in sterile water.

M. smegmatis mc^2155 (pJV53) was grown and induced for recombineering functions as described previously (30). phAE87::*hsp60-EGFP* DNA and the targeting substrate were co-electroprated in the induced electrocompetent cells, cells were recovered for 2 hours at 30^{o}C and plated in an infectious center assay. Screening of plaques for the presence of the insertion was done by PCR using a forward primer that anneals to the STAGII sequence (5´-gcttggagccacccgcagttc- 3´) and a reverse primer (5´-gaccgcaaccatgccggtgcgg-3´) that anneals to the sequence of gp10 (downstream of gp9 in the TM4 genome) giving an amplicon of 500bp.

Construction of a plasmid expressing STAG-gp9 of TM4. The major capsid protein gene (gp9) of TM4 containing a StrepTag II plus two amino acids used as a linker was cloned in pNIT

plasmid, a nitrile-inducible gene expression vector (31). DNA present in primary positive plaques from the BRED experiment described above was used as a template in a PCR reaction using the following primers gp9STAGF: 5´-aattccatatggctgacatttcacgcgcc-3´(containing a NdeI site underlined) and gp9STAGR: 5´-ttacaaagcttctatttttcgaactgcgggtggct-3´(containing a HindIII site underlined). The amplicon was digested with NdeI-HindIII and cloned in pNIT digested with the same restriction enzymes.

The resultant plasmid (pNIT gp9-StrepTag) was used to transform *M. smegmatis* mc^2155 electrocompetent cells and Kan^R colonies were obtained after incubation for 3 days at 37 °C. Resistant clones were grown to an OD_{600nm} of 0.3 in TH9-ADC media and protein expression induced by addition of 28 mM of ε-caprolactam (SIGMA, St Louis, USA). Expression of STAG-gp9 at different time points was checked by Western Blot using a monoclonal anti-STAG antibody HRP conjugated (StrepMAB Classic-HRP, Cat No 2-1509-001, IBA GmbH, Goettingen, Germany) and Pierce ECL chemiluminiscent substrate (Thermo Fisher Scientific Inc., Rockford, IL USA).

Preparation of phAE87::*hsp60-EGFP* stocks containing STAG-gp9 particles. Phage stocks were prepared using a plaque assay. Briefly, cultures of *M. smegmatis* mc^2155 (pNIT gp9-STAG) were grown until an OD_{600nm} of 0.3 in TH9-ADC media when ε-caprolactam (28mM) was added. When cells reached an OD_{600nm} of 1, they were infected with phAE87::hsp60-EGFP and adsorbed for about 15 min at room temperature. After that, bacteria-phage mixes were included in MBTA top agar containing ε-caprolactam and poured on top of TH10 plates containing Kanamycin. Infections were done in order to obtain about 5000-10000 plaques per plate (150 mm plates) with a total of 20 plates prepared. Plaques were visualized after incubation for about 48 hours at 30°C. Plates were flooded with phage buffer (10 ml) and left standing for 2 hours at 37 °C or overnight at 4°C. The buffer was collected and centrifuged to

remove cells and debris and the supernatant was filtered using a Stericup® Filter Unit (EMD Millipore, Billerica, MA, USA). Filtered supernatants were subjected to ultracentrifugation at 31,000 g using a Ti45 rotor in a Beckman Coulter Optima L90K for phage concentration and removal of unincorporated STAG-gp9 capsid proteins. Phage pellets were resuspended in approximately 1 ml of phage buffer and PFU (plaque forming units) ml^{-1} were calculated using a plaque assay. Titers of 10^{12} PFU ml^{-1} were obtained. The resulting phage preparation was designated STAG gfpϕ.

Western Analysis. Forty µl of a concentrated phage stock were mixed with 25 µl of water and frozen in dry ice. The frozen mixture was rapidly thawed and mixed by vortexing. This process was repeated twice and the mixture was then heated to 75°C for 3-4 min. Samples were boiled for 3 min in sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 20% Glycerol, 5% ß-mercaptoethanol, 0.1% Bromophenol Blue) and loaded onto 7% SDS-polyacrylamide gels. Proteins were electrotransferred to a PVDF membrane for 3 hours and visualized using a monoclonal anti-STAG antibody HRP conjugated (StrepMAB Classic-HRP, Cat No 2-1509-001, IBA GmbH, Goettingen, Germany) and Pierce ECL chemiluminiscent substrate (Thermo Fisher Scientific Inc., Rockford, IL USA). A cell extract of induced *M. smegmatis* mc^2155 (pNIT gp9-STAG) cells was used as a control.

Enzyme-linked immunosorbent assays (ELISA). StrepMAB-Immo coated microplates (Cat.no: 2-1521-001, IBA GmbH, Goettingen, Germany) that bind STAGII were used. Serial dilutions of the phage stock were done in binding buffer (25 mM Tris-HCl, 2 mM EDTA, 140 mM NaCl, pH 7.6) and incubated for 1 hour at room temperature. After that, three washes with washing buffer (binding buffer supplemented with 0.05% Tween- 20) were done to remove unbound phage. Two hundred µl of a 1:5000 dilution of StrepMAB Classic-HRP were added and incubated for 1 hour at room temperature. After four washes with washing buffer, 100 µl of

SureBlue Reserve TMB Microwell Peroxidase Substrate (KPL Inc., Gaithersburg, Maryland, USA) were added and left at room temperature until blue color development. Reactions were stopped by addition of an equal volume of TMB Stop Solution to the microwell plate and absorbance read at 450 nm.

Inmunoelectronmicroscopy. Phage preparations were absorbed to a 400 Mesh, support film, carbon coated EM grid (Ted Pella # 01814-F) previously glow-discharged and rinsed with phage buffer. Grids were blocked with BSA 50 mg ml-1 for 5 mins, rinsed with washing buffer (50 mM Tris HCl pH 7.5, 0.1% Tween 20%) and incubated for 5 mins with the primary antibody (Rabbit Anti-Strep-tagII Polyclonal antibody, Genscript, Piscataway, NJ, USA) diluted 1:500 in the same buffer supplemented with 0.5% BSA. After rinsing with washing buffer, grids were incubated with the secondary antibody (Gold labeled anti-Rabbit IgG, KPL Inc., Gaithersburg, Maryland, USA) diluted 1:1000. The grid was rinsed with washing buffer and stained with a 1% uranyl acetate solution before examination of several fields under the electron microscope.

Efficiency of capture of free phage particles using Strep-Tactin coated magnetic beads. Twenty-five µl of MagStrep "type 2HC" beads (Cat. No. 2-1612-002 IBA GmbH, Goettingen, Germany) were mixed with different dilutions of STAG gfpϕ, gfpϕ or D29. Beads and phages were incubated at 4°C for 30 minutes with occasional mixing. Beads were separated using a magnetic separator and washed six times with 200 µl of Buffer W (100mM Tris-HCl pH8, 150 mM NaCl, 1 mM EDTA). Supernatant (fraction obtained after magnetic removal of the beads) and washes were combined to calculate the amount of unbound phage. PFU ml^{-1} were calculated using a plaque assay. Different amount of beads were tested to optimize the beads/phage ratio.

Recovery of phage-bacteria complexes using Strep-Tactin coated magnetic beads. *M.*

smegmatis cells were grown to an OD_{600nm} of 1 in TH9+ADC in the absence of Tween**.** Approximately 250 µl of cells (about 2.5 x 10^7 cells) were infected with STAG gfpϕ or gfpϕ at a MOI (multiplicity of infection) of 100. Incubation was done standing for 10 minutes at room temperature and shaking for 3.5 hours at 37°C cells. After that, cells were fixed with an equal volume of paraformaldehyde 4% (Cat No HT5011, Sigma, St. Louis, USA) for 30 min at RT. Fifty µl of the fixed cells suspension (approximately 10^6 cells) were incubated with 50 µl of MagStrep "type2HC" beads (Cat. No 2-1611-002/006, IBA GmbH, Goettingen, Germany) for 30 min at room temperature on a rocking platform. Beads were separated using a magnetic separator and washed three times using buffer W (100 mM Tris/HCl pH 8, 150 mM NaCl, 1mM EDTA) according to manufacturer instructions. At this step either 5 µl of beads were directly spotted on top of a slide for examination or phage-bacteria complexes were eluted from beads using Buffer BE (100 mM Tris/HCl pH 8, 150 mM NaCl, 1mM EDTA, 2 mM D-biotin) and eluted cells observed by epifluorescence microscopy.

Microscopy and settings. A fluorescence microscope (Axiostar Plus; Carl Zeiss) with a 40X objective and a 100X objective with oil immersion and phase contrast was used. Fluorescent images were acquired using an AxioCam MRc5 camera (Carl Zeiss) and Carl Zeiss AxioVision Rel. 4.6 software. In all experiments the same exposure time was used. For detection of EGFP fluorescent protein the filter CLON ZsGreen1 (42002- HQ 470/30X, HQ 520/40m, Q495LP) from Chroma Technology Corporation was used. Image processing was done using Adobe Photoshop CS2 (Adobe Systems Incorporated) when brightness and contrast were modified the exact same settings were used for comparable images.

RESULTS

Modification of the TM4 capsid gene is deleterious to phage growth.

Initially, we attempted to construct a recombinant derivative of TM4 in which a Strep II tag (STAG) consisting of eight amino acids and a two-residue linker was added to the extreme C-terminus of the TM4 capsid protein (gp9). By extrapolating from HK97 structural studies (25, 32), we reasoned that a short addition to the C-terminus might be tolerated for capsid assembly, and exposed on the capsid surface. Our approach was to use the previously described Bacteriophage Recombineering using Electroporated DNA (BRED) strategy (29, 33) to engineer a modification to TM4 gene *9* (Fig. 2A). Following co-electroporation of TM4 DNA and a 200 bp mutagenic substrate into recombineering *M. smegmatis* cells, plaques were recovered in an infectious center assay, and screened for the presence of the mutant addition. We were successful in detecting the presence of the mutant in pools of plaques (Fig. 2B), although it was present at low frequency [<1 in 100 plaques; in other BRED experiments typically ~10% of individual plaques are mixed, and contain both wild-type and mutant alleles (29)]. However, even after multiple rounds of plaque purification and screening we were unable to purify a homogenous mutant derivative. A simple interpretation is that the mutant can be constructed, but cannot be purified to homogeneity because the recombinant protein is not tolerated if it represents all of the 415 subunits in the assembled capsid.

Addition of an affinity tag to the TM4 capsid through mixed assembly

Because we were able to identify the mutant allele in at least some phage pools, we reasoned that the C-terminal addition could be tolerated if it is present in only a subset of subunits within a particle. We therefore tested the possibility of a mixed assembly, where the recombinant form of the protein is expressed from a plasmid during the process of phage infection and growth, and can potentially co-assemble with wild-type capsid protein. We constructed a plasmid expression system in which a TM4 gp9-STAG fusion (expected size: 33 kD) is expressed from the inducible pNit promoter (Fig. 3A) (31), and demonstrated using anti-STAG antiserum that

the protein was well-expressed following induction with ε-caprolactam (Fig. 3B). Interestingly, only an ~33 kD protein was observed by SDS-PAGE (Fig. 3C) indicating that while procapsids may assemble, they do not progress to form mature capsids that are covalently crosslinked, as seen in mature TM4 particles (23).

Previously we described construction of a recombinant TM4 derivative carrying a GFP reporter (phAE87::*hsp60-EGFP*, abbreviated gfpϕ) that yields fluorescent *M. smegmatis* or *M. tuberculosis* cells following infection (18). We prepared lysates of gfpϕ on the induced gp9-STAG expressing strain, and the phage particles were purified and concentrated; the resulting phage preparation we referred to as STAG-gfpϕ. Phage stock titers from gfpϕ (prepared in the control strain) or STAG-gfpϕ (prepared in the gp9-STAG expressing strain) were comparable showing that phage production was not compromised in the STAG-gp9 expression strain and that particles generated were of equivalent infectivity. To determine if the tag was incorporated into the assembled particles we used anti-STAG antiserum in a Western blot of whole phage particles (Fig. 4A). A clear signal was observed, but only at high molecular weights that presumably correspond to crosslinked capsomers (Fig. 4A). No signal was observed at a molecular weight (33 kDa) corresponding to the unassembled gp9 subunit. Thus gp9-STAG capsid subunits are incorporated as crosslinked subunits into mature virions. The STAG tag was also clearly detectable by ELISA analysis, and a signal greater than background was readily detected from 10^9 particles (Fig. 4B). Finally, we could readily detect the STAG in the phage particles by immunoelectronmicroscopy (Fig. 4C). Because it is difficult to apply these methods quantitatively to these samples, we do not have an accurate determination of the proportion of particles containing at least one tag, or the average number of STAGs incorporated into each particle.

Affinity capture of the tagged phages was assayed using magnetic anti-STAG beads (Table 1). Plaque forming units were calculated in initial samples, in supernatants (after removal of the beads) and in washes. Approximately 95% of STAG-gfpϕ particles were captured. Surprisingly, an unexpectedly large proportion (~80%) of phage particles propagated without STAG-gp9 were also captured, presumably because of Strep-Tactin cross reactivity with native TM4 proteins. This appears to be specific to TM4, as this was not observed with the unrelated phage D29 (Table 1).

STAG-affinity capture of TM4-*M.smegmatis* complexes

A potential utility for STAG-TM4 particles is for capture of phage-bacterial complexes from sputum for diagnostic purposes (Fig. 5A). To test whether STAG gfpϕ particles could be used to capture mycobacterial cells, we prepared a lysate as described above and used this to infect *M. smegmatis* (Fig. 5B). A control lysate of the same phage grown on wild-type *M. smegmatis* was also used. The same number of cells and MOI were used for further comparison. The phage-bacterial complexes were then fixed, captured using magnetic anti-STAG beads, the beads collected, the complexes eluted, and examined by fluorescent microscopy (Fig. 5B). Using a high multiplicity of infection (100) fluorescent cells captured with the STAG gfpϕ particles were readily observed, and these represented about 10% of initial input cells. We were also able to microscopically observe fluorescence by examining the beads directly without elution (Fig. 5C). We have not been able to substantially increase the proportion of captured cells by altering the multiplicity of infection, incubation times, or other reaction conditions, and may reflect an inherent limitation resulting from relatively poor adsorption, as observed with other mycobacteriophages (34, 35). We also cannot exclude the possibility – although we consider it unlikely – that the virion protein ghost is displaced from the cell surface once DNA injection is complete.

DISCUSSION

Addition of affinity tags to bacteriophage particles is useful for a variety of approaches including antigen delivery for vaccine development (36, 37), for capture of phage-bacterium complexes (38), and mechanistic dissection of the process of phage adsorption and infection (39, 40). Non-specific labeling of the phage particles such as addition of fluorescent quantum dots to biotinylated particles is effective but a subset of the modifications are likely to specifically interfere with adsorption and DNA injection (40). Phages such as T4 and λ have head decoration proteins (Hoc and Soc for T4, D for λ) that can be modified – including addition of a fluorescent tag (39) – with little or no reduction in infectivity. Although few phage virions have been structurally defined as well as λ and T4, genome analyses suggest that most phages with siphoviral morphologies likely don't have head decoration proteins, and thus cannot be modified simply using this approach.

Modification of the capsid subunit itself offers an alternative approach. Comparative genomic studies reveal examples in which closely related capsid subunits differ in their lengths as a result of C-terminal extensions (3, 41), suggesting that a C-terminal fusion – especially a small one – would be well tolerated.

A phage display system based on bacteriophage T7 is available (http://www.merckmilliporechina.com/promart/library/3Function/Novagen-T7Select-phagedisplay-system.pdf). This system has the capacity to display peptides up to 50 amino acids long in high copy number (415 per particle), and peptides or proteins up to 1200 amino acids in low copy number (0.1–1 per particle). The T7 capsid protein is normally made in two forms, 10A (344 aa) and 10B (397 aa) with 10B produced by a translational frameshift of 10A;

thus 10B contains most of the sequence of 10A with 52 extra amino acids from the alternate frame added onto the C terminus. In this phage, functional capsids can be composed entirely of either 10A or 10B, or of various ratios of the proteins. Coding sequences for the peptides or proteins to be displayed are cloned in a vector following amino acid 348 of the 10B protein, and because the natural translational frameshift site within the capsid gene has been removed, only a single form of capsid protein is made.

It is thus somewhat surprising that the 10 aa STAG tag (plus linker) appears not to be tolerated as a C-terminal addition to the TM4 capsid as a genetically homogenous recombinant phage particle. We note that capsid assembly requires a complex and well-coordinated ballet of conformation changes (42), and presumably even this small tag can interfere with the capsid assembly when present in all of the subunits. It is plausible that C-terminal capsid fusions are tolerated in other mycobacteriophage genomes, but to our knowledge this is the first time that attempts to do so have been described.

Growth of phage particles on a strain expressing the recombinant capsid-STAG fusion provides opportunities to form mixed particles containing both native and fusion forms of the capsid protein. Capsid assembly with the fusion protein does not generally appear to inhibit phage growth, and phage titers recovered are similar to those propagated on a wild-type strain. Western analysis and immunoelectron microscopy suggest that a substantial proportion of the particles contain at least some STAG-tagged subunits, although precise quantification has proven difficult. Nonetheless, the approach may be of broad applicability for addition of tags to other types of phages and phages of other hosts, especially where it is suspected that no head decoration proteins are available. This approach is also attractive as a variety of alternative recombinant phages (such as those containing reporter genes or specific mutations) can be propagated with STAG-labeled capsids using a single capsid-STAG expressing strain.

There are a variety of potential applications for STAG tagged phage particles. One application is for the capture of phage-bacterial complexes in diagnostic use of reporter phages, in which reporter genes such as firefly luciferase or gfp gene is used (17, 18). This could be of particular use in the diagnosis of tuberculosis, where recovery of bacteria from sputum that are competent for phage infection presents a substantial impediment (19). Infection with STAG tagged reporter phage particles followed by recovery of the complexes provides a plausible solution, although we note that the efficiency of recovery in the studies reported here was relatively poor. This may reflect the observations reported for several mycobacteriophages – including TM4 – that absorption can be relatively inefficient (34, 35, 43). However, at least for some phages, mutants can be isolated with enhanced adsorption and this might provide a strategy for improving the efficiency of recovery. Many alternative applications and configurations can be envisaged, including attachment of the particles to a solid surface and monitoring the capture and assessment of captured bacterial hosts (44).

ACKNOWLEDGMENTS

This work was supported by US National Institutes of Health (FIRCA-BB) R03TW008926 to GH and MP (LMICC), ANPCyT PICT2009-0095 and CONICET PIP 2011-0222 to MP. LR is a doctoral fellow of CONICET (Consejo Nacional de Investigaciones Científicas y Tecnológicas, Argentina). We would like to thank Robert Duda and Charles Bowman for their help with the inmunoelectronmicroscopy experiments.

References

1. **Hatfull GF.** 1994. Mycobacteriophage L5: A toolbox for tuberculosis. ASM News. **60:**255-260.
2. **Hatfull GF.** 2010. Mycobacteriophages: genes and genomes. Annu Rev Microbiol **64:**331-356.
3. **Hatfull GF.** 2012. The secret lives of mycobacteriophages. Adv Virus Res **82:**179-288.
4. **Hatfull GF.** 2012. Complete Genome Sequences of 138 Mycobacteriophages. J Virol **86:**2382-2384.
5. **Pedulla ML, Ford ME, Houtz JM, Karthikeyan T, Wadsworth C, Lewis JA, Jacobs-Sera D, Falbo J, Gross J, Pannunzio NR, Brucker W, Kumar V, Kandasamy J, Keenan L, Bardarov S, Kriakov J, Lawrence JG, Jacobs WR, Hendrix RW, Hatfull GF.** 2003. Origins of highly mosaic mycobacteriophage genomes. Cell **113:**171-182.
6. **Bardarov S, Bardarov Jr S, Jr., Pavelka Jr MS, Jr., Sambandamurthy V, Larsen M, Tufariello J, Chan J, Hatfull G, Jacobs Jr WR, Jr.** 2002. Specialized transduction: an efficient method for generating marked and unmarked targeted gene disruptions in Mycobacterium tuberculosis, M. bovis BCG and M. smegmatis. Microbiology **148:**3007-3017.
7. **Bardarov S, Kriakov J, Carriere C, Yu S, Vaamonde C, McAdam RA, Bloom BR, Hatfull GF, Jacobs WR, Jr.** 1997. Conditionally replicating mycobacteriophages: a system for transposon delivery to Mycobacterium tuberculosis. Proc Natl Acad Sci U S A **94:**10961-10966.
8. **Lee MH, Pascopella L, Jacobs WR, Jr., Hatfull GF.** 1991. Site-specific integration of mycobacteriophage L5: integration-proficient vectors for Mycobacterium smegmatis, Mycobacterium tuberculosis, and bacille Calmette-Guerin. Proc Natl Acad Sci U S A **88:**3111-3115.

9. **Morris P, Marinelli LJ, Jacobs-Sera D, Hendrix RW, Hatfull GF.** 2008. Genomic characterization of mycobacteriophage Giles: evidence for phage acquisition of host DNA by illegitimate recombination. J. Bacteriol. **190:**2172-2182.

10. **Pham TT, Jacobs-Sera D, Pedulla ML, Hendrix RW, Hatfull GF.** 2007. Comparative genomic analysis of mycobacteriophage Tweety: evolutionary insights and construction of compatible site-specific integration vectors for mycobacteria. Microbiology **153:**2711-2723.

11. **Huff J, Czyz A, Landick R, Niederweis M.** 2010. Taking phage integration to the next level as a genetic tool for mycobacteria. Gene **468:**8-19.

12. **Freitas-Vieira A, Anes E, Moniz-Pereira J.** 1998. The site-specific recombination locus of mycobacteriophage Ms6 determines DNA integration at the tRNA(Ala) gene of Mycobacterium spp. Microbiology **144:**3397-3406.

13. **Donnelly-Wu MK, Jacobs WR, Jr., Hatfull GF.** 1993. Superinfection immunity of mycobacteriophage L5: applications for genetic transformation of mycobacteria. Mol Microbiol **7:**407-417.

14. **Brown KL, Sarkis GJ, Wadsworth C, Hatfull GF.** 1997. Transcriptional silencing by the mycobacteriophage L5 repressor. Embo J **16:**5914-5921.

15. **Sarkis GJ, Jacobs WR, Jr., Hatfull GF.** 1995. L5 luciferase reporter mycobacteriophages: a sensitive tool for the detection and assay of live mycobacteria. Mol Microbiol **15:**1055-1067.

16. **Pearson RE, Jurgensen S, Sarkis GJ, Hatfull GF, Jacobs WR, Jr.** 1996. Construction of D29 shuttle phasmids and luciferase reporter phages for detection of mycobacteria. Gene **183:**129-136.

17. **Jacobs WR, Jr., Barletta RG, Udani R, Chan J, Kalkut G, Sosne G, Kieser T, Sarkis GJ, Hatfull GF, Bloom BR.** 1993. Rapid assessment of drug susceptibilities of

Mycobacterium tuberculosis by means of luciferase reporter phages. Science **260:**819-822.

18. **Piuri M, Jacobs WR, Jr., Hatfull GF.** 2009. Fluoromycobacteriophages for rapid, specific, and sensitive antibiotic susceptibility testing of Mycobacterium tuberculosis. PLoS ONE **4:**e4870.

19. **Jain P, Hartman TE, Eisenberg N, O'Donnell MR, Kriakov J, Govender K, Makume M, Thaler DS, Hatfull GF, Sturm AW, Larsen MH, Moodley P, Jacobs WR, Jr.** 2012. phi(2)GFP10, a high-intensity fluorophage, enables detection and rapid drug susceptibility testing of Mycobacterium tuberculosis directly from sputum samples. J Clin Microbiol **50:**1362-1369.

20. **Timme TL, Brennan PJ.** 1984. Induction of bacteriophage from members of the Mycobacterium avium, Mycobacterium intracellulare, Mycobacterium scrofulaceum serocomplex. J Gen Microbiol **130:**2059-2066.

21. **Jacobs WR, Jr., Tuckman M, Bloom BR.** 1987. Introduction of foreign DNA into mycobacteria using a shuttle phasmid. Nature **327:**532-535.

22. **Pope WH, Ferreira CM, Jacobs-Sera D, Benjamin RC, Davis AJ, DeJong RJ, Elgin SCR, Guilfoile FR, Forsyth MH, Harris AD, Harvey SE, Hughes LE, Hynes PM, Jackson AS, Jalal MD, MacMurray EA, Manley CM, McDonough MJ, Mosier JL, Osterbann LJ, Rabinowitz HS, Rhyan CN, Russell DA, Saha MS, Shaffer CD, Simon SE, Sims EF, Tovar IG, Weisser EG, Wertz JT, Weston-Hafer KA, Williamson KE, Zhang B, Cresawn SG, Jain P, Piuri M, Jacobs WR, Jr., Hendrix RW, Hatfull GF.** 2011. Cluster K Mycobacteriophages: Insights into the Evolutionary Origins of Mycobacteriophage TM4. PLoS ONE **6:**e26750.

23. **Ford ME, Stenstrom C, Hendrix RW, Hatfull GF.** 1998. Mycobacteriophage TM4: genome structure and gene expression. Tuber Lung Dis **79:**63-73.

24. **Rybniker J, Kramme S, Small PL.** 2006. Host range of 14 mycobacteriophages in Mycobacterium ulcerans and seven other mycobacteria including Mycobacterium tuberculosis--application for identification and susceptibility testing. J Med Microbiol **55:**37-42.

25. **Duda RL, Martincic K, Xie Z, Hendrix RW.** 1995. Bacteriophage HK97 head assembly. FEMS Microbiol Rev **17:**41-46.

26. **Rao VB, Black LW.** 2010. Structure and assembly of bacteriophage T4 head. Virol J **7:**356.

27. **Li Q, Shivachandra SB, Leppla SH, Rao VB.** 2006. Bacteriophage T4 capsid: a unique platform for efficient surface assembly of macromolecular complexes. J Mol Biol **363:**577-588.

28. **Snapper SB, Melton RE, Mustafa S, Kieser T, Jacobs WR, Jr.** 1990. Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. Mol Microbiol **4:**1911-1919.

29. **Marinelli LJ, Piuri M, Swigonova Z, Balachandran A, Oldfield LM, van Kessel JC, Hatfull GF.** 2008. BRED: a simple and powerful tool for constructing mutant and recombinant bacteriophage genomes. PLoS ONE **3:**e3957.

30. **van Kessel JC, Hatfull GF.** 2007. Recombineering in *Mycobacterium tuberculosis*. Nature Methods **4:**147-152.

31. **Pandey AK, Raman S, Proff R, Joshi S, Kang CM, Rubin EJ, Husson RN, Sassetti CM.** 2009. Nitrile-inducible gene expression in mycobacteria. Tuberculosis (Edinb) **89:**12-16.

32. **Conway JF, Wikoff WR, Cheng N, Duda RL, Hendrix RW, Johnson JE, Steven AC.** 2001. Virus maturation involving large subunit rotations and local refolding. Science **292:**744-748.

493 33. **Marinelli LJ, Hatfull GF, Piuri M.** 2012. Recombineering: A powerful tool for
494 modification of bacteriophage genomes. Bacteriophage **2:**5-14.
495 34. **Barsom EK, Hatfull GF.** 1996. Characterization of Mycobacterium smegmatis gene that
496 confers resistance to phages L5 and D29 when overexpressed. Mol Microbiol **21:**159-
497 170.
498 35. **Jacobs-Sera D, Marinelli LJ, Bowman C, Broussard GW, Guerrero Bustamante C,**
499 **Boyle MM, Petrova ZO, Dedrick RM, Pope WH, Science Education Alliance Phage**
500 **Hunters Advancing G, Evolutionary Science Sea-Phages P, Modlin RL, Hendrix**
501 **RW, Hatfull GF.** 2012. On the nature of mycobacteriophage diversity and host
502 preference. Virology **434:**187-201.
503 36. **Peachman KK, Li Q, Matyas GR, Shivachandra SB, Lovchik J, Lyons RC, Alving**
504 **CR, Rao VB, Rao M.** 2012. Anthrax vaccine antigen-adjuvant formulations completely
505 protect New Zealand white rabbits against challenge with Bacillus anthracis Ames strain
506 spores. Clin Vaccine Immunol **19:**11-16.
507 37. **Sathaliyawala T, Rao M, Maclean DM, Birx DL, Alving CR, Rao VB.** 2006. Assembly
508 of human immunodeficiency virus (HIV) antigens on bacteriophage T4: a novel in vitro
509 approach to construct multicomponent HIV vaccines. J Virol **80:**7688-7698.
510 38. **Tolba M, Minikh O, Brovko LY, Evoy S, Griffiths MW.** 2010. Oriented immobilization
511 of bacteriophages for biosensor applications. Appl Environ Microbiol **76:**528-535.
512 39. **Alvarez LJ, Thomen P, Makushok T, Chatenay D.** 2007. Propagation of fluorescent
513 viruses in growing plaques. Biotechnol Bioeng **96:**615-621.
514 40. **Edgar R, Rokney A, Feeney M, Semsey S, Kessel M, Goldberg MB, Adhya S,**
515 **Oppenheim AB.** 2008. Bacteriophage infection is targeted to cellular poles. Mol
516 Microbiol **68:**1107-1116.
517 41. **Hatfull GF.** 2006. Mycobacteriophages, p. 602-620. *In* Calendar R (ed.), The
518 Bacteriophages. Oxford University Press, New York, NY.

519 42. **Hendrix RW, Johnson JE.** 2012. Bacteriophage HK97 capsid assembly and
520 maturation. Adv Exp Med Biol **726:**351-363.
521 43. **Piuri M, Hatfull GF.** 2006. A peptidoglycan hydrolase motif within the
522 mycobacteriophage TM4 tape measure protein promotes efficient infection of stationary
523 phase cells. Mol Microbiol **62:**1569-1585.
524 44. **Bennett AR, Davids FG, Vlahodimou S, Banks JG, Betts RP.** 1997. The use of
525 bacteriophage-based systems for the separation and concentration of Salmonella. J
526 Appl Microbiol **83:**259-265.
527 45. **Soding J.** 2005. Protein homology detection by HMM-HMM comparison. Bioinformatics
528 **21:**951-960.
529
530

	Number of infectious phage particles/ml		
Bacteriophage	**Initial**	**Unbound** (supernatant and washes)	**Particles bound (%)**
D29	$6.55 \times 10^7 \pm 7.07\ 10^5$	$6.34 \times 10^7 \pm 1.48\ 10^5$	3.2 ± 1.2
gfpϕ	$4.25 \times 10^8 \pm 3.54\ 10^7$	$8.5 \times 10^7 \pm 3.54\ 10^7$	80.2 ± 6.6
STAG gfpϕ	$6.10 \times 10^9 \pm 1.41\ 10^8$	$4.28 \times 10^8 \pm 1.59\ 10^8$	92.9 ± 2.77

Table 1. Capture of bacteriophage using Strep-Tactin coated magnetic beads.

Data (means ± standard deviations for 3 experiments) represents the number of plaque forming units per ml in the initial sample and in the supernatant (fraction obtained after magnetic removal of the beads) and washes.

538 **Figure Legends**

539

540 **Figure 1. HHPred alignment of TM4 gp9 with the HK97 gp5 major capsid subunit.** TM4
541 gp9 was used as a query sequence (Q) in a HHPred search using standard parameters (45),
542 and the top two hits (probability 100%) were matches to the cleaved mature capsid subunit, and
543 to the uncleaved procapsid (T), the second of which is shown here. Consensus sequences and
544 secondary structure predictions (ss) are shown. The positions of the lysine and asparagine
545 residues (positions 169 and 356 respectively in HK97 gp5) participating in covalent crosslinking
546 in HK97 gp5 are shown by arrows. The position corresponding to the latter is an aspartic acid
547 residue in TM4 gp9.

548

549 **Figure 2. Addition of a STAG to the capsid protein using BRED.**
550 **A**. Schematic representation of the strategy used to add a Strep-tag II (STAG) to the C-terminus
551 of gp9 of TM4 using BRED (see Material and Methods for details).
552 **B**. Screening by PCR for the insertion of the STAG in gp9. Pools of 10 primary plaques (lanes
553 P1-P11) were screened by PCR using a forward oligo that anneals to the STAG II sequence
554 and a reverse oligo that anneals to gp10 sequence. In the presence of a positive plaque the
555 expected size product is 500 bp. Fld: A flooded plate from a BRED experiment was used as a
556 positive control to demonstrate the presence of the phage mutant in the population. TM4: DNA
557 of TM4 used as a negative control for the PCR reaction.

558

559 **Figure 3. Expression of STAG-tagged TM4 capsid subunit, gp9.**
560 **A**. Schematic representation of pNIT gp9-STAG. **B**. Growth curve of *M. smegmatis* mc^2155
561 (pNIT gp9-STAG). Cells were grown to an OD_{600nm} of 0.3 when ε-caprolactam was added.
562 Arrows indicate the different time points when gp9-STAG expression was checked by Western

blot. Diamonds: induced cells; squares: non-induced cells. **C**. Expression of gp9-STAG was checked by Western blot. Lanes 1 to 3, induced cells; lanes 4 to 6, non-induced cells. Samples were removed after 1 hour (1 and 4), 3hs (2 and 5) and 24 hours (3 and 6) of addition or not of the inducer.

Figure 4. Incorporation of an affinity STAG to the phAE87::*hsp60-EGFP* capsid.

The presence of the tag in the capsid of the phage particles was checked by: **A.** Western blot. Whole phage particles of: **1.** phAE87::*hsp60-EGFP* [gfpϕ], **2.** phAE87::*hsp60-EGFP* amplified in *M. smegmatis* mc^2155 (pNIT gp9-STAG) [STAG gfpϕ] or **3.** Cell extract of *M. smegmatis* mc^2155 (pNIT gp9-STAG) were subjected to SDS-PAGE and revealed by chemiluminiscence using an HRP labeled anti STAG antibody. **B.** ELISA. Dilutions of: **1.** gfpϕ or **2.** STAG gfpϕ were applied to plates coated with an anti STAG antibody. From left to right: 1×10^{10}, 5×10^{9}, 2.5×10^{9}, 1.25×10^{9} phage particles respectively were adsorbed to the plate. After washing, the capture of the phage particles was checked using an HRP labeled anti STAG antibody in a colorimetric assay. **C.** Electroinmunomicroscopy. Phage particles were adsorbed to a grid and incubated with a rabbit anti-STAG primary antibody and a gold- labeled anti-rabbit secondary antibody. The grid was stained with uranyl acetate and several fields were examined under the electron microscope. Scale bar: 100nm

Figure 5. STAG affinity capture of phage-bacteria complexes.

A. Schematic representation of the protocol used for infection of *M. smegmatis* with STAG gfpϕ and recovery of phage-bacteria complexes using Strep-Tactin coated magnetic beads. **B.** Fluorescence micrograph images after elution of phage-bacteria complexes from Strep-Tactin coated magnetic beads. Cells were infected with STAG-gfpϕ (left panel) or gfpϕ (right panel). Cells were observed using 400X magnification. **C**. Fluorescence micrograph (left panel) and phase contrast (right panel) images from Strep-Tactin coated magnetic beads after recovery of

588 phage-bacteria complexes. Cells were observed using 1000X magnification.

589

590

591