

Fully synthetic chassis for the rapid engineering of Mycobacteriophages with therapeutic potential

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Multidrug Resistant Mycobacteria: Targets for Phage-Based Therapy

Mycobacteria are found ubiquitously in the environment.

In addition to the causative agent of tuberculosis, Mycobacteria (e.g. *M. abscessus*, *M. avium*) are opportunistic pathogens associated with hospital acquired infections and infection of the immune-compromised.

High incidence of multidrug resistant Mycobacteria makes these pathogens potential targets for phage-based therapy.



Micrograph of mycobacteria, photo credit: NIAID cc-by-2.0.



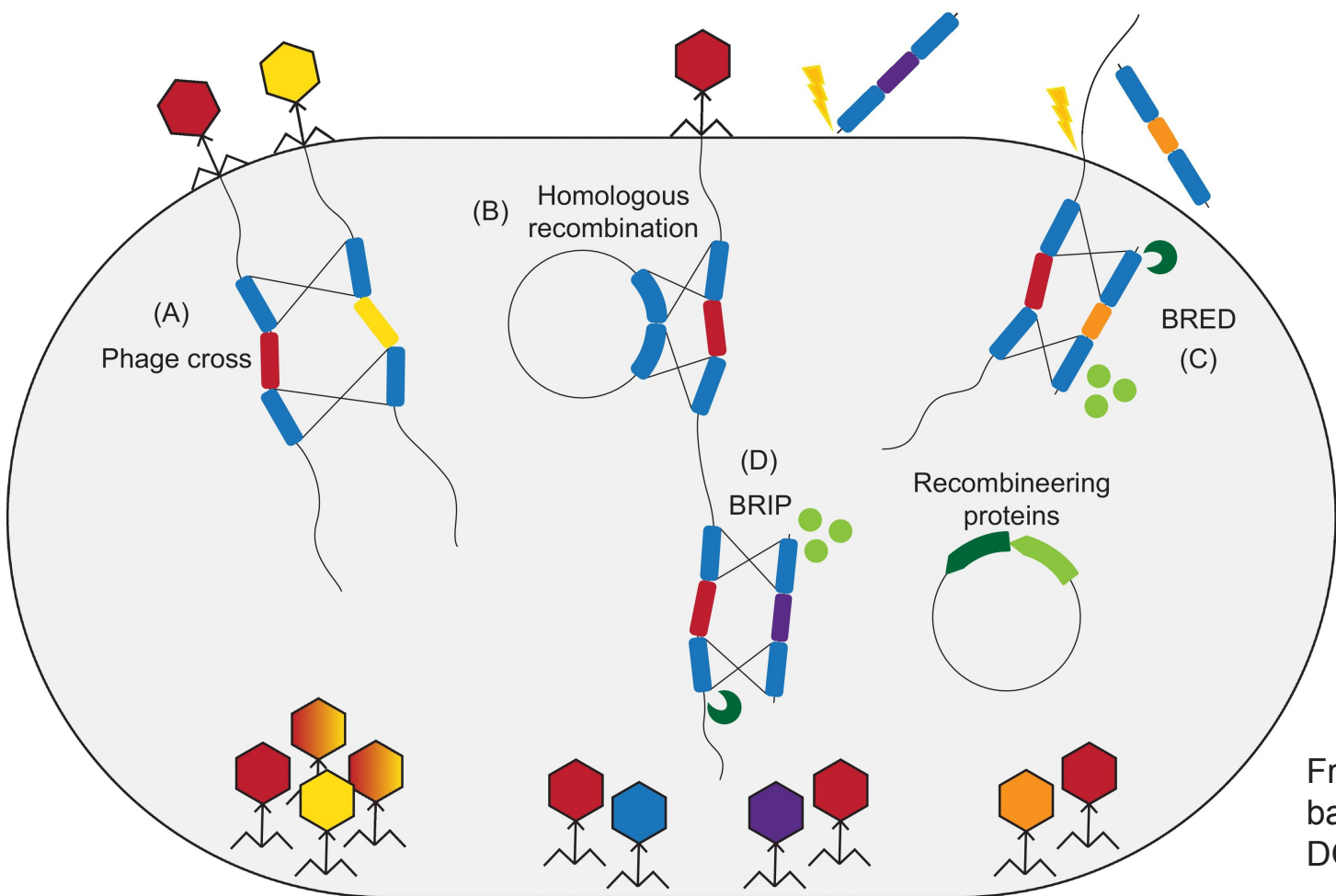
An undergraduate lab program to identify environmental phages has generated a vast collection of mycobacteriophage strains and genome sequences.

Research Challenges:

- High GC content (60-70%) complicates PCR amplification and DNA synthesis.
- Limited genetic tools compared to *E. coli*.
- Large phage genomes (50 kb+).

Hatfull "Actinobacteriophages: Genomics, Dynamics, and Applications" doi.org/10.1146/annurev-virology-122019-070009
Hanauer et. al. "An inclusive Research Education Community (iREC): Impact of the SEA-PHAGES program on research outcomes and student learning" doi.org/10.1073/pnas.1718188115

Phage Engineering Challenges: Rare Events and Complex Screens

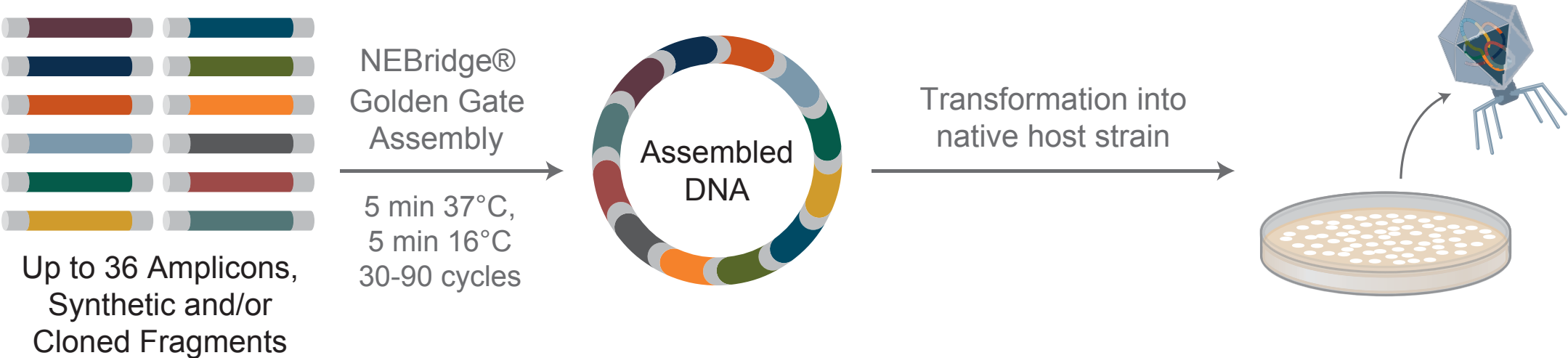


From: Mahler et. al., "Approaches for bacteriophage genome engineering," DOI: 10.1016/j.tibtech.2022.08.008

In vivo Recombineering methods require:

- Simultaneous transformation of donor DNA and phage infection
- Rare recombination events within short replication windows
- Specialized host strains
- Extensive screening/selection/counterscreening required
- Modification of one genome region at a time
- Weeks to months to isolate desired variants

Streamlined Phage Engineering: Genome Synthesis via Golden Gate Assembly



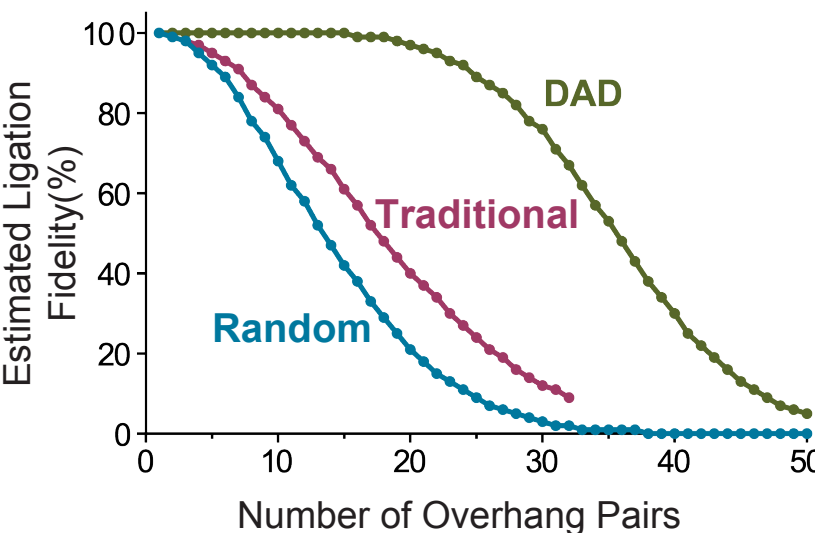
Pryor et. al., "Rapid 40 kb Genome Construction from 52 Parts through Data-optimized Assembly Design," doi.org/10.1021/acssynbio.1c00525
Sikkema et. al. "High-Complexity One-Pot Golden Gate Assembly" doi.org/10.1002/cpz1.882

Advantages of High-Complexity Golden Gate Assembly (HC-GGA) genome synthesis:

- Break phage genomes into 1-5kb fragments – easily amplified & cloned
- Direct transformation into host no secondary passage for construct propagation
- Minimal screening - low rates of misassembly, SNPs, indels
- Genome engineering via plasmid part modification
- Modify multiple regions simultaneously
- Online tools to support design: <https://goldengate.neb.com/#/>
<https://ligasefidelity.neb.com/>

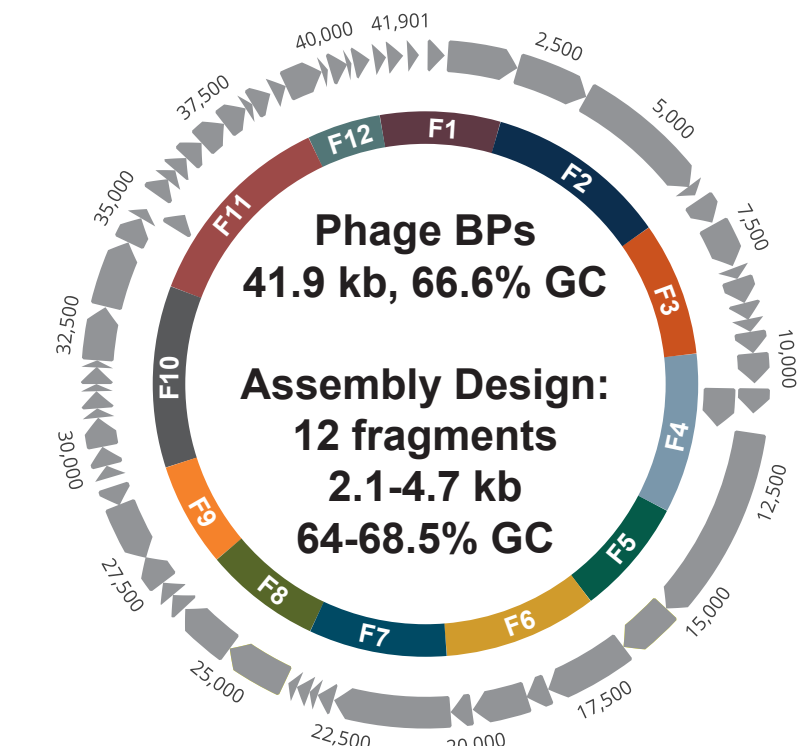
Data-assisted Assembly Design (DAD) Applies Comprehensive Data to Enable HC-GGA

Accurate assembly of 30+ parts!



Design, Synthesis, Assembly and Rescue of Synthetic Phage BPs

We have applied HC-GGA to build fully synthetic mycobacteriophages.

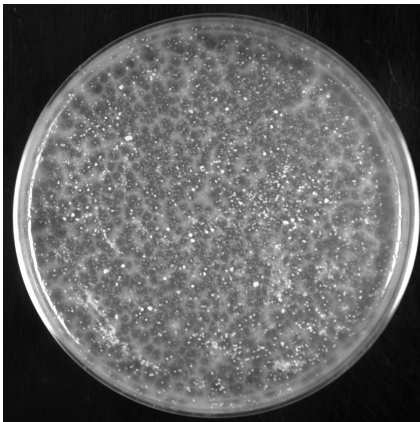


In silico designed parts rated too complex for phosphoramidite synthesis by multiple vendors

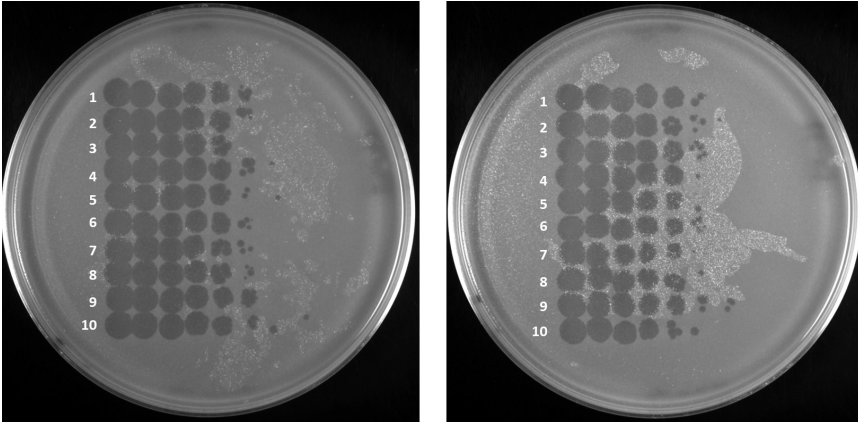
Ansa Biotechnologies synthesized all fragments without issue.

Cloned & sequence verified parts permit easy generation of high-quality DNA for GGA

Synthetic BPs Plaques



Phage Fecundity Titration wt BPs Syn BPs

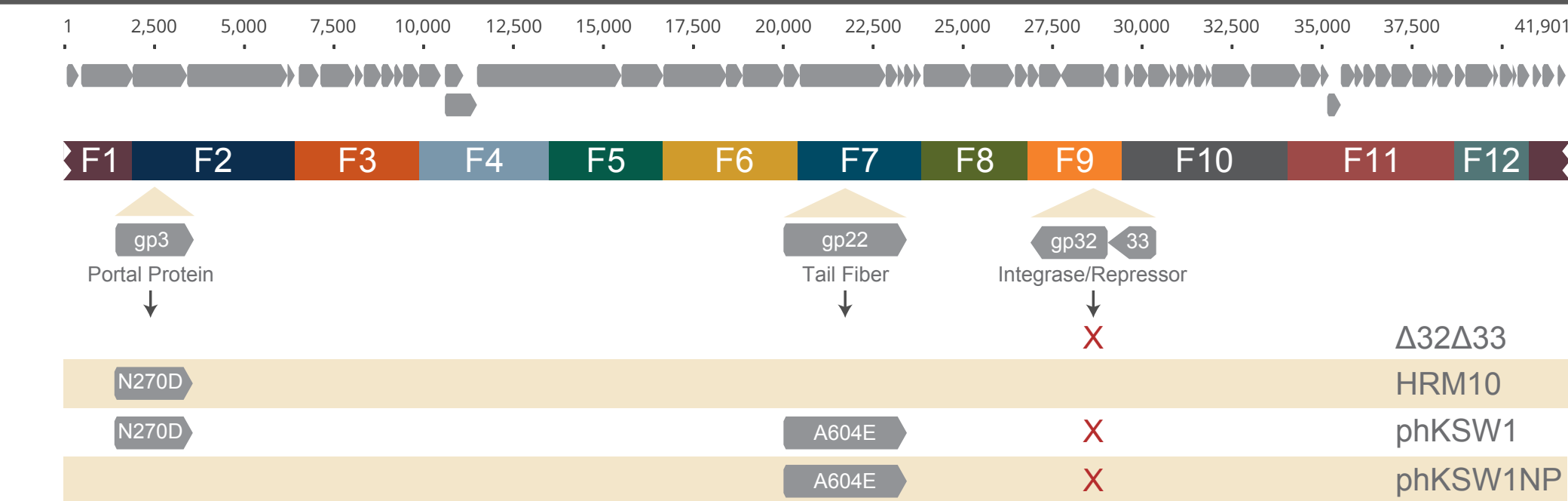


20µl Golden Gate Assembly reaction were purified using a column-based clean up kit and eluted with 10µl elution buffer (10mM Tris-HCl (pH8.5), 0.1mM EDTA). 1 - 3µl were transformed into 200µl *M. smegmatis* strain mc²155 competent cells, and recovered for 2 hours before plating.

Isolated plaques were picked using a P1000 tip. The extracted agar piece was resuspended in 100µl of 1mM CaCl₂-containing phage buffer and incubated for 24 hours at room temperature for phage release from the agar piece to obtain phage lysate. The lysate was serially diluted (10⁻¹ to 10⁻⁸), and 2µl of each dilution was spotted on *M. smegmatis* strain mc²155 lawns to determine titer.

12/12 sequenced plaques were verified 100% sequence correct. Titers are identical to true wt phage.

Deletions and Point Mutants Easily Generated by Plasmid Mutagenesis

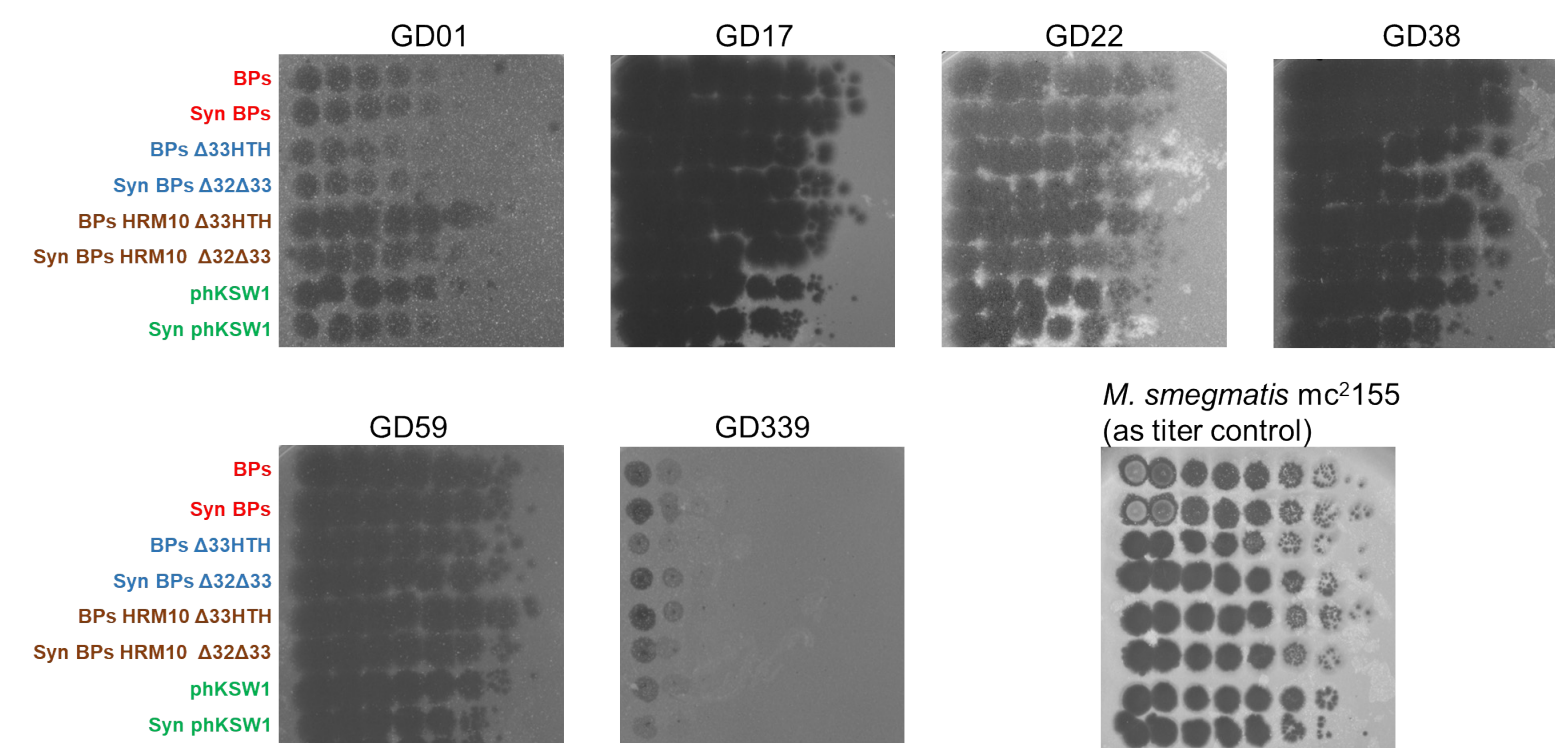


Mutations chosen based on previously characterized variants (Wetzel et. al., <https://doi.org/10.1038/s41564-023-01451-6>).

Changes were introduced into plasmid parts F2, F7, and F9 via classic mutagenesis methods, combined with remaining parts and assembled as wt by HC-GGA.

20/22 sequenced plaques 100% sequence correct, two contained single base insertions.

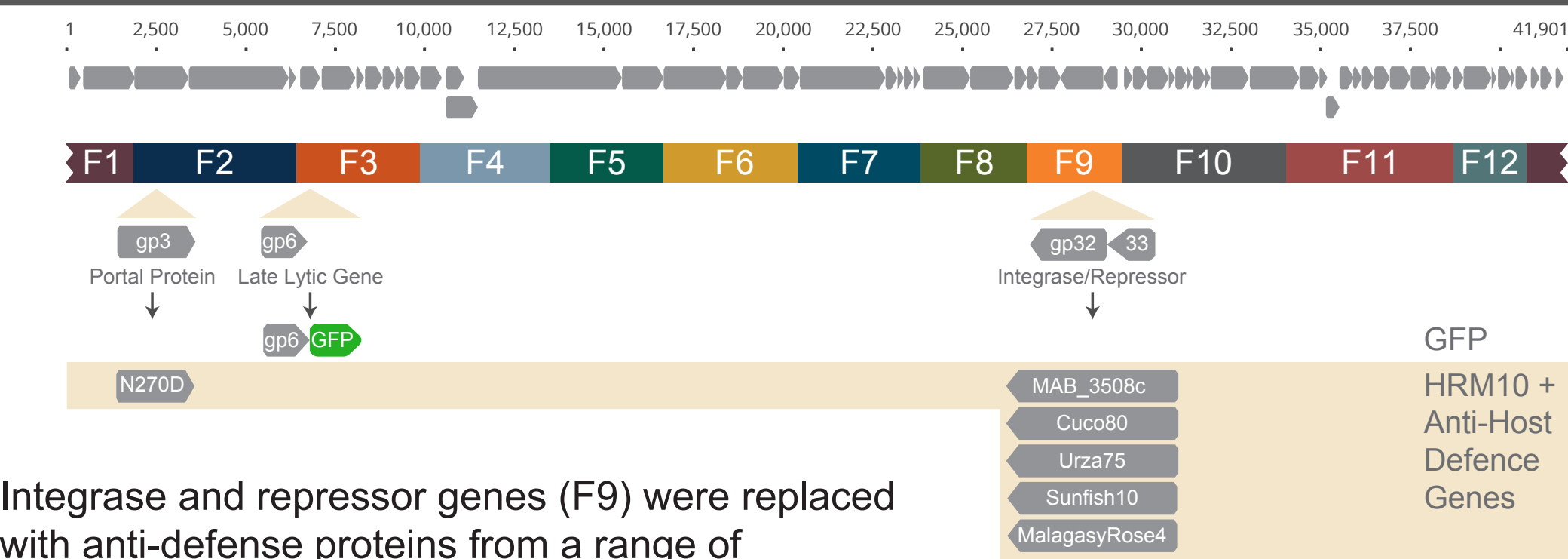
Phage Efficiency of Plating Across M. abscessus Strains



The phage lysates were serially diluted (10⁻¹ to 10⁻⁸), and 2.5µl of each dilution was spotted on lawns of different *M. abscessus* strains to determine titer.

In all cases, synthetic phage matches fecundity of wild type/natural variants.

Insertion of Anti-Defense Genes: Laying the Groundwork for Therapeutics



Integrase and repressor genes (F9) were replaced with anti-defense proteins from a range of mycobacteriophages – aiming to create obligatory lytic strains with broad host range as candidate therapies against mycobacterial infections.

Characterization of these chimeric stains is ongoing!