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

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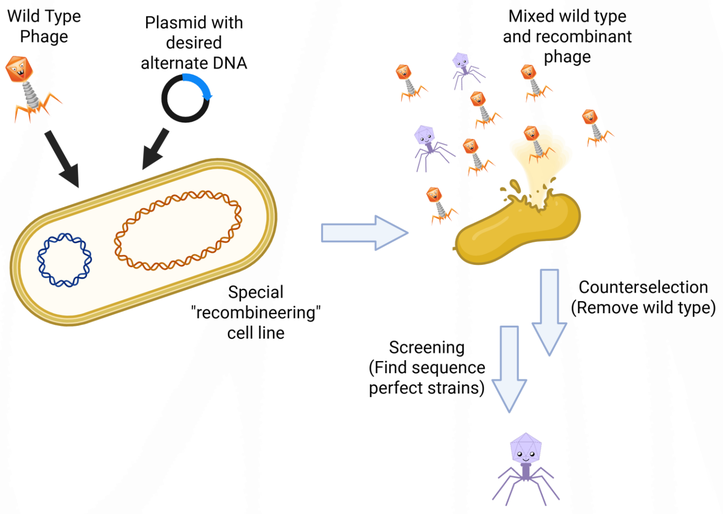
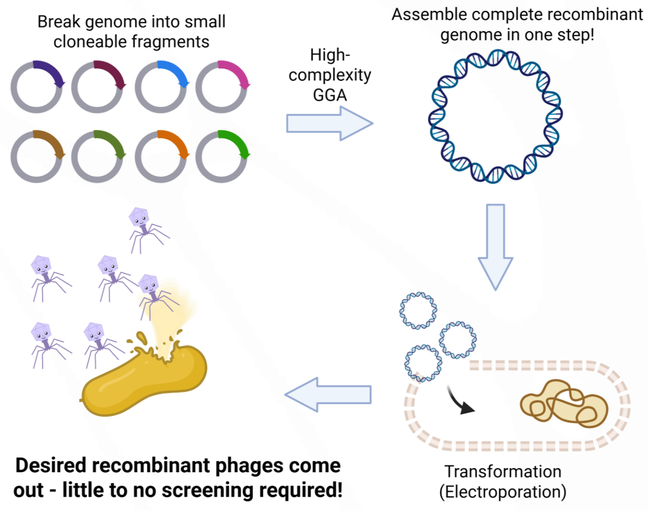
Phage Engineering Challenges: Rare Events and Complex Screens



***In vivo* Recombineering methods require:**

- Requires production of host strain with plasmid containing recombination target
- Rare recombination events within short replication windows
- Extensive counterselection and screening
- Modification of one genome region at a time
- Weeks to months to isolate desired variants

Streamlined Phage Engineering: Genome Synthesis via Golden Gate Assembly



Advantages of direct genome synthesis:

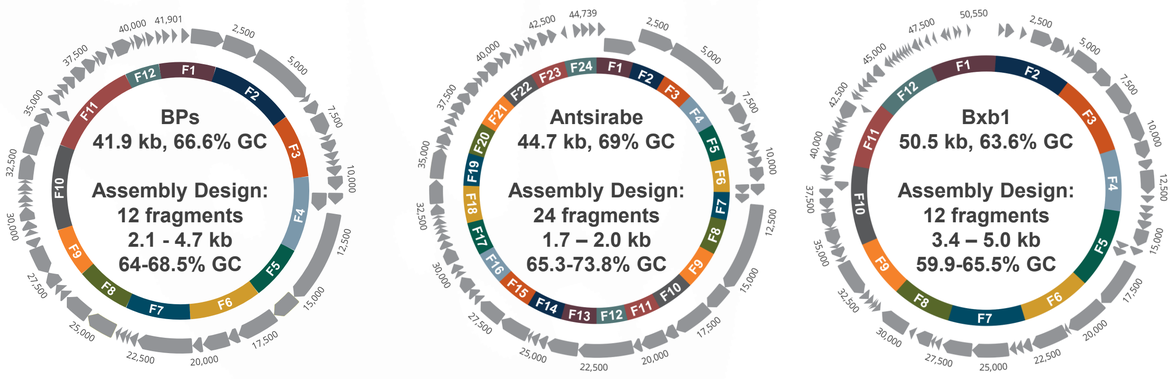
- Assemble up to 36 parts in a single assembly reaction
- Break phage genomes into 1-5kb fragments – easily amplified & cloned
- Direct transformation of assembly reaction with no secondary passage
- Minimal screening - low rates of misassembly, SNPs, indels
- Genome engineering via part modification
- Modify multiple regions simultaneously
- Online tools to support design: https://goldengate.neb.com/#!/ https://ligasefidelity.neb.com/

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

Mycobacterium species, and the phages that prey upon them, are found ubiquitously in the environment. Outside of the causative agent of tuberculosis, most *Mycobacterium* species are harmless to humans while a subset can cause opportunistic infections, colonizing medical equipment and infecting immune compromised patients. There is interest in the study and engineering of Mycobacterial phages as tools for studying the biology of these organisms, and potentially as therapeutics targeting recalcitrant Nontuberculosis Mycobacteria (NTM) infections. Mycobacteria and Mycobacterial phages are also notable for their high GC genomes, typically between 65-70% overall genomic GC content which can make amplification, sequencing, and especially *de novo* synthesis of the DNA of these organisms challenging creating difficulties in the generation of recombinant phages with defined genomic changes.

Here we report the generation of fully synthetic engineering systems for the Mycobacterial phages BPs (41,901bp, 66.6% GC), Antsirabe (44,739bp, 69.0% GC), and Bxb1 (50,550bp, 63.6% GC). The genomes were domesticated of Type IIS sites (BPs: 18 sites, Antsirabe: 2 sites, Bxb1: 49 sites), divided into twelve or twenty-four parts, synthesized, and assembled into clonal fragments in *E. coli* vectors. The fragments could then be assembled into complete a phage genome in a single Golden Gate reaction step and rescued by direct transformation into *Mycobacterium smegmatis.* This method reliably produced recombinant plaques with essentially perfect fidelity and minimal screening requirements. The assembly systems were then applied to the rapid genetic manipulation of the phages, with recombinant phages containing gene deletions, insertions, and point mutations. Phenotyping demonstrates the ability to affect host range and alter the lytic/lysogenic cycle. These systems represent a model for simplified phage molecular biology, with applications in the development of new therapeutic systems targeting NTM infections.

Synthetic Mycobacteriophage Assembly Designs

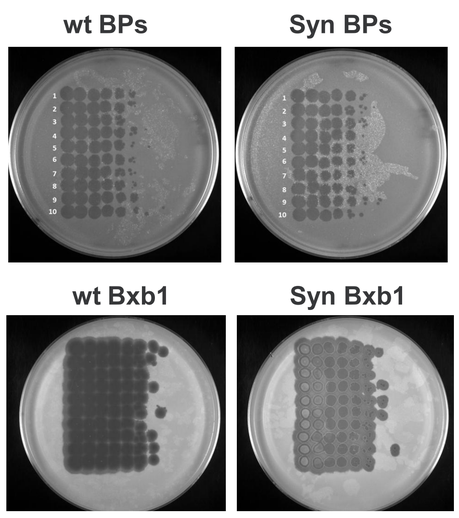


Direct Transformation of Assembly Reactions Results in Plaques of Synthetic Phage

HC-GGA reactions using BsaI-HFv2 NEBridge Golden Gate Enzyme Mix (BPs and Antsirabe) or BsmBI-v2 NEBridge Golden Gate Enzyme Mix (Bxb1) and 3nM each fragments were carried out using a 15-hour cycling protocol (90 cycles of 5 min 37/42°C → 5 min 16°C then 5 min, 60°C). Reactions were purified by column cleanup and transformed into *M. smegmatis strain mc²155* by electroporation before plating.

12/12 sequenced BPs and 5/5 Antsirabe (Bxb1 in sequencing progress) plaques were verified 100% sequence correct.

wt and Synthetic BPs Show Similar Fecundity

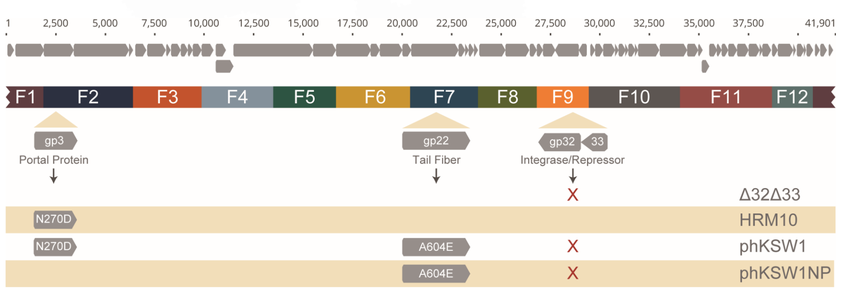


Isolated plaques were picked and recovered in 1mM $CaCl_2$-containing phage buffer. Recovered lysate was serially diluted (10^{-1} to 10^{-8}) and spotted on *M. smegmatis strain mc²155* lawns to determine titer. True wt and synthetic domesticated wt (Syn) BPs and Bxb1 show similar titer results.

Measured titers (average of 10 plaques)
wt BPs: 2.2 x 10^8 particles/plaque
Syn BPs: 1.3 x 10^8 particles/plaque

wt Bxb1: 3.7 x 10^9 particles/plaque
Syn Bxb1: 2.3 x 10^9 particles/plaque

Deletions and Point Mutants via Plasmid Mutagenesis

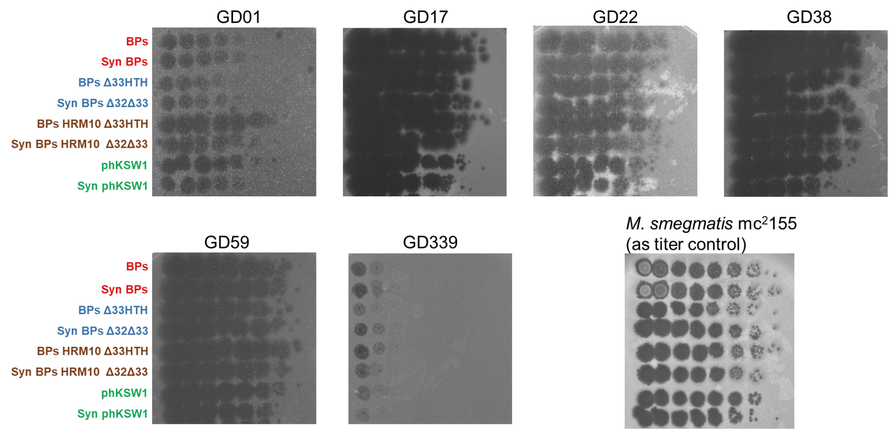


Mutations of BPs were chosen based on previously characterized variants from Wetzel et. al., (PMID: 37644325)

Changes were introduced into plasmid parts F2, F7, and F9 via standard mutagenesis methods.

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

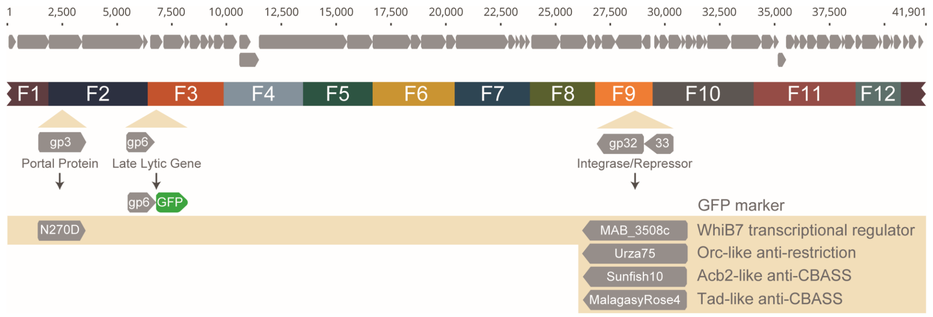
BPs Strain Efficiency of Plating (EOP) across *M. abscessus* strains



Serially diluted phage was spotted on lawns of different *M. abscessus* strains to determine titer.

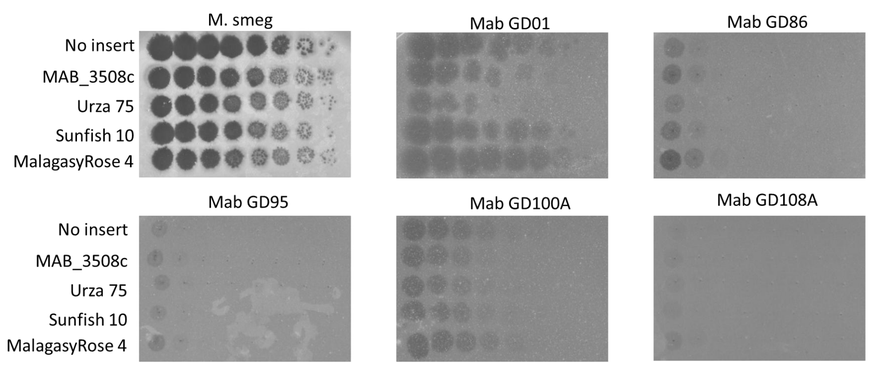
In all cases, synthetic phage matches fecundity of the undomesticated variant

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



The integrase and repressor genes (F9) were replaced with anti-defense genes aiming to create obligatory lytic strains with broad host range as candidate therapies against Mycobacterial infections.

Anti-Defense BPs Strains EOP across *M. abscessus* strains



Serially diluted phage was spotted on lawns of different *M. abscessus* strains to determine titer.

No host range changes noted for initial round of synthetic variants.