Application of a fully synthetic golden gate assembly system to the rapid and flexible engineering of *Pseudomonas aeruginosa* phage ϕKMV

Andrew P. Sikkema[1], Kaitlyn E. Kortright[2], Jyot Antani[2], Matthew Davidson[2], Max Hopkins[3],Benjamin Newman[1], Vladimir Potapov[1], Hemaa Selvakumar[3], Cecilia Silva[1], S. Kasra Tabatabaei[1], Robert McBride[3], Paul E. Turner[2], **Gregory J. S. Lohman**[1]

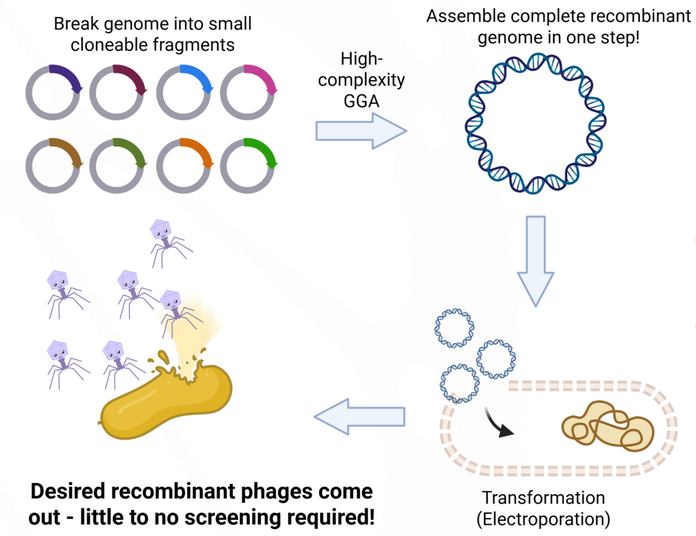
[1] New England Biolabs, Research Department, Ipswich, MA
[2] Yale University, Department of Ecology and Evolutionary Biology, New Haven, CT
[3] Felix Biotechnologies

Abstract

Bacteriophages have potential applications in human and veterinary medicine, agriculture, food safety, and biosecurity. Phage therapy, the application of phages to treat antibiotic-resistant bacterial infections, typically requires screening of environmental phages against the infecting strains for each individual patient, a laborious process that hinders development of standardized treatments. To overcome these limits, a robust genomic engineering platform is required which permits rapid and dependable phage mutagenesis, including alteration of large stretches of DNA, and altering multiple regions simultaneously.

We developed an engineering platform for the bacteriophage ϕKMV, which targets the important opportunistic human pathogen *Pseudomonas aeruginosa*. This system takes advantage of High Complexity Golden Gate Assembly (HC-GGA) design to divide the ϕKMV genome into fragments small enough to be conveniently obtained as synthetic DNA, and, through selection of breakpoints to separate promoters from toxic genes, enable all but one fragment to be maintained in *E. coli* propagatable vectors. These fragments are assembled in a high accuracy, one-pot reaction, with clonal plaques after transformation. The system has been applied to the production of point mutations, incorporation of fluorescent reporter genes and other inserts, deletion of a non-essential gene, and functional domain swaps within tail fiber genes with downstream effects on host range. All genome edits are accomplished through simple manipulation of plasmid parts through classic molecular biology or substitution of small synthetic parts, bypassing laborious recombineering strategies for phage engineering. Assemblies can be performed in parallel, going from parts to plaques in <1 week, with very low error rates (< 1 in 100,000 bp) necessitating minimal screening.

Streamlined Phage Engineering: Genome Synthesis via High-Complexity Golden Gate Assembly (HC-GGA)



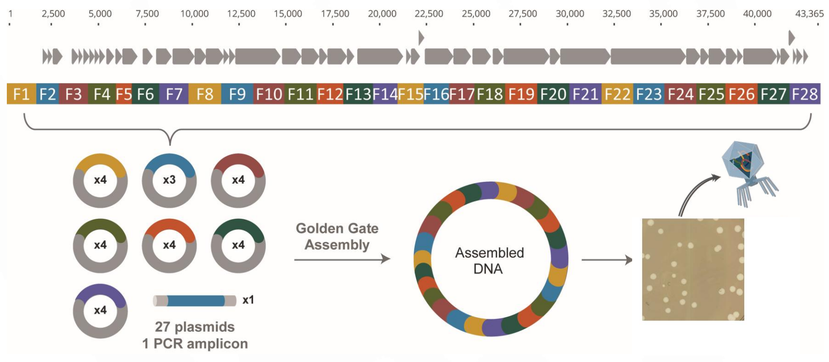
Pryor et. al., "Rapid 40 kb Genome Construction from 52 Parts through Data-optimized Assembly Design," doi.org/10.1021/acssynbio.1c00525

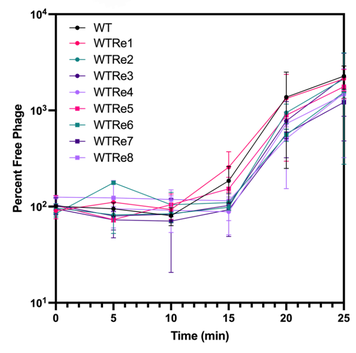
Sikkema et. al. "High-Complexity One-Pot Golden Gate Assembly" doi.org/10.1002/cpz1.882

Advantages of 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 into host, no secondary passage for construct propagation
- 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/

Synthetic ϕKMV Assembly and Reboot

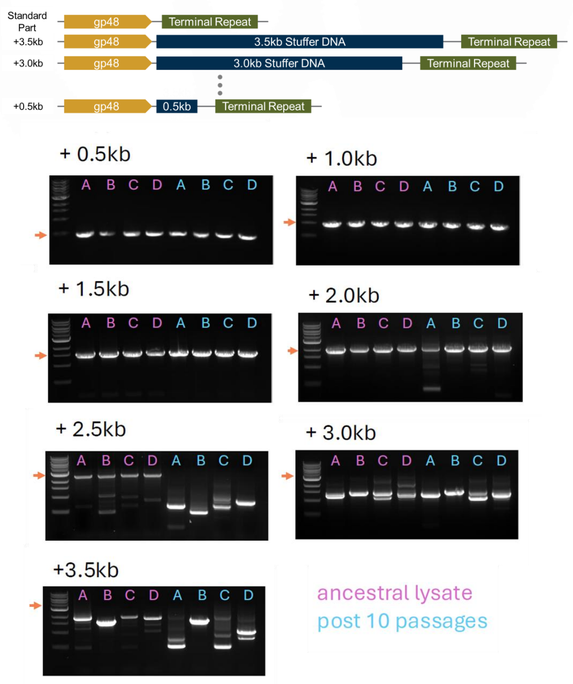




The ϕKMV genome was divided *in silico* into 28 parts; 27 were obtained as cloned fragments, one region (F2, which contains the early promoters) could not be cloned in *E. coli* and was used as a PCR-amplified gBlock. HC-GGA reactions using BsmBI-v2 NEBridge Golden Gate Enzyme Mix and 3nM of each fragment were carried out using a 15-hour cycling protocol ((42°C, 5 min → 16°C, 5 min) x 90 cycles → 60°C, 5 min). 1 µL of assembly was transformed into 50 µL electrocompetent NEB 10β *E. coli*, mixed with PA01, and plated. Individual plaques were picked for propagation and sequencing.

65% of plaques were fully correct, with no SNPs or indels. 100% of the observed errors occurred in the non-clonal F2. Eight plaques were propagated and showed identical growth phenotypes to true wt.

DNA Insertions Define ϕKMV Genome Size Limits



Modified F28 parts were generated containing between +0.5 and +3.5 kb of arbitrary DNA inserted between gp48 and the terminal repeat. These fragments were substituted into the standard assembly to generate phages with expanded genome size.

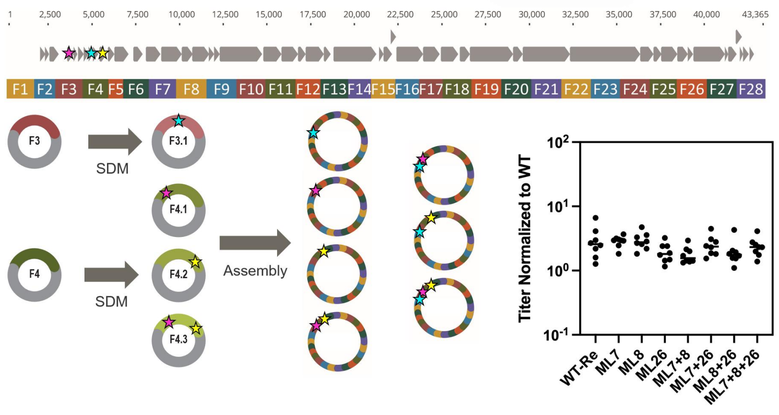
Plaques were generated for each variant. PCR analysis of the insert region showed stable insertion up to +2 kb on initial rescue, with evidence of deletions for the +2.5 kb variant and no +3 or +3.5 kb phages recovered with the full insert.

For each variant, four plaques were passaged 10 times; +0.5, +1.0, and +1.5 kb inserts remained stable, +2.0 kb showed some population with deletions, and all +2.5 kb and greater strains had lost significant portions of the insert.

Sequencing confirmed deletions were predominately within the inserted stuffer DNA, a single plaque contained a deletion of stuffer DNA and gp47-48.

ϕKMV can stably accept 1.5 – 2 kb insertions

Point Mutations Inserted via Plasmid Mutagenesis



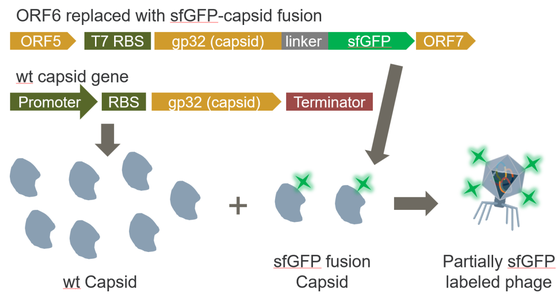
Point mutations were introduced into ϕKMV by modification of individual plasmid parts via site-directed mutagenesis (SDM).

Modified parts were then combined singly, doubly, or as a combinatorial library with the rest of the assembly parts to construct modified genomes.

All desired ϕKMV mutants were obtained with a 100% success rate of inserting the desired change, with a similar error rate (~1 SNP/indel per three plaques) to WT assembly.

All mutants had similar titers to wt.

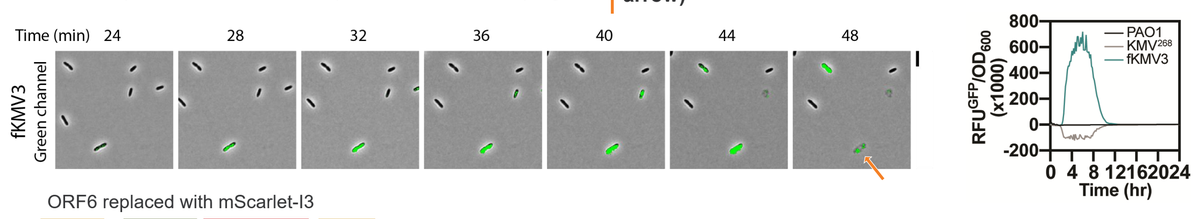
Fluorescent Reporters allow Visualization of Infection and Individual Phage Particles



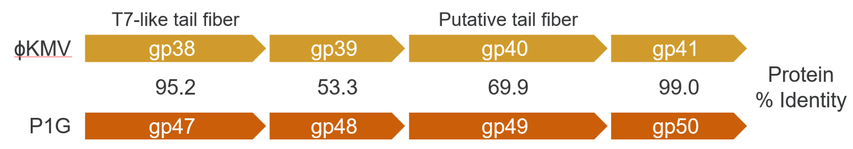
fKMV3 – capsid fused GFP permits visualization of replication and phage particles post lysis

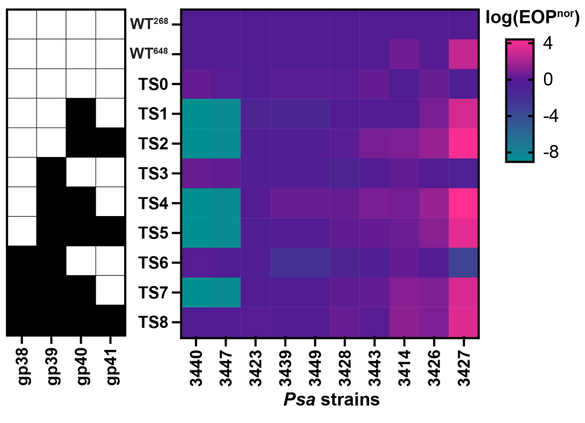
ϕKMV has a single capsid protein; attempts to fuse domains to this protein failed to produce viable phage. As an alternative, we created a minor capsid by duplicating gp32 and inserting it in place of the early gene ORF6, which has a premature stop codon. The second capsid gene is fused to sfGFP and produced GFP labeled phage.

GFP expression could be measured during infection and labeled phage particles can be observed after lysis (orange arrow)



Tail Fiber Swaps Result in Altered Host Range

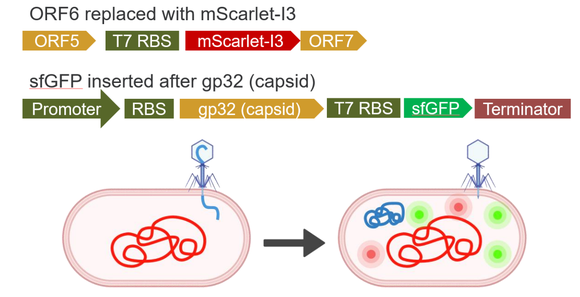




Pseudomonas phage vB_PaeP_P1G, closely related to ϕKMV, shows differences in the ϕKMV tail fiber region (gp38-41). Different combinations of these four genes were generated using the ϕKMV HC-GGA system via part swaps in F25 and F26. Plaques for every tail swap were generated in PA01 (TS 1-8, white indicates presence of the ϕKMV sequence, black the P1G variant).

Efficiency of plaquing (EOP) assays across ten other *P. aeruginosa* strains. For some, tail swaps had little effect on EOP. However, TS1, 2, 4, 5, and 7 did not plaque on strains 3440 and 3447 and showed increased ability, along with TS8, to plaque on strain 3427 as compared that of WT^{268}. These results seem to be driven by the presence of the alternative gp40. Interestingly, TS8, which has the full contingent of alternative gp38-40 shows no decrease in plaquing on 3440 and 3447.

fKMV10 – mScarlet and sfGFP genome insertions allow two color visualization of phage replication



An mScarlet-I3 gene and T7 RBS were inserted in the early genes in place of ORF6, and an RBS/sfGFP gene was inserted in the structural proteins after gp32. These changes increase the genome by a net size of 1132 bp.

Both proteins could be observed during phage infection. We expected to see mScarlet expressed first, followed by GFP late in infection. Instead, we observed simultaneous expression of both genes.