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2022 SEA Symposium Abstract

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An Application of Mycobacteriophage Genome Engineering using Bacteriophage Recombineering with Electroporated DNA (BRED) and CRISPR Cas-9 Systems

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The increasing occurrence of antibiotic-resistant pathogens remains a global threat to public health, spurring research to develop alternative treatments for infectious bacteria. Phage therapy, the targeted use of bacteria-specific viral particles called bacteriophages, has emerged as a promising antibiotic alternative. Our lab studies mycobacteriophages – phages specific to bacteria of the genus *Mycobacterium*, which includes infamous, antibiotic-resistant pathogens such as *M. tuberculosis* and *M. abscessus*. For the use of many mycobacteriophages in phage therapy treatments, gene deletions are often necessary to ensure host lethality. Previous methods for mutant generation used a technique called bacteriophage recombination with electroporated DNA (BRED), which used electrocompetent host cells equipped with recombination machinery, genomic phage DNA, and a synthetic DNA g-block to generate mutants. This process proved inefficient with recalcitrant recovery of some deletions, requiring extensive screening of plaques. To improve the process of genetic modifications with mycobacteriophages, greater precision and improved screening has recently been achieved through the combined use of BRED with a mycobacteria-efficient *Streptococcus* *thermophilus* CRISPR Cas-9 gene knockdown system for selection against the parent phage (CRISPY-BRED). Here we present an application of the CRISPY-BRED technique for the deletion of the immunity repressor gene in the mycobacteriophage known as Mufasa. The Cas-9 system involves a plasmid containing the modified, nuclease-dead Cas9 gene, a sgRNA fused to an anhydrotetracycline inducible promoter, a kanamycin resistance gene, an *E. coli* replication origin, and an attP site for chromosomal integration in mycobacteria. The identification of a Protospacer Adjacent Motif (PAM) within the mycobacteriophage immunity repressor gene allowed for the design of a 20 bp sgRNA targeting sequence from the phage DNA. With host cell transformation of this CRISPR Cas9 plasmid programmed to target the parent phage immunity repressor and inhibit overall parent phage gene expression, straightforward mutant recovery should be achievable.