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Functional analysis of conserved hypothetical genes in the cluster K bacteriophage Hammy

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The genus *Mycobacterium* encompasses diverse saprophytic and commensal species, as well as serious pathogens such as *M. tuberculosis* and *M. leprae*. Bacteriophages play a crucial role in the evolution of mycobacteria and provide insights into the genetics and physiology of this economically important group of organisms. Mycobacteriophages also attract a lot of recent interest as potential therapeutic agents for the treatment of multidrug-resistant tuberculosis. To date, over 1,700 bacteriophages that infect *Mycobacterium* were characterized through genome sequencing and grouped into 29 clusters based on genetic similarity. In addition to well-characterized genes that encode structural, regulatory, DNA metabolism, and lytic proteins, all mycobacteriophages genomes carry numerous conserved hypothetical genes. The specific functions of these genes remain unknown, and only a few proteins have been expressed and studied experimentally.

In this study, we performed a functional analysis of Hammy, a K cluster mycobacteriophage with a 62-kb genome that encodes 95 predicted protein-coding genes. Fifty of these genes are homologous to viral proteins of known function, while the role of the remaining 45 genes is currently unknown. We employed a combination of high-fidelity PCR and Gibson assembly to clone 81 Hammy gene into the broad-host-range plasmid vector pSMEG-ExT (85% overall success rate). The resultant recombinant plasmids were electroporated into *M. smegmatis* mc2 155 and screened for cytotoxicity in the presence of the inducer anhydrotetracycline. The screen identified several cytotoxic genes, which are variably present in members of the K cluster (immediate relatives of Hammy) and other phages that infect *Mycobacterium* and *Gordonia*. Four cytotoxic genes (32, 34, 50, and 56) were subjected to bacterial two-hybrid analysis to identify the host proteins targeted by Hammy. The two-hybrid analysis identified several *M. smegmatis* proteins targeted by the cytotoxic gene 56. One of these targets, malate synthase, was previously identified during the two-hybrid analysis of bacteriophage ϕKMV and *Pseudomonas aeruginosa*. Results of this study will help to elucidate the role of poorly characterized viral genes in the biology of phages that infect *M. smegmatis*, *M. tuberculosis*, and closely related bacteria.