# Investigating Gene Functions in Mycobacteriophage Island3

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## Abstract

Island3 is a temperate Cluster I1 mycobacteriophage that infects *Mycobacterium smegmatis* mc$^2$155. Its genome consists of 76 protein-coding genes, only 17 of which have known functions. Towards the goal of identifying additional gene functions, we amplified, cloned, and assayed 14 genes for host cytotoxicity and the ability to render the host resistant to infection by other phages (defense). We analyzed genes 10, 11, 12, 13, 14, 15, 21, 22, 25, 50, 51, 57, 60, and 61 and concluded that none of these genes exhibited either host cytotoxicity or defense against phage infection. We are in the process of assaying the remaining genes of Island3.

## Introduction

Island3 is a temperate, cluster I1 mycobacteriophage isolated on *Mycobacterium smegmatis* mc$^2$155 from enriched soil in Pittsburgh, PA in 2008. It has a genome length of 47,287 bp consisting of 76 annotated genes, of which only 17 have known functions (Pope et al., 2020 and PhagesDB, 2021).

We performed a series of experiments to discover functions of 14 of the 76 genes in Island3. We amplified our genes, cloned each into a pExTra plasmid under the control of an anhydro-tetracycline (aTc) inducible promoter followed by the reporter gene mCherry, and electroporated into *M. smegmatis*. These constructs were used to assay for cytotoxicity and the ability to defend against bacteriophage infection (defense).

We tested 14 genes of which none showed cytotoxicity or defense. Our long-term goal is to assay all 76 genes from Island3.

## Methods and Materials

**Amplification and Cloning**

We started our workflow (Figure 1a) by amplifying genes of interest directly from Island3 high titer lysate using Q5 master mix (New England BioLabs (NEB), Ipswich, MA) for 30s/kb. We analyzed samples on gels. If products produced a single band, they were column purified (Zymo Column Purification Kit, Irvine, CA). If products produced multiple, well-separated bands, they were gel purified (Zymoclean gel recovery kit, Irvine, CA). We eluted the DNA with water and measured DNA concentration using the Nanodrop. Purified phage inserts were cloned into pExTra plasmid using isothermal assembly with NEBuilder HiFi DNA Assembly Master Mix (NEB, Ipswich, MA) and transformed into chemically competent *E. coli* (Figure 1b). We plated each transformation reaction on plates containing 50 µg/ml kanamycin. We verified the constructs by amplifying directly from 4 colonies per gene using Taq polymerase (NEB, Ipswich, MA) for 1 min/kb. We purified chosen constructs using GeneJET Plasmid Miniprep Kit (Thermoscientific, Waltham, MA).

**Electroporation of *M. smegmatis***

We transformed our genes into electrocompetent *M. smegmatis* by incubating plasmid with cells on ice for 1 minute. We added 3x volume cold water, put the mixture into a 1mm cuvette (Bulldog Bio, Portsmouth, NH). Cells were electroporated (GenePulser Xcell, BioRad, Hercules, CA) at 2.5 kV, 500 Ω, 10 µF. After a two-hour recovery in 7H9 complete with Tween, we plated on 7H10 plates with 10 µg/ml of kanamycin (kan10) and incubated plates for 5 days at 37°C (Figure 1c).

**Phenotypic Assays**

We tested 3 colonies/gene for cytotoxicity by diluting, spotting on 7H10 kan10, 7H10 kan10 aTc10 ng/ml, and 7H10 kan10 aTc100 ng/ml (iba, Goettingen, DE), and incubating for 5 days at 37°C. Simultaneously, 2 colonies/gene were tested for defense by growing in 7H9 kan10 complete with Tween for 2 days at 37°C. These liquid cultures were mixed with top agar to form a bacterial lawn, on which phages Island3, Larva, Roots, and Beezlebub were spotted. The plates were incubated at 37°C for 2 days.

Note: We followed the SEA-GENES protocols (SEA-GENES Protocol Handbook) for most of the protocols and mentioned our modifications above.

## Results

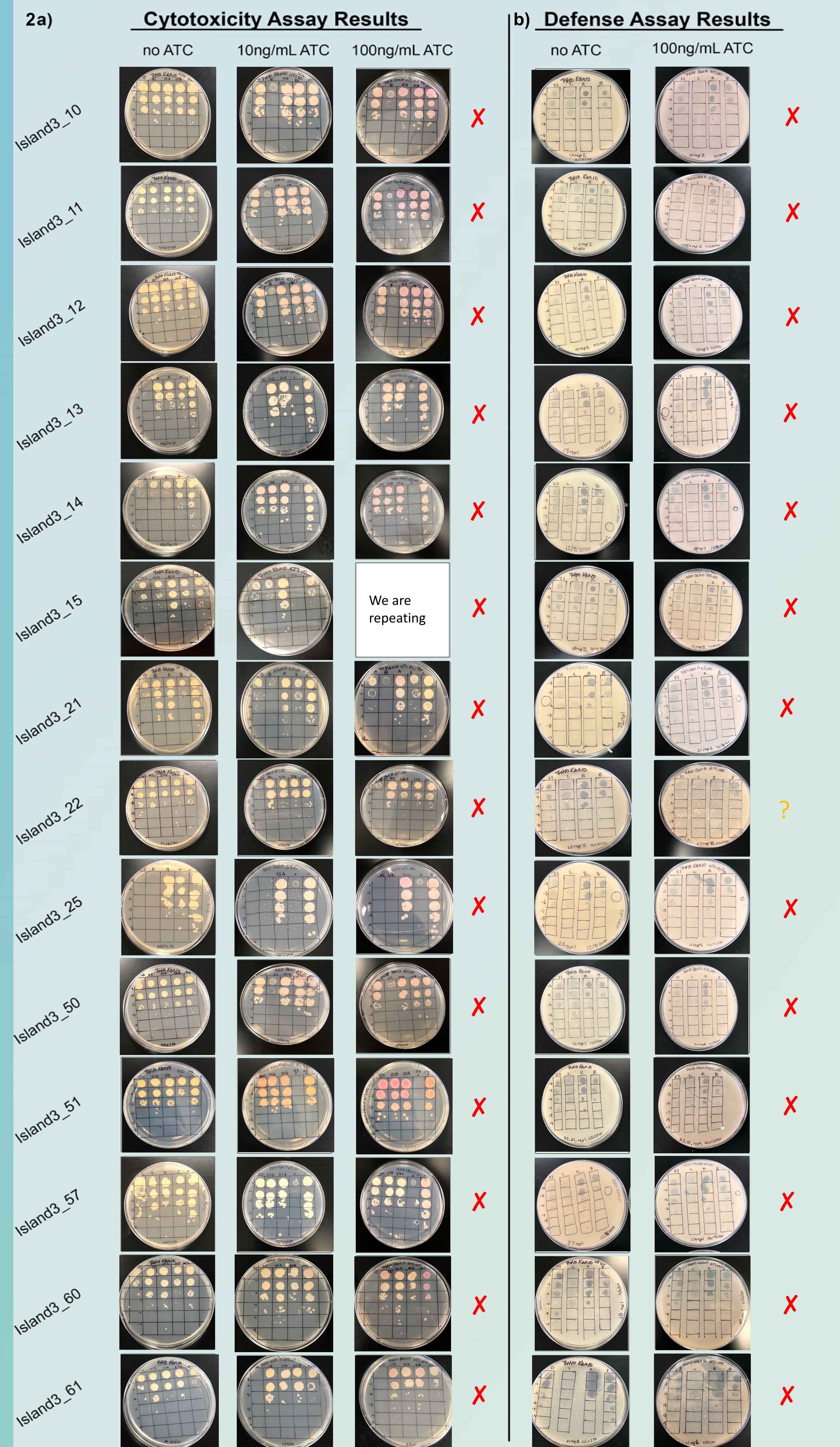


**Figure 2:** Plate images of the cytotoxicity and defense assay results. For the cytotoxicity assay, each of the plates contained 0, 10, and 100 ng/mL of the inducer aTc (2a). For each plate, we included triplicates of each clone, a positive cytotoxic control, and a negative control all diluted tenfold to $10^{-5}$. For the defense assay results, plates with 0 and 100 ng/mL aTc are shown (2b). Each plate contained a *M. smegmatis* clone lawn on which we spotted Island3, Larva, Roots, and Beelzebub from $10^{-4}$ to $10^{-9}$.

## Experimental Process

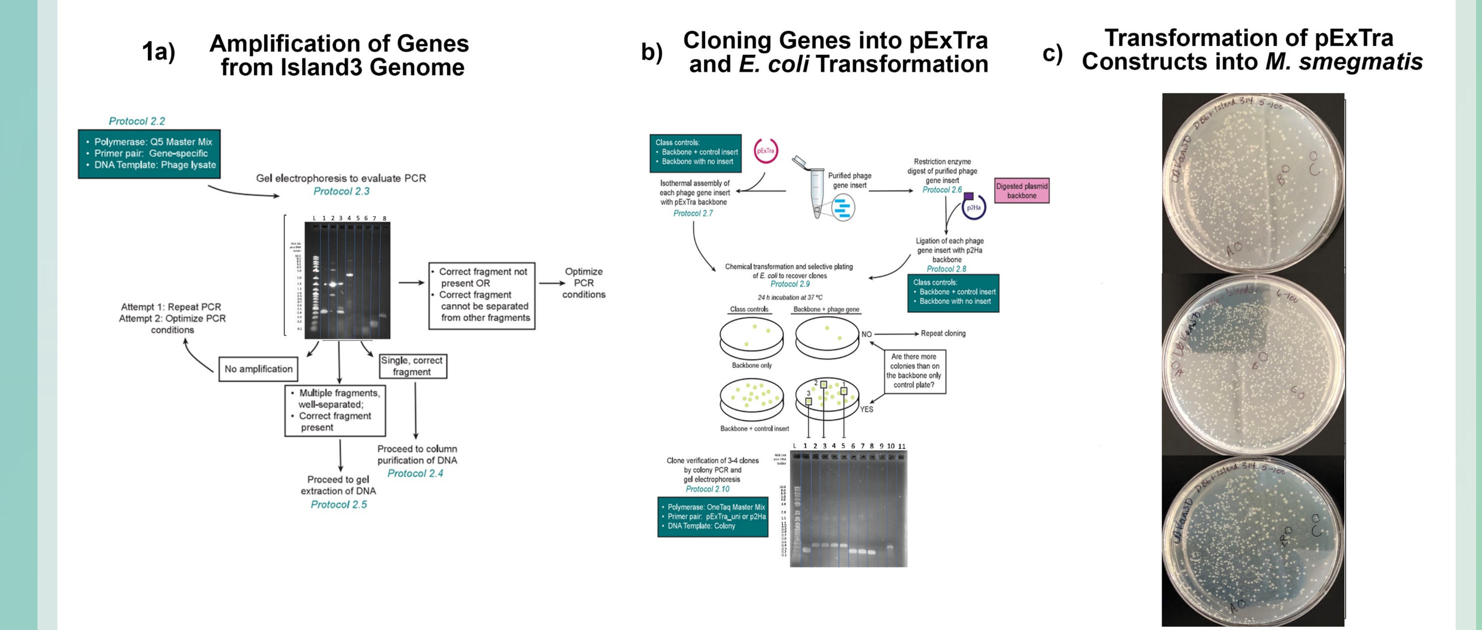


**Figure 1:** Partial experimental outline split into three main steps. The first step of the experimental process was to amplify the genes using PCR, verify products using gel electrophoresis, and purify the PCR products by column or gel purification (1a). The second step included cloning the gene into the pExTra backbone using isothermal assembly and confirming the cloning using PCR with backbone specific primers (1b). We then transformed the products into chemically competent *E. coli* NEB5α (1b). For the third step, we electroporated the pExTra constructs into *M. smegmatis,* and we provided three images of transformation plates (1c).

## Discussion

To examine the functions of 14 genes from Island3, we cloned each gene into the pExTra plasmid to assay for a cytotoxicity or defense phenotype in *M. smegmatis*. Since the phage genes were controlled by an aTc inducible promoter, we hypothesized that if the gene product was cytotoxic, induction using aTc would result in reduced growth of the *M. smegmatis* clones with the gene. We did not find any reduced growth with aTc induction for any of the *M. smegmatis* clones; therefore, none of the assayed gene products were cytotoxic (Figure 2a). We are in the process of repeating the results for gene 15. For the defense assay, we hypothesized that if an individual Island3 gene product conferred defense to an infection with either temperate phages Island3 and Larva, or the lytic phages Roots and Beelzebub, then the plaque sizes would be smaller, less numerous, and would exhibit a decreased apparent titer compared to controls. We did not identify any gene products that conferred defense; however, our results were inconclusive for gene 22 (Figure 2b). Further experimentation is underway to assay the functions of additional Island3 phage genes with the goal of analyzing all 76 Island3 genes for cytotoxicity and defense.

## References and Acknowledgements

SEA-GENES Protocol Handbook

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