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Investigating Cytotoxicity and Defense functions of bacteriophage Larva genes in host *Mycobacterium smegmatis*

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Abstract It is estimated that approximately 75% of gene functions within any given phage cluster remain unknown. Two phage traits that can be measured phenotypically were investigated: cytotoxicity, which causes host cell lysis, and superimmunity, which results in protection of the host from infection by similar phages. Five genes (35, 42, 46, 49, and 59), from a subcluster K5 bacteriophage named Larva, were amplified by the polymerase chain reaction (PCR) and assembled into a cloning plasmid (pExTra) using isothermal assembly. Initially, chemical transformation of *Escherichia coli* with the pExTra + gene insert, enabled amplification of the plasmids. Clonal PCR verified the presence of the inserts that were selected for on Kanamycin plates. Host bacteria *M. smegmatis* cells were electroporated with pExTra + insert and the inducer molecule anhydro-tetracycline (aTc) was used to induce expression of the relevant genes. The expression of Larva genes 35, 42, 46, and 59 were non-toxic to the host. However, Larva gene 49 was toxic, causing the bacteria to lyse. Evidence of this was further revealed in the defense assay where expression of gene 49 caused an absence of bacterial lawn growth. The expression of Larva genes 42 and 59 highlight a defense mechanism associated with these gene products that protects the host from attack by other phage in the same cluster. Further work to elucidate which host-parasite proteins are in contact to cause the phenotypic changes will be investigated using protein-protein interaction (PPIs) assays and hopefully reveal important clues towards understanding phage gene function.

Introduction

Members of the National SEA-PHAGE Research Project (including students at Winthrop University) have discovered, sequenced and annotated thousands of novel bacteriophages from the environment. Bioinformatics has been used to group and cluster many of the homologous gene sequences that have been discovered but still, the majority of this genetic information does not align with any known or previously characterized protein or function and remains an intriguing mystery. In response to this challenge, HHMI has sponsored the SEA-GENES project that endeavors to discover previously unidentified phage gene functions. How phages interact with their host bacteria and the genes that function to drive this process will reveal important clues to an ancient host-parasite evolutionary process. The project also hopes to uncover novel gene functions that may have antibacterial, medical, and bioengineering applications. This work begins by investigating those genes that may be involved in the attack and defense mechanisms utilized by phages during their infection cycle. In the annotated phage Larva, one area that has been under investigation by the GENES team is a small cluster of genes located near the right end of its genome. Our investigations aim to add to the growing evidence of gene functions within this area of Larva's genome as well as perfect the techniques used for elucidation of gene function. Molecular cloning of the genes of interest, followed by transformation of bacterial host *Mycobacterium smegmatis* (*M. smegmatis*) enabled each chosen gene to be individually tested in cytotoxicity and defense assays. These phenotypic assays give hints to how the chosen genes function in the lysing of the cell during lytic replication, or in defense against superinfection by similar phages during lysogeny. These are the initial steps by Winthrop's team of SEA-GENES undergraduates towards revealing the functions and potential discovery of novel gene functions that complement the immense amount of data generated by the SEA-PHAGES research.

Methods & Materials

Polymerase Chain Reaction: Template DNA of each chosen gene* (Figure 1) was amplified using specific primers and Q5 polymerase in a thermocycler. The PCR reaction amplifies the number of each gene sequence exponentially. Gel electrophoresis was used to confirm the success of the PCR reaction (Figure 4). The correct sized amplicons were purified in preparation for cloning into the pExTra plasmid (Figure 2).

Isothermal Assembly: Gene inserts were combined with linearized pExTra plasmid (containing forward and reverse sequences to be added to the gene insert ends and used as universal primers downstream) by isothermal assembly of complementary sticky ends. This process resulted in plasmids ready for transformation into competent *Escherichia coli* (*E. coli*).

Chemical Transformation: Heat shock methodology was used to transform the pExTra plasmids + gene insert into *E. coli*. After allowing cells to recover, the cells were plated and selected for on kanamycin (50µg/mL).

Clone Verification: Several colonies were picked and universal primers were used in Taq polymerase PCR to amplify the pExTra + gene insert DNA. Gel electrophoresis was used to confirm which colonies contained the correct sized gene (Figure 5). One verified colony for each gene was grown overnight in a liquid broth to increase the number of plasmids.

Plasmid Purification: High density cultures were lysed, and unwanted cell structures and bacterial chromosome material were removed. Plasmids were bound to a column (Zymo), washed and eluted.

Electroporation: pExTra plasmids were transformed into *M. smegmatis* (Figure 3) through electroporation. After allowing cells to recover, cells were plated and selected for on kanamycin (20µg/mL).

Cytotoxicity Assay: Three replicates of transformed *M. smegmatis* were plated in serial dilutions on kanamycin (20µg/mL) plates containing either 0ng/mL anhydro-tetracycline (aTc), 10 ng/mL aTc or 100ng/mL aTc. Positive and negative phage Fruitloop controls were included for comparison (Figures 6 and 7). aTc induced expression of each gene insert, allowing any toxicity traits to be observed.

Defense Assay: Lawns of transformed *M. smegmatis* were grown on kanamycin (20µg/mL) plates with 0ng/mL aTc and 100 ng/mL aTc. Phage lysate from phages Larva, ExplosioNervosa, and D29 were applied to the lawns in serial dilutions along with a negative control (Figure 8). The aTc induced gene expression, allowing any superimmunity defense to be observed.

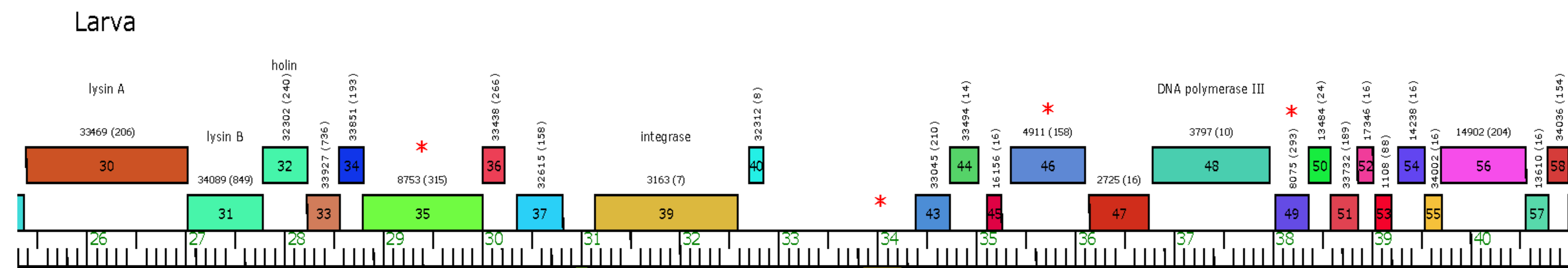


Figure 1. Larva annotated genome

Results

After transformation into *M. smegmatis*, expression of the genes were triggered by the inducible Tet promoter (using the addition of tetracycline to the plates) and observed through the reporter gene mCherry (that turns the cells pink when the gene under investigation is expressed). Cytotoxicity traits were observed in Larva 49 (Figure 6) as seen by the reduction in number of colonies able to survive when the gene was induced, but not the other genes tested so far (example Larva 59 shown in Figure 7). Superimmunity defensive traits were observed against phage Larva (and possibly phage D29), in *M. smegmatis* transformed with Larva 42 and possibly 59 (Figure 8) with a decrease in lysis observed on the plates when the gene expression was induced.

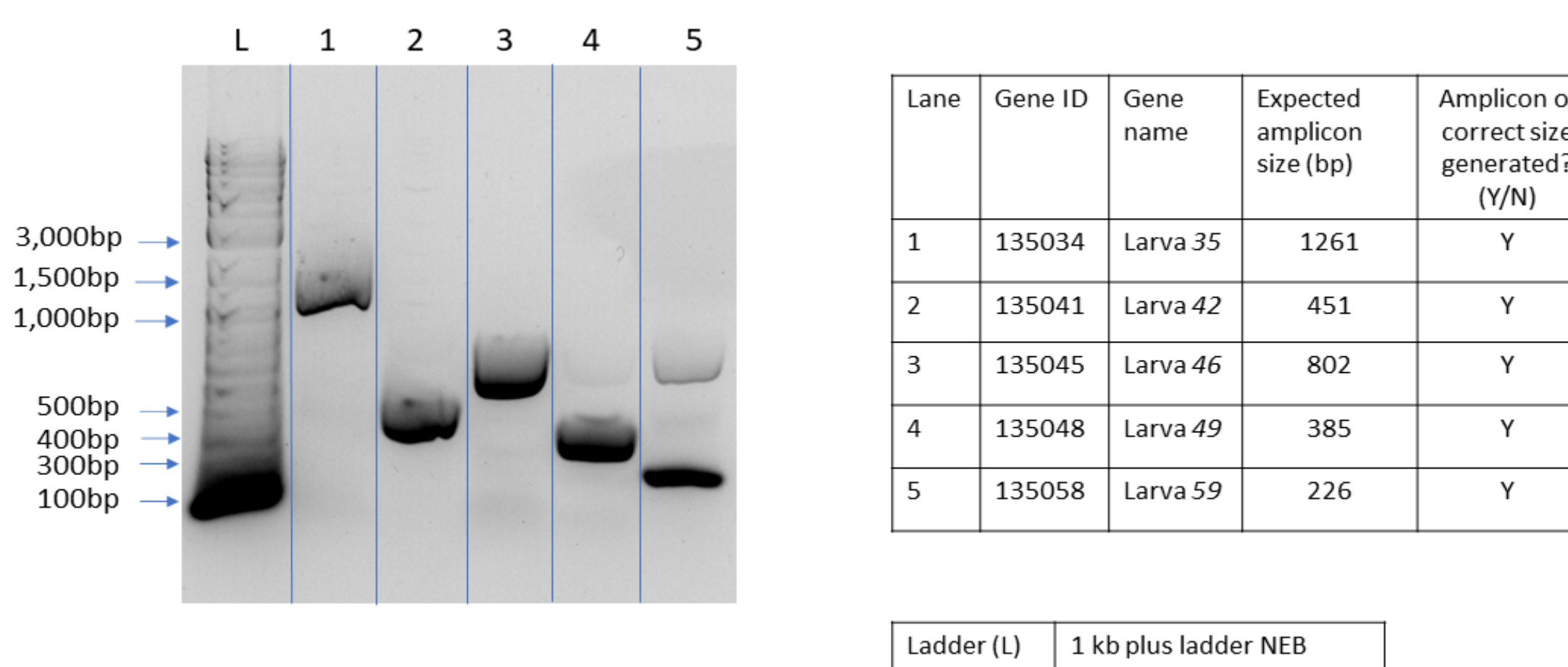


Figure 4. PCR Verification Data Card

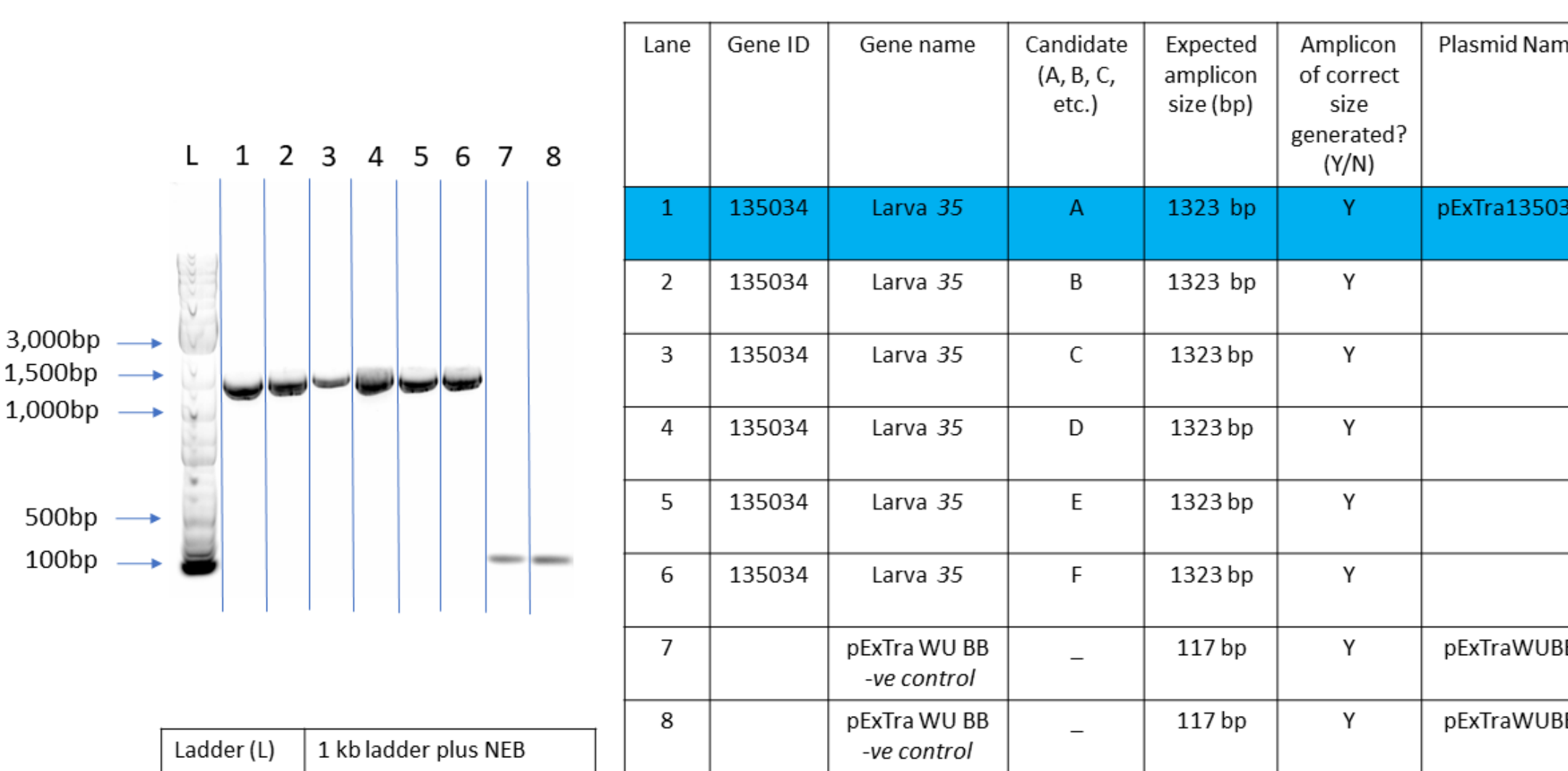


Figure 5. Clone Verification Data Card for Larva 35

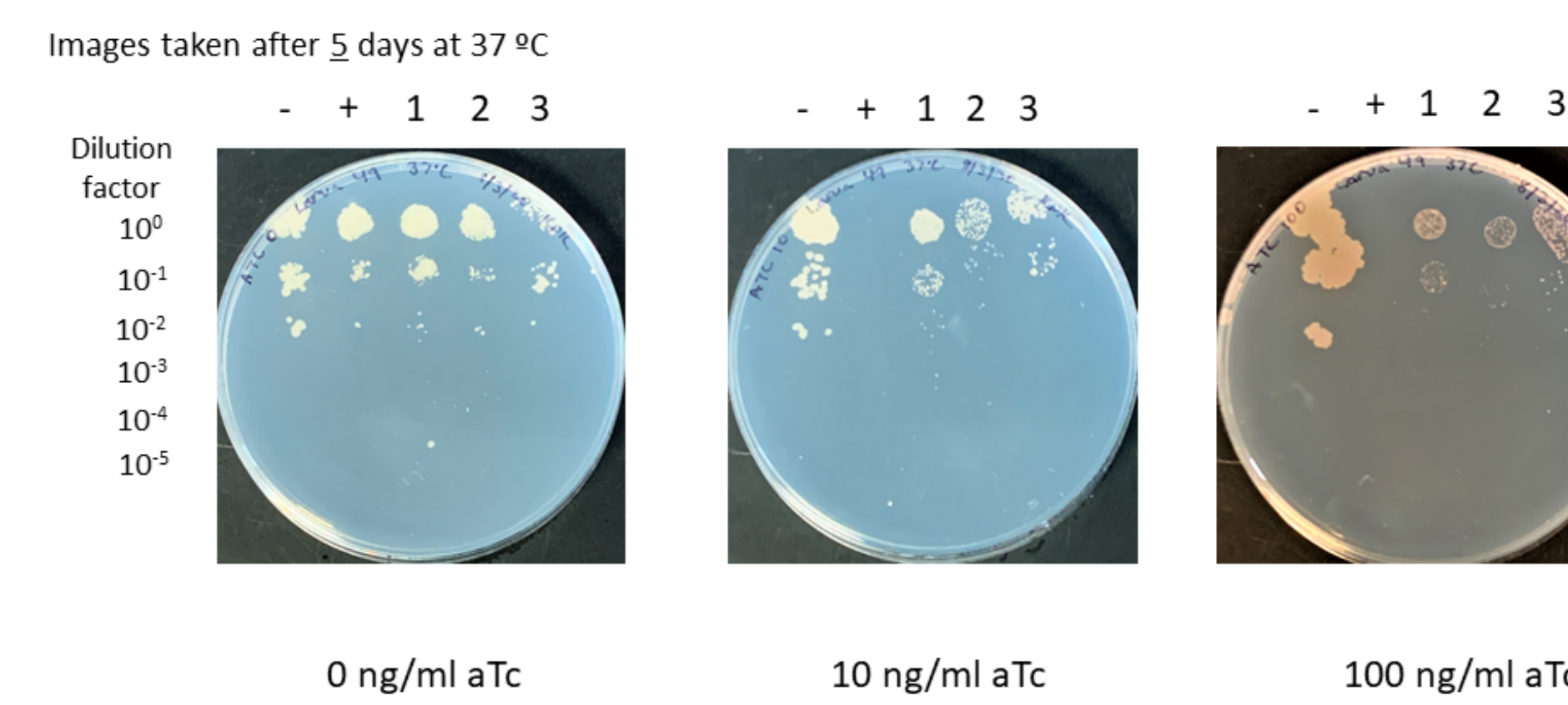


Figure 6. Cytotoxicity Data Card Larva 49

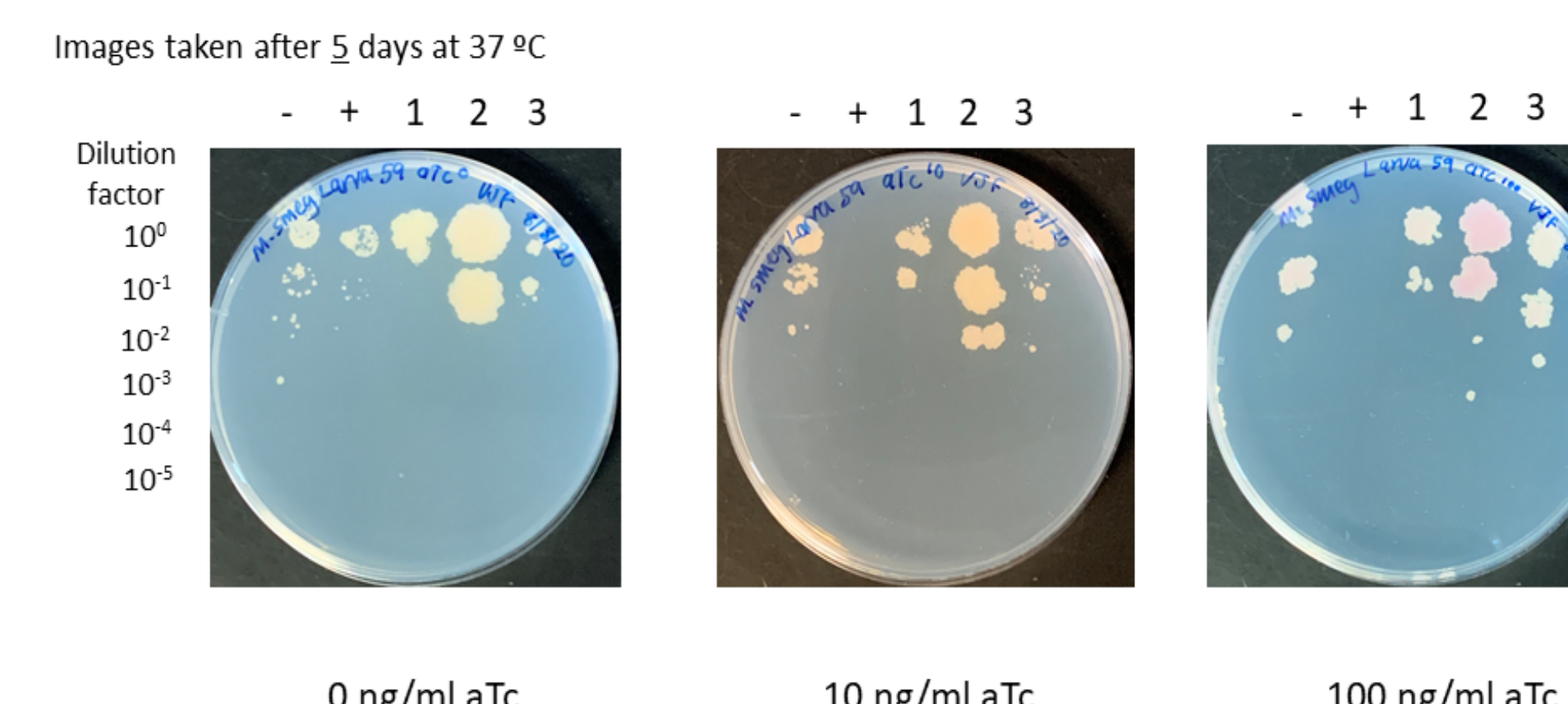


Figure 7. Cytotoxicity Data Card Larva 59

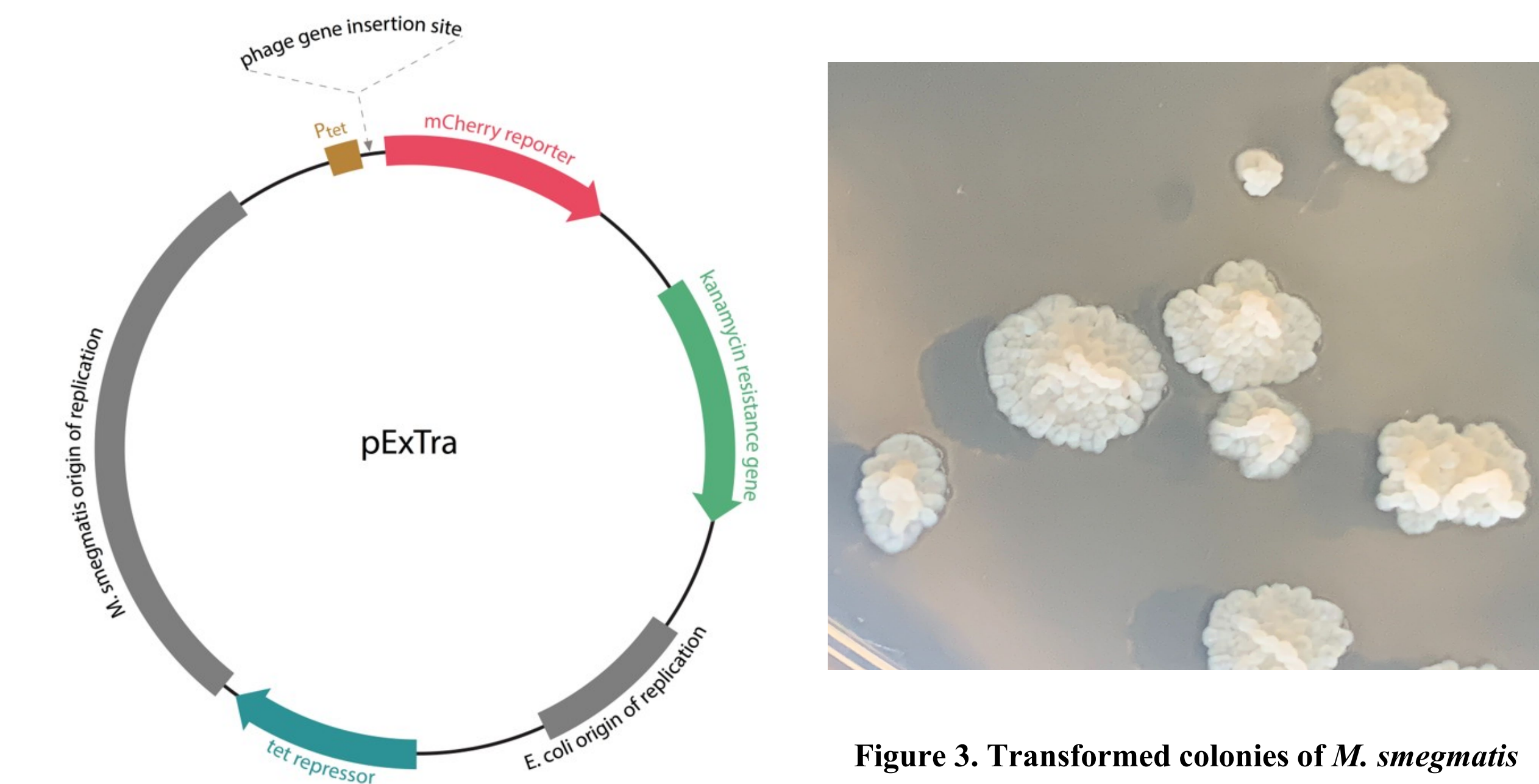


Figure 3. Transformed colonies of *M. smegmatis*

Figure 2. pExTra

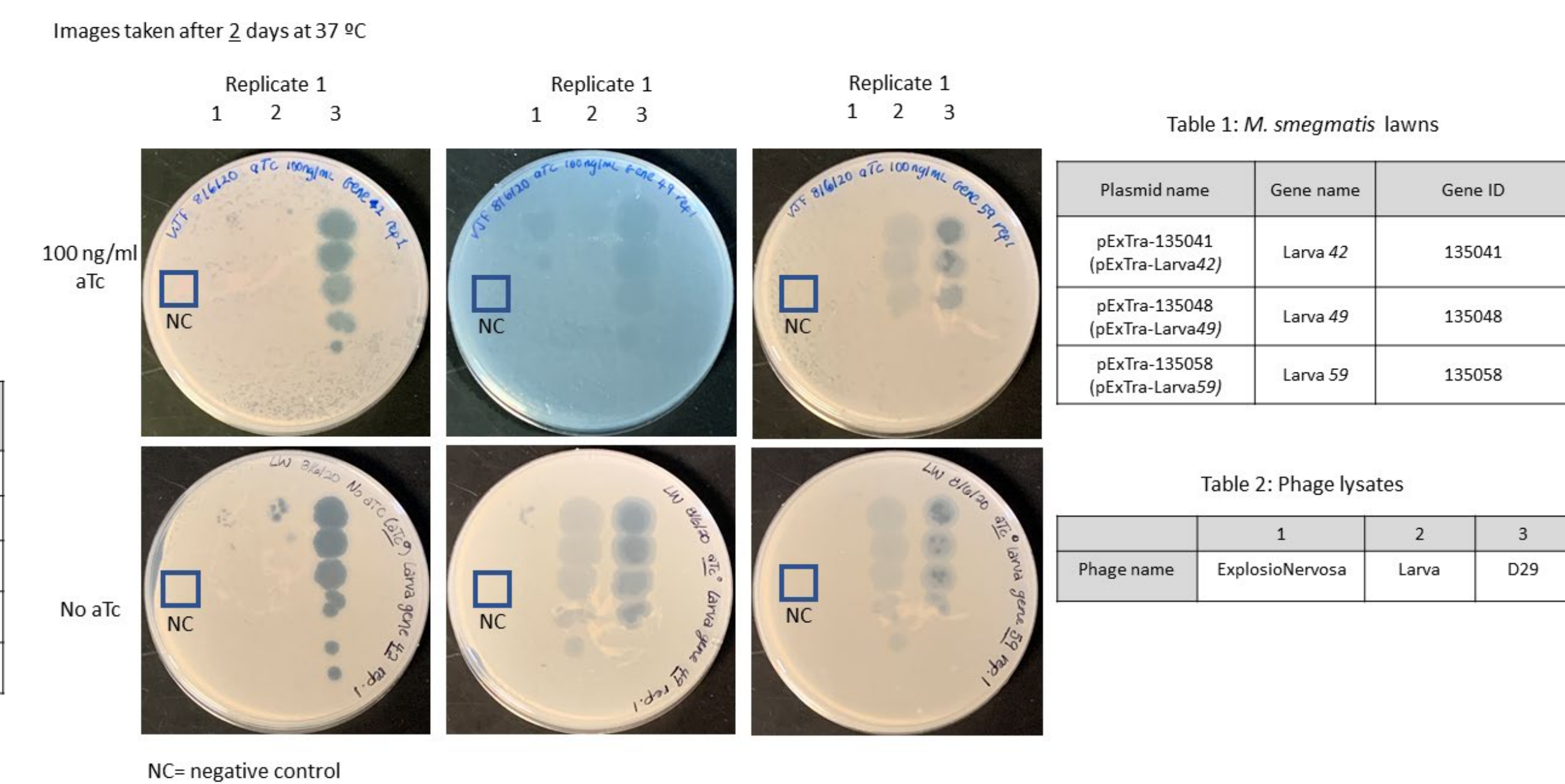


Figure 8. Defense Assay Data Card Larva 42, 49 and 59

Discussion

Expression of Larva 49 reveals a cytotoxic function associated with this gene since when induced in its host (*M. smegmatis*) the bacterial cells lysed. Superimmunity defense traits associated with expression of Larva 42 and 59 reveals that these genes may function in allowing phage Larva to protect its bacterial host from other similar bacteriophages in its cluster. While this study provides evidence pointing to the characterization of these genes, the mechanism enabling Larva 49 to lyse the host cell, and for Larva 42 and 59 to prevent superinfection is unclear. However, protein-protein interaction assays using the genes' protein products interacting with other proteins belonging to its host *M. smegmatis*, may give insight to these toxic and defense mechanisms in future studies. Characterizing the functions of genes in this way is one of the first steps in unravelling the mechanisms used during host-parasite evolution that has continued for more than three billion years.

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