

Systematic analysis of gene toxicity in Mycobacteriophage Bromden

Nicolas Toex

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Department of Biochemistry, Microbiology, and Immunology
Faculty of Medicine
University of Ottawa

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Abstract

With an estimated population of 10^{31} individuals, Bacteriophages are the most abundant form of life present on this planet. This abundance can be observed in the diversity of their genome: over 70% of genes present in newly sequenced bacteriophages remain without an unknown function.

To reduce this gap in knowledge, I screened each gene in the phage Bromden genome for cytotoxicity when expressed individually in its host, *Mycobacterium smegmatis*. To perform this screen, I created a plasmid library that allows the inducible expression of each Bromden gene.

I found 42 genes affecting the growth of the host bacteria which will be pursued in the laboratory to understand the underlying mechanism behind the toxic phenotype. Two genes were found to be extremely toxic, and I describe additional experiments I am pursuing to study them. In addition, I screened the uOttawa Mycobacteriophage library for host range capabilities onto *Mycobacterium tuberculosis*.

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Outside of the lab, I would like to give a tribute to the Friday overnight team from VCRT. From Caitlin and Ben (OG team) to Tracy and Jordan, passing by Emilyne, Xime and Mathilde, you guys always made sure that my Friday overnight would be fun and find ways to solve small and big problems.

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List of Abbreviations

AD supplement - Albumin Dextrose supplement

AMR - antimicrobial resistance

aTc - Anhydrotetracycline

CsCl - Cesium Chloride

CHX - Cyclohexamide

E. Coli - *Escherichia coli*

Kan - Kanamycin

Kb – Kilo base pairs (1kb = 1000 nucleotides).

gp – gene product

NKF – No Known Function

MOI- multiplicity of infection

M. tb – *Mycobacterium tuberculosis*

M. smeg – *Mycobacterium smegmatis*

OD₆₀₀ – optical density at 600nm

Pfu – plaque forming unit

SEA- Science Education Alliance

SEA-GENES - Science Education Alliance-Gene function Elucidation by a Network of Emerging Scientists

SEA-PHAGES - Science Education Alliance-Phage Hunters Advancing Genomics and Evolutionary Science

Introduction

The AMR problem

The World Health Organisation refers to antimicrobial resistance (AMR) as an “[occurrence where] bacteria, viruses, fungi and parasites change over time and no longer respond to medicines, making infections harder to treat and increasing the risk of disease spread, severe illness, and death”^{1,2}. This phenomenon is driven by acquired resistance^{3–5}, a natural process where bacteria can acquire new genetic material via random mutations and horizontal gene transfers^{4,6}, increasing host diversity and resilience against environmental constraints (pH, temperature, nutrients)^{5,7,8}. Alongside natural selection, acquired resistance allows the most resilient host to survive, while the most susceptible one will be killed. The discovery and wide use of antibiotics in our society added intense pressure for bacterial population to adapt themselves, leading to the apparition of AMR^{1,6,9–12}.

According to the latest reports, diseases caused by bacteria such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Mycobacterium tuberculosis* are a global health crisis, becoming more and more prominent in our societies^{2,6,11,13–15}. In 2018, 5 million excessive deaths, including 14,000 in Canada, were attributed to AMR, and this number is expected to grow for years to come^{11,14,16–19}. It’s been estimated that AMR will kill more people in 2050 than cancer and diabetes combined⁹. This poses a serious threat to world populations, both from a health and economic standpoint^{14,19}, as there is currently limited to no (multi-drug resistance) options to combat such infections due to their resistant nature to available antibiotics. As a result, finding alternatives is vital to ensure the proper treatment of bacterial diseases that fail to respond to traditional therapies^{2,11,14–16,20}. This is especially important as our world emerges from the COVID-19 pandemic and prepares for potential future outbreaks of bacterial origin^{21,22}.

A solution: bacteriophages

One of these alternative treatments could be bacteriophages, viruses that infect bacteria^{23–25}. Although initially discovered in 1915 by Frederick W. Twort and formally described in 1917 by Félix d'Hérelle, phages were quickly overshadowed by the discovery and widespread use of modern antibiotics during World War II^{16,23,26,27}. However, the rise of AMR brought the light back on bacteriophages as possible allies, with successful uses on patients infected by drug-resistant bacteria^{20,23–32}. Phage-based treatments are currently used as last resort in North America, due to the phage specificity and individual regulatory approval required³³. Those treatments can be used alone (phage cocktail) or in conjunction with antibacterial agent, amplifying its effect by weakening the host^{28–30,34}.

Phage diversity

With an estimated population of 10^{31} at any given moment²⁴, phages are believed to be the largest reservoir of genetic diversity present on Earth^{35,36}. Even though this number accounts for clonal/copies of an identical phage, this number remains impressive: metagenomic predictions estimate that there would be at least 100 million (10^8) distinct virion genomes^{37,38}. This abundance makes phages the most common entity present on this planet, by being orders of magnitudes more abundant than bacteria, and it's believed that every bacterium has a phage that can infect it^{35,39–41}. According to PhagesDB, the largest public database of phages, only ~26,000 phages have been isolated and about 4,900 have been sequenced⁴². This database also shows a large bias towards certain bacterial genera: 2,402 *Mycobacterium* phages have been isolated and sequenced, whereas only 375 *Streptomyces* phages have been studied⁴². This unbalance can be explained by the larger therapeutic application available for *Mycobacterium*^{24,43}, ease of work^{44,45}, but also by the work of Science Education Alliance (SEA), and their program Phage Hunters Advancing Genomics and

Evolutionary Science (PHAGES), allowing for the collection and characterization of phages by undergraduates around the world⁴⁶. As a result, many phages still have to be uncovered!

The diversity of phages is one of their advantages in evading AMR. While the bacteria are actively mutating and finding ways to survive longer in their environment, phages are actively countering their efforts by doing the same thing. This predator-prey dynamic puts intense pressure on populations⁴⁷. This can be seen in oceans, where phages kill 40% of all bacteria present on a daily basis²³, allowing for the release of nutrients for neighboring organisms^{23,47}.

Phage diversity is also one of the biggest challenges while studying them. Phages' genome can range from a few thousand base pairs to almost 500kbp^{23,48}, and unlike bacteria, no single gene is conserved across all phages²³. The latest genome characterizations show that more than 77% of phage genes have no known functions (NKF)⁴⁹⁻⁵¹. In addition to the large percentage of NKF genes, numerous phage genes with a known function are either poorly or not characterized in phages⁵⁰⁻⁵⁴. This shallow understanding makes phages the dark matter of the biological field, as they remain largely unexplored despite their vast presence and potential significance⁵⁵. This knowledge gap limits our ability to fully realize the potential of phage therapy²⁹.

Phage life cycles

As phage genes remain largely unexplored, we can classify them into two broad categories corresponding to the phages' two distinct life cycles: the lytic cycle and the lysogenic cycle. This classification offers a basic but effective way to understand the gene functions in each lifecycle. Essentially, it can be assumed that most genes are associated with either one lifecycle or the other, with only a few potentially involved in both cycles⁵⁰. Phages can switch between these

two cycles depending on specific environmental conditions, contributing to the complexity and diversity of their gene functions

a) Lytic life cycle (killing the host)

During the lytic life cycle, the phage rapidly kills its host. After injecting its genetic material into the host, the bacteriophage hijacks the host machinery to replicate itself in addition to produce all structural protein required for encapsulating the newly formed viral genome^{47,56}. This cycle is repeated until the spreading of newly created virions into the environment, following the creation holes in the bacterial cell membrane and wall, repeating this cycle with neighbouring cells^{12,47,51}. Phages that undergo only the lytic cycle are believed to be essential for effective and safe phage therapy, as they completely clear the host^{20,28–30}. As a result, both phage therapies (by Dr. German in Toronto for an UTI⁵⁷ and Dr. Azad in Ottawa for a prosthetic joint infection⁵⁸) that have been approved by Health Canada were using purely lytic phages.

b) Lysogeny life cycle (protecting the host)

Upon infection, phages can become dormant by inserting their genomes in the host genetic material^{12,47,50,59}, by a phenomenon called as lysogeny. Once integrated into the host genome, the phage is known as a prophage, and the host is a lysogen^{41,56}. Main reasons to enter lysogeny include low host cell density (killing more hosts that could lead to its eradication)⁴⁷ and genetic compatibility (presence of specific integration sites)⁶⁰.

Lysogens express genes needed to maintain lysogeny and often also express genes that may benefit the host, including providing defense against reinfection with another phage (Superinfection immunity)^{12,47,60,61}. Furthermore, lysogeny may offer the transfer of (antibiotic resistance) genes between bacterial cells in a process called transduction^{12,23,61}.

Phages can excise from the host genome and enter the lytic cycle spontaneously or when environmental condition changes, including the activation of the SOS response within the cell⁶⁰, the presence of stressors⁴⁷ and DNA damaging agents inside the host^{12,60}, by a process known as induction^{47,60}. In a therapeutic setting, lysogenic phages may be risky if they provide immunity to the pathogenic bacteria against themselves or lead to the transduction of antimicrobial resistance genes to the host⁶⁰. Although this would seem like a dangerous drawback, recent work has shown *E. coli* lysogenic phages synergize effectively with antibiotics^{62,63}, a quality that is desired in phage therapy.

Phages outside the AMR scope

Stepping away from the AMR applications, phages have a lot to offer to many aspects of science. From understanding the humongous marine biome^{47,64}, the human microbiome and its role in the child development⁶⁵, to their possible role to accelerate wound healing³⁶ and extend the produce lifespan via its incorporation in food packaging^{66–68}, the field of phage biology is in constant progression.

Phages have played a key role in our understanding of fundamental biology. Their use in laboratories allowed the creation of bacterial models suitable for plasmid transformation^{23,44,69}. Phages were also the first sequenced organisms, thanks to their small genome and short turnaround time (only a few hours to obtain billions of newly formed virions)²³. The most famous biotechnology application of a fundamental phage process is the discovery of the CRISPR-Cas9 system, a now well-known genome editing tool, capable of excising and modifying DNA sequences^{70,71}. This system is used by bacteria, where it acts as a system of defense against phage infection^{70–73}, while phages use it to reduce the host immune response during infection^{74–76}. CRISPR characterization led to the award of the 2020 Nobel Prize to

Emmanuelle Charpentier and Jennifer Doudna, the researchers who uncovered CRISPR secret⁷³. Although studied in bacteria, CRISPR genome editing is used regularly in the study of fundamental mechanisms in model systems of cell, developmental and organismal biology^{72,73,77}. Examples of concrete CRISPR technology applications include curing patients with genetic diseases such as sickle cell disease and β -thalassemia⁷⁸, and stopping malaria infections by neutering carrying mosquitos⁷⁹. Those examples are only a few among the huge power that CRISPR has, as many ethical questions are raised by this tool and its possible off-target effect, genetic enhancement, and ability to modify the genetic material over multiple generations of living organisms^{80,81}.

Altogether, the abundance of NKFs reflects the many unknowns in phage biology and poses significant challenges to phage therapy. The limited understanding of phage life cycles leaves many questions unanswered, especially in terms of gene expression, regulatory mechanisms, and phage-phage interactions. Furthermore, other phage processes may be as interesting and useful as CRISPR but haven't been characterized yet with our current lack of understanding on the phage.

What has been done so far to understand phage gene functions?

The study of gene function is pivotal to expanding our understanding of phage biology. This study necessitates the use of biological techniques specifically designed to assess gene expression, such as forward and reverse genetics. Forward genetics involves the examination of a genotype through the observation of the entire phenotype⁸². Conversely, reverse genetics uses the DNA sequence to comprehend the phenotype and the mechanisms that activate or suppress specific genes⁸². Both techniques have been employed in the past to characterize genes in phages^{49-51,83,84}.

Reverse genetics approaches have been used more often, as they target specific genes and regions in phages^{49,50}. Two studies have used a systematic approach, allowing for screening of the entire genomes of two phages^{51,84}. Despite current efforts, complete characterization of all phage genomes will take time. According to phagesDB.org^a, there are over 480,000 genes, divided into 30,707 phams (gene “phamilies”; each phage gene product is grouped with similar DNA sequences) coming from the 4,864 phages that have been sequenced and annotated on the platform⁴². This enormous number underscores the vast diversity of phages and the significant amount of work required to characterize all Actinobacteriophage gene families.

Understanding this massive gap in the field, the SEA consortium, created a new program called Gene function Elucidation by a Network of Emerging Scientists (GENES)⁸⁵. This program aims to reduce the gap in our knowledge by coordinating efforts and providing partner schools with 1 phage to analyze, screen and characterize its gene functions⁸⁵. This is not the first time the SEA team tackled a large problem in the phage field. Over the last 17 years, SEA helped schools around the world to discover, isolate, and characterize phages (using restrictions enzymes, electron microscopy, sequencing, and bioinformatics annotation) with the Science Education Alliance-Phage Hunters Advancing Genomics and Evolutionary Science (SEA-PHAGES) program^{46,86}. This broadly successful program has isolated over 23,700 phages and sequenced and annotated over 4,000 of them⁴², while also exposing thousands of undergraduate researchers to the world of microbiology, lab work and bioinformatics^{46,86}.

The Rudner lab joined the SEA-PHAGES curriculum in 2019, and in collaboration with the Translational and Molecular Medicine program (TMM), taught over 400 students the rudiments

^a As of March 14, 2024

of phage hunting and genome annotation. So far, 359 phages have been discovered, using 10 bacterial hosts, with more work conducted on 41 phages, that were sequenced and annotated. With the success of its SEA-PHAGES course, the University of Ottawa Phage Hunting team had the opportunity to join the SEA-GENES program in 2022 and I have led our efforts in the complete characterization of the Mycobacteriophage Bromden^b

Bromden: Our focus

Bromden is a virus infecting *M. smegmatis mc²155*, discovered in Westland, Michigan, USA, by Hannah E Cizauskas in 2017^{42,87}. Following its sequencing and characterization, Bromden is composed of 118 genes + 13 tRNAs (Figure 1)⁸⁷⁻⁸⁹, and was found to be part of the poorly characterized L4 cluster, a group of phages having a nucleotide sequence similarity that spans more than 50% of their genome lengths and have closely related gene contents^{90,91}. 67 of Bromden's genes (57%) are NKFs. This phage was sent to us by Howard Hughes Medical Institute (HHMI) for further characterization over the summer of 2022. Bromden was imaged and showed structural characteristics of a *Siphoviridae* morphology, with a long (~300nm) and thin noncontractile tail (Figure 2).

Bromden's host, *M. smegmatis mc²155* (*M. smeg*), is a non-pathogenic bacterium commonly found in soil and freshwater environments around the globe^{24,28,44,45,92}. This Bacillus-shaped bacteria forms colonies in 3-5 days^{44,92} and possesses key attributes of the Mycobacterium genus, sharing more than 95% of its genes with *M. tuberculosis*^{44,45}. The strain mc²155 was engineered in 1990, creating a strain amenable to genetic analysis and a model system for understanding

^b You can learn more about Bromden using the following link: <https://phagesdb.org/phages/Bromden/>

mechanisms of mycobacterial infection⁴⁴. With its fast-growing abilities and similarities with *M. tuberculosis* (*M. tb*) and other pathogenic *Mycobacterium*, *M. smeg* quickly became a popular tool for lab around the world to study the biology of *Mycobacteria*. Work on mc²155 helped elucidate the pharmacological actions of the *M. tb* drugs Isoniazid, Ethionamide, and Ethambutol. From a clinical standpoint, mycobacteriophages previously discovered in *M. smeg* were able to be used against other *Mycobacteria* such as *M. tuberculosis* or *M. abscessus*^{20,24,25,28–30,45,93}.

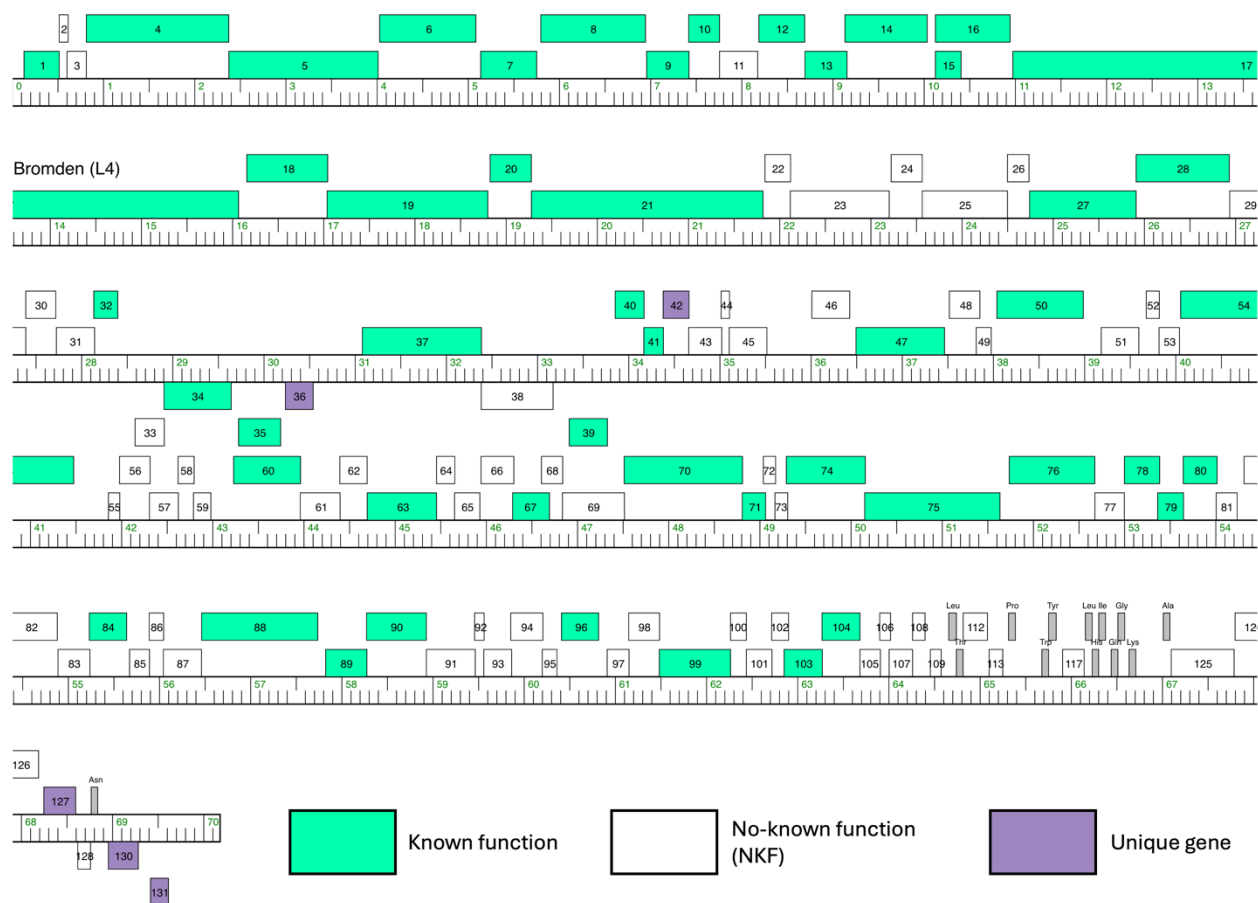


Figure 1. Simplified gene map of the genes present in Bromden. Obtained using phamerator.org⁹⁴, colored using phagesDB.org⁴² and NCBI GenBank⁹⁵ information from Bromden record (Accession MH576973.1). Shown in green are the genes with a known function, in white genes with no known function (NKF), in purple are genes solely present in this phage and in grey small boxes are tRNA. A list of the functions of the genes is available in Table 3.

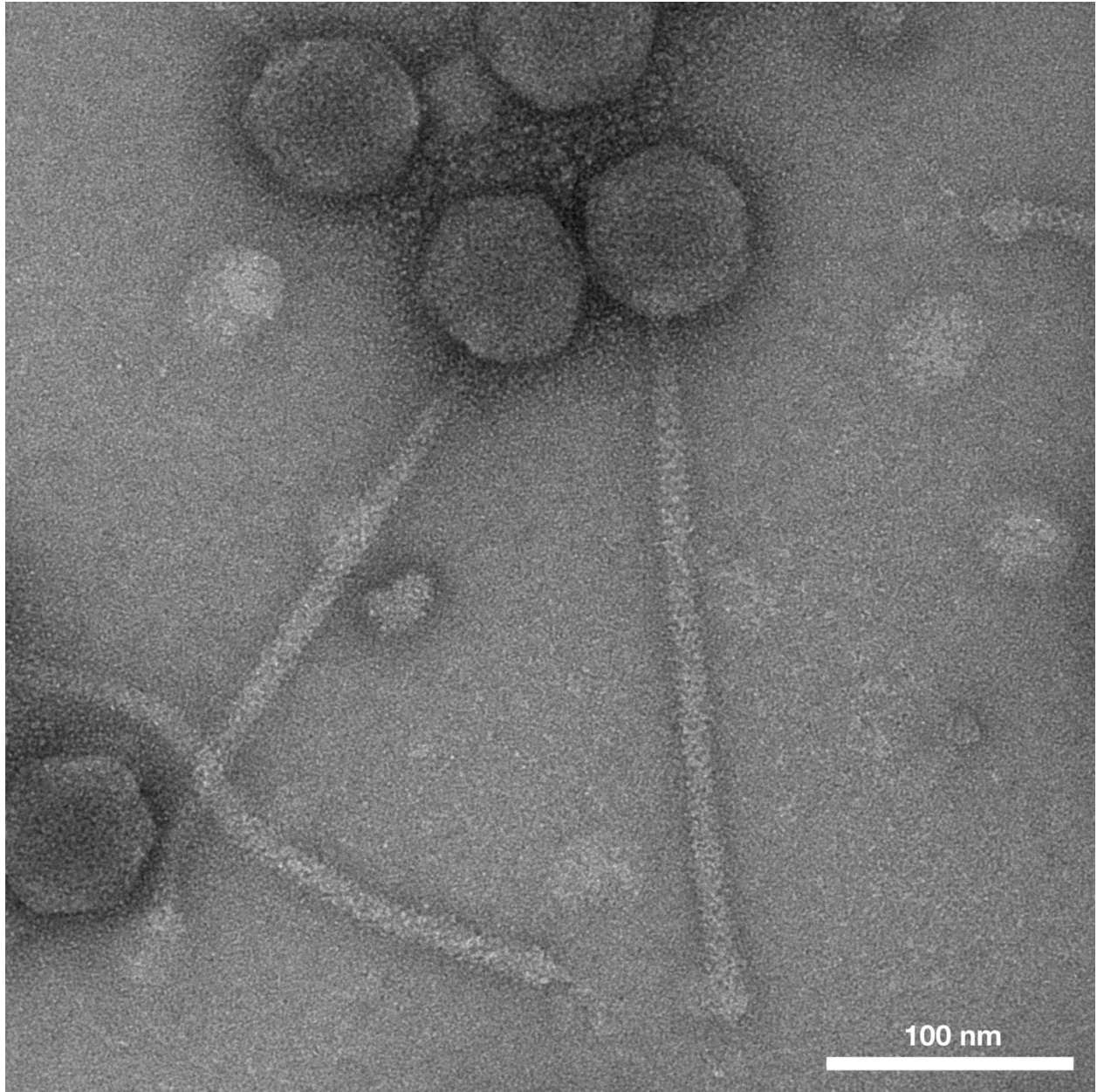


Figure 2. Transmission electron microscope picture of Mycobacteriophage Bromden. This picture was taken using the transmission electron microscope (TEM) of the TEM core facility of the University of Ottawa (Settings: 120kV). The Bromden grid was prepared using a high-titer Bromden lysate, left to set for 10 min, rinsed twice with filtered mqH₂O, and stained with 1% uranyl acetate. Dimensions of Bromden's tail are 290 ± 25 nm tall by 11 ± 2 nm wide. Bromden's capsid size is 65 ± 3 nm.

Aims of this project:

By systematically characterizing Bromden's genes, I aim to increase our knowledge about the functions of phage genes in killing their host and mediating superinfection immunity and to gain a broader understanding of phage genome organization and the conservation of phage functions.

How to do it?

Ectopic gene expression or overexpression is a powerful tool used in fields such as cancer research, cellular metabolism and mechanistic studies. Using plasmids that contain inducible promoters, I can modulate the level of expression of a specific protein of interest inside a living cell and analyze the phenotypic consequences. This tool was first developed for work in the model eukaryote, *Saccharomyces cerevisiae*, similar strategies have been used for studies in bacteria, mammalian cells and phages^{50,51,96}.

Using gene overexpression inside the host, I assayed two phenotypes: cytotoxicity and immunity to infection by Bromden or other closely related phages. Performing these assays allowed me to observe the impact of individual Bromden genes on *M. smeg*, resulting in toxicity towards the host or protection against phage infection. Furthermore, completing those assays offered me a quickly identify genes of interest (showing a toxic or defense phenotype) for further mechanistic studies. Two other complete genome screens of Mycobacterial phages, Waterfoul and Hammy^{51,84}, have already been completed. However, Bromden has only one pham overlap with these phages, so I anticipate that this work will generate a unique dataset to deepen our understanding of phage biology.

Methods

Most of the methods used for this thesis are modified versions from the SEA-GENES guide, written by Danielle Heller et al. You can access the guide using the following link:

<https://seagenes.helpdocsonline.com/home>. Note that some of the protocols might only be present on the “instructor version”, accessible on the following webpage:

<https://seagenesinstructorguide.helpdocsonline.com/home>

1) Growth conditions

a) Phage buffer

Phage buffer (10 mM Tris, 10 mM MgSO₄, 68 mM NaCl, 1 mM CaCl₂ and 10% glycerol) was used to make serial dilution of phage lysates. Phage lysates used for this experiment were highly concentrated ($>10^{10}$ pfu/mL).

b) *M. smegmatis* growth

In liquid cultures, *M. smegmatis* mc²155 was grown in Middlebrook 7H9 broth supplemented with 10% AD supplement (2% w/v Dextrose, 145mM NaCl, 5% w/v Albumin Fraction V), 0.05% Tween80, and 10 µg/ml cycloheximide (CHX). Cells were incubated at 37°C at a speed of 220rpm. Flasks of bacteria were then kept stationary at room temperature following their saturation. Tween80 was removed from the growth media by inoculating a smaller culture of 7H9 broth with 10% AD supplement and 10 µg/ml CHX, as tween80 is shown to impact phage infection^{97–99}.

On plates, *M. smegmatis* mc²155 was grown at 37°C on Middlebrook 7H10 agar plates supplemented with 10% AD supplement and 10 µg/mL CHX. Top Agar was made using Middlebrook 7H9 media, supplemented with 0.4% Agar, 0.2% Glycerol and 0.1mM of CaCl₂

c) Selection of *M. Smegmatis* + pExtra induction (Cytotoxicity and Immunity assay)

Electroporated cells were recovered in Middlebrook 7H9 neat liquid media. *M. smegmatis* mc²155 cells were selected using Middlebrook 7H10 agar plates supplemented with 10% AD supplement, 10µg/ml CHX and 10µg/ml Kan. The same plates were optionally containing Anhydrotetracycline (aTc) at the concentration of 10ng/ml (aTc₁₀) or 100ng/ml (aTc₁₀₀) to modulate the plasmid induction during the Cytotoxicity and Immunity assay. Top Agar was used for the Immunity assay and made using 7H9 media, supplemented with 0.4% Agar, 0.2% Glycerol, 0.1mM of CaCl₂ and optionally 100ng/ml aTc if the plates used contained the inducer.

d) *E. coli* growth

E. coli cells were recovered in LB media (1% Tryptone, 1% NaCl, 0.5% Yeast extract) and plated in LB+Kan plates (1% Tryptone, 1% NaCl, 0.5% Yeast extract, 1.5% Agar and 50µg/ml Kan).

e) *M. tuberculosis* growth

M. tuberculosis was grown at 37°C in Middlebrook 7H9 medium supplemented with 0.2% glycerol, 0.05% Tween-80, 10% OADC, 24 µg/ml D-pantothenic acid, and 50 µg/ml L-leucine, at a low shaking speed (~50 rpm). Flasks of bacteria were then kept stationary at room temperature following their saturation.

2) Phage work

a) Phage titer (spot assay)

A lawn of *M. smeg mc²155* was created on a 7H10 plate with 250µl of bacterial culture and 3.5ml of Top Agar. Phage lysates are diluted 1:10 in phage buffer and spotted as 5µL on the bacterial lawn. The plate is incubated for 2 days at 37°C. The resulting titer was calculated using the following formula:

$$titer \left(\frac{pfu}{ml} \right) = \frac{\# \text{ of plaque forming units}}{\text{amount of phage (5}\mu\text{l)}} * \frac{1}{\text{dilution factor}}$$

b) Phage amplification (webbed plates)

Stock lysates used for this project had a known titer, obtained via a spot assay. I used those stocks to obtain a lawn of bacteria with very high density of plaques, also known as webbed plate. The number of phages needed to create a webbed plate depends on plaque size and is determined empirically. Webbed plates are flooded with 8ml of phage buffer and left untouched for 2h before harvesting + filtration with a 0.22µm filter. The resulting lysate was then spot-tested to ensure the phage concentration.

c) Cesium Chloride (CsCl) extraction

Using a large quantity of phage lysate (>100mL), treated with DNase (1.5ng/ml) and RNase A (15ng/ml), the prepared phage is spun for 3h at 30,000xg to create a phage pellet. After carefully discarding the supernatant, the pellet is resuspended in 10mL of phage buffer and 7.5g of CsCl. This solution is mixed until the CsCl fully dissolves, transferred in Quick-Seal tubes and spun for 24h at 45,000xg to create a gradient. After 24h, a white band, containing all the phage, will appear in the middle of the tubes and has to be extracted using a needle and a

syringe. The extracted phage band will then be dialyzed in phage buffer to remove any leftover CsCl molecules present in the extracted liquid.

d) DNA extraction using Phenol/Chloroform

500 μ L of CsCl purified phage is added in a phase-lock tube. A 1:1 ratio of phenol/chloroform is added to the tube and vortexed for 1 minute. The tube is then spun at max speed for 5 minutes in a microcentrifuge, creating 2 phases in the tube (top: aqueous; bottom: organic). The top (aqueous) phase is transferred in a fresh tube and a 1:1 ratio of Chloroform is added to this new tube. After vortexing for 30 seconds and spinning for 2min, the top layer is again extracted. Note that a possible whitish phase can be present between both phases and should be avoided.

DNA is precipitated from the aqueous extract using 1/10th volume of 3 M NaOAc and 2.5 volumes of 100% ethanol. After an overnight incubation at 4°C and a 5-minute spin, the supernatant should be discarded, and the pellet should be washed in 70% ethanol. Following this wash, the pellet should be spun again and left to dry in the fume hood. The final pellet should be dissolved in 50 μ L of mqH₂O. Incubation at 37°C and/or 55°C can help the pellet dissolution.

3) Assembly in pExTra

a) Controls

To create our library, I used the pExTra plasmid library, created by Danielle Heller and received throughout the SEA-GENES program⁵¹. All plasmids possess the same key characteristics, explained in Figure 3 . pExTra01 contains no gene insert, offering antibiotic resistance to the host and turning pink when the inducer is present. I also used pExTra02 and pExTra03 plasmids, derived from the work on Fruitloop gp52 from Graham Hatfull⁵⁰. pExTra02 contains the insert of this known toxic gene, while pExTra03 contains a mutated version (I70S) of the same insert.

b) Backbone

To make my stock of backbone, pExTra01 was amplified by PCR, using the NEB 2xQ5 HotStart Master Mix with the primers pExTra_F and pExTra_R (See Table 4). PCR protocol guidelines are available in the Table 8. PCR products were run on a 1% agarose gel, ensuring the product size and the 6kb fragment was gel extracted using the Zymo Gel Extraction kit, following the manufacturer's instructions. After elution and DNA quantification by Nanodrop One, the backbone concentration was adjusted to 25ng/μL for downstream application.

c) Insert cloning

Each Bromden gene was PCR-amplified with 2xQ5 HotStart Master Mix (NEB) using ~20ng of Bromden genomic DNA as a template, using the guidelines present in Table 8. Forward primers are complementary to the first 15–25 bp of the gene sequence, introduce a uniform ATG start codon, and all oligos contain a 5' 21bp RBS-containing sequence that is complementary to one end of the backbone fragment and mediates assembly of the plasmid. Reverse primers are

complementary to the last 15–25 bp of the gene sequence, introduce a uniform TGA stop codon, and all oligos contain a 5' 25bp sequence that is complementary to one end of the backbone fragment and is needed for assembly of the plasmid.

Traditional troubleshooting steps, such as addition of 2% DMSO and increasing the annealing temperature (between 60°C and 68°C) were used to help overcome any PCR cloning issues.

Each PCR product was run on a 1% agarose gel and imaged using Bio-Rad ChemiDoc imager to confirm size. PCR products were then purified using Thermo Scientific GeneJET PCR purification kit for PCR with a single product/band or using Thermo Scientific GeneJET gel extraction kit for PCR producing more than one product, following the manufacturer's instructions.

d) Isothermal assembly and transformation in *E. coli*

Linearized pExTra plasmid was assembled with each gene insert by isothermal assembly, using HiFi 2X MasterMix (NEB) or 2X Isothermal DNA Assembly Mix (uOttawa GEM core). A 2:1 insert-to-backbone ratio was used and incubated with the assembly mix for 45min at 50°C. 2 µL of the isothermal assembly was transformed into 25 µL of chemically competent *E. coli* cells (NEB 5- α F'I^Q) by incubating on ice for 30 min, then shock at 42°C and recovery for 1 hour at 37° in 1ml of liquid LB media. Recovered cells were plated on LB agar + 50 µg/ml Kanamycin (Kan) incubated overnight at 37°C.

e) Clone Verification

Three colonies were tested for successful assembly of the plasmid by picking and resuspending the colony in 100uL of mqH₂O, and using 1uL as a template for PCR with pExTra_UniF and pExTra_UniR primers (Table 4) using OneTaq 2X MasterMix with Loading Dye (NEB). PCR was run on a 1% 1xTAE agarose gel and imaged using Bio-Rad ChemiDoc imager to confirm the correct insert size.

f) Plasmid isolation and archiving

Two confirmed clones were grown overnight in LB + 50 mg/ml Kanamycin and the plasmids were isolated using the Zyppy Plasmid Miniprep kit (Zymo Research), following the manufacturer's instructions. Plasmids DNAs were stored at -20°C until use for electroporation or sequencing. Permanent stocks of bacteria harbouring each clone were created by mixing 700μL of overnight bacterial culture with 300μL of 40% glycerol in a Cryo-tube, before being stored at -80°C.

g) Sequencing

Plasmids were sequenced using Sanger sequencing (Azenta Life Science). Small genes (<800bp) and medium genes (800bp-1500bp) were sequenced using sequencing primers pExTra_seqF and pExTra_uniR (Table 4); longer genes (>1500bp) were also sequenced with internal sequencing primers designed using Primer3(v.4.1.)^{100–102} and listed in Table 7. Plasmids containing gp17, gp23 and gp72 were sequenced using whole plasmid sequencing (Plasmidsaurus). Sequences were confirmed with the SnapGene software (www.snapgene.com) and uploaded on GenesDB (genesdb.org) for public access.

4) Preparation of competent *M. smegmatis* *mc*²*155*

A fresh streak of *M. smegmatis* on a 7H10 agar plate was grown for five days at 37°C and a single isolated colony was inoculated in 5mL of 7H9 supplemented liquid media. This culture was grown for ~16h with shaking at 37°C to reach an OD₆₀₀ value between 1 and 3. The starter culture was used to inoculate 7H9 supplemented media (~1:150) to an OD₆₀₀ value of 0.005 in the final volume.

Cells were grown overnight to late log phase (OD₆₀₀ ~0.8), incubated on ice for 2 hrs, and pelleted by centrifugation at 3000xg for 20min. Note that cells with an OD₆₀₀ > 1.0 produce cells with low competency. The supernatant was discarded, and cells were resuspended in 400mL of 10% ice-cold glycerol. Centrifugation and resuspension were repeated two times and after the final pelleting, the cell was resuspended in 1:250 of the original culture volume and aliquoted in 1.5ml tubes to be stored at -80°C.

5) Transformation in *M. smegmatis* *mc*²*155*

For the transformation of genes in *M. smegmatis* *m*²*155*, 1 µL of plasmid was mixed with 25 µL of electrocompetent cells. This mixture was loaded in a 1mm electroporation cuvette, mounted in the MicroPulser Electroporator (Bio-Rad), set on 1.8 kV (manual voltage). Successful transformation correlates with a time constant between 4 and 6ms. Electroporated cells were recovered in 1mL of 7H9 neat media, incubated for 2h at 37°C and plated on 7H10 agar plates supplemented with 10 µg/ml Kanamycin (Kan).

6) Cytotoxicity Assay

Three transformed *M. smeg* colonies were picked using a sterile toothpick and dissolved in 400 μ L of 7H9 neat media. In addition, 1 positive control (pExTra02) and 1 negative control (pExTra03) colonies were prepared.

The bacterial suspensions were pipetted into one column of a sterile flat-bottomed 96-well plate and serial diluted 1:10 (from 10^0 to 10^{-5}) by mixing 30 μ L of cells in 270 μ L of 7H9 neat media.

A 48-prong frogger (Dan-Kan Corp.) was used to transfer $\sim$ 2-3 μ L of serial diluted cells from the 96-well plates to 7H10 plates supplemented with Kanamycin and containing no aTc, aTc₁₀ and aTc₁₀₀. Spots of bacteria were left to dry for 15 minutes in the dark, before being flipped and incubated in the dark for 5 days at 37°C. Plates were imaged using a ring light and a smartphone. All cytotoxic genes were re-tested to ensure reproducibility.

7) Immunity Assay

Following the electroporation of the genes, two colonies for each gene were dissolved in 5mL of 7H9 complete liquid media, before being incubated for 2 days at 37°C. In parallel, 2 colonies from the pExTra01 transformations were also prepared for this experiment to serve as a control.

To create the bacterial lawn required for a spot test, two 400 µL aliquot per saturated cultures were spun at 3000xg for 5 minutes for each tested colony, pelleting the cells, before resuspending them in 7H9 neat liquid media, removing the detergent from the culture. This step is important as the presence of Tween-80 could have an impact on the phage infection⁹⁷⁻⁹⁹. Each aliquot was then plated on 7H10 agar plates, containing either no aTc or aTc₁₀₀, using 3.5 mL of 7H9 top agar (containing aTc if the plate contained aTc), and left to harden for 15 min under a box to avoid light contact.

High-titer phages lysate from Bromden, Archie, LeBron, and Finnry were pipetted into one column of a sterile flat-bottomed 96-well plate and serial diluted 1:10 (from 10⁰ to 10⁻⁵) by mixing 30 µL of lysate in 270 µL of phage buffer.

A 48-prong frogger (Dan-Kan Corp.) was used to transfer ~2-3µL of the diluted lysates from the 96-well plates to bacterial lawns prepared earlier. Spots were left for 15 minutes under a box, allowing for the liquid to sink in the plate media, before being flipped and put in a box for 3-day incubation at 37°C. Data was then collected by imaging them using a ring light and a smartphone. Efficiency of plating (E.O.P.) was calculated using the following formula:

$$E.O.P. = \frac{\text{phage titer on aTc plates}}{\text{phage titer on control plates}}$$

8) Host Range on *M. tuberculosis*

a) Strains used

M. tuberculosis H37Rv-derived auxotroph strain *mc*²6206 (Δ *panCD* Δ *leuCD*)^{103–105} (*M. tb*) and a bioluminescent *M. tuberculosis mc*²6202 recombined with pMV306hsp + LuxG13 (*M. tb lux*)^{105,106}, graciously offered by Dr. Jim Sun, and *M. smegmatis mc*²155 (*M. smeg*) were used for this host range of the Mycobacterium phages isolated by the Rudner lab and the TMM discovery lab prior to June 2023.

b) Host range assay

To create the bacterial lawn required for a spot test, I used 400 μ L of my saturated cultures, and spun them at 3000 xg for 5min, pelleting the cells, before resuspending them in 7H9 neat liquid media. This removed the presence of Tween-80, which could have an impact on the phage infection^{97–99}. The cells were then plated on their respective 7H10 agar plates using 3.5 mL of 7H9 top agar and left to harden for 15 minutes.

Mycobacteriophages lysates varied between 10⁸ pfu/ml and 10¹² pfu/ml and were pipetted into one column of a sterile flat-bottomed 96-well plate and serial diluted 1:10 (from 10⁰ to 10⁻⁵) by mixing 30 μ L of lysate in 270 μ L of phage buffer. A 48-prong frogger (Dan-Kan Corp.) was used to transfer ~2-3 μ L of the diluted lysates from the 96-well plates to bacterial lawns prepared earlier. Plates were left untouched for 10 minutes to let the spot soak throughout the agar, before being flipped and transferred in the 37°C incubator. *M. smeg* plates were imaged 2 days following the phage infection, while *M. tb* waited for a total of 7 days before being imaged using Bio-Rad ChemiDoc imager.

9) Bioinformatics

a) Protein folding in-silico

I used ChimeraX^{107–109}, version 1.7.1, and its structure prediction function, using ColabFold¹¹⁰, a modified version of AlphaFold2 running on Google Colab servers. All settings were set to default. The resulting structure was saved as a .pdb file and compared against Protein Data Bank using the Dali server¹¹¹.

b) Creation of STOP codon + gp75 mutants

Using Bromden's sequence available on NCBI (accession MH576973) inside the SnapGene software (www.snapgene.com)¹¹² with the primers for each gene (Table 5), I modified the codon next to the start site of the gene to TGA, a stop codon. Gp75 mutants followed similar steps, whereas mutation sites were dictated by the sites found during our research. Those mutants followed the same pipeline as normal genes to create our plasmid library.

Results

1. Library construction

1.1. Completion of the library construction

With the help of the 11 undergraduate students and a project-based MSc student, I was able to create a plasmid library of all 131 genes present in Bromden. This library used the pExTra plasmid, a reliable vector able to be expressed both in *M. smeg*, Bromden's host, and *E. coli* for plasmid propagation. pExTra's key characteristics are shown in the schematic presented in Figure 3. This plasmid was created by Danielle Heller and contains various characteristics that makes it ideal for our assay. First, pExTra has an anhydrotetracycline-inducible promoter, P_{tet} , present upstream from the gene insertion site, allowing for control on the plasmid induction and thus, gene expression. Upon normal conditions, P_{tet} is repressed by the presence of a Tetracycline repressor cassette, Tet_R . Downstream from the gene insertion site, a mCherry transcriptional reporter enables visual confirmation of the protein formation in addition to a Kanamycin Resistance cassette (Kan_R), allowing for selection. A full map of the pExTra01 plasmid is available at the following webpage: [link](#)

Figure 3. Simplified schematics of pExTra plasmid. Made using BioRender. Presents the characteristics present on the pExtra plasmid.



The genes were assembled with the pExTra backbone using isothermal/Gibson assembly¹¹³, using the workflow present in Figure 4. Homology regions upstream and downstream were added to the primers used to clone each gene, similar to the one present on both ends of the backbone. Those regions are targeted by an exonuclease that digests each end, creating overhangs that allow for gene-backbone junction, which will be completed by the action of a DNA polymerase, filling up the gaps and DNA ligase linking back the strands together.

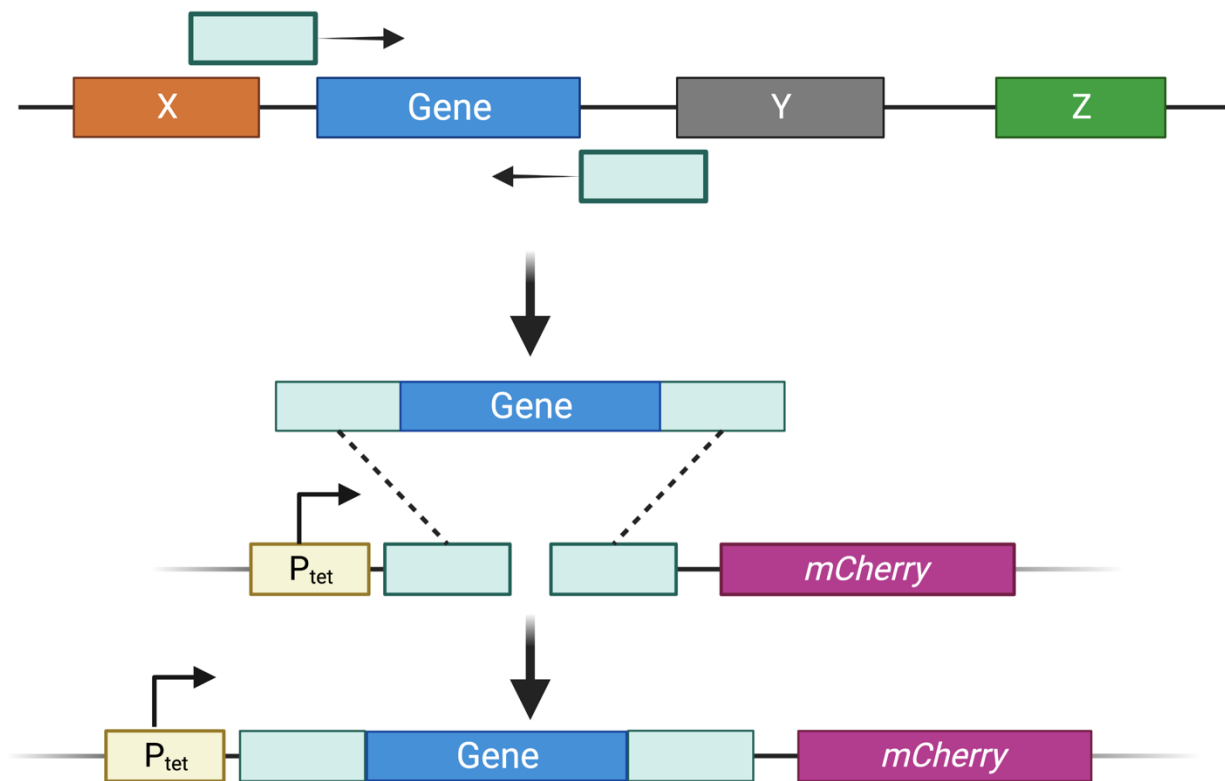


Figure 4. Schematics of the PCR amplification and isothermal assembly. Made using BioRender. Specific PCR primers are used to copy a gene of interest (dark blue). The PCR product contains a 23-bp homology region on his 5' and 3' end (present in light blue). Following digestion by an exonuclease, overhangs of homology regions on the PCR product and linear backbone can align and assemble into a plasmid containing the gene of interest.

We documented the PCR amplification and confirmation of plasmid clones using agarose gels and constructed data cards for every gene. Using the gene size, I was able to quickly confirm our constructs or take the appropriate steps if required. Data cards have been uploaded on GenesDB, allowing for public access of our research (<https://genesdb.org/phage/Bromden>).

1.2. Sequencing

All plasmids constructed have been sequenced using Sanger sequencing and proofread with SnapGene¹¹². Most of our plasmid collections (107/118) were confirmed, requiring only one round of sequencing. Others, such as the plasmids containing Bromden gp12, 27, 50, 56, 57, 72, 76 and 84 had to be repeated due to a poor number of reads or low confidence during their first round of sequencing. Plasmids containing gp17, 23 and 72 could not be sequenced using Sanger sequencing, thus, whole plasmid sequencing was performed and confirmed the successful construction of our plasmids. All the sequencing data has been uploaded to the GenesDB platform for public access. Furthermore, this plasmid library has been archived at uOttawa and the Howard Hughes Medical Institute, allowing other schools part of the SEA-GENES program to access the library collection for future experiments.

Only a single mutation was detected in all 118 plasmids, as demonstrated in Figure 5. Gp1 contains a Cytosine to Tyrosine substitution resulting in an A88V change in the protein sequence from Bromden genome reported in GenBank. This mutation was found in multiple clones, and I hypothesize that it reflects a mutation that occurred during the amplification of the Bromden phage. The A88V mutation lies within an alpha helix and AlphaFold2¹¹⁰ predicted structures of the wild type and mutant protein did not indicate any structural changes resulting from this amino acid, as shown in Figure 6. Direct sequencing of the phage genomic DNA used in library construction could confirm this change within Bromden.

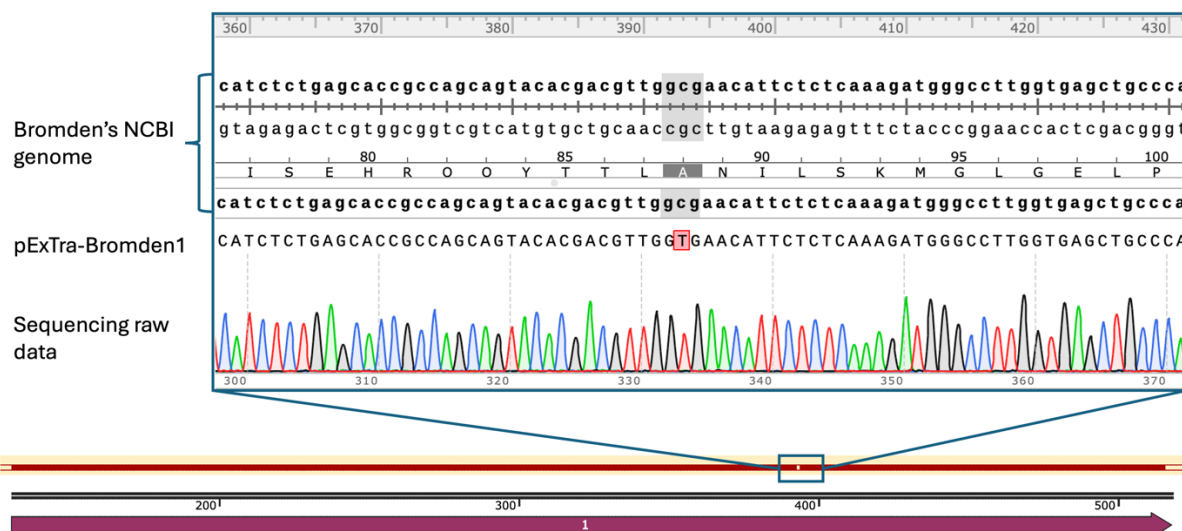
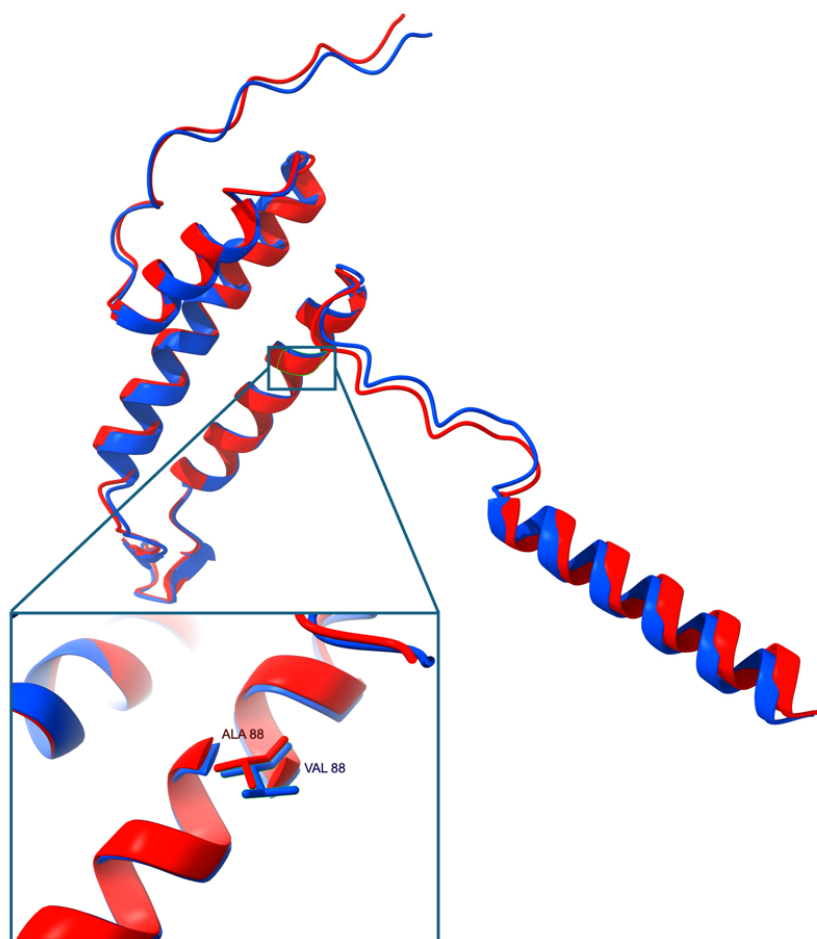


Figure 5. C393T nucleotide change on pExTra-Bromden1 compared to its wild-type lysate. Obtained using SnapGene¹¹². pExTra-Bromden1 was sequenced using Sanger Sequencing with the pExTra_SeqF primer (see Table 4 for sequence). I compared the sequence with Bromden's sequence, available on NCBI (accession MH576973) on SnapGene using the alignment tool built-in in the software. This mutation was found in two independent clones, which I hypothesize represent sequence drift in the Bromden genome.

Figure 6. Protein folding of Bromden's gp1 and its mutant A88V found in our plasmid. Obtained using ChimeraX and its AlphaFold capabilities. Sequences of the protein were obtained throughout Bromden's NCBI entry (gene ID: 63209946) and the sequencing data obtained from Azenta (Raw file not included, but base pair modification shown in Figure 5). Both proteins were folded using the same settings (default), and superimposed using the matchmaker function. Residues were manually highlighted and labelled using the select and label option.



2. Cytotoxicity Assays

Our library of containing every Bromden genes was successfully tested for cytotoxicity. All the raw data of the cytotoxicity assay is available at the end of this thesis, under the supplement chapter. For this experiment, I followed the logic outlined in Figure 7. Figure 8 presents a gene map summarizing our results, colour-coded to the cytotoxicity levels observed. Table 1 summarizes the results in a table format.

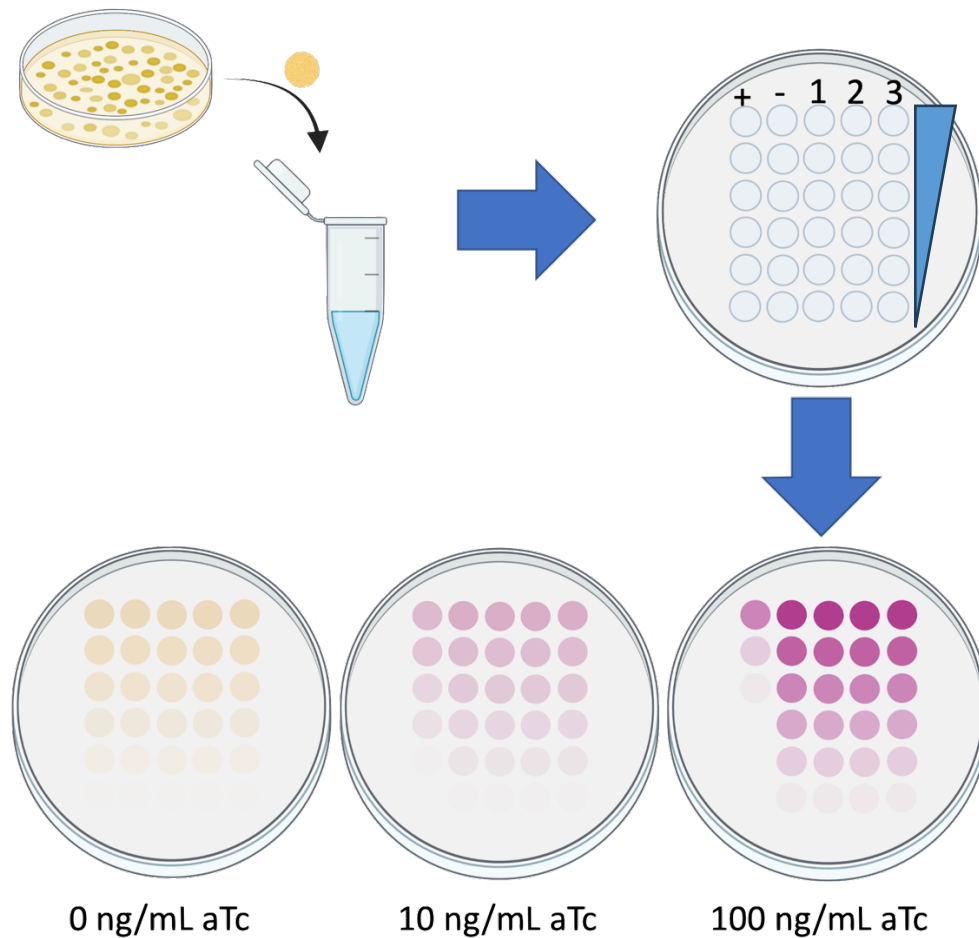


Figure 7. Graphical abstract of the Cytotoxicity assay. Created using BioRender.com. Offers a visual aid for the experimental design described in Methods. Following electroporation, 3 colonies containing the tested gene (labelled as 1,2,3 on the plate) alongside a positive (+, toxic, pExTra02) and negative control (-, non-toxic, pExTra03) were picked, dissolved and serial diluted in media. Those dilutions were plated on 7H10+Kan with various level of aTc, creating various level of induction of the protein. As our plasmid contains mCherry, we are expecting an increase in the level of pinkiness of the colonies following higher induction levels.

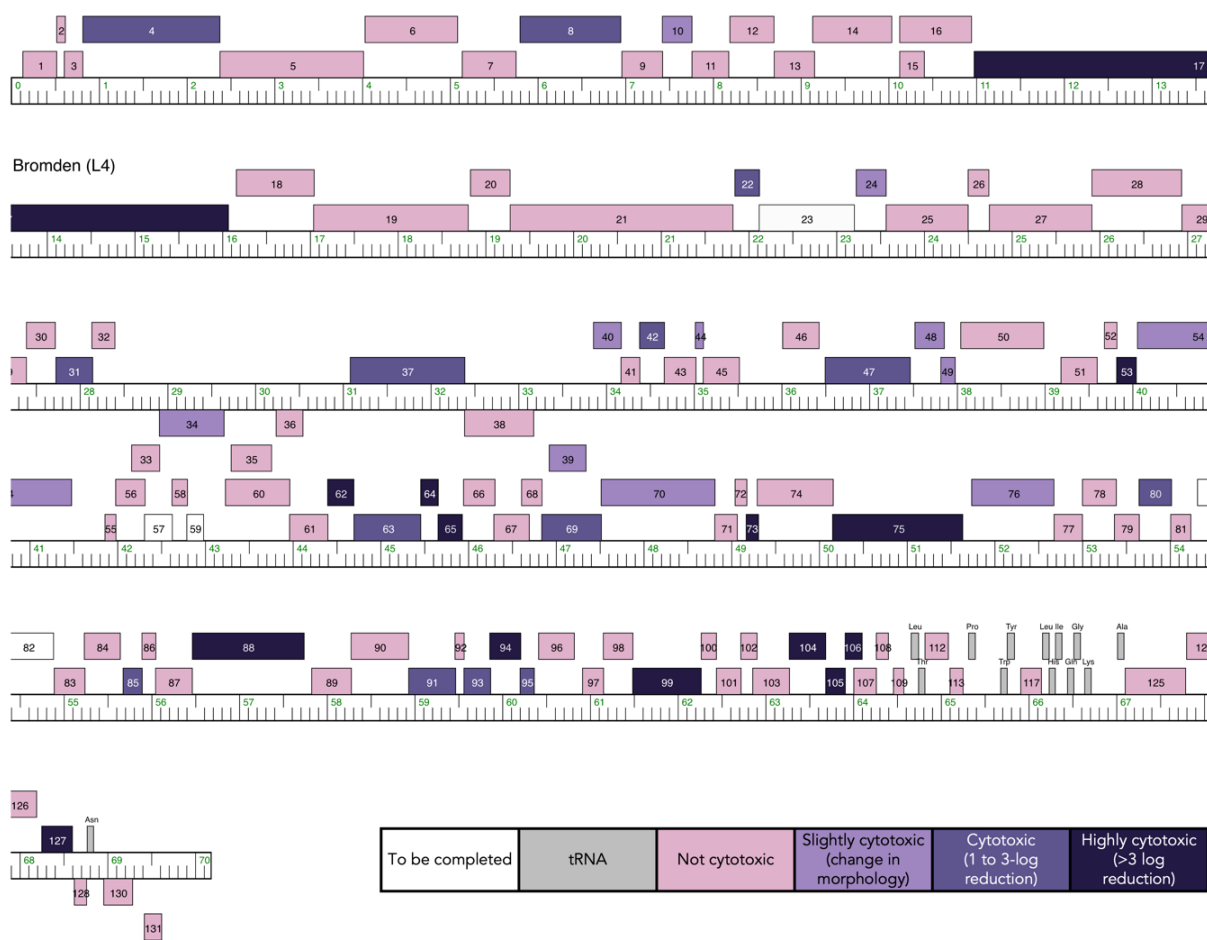


Figure 8. Gene map of Bromden and his cytotoxic genes. Based on Bromden's map from Pharmerator.org and modified using results from Table 1 (uses the same legend). Results were quantified after obtention of the data card for cytotoxicity.

Table 1. Results of our Cytotoxicity screening for Bromden. This is an alternative view to Figure 8, adding the gene function present on NCBI GenBank ⁹⁵ Bromden record (Accession MH576973.1).

Gene number	Function	cytotoxicity	Gene number	Function	cytotoxicity	Gene number	Function	Cytotoxicity
1	Terminase, small subunit		46	NKF		91	NKF	
2	NKF		47	Metallophosphoesterase		92	NKF	
3	NKF		48	NKF		93	NKF	
4	Terminase, large subunit		49	NKF		94	NKF	
5	Portal protein		50	Band-7-like membrane protein		95	NKF	
6	Capsid maturation protease		51	NKF		96	HNH endonuclease	
7	Scaffolding Protein		52	NKF		97	NKF	
8	Major capsid protein		53	NKF		98	NKF	
9	Head to tail adaptor		54	RNA-binding protein		99	DnaQ-like DNA polymerase III subunit	

10	Head-to-tail stopper		55	NKF		100	NKF	
11	NKF		56	Membrane protein		101	NKF	
12	Tail terminator protein		57	NKF		102	NKF	
13	HNH endonuclease		58	NKF		103	HNH endonuclease	
14	Major tail protein		59	NKF		104	endonuclease VII	
15	Tail assembly chaperone		60	Exonuclease		105	NKF	
16	Tail assembly chaperone		61	Endonuclease VII		106	NKF	
17	Tape measure protein		62	NKF		107	NKF	
18	Minor tail protein		63	ERF family ssDNA binding protein		108	NKF	
19	Minor tail protein		64	Erf-like ssDNA annealing protein		109	NKF	
20	Minor tail protein		65	NKF		110	tRNA-Leu	
21	Minor tail protein		66	NKF		111	tRNA-Thr	
22	NKF		67	HNH endonuclease		112	NKF	
23	NKF		68	NKF		113	NKF	
24	NKF		69	NKF		114	tRNA-Pro	
25	Hydrolase		70	DnaB-like replicative helicase		115	tRNA-Trp	
26	NKF		71	Transcriptional regulator WhiB-like		116	tRNA-Tyr	
27	Lysin A		72	NKF		117	NKF	
28	Lysin B		73	NKF		118	tRNA-Leu	
29	Holin		74	Phosphoribosyl transferase		119	tRNA-His	
30	Holin		75	Nicotinate ribosyl-transferase		120	tRNA-Ile	
31	Membrane protein		76	Polynucleotide kinase		121	tRNA-Gln	
32	NrdH glutaredoxin		77	NKF		122	tRNA-Gly	
33	Membrane protein		78	RusA-like resolvase (endonuclease)		123	tRNA-Lys	
34	Nucleotidyl transferase		79	WhiB transcriptional factor		124	tRNA-Ala	
35	Lsr2-like DNA bridging protein		80	WhiB transcriptional factor		125	Nuclease	
36	NKF		81	NKF		126	NKF	
37	Tyrosine integrase		82	DNA binding protein		127	NKF	
38	Membrane protein		83	NKF		128	NKF	
39	Immunity repressor		84	SsDNA binding protein		129	tRNA-Asn	
40	Cro		85	NKF		130	NKF	
41	Excise		86	NKF		131	NKF	
42	NKF		87	NKF		Legend To be done Slightly cytotoxic (change in morphology) Highly cytotoxic (>3 fold) Non-cytotoxic Cytotoxic (1-3 fold) tRNA		
43	NKF		88	RNA ligase				
44	Membrane protein		89	HNH endonuclease				
45	NKF		90	ClpP-like protease				

In total, I observed that 42 genes (34%) are impacting the host, ranging from a slight modification in the colony size (gp10, slightly cytotoxic) to a large reduction in the number of colonies produced (gp62, highly cytotoxic). This result is in accordance with previous results found by Heller and Amaya ^{51,84}, finding respectively 33% and 26% of genes impacting the host development. Among the toxic genes present in Bromden, 14 impaired the bacterial growth of *M. smeg*, in a moderate manner (cytotoxic, fold reduction of the colony growth by 1 to 3-log), while 14 genes impacted the bacteria by reducing colony formation >3-log (highly cytotoxic). Examples for moderate and high level of cytotoxicity are presented in Figure 9.

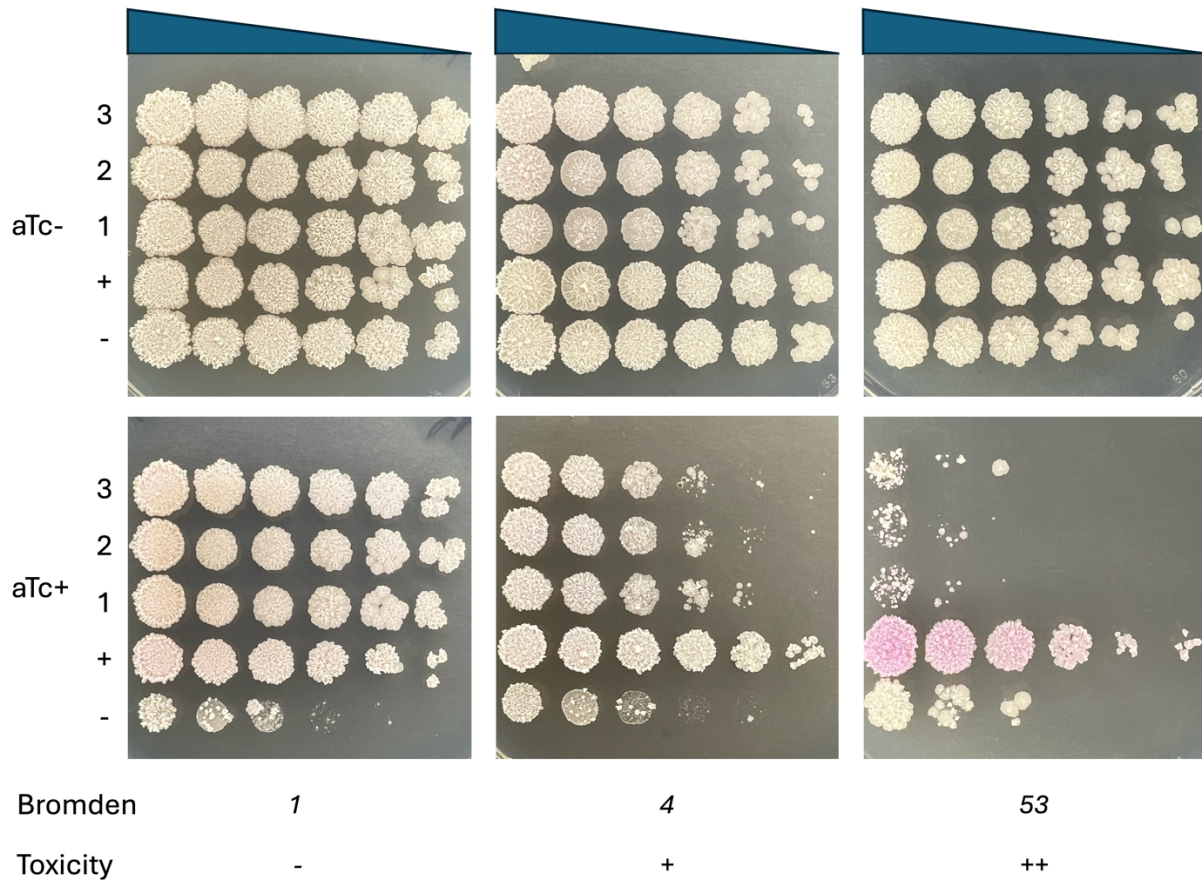


Figure 9. Cytotoxicity results for Bromden_1; _4 and _53. The top row represents *M. smeg* cells including the gene of interest without induction (aTc 0ng/ml) while the bottom row contains bacterial colonies with the induced plasmid (aTc 100ng/ml). Positive (pExTra02), negative control (pExTra03), and 3 colonies candidate per tested gene (1,2,3) are present on each plate. Bromden_1 is non-toxic as it exhibits no morphological changes, while Bromden_4 is cytotoxic, reducing the colonies formed by a factor of 1-2-fold when induced. Bromden_53 is highly cytotoxic as it greatly reduces the number of bacterial colonies formed when induced by 4-5-fold.

2 genes attracted my attention, due to their impact on the host before their induction.

Bromden gp127 (gene function: NKF) was also able to impact the bacterial growth of *M. smegmatis*, reducing the colony to the size of grain of salts (Figure 10) prior to induction. This reduction was reproduced four times and led to contradicting results after plasmid induction.

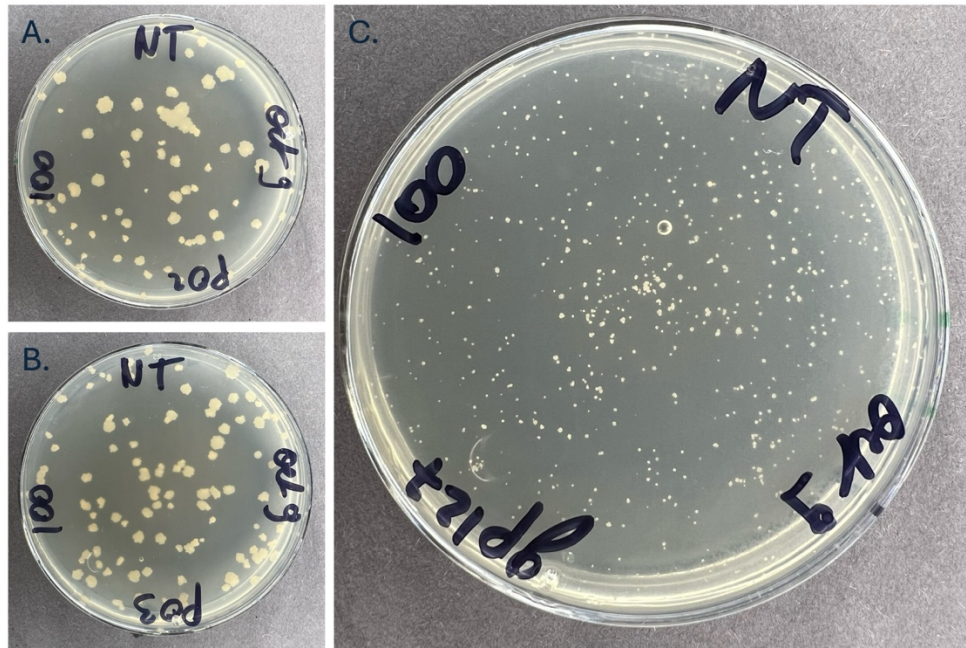


Figure 10.
Transformation in *M. smegmatis* of gp127.
pExTra02 (non-toxic control, panel A.) and pExTra03 (toxic control, panel B.) showed normal *M. smeg* morphology, while gp127 (NKF, panel C.) seems to be impacted by some toxicity prior to his induction by aTc, resulting in a large colony size reduction for the surviving cells.

Meanwhile, Bromden gp75 (gene function: Nicotinate Ribosyltransferase) was found to be extremely cytotoxicity, killing *M. smeg* before the formation of colonies (Figure 11). Despite repeating the plasmid construction and making multiple *M. smeg* transformations, no transformants were able to be recovered.

In her study of the phage Waterfoul, Heller had a similar problem during the *M. smeg* transformation of pExTra containing Waterfoul_8 and _86⁵¹. Despite multiple trials, Heller was unable to obtain viable survivors for those 2 genes following their electroporation, prior to their induction. She hypothesized that these issues could be linked to the leakiness of the promoter P_{tet} may prevent the transformation of plasmids containing highly cytotoxic genes. As a result, I tested this hypothesis by creating a nonsense mutant of those genes, adding a STOP codon

(TAG) next to the start site present at the beginning of the protein (See Table 6). After transformation in *M. smeg*, I was able to recover non-toxic transformants for gp75, confirming the hypothesis (Figure 11).

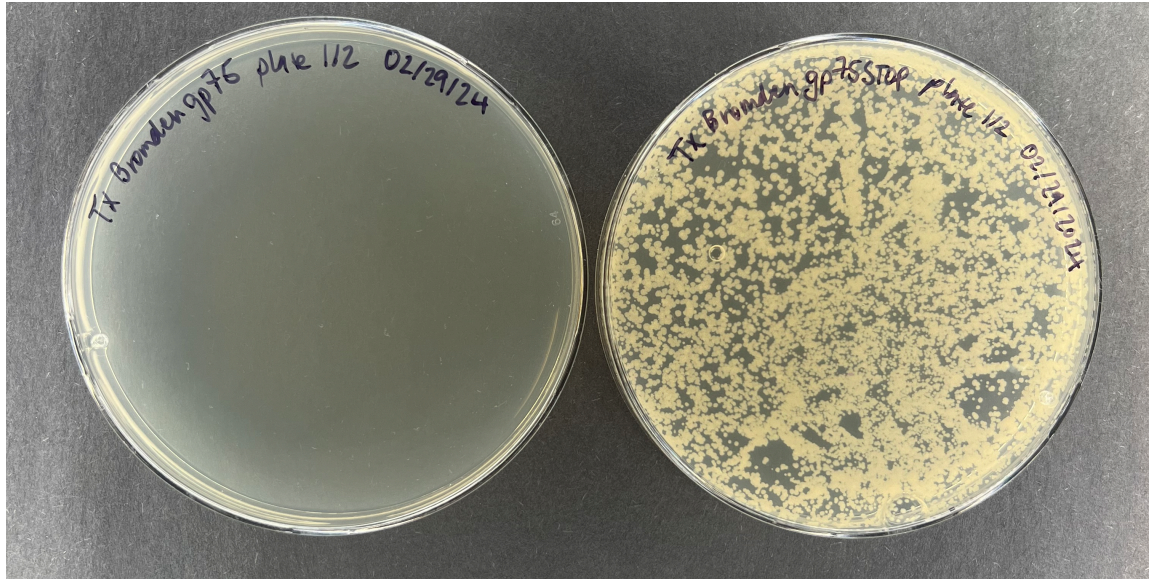


Figure 11. gp75STOP alleviates the toxicity of gp75. Bromden's gp75 doesn't allow for transformation in *M. smeg*, leading to the death of the host prior to his induction. The addition of a STOP codon allows for full recovery of the host.

3. Immunity Assays

Immunity assays have been performed on 14/118 Bromden genes. Figure 12 shows the pipeline used for this project. After transforming our plasmid containing a gene of interest in *M. smeg*, a colony is picked to grow in liquid media. This liquid culture will be used to create a bacterial lawn on plates containing no inducer or 100 ng/ml of aTc, allowing us to test the Bromden's genes implication during phage infection and any possible immunity that those could confer. For our assay, I decided to focus on Bromden and closely related phages coming from neighboring clusters, being Archie (L2), LeBron (L1) and Finnry (L3). This would allow us to explore the possibility of immunity that Bromden genes could offer to closely related phages or if immunity is restricted to autoimmunity.

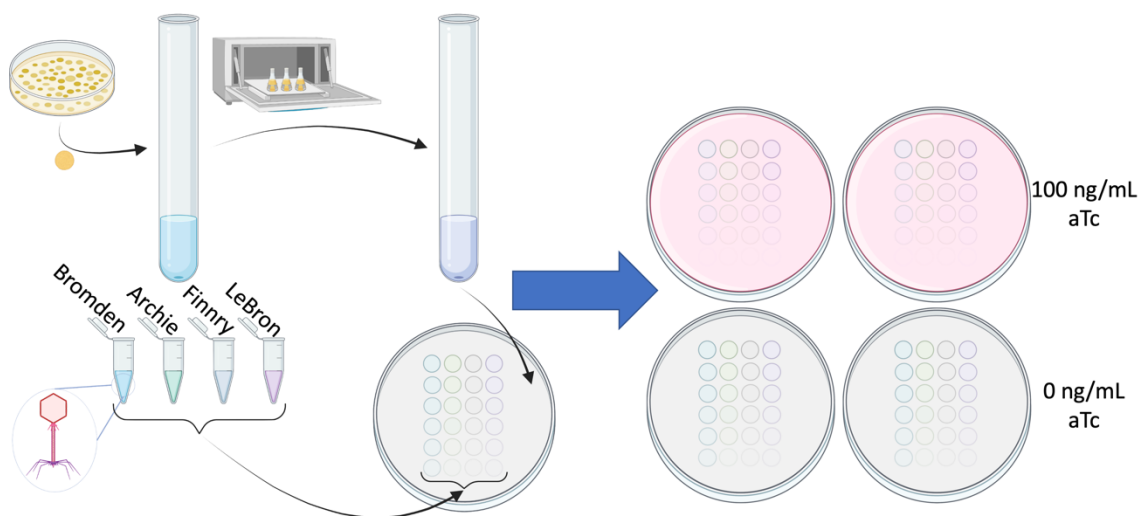


Figure 12. Graphical abstract of the Immunity assay. Created with BioRender.com. Offers a visual aid for the experimental design described in Methods. Following electroporation of the plasmid, a colony of the transformed gene was dissolved in media and left to grow until saturation. Upon saturation of the culture, a bacterial lawn was created using the culture and Top Agar. Following the creation of the lawn, phages (Bromden, Archie, Finnry and LeBron) were serially diluted and plated onto the lawn. This was repeated twice on plates containing no inducer (aTc 0ng/ml) and twice on plates containing 100ng/ml of aTc, allowing for activation of the plasmid.

Data obtained for this assay is available under Supplemental 2. Results showed a lot of variability in the infection and lawn formation, with poor Bromden infection on a bacterial lawn at 37°C, which is even worse when the plasmid is induced. Figure 13 illustrates this poor infection during our assay with the empty pExTra plasmid, p01. The temperate phenotype that Bromden seems to have makes it difficult to determine its efficiency of plating (E.O.P.), our main measurement for coming out of this experiment.

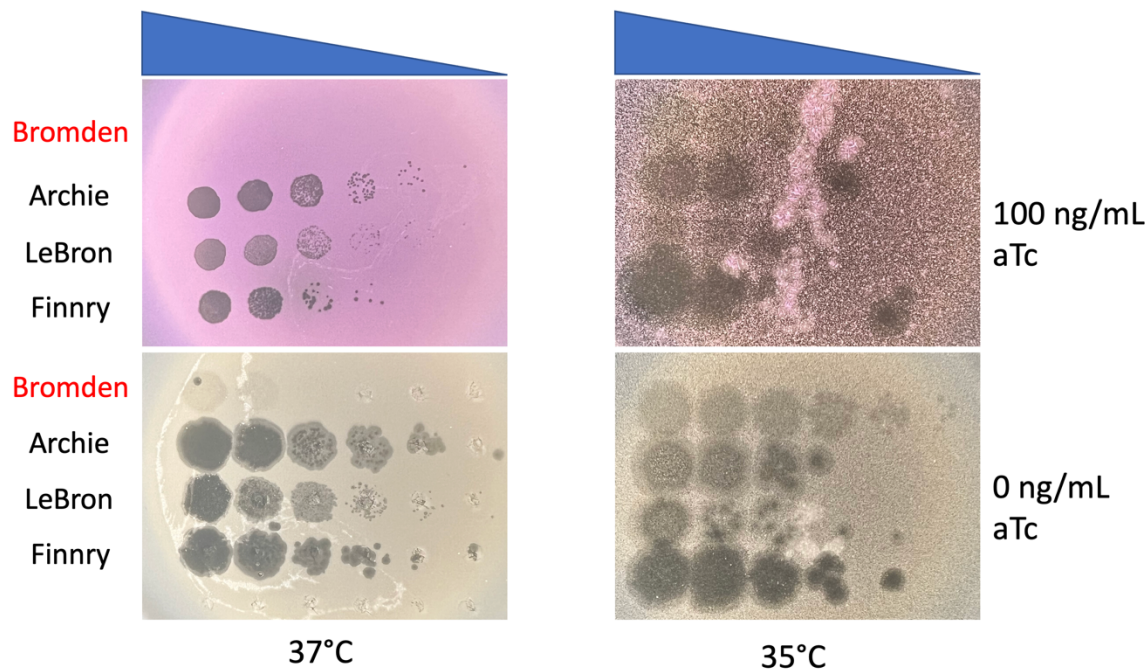


Figure 13. Immunity assay for p01 (empty plasmid vector). This immunity seems to be even worse following the induction of the plasmid. Experiments were repeated at lower temperatures, as Bromden was isolated at 34°C, leading to bacterial defect in the growth of the bacterial lawn of the p01 plasmid.

4. Host range with *Mycobacterium tuberculosis*

As *Mycobacterium smegmatis* shares 95% of its genome with *Mycobacterium tuberculosis*⁴⁵, and previously isolated Mycobacteriophages were able to infect more than one host^{20,24,25,28–30,114}, I explored the therapeutical potential of phages that have been isolated by our lab and the TMM discovery lab (total of 27 viruses) for infection against this pathogenic bacterium, using an attenuated strain of *Mycobacterium tuberculosis* (*M. tb*) from Dr. Jim Sun. To do so, I used a similar technique to the immunity assay, where a bacterial lawn of *M. smeg* or *M. tb* was infected by various dilutions of the phages in our possession. Data collected during this assay is presented in Figure 14, and simplified in Table 2.

Table 2. Infection of phage inventory over various *Mycobacterium* hosts. Results were compiled and classified by the last dilution where a plaque was formed. The number present in the first column corresponds to the phage number on the bacterial lawn for Figure 14.

#	phage name	<i>M. smeg</i>	<i>M. tb</i>	<i>M. tb lux</i>	#	phage name	<i>M. smeg</i>	<i>M. tb</i>	<i>M. tb lux</i>
1	Pilon				15	Doppler			
2	Lebron				16	Rondoudou			
3	Finnry				17	Aishwareya			
4	Baddie				18	LovelyOne			
5	Bromden (mutant)				19	Tinish			
6	Pheedme				20	Mimas			
7	Daeun's phage				21	Polkaroo			
8	BabyTango				22	Greenboro			
9	Gahunia				23	Amalu272			
10	Falooda				24	Archie			
11	Guerriero				25	Raquel			
12	Chloris				26	WT Bromden lysate			
13	Baghrir				27	WT Bromden CsCl			
14	Oksu								
Plaque formed at 10 [^]	0	1	2	3	≥4				

As shown in table 2, all phages present in our collection showed decreased, to no level of infection towards *M. tb* compared to their infection in *M. smeg*. The phage Falooda showed great potential as a possible therapeutic agent against *M. tb*., as well as the phage Baddie, Pheedme, Finnry, Amalu272 and LovelyOne, with the formation of plaques up to the 10⁻⁴ dilution, with E.O.P ranging from 0.001 to 0.05.

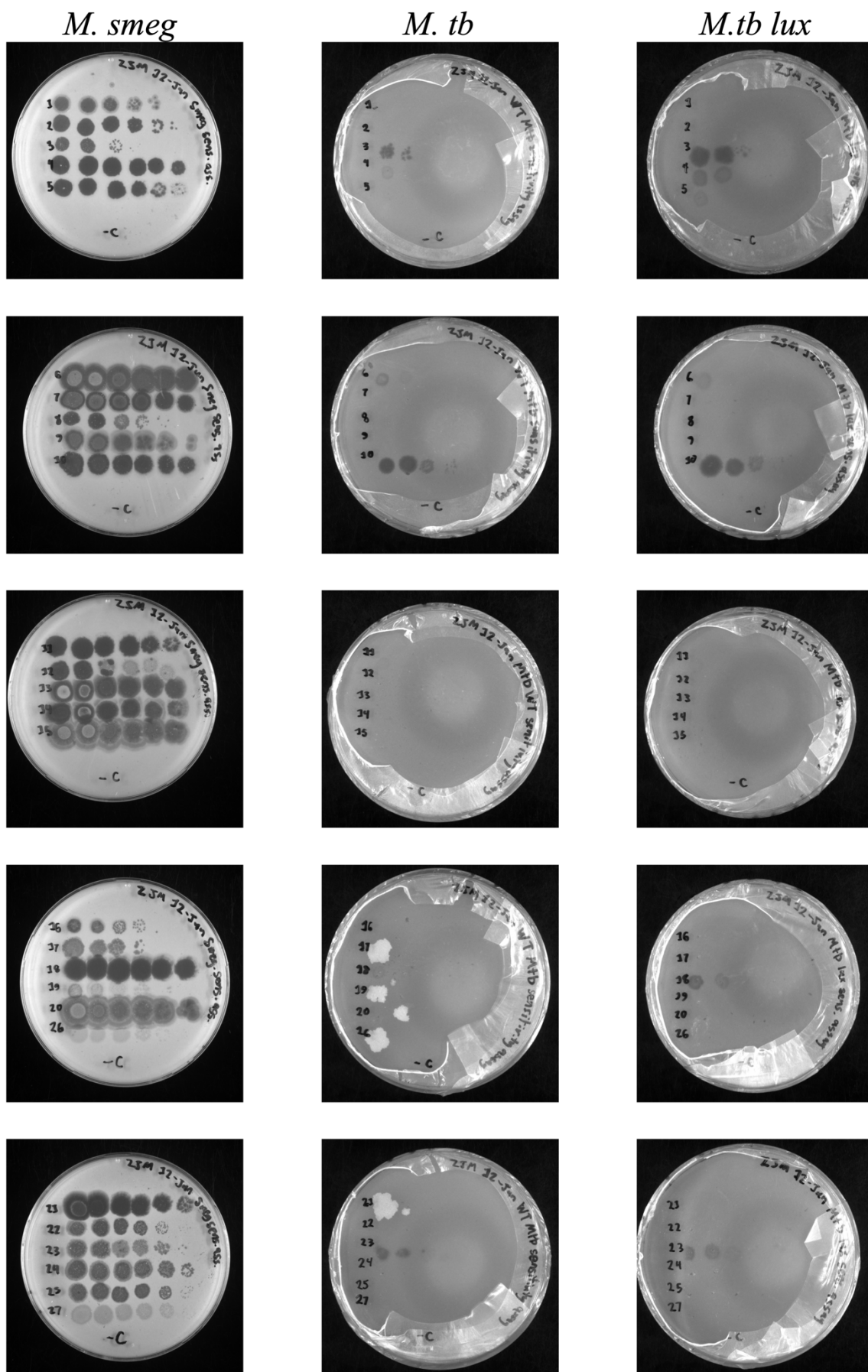


Figure 14. Raw data from the host range experiment in *M. tb* The phage linked to every number is available in Table 2. On the left column is our *M. smeg* infection, at the center is the *M. tuberculosis* and column on the right shows *M. tuberculosis-lux* infection.

Discussion

1. Cytotoxicity

What is cytotoxicity?

Cytotoxicity refers to the toxic effect that some genes can express toward host bacteria. This toxic effect is usually directly related to the lytic cycle happening during the phage infection. There can be multiple causes of cytotoxicity, as shown in Figure 15. Notably, phage proteins can have a direct impact on the host genome (such as DNA binding agents and nucleases)^{51,84}, within pathways (Wag31-Fruitloop gp52 interaction created an environment prone to further phage infection)⁵⁰ or via an indirect action (protein aggregation)⁴⁹. Therefore, determining whether a gene is cytotoxic could help us better understand phage attack and bacterial lysis. Identifying toxic genes and understanding their interaction with the host could be used to find new therapeutic targets that could be used to create better therapies.

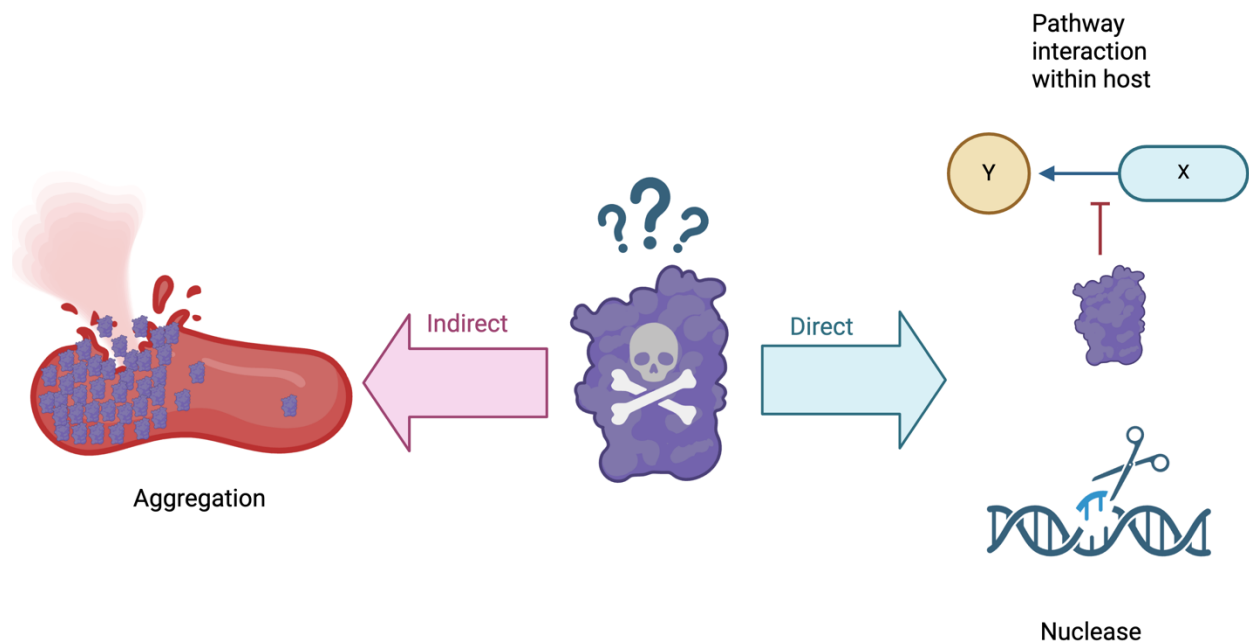


Figure 15. Schematics of main causes that make a gene cytotoxic. Figure made with Biorender.com. Based on previous results obtained by genome analysis^{49–51,84} and summarized as a drawing. The protein (in purple) can have indirect effects in the host (in red), or direct effect such as Pathway interaction within the host, or nuclease action.

Cytotoxicity in Bromden

This screening found that 14 Bromden genes moderately impacted bacterial growth of *M. smeg*, and 14 genes severely impacted the host bacteria.

Among the 14 genes showing high levels of cytotoxicity, 9 have currently no predicated function and would require some further study to understand the mechanisms behind their action. Only 2 out of the 10-nuclease domains present in Bromden exhibited high levels of toxicity, being gp104, endonuclease VII and gp99, DnaQ-like DNA polymerase III subunit. Our result for Bromden's DnaQ-like DNA polymerase III subunit contradict those obtained by Heller with the phage Waterfoul⁵¹, but confirm the result from Amaya with their observation in the phage Hammy⁸⁴. Further work will be required to understand the mechanisms and cause of cytotoxicity for this polymerase. Amaya hypothesized that this toxicity might be phamily-specific but seems to be shared with Bromden's gp99 phamily, which might relate to a common ancestor between both phages⁸⁴. A quick Blastn comparison, confirms that Bromden shares more bp identity with Hammy (95% percent identity) than Waterfoul (about 70%). Among the other highly toxic genes, we find DNA/RNA repair agents, such as gp64, Erf-like ssDNA annealing protein, and gp88, an RNA ligase, which have previously been found to be toxic when being overexpressed within *E. Coli*¹¹⁵.

Discussing now about the genes showing moderate levels of cytotoxicity, I observed that 9 out of 14 have no predicted function. Among the 5 cytotoxic genes with a predicted function, we have gp4, Terminase large subunit, implied in the DNA packaging of the phage into its capsid^{116,117}, gp8, Major capsid protein, a structural protein forming the phage capsid structure, gp47, Metallophosphoesterase, a poorly characterized phage function¹¹⁸ and gp63, ERF family ssDNA binding protein, a protein implied in the linearization of the phage genome prior to

infection¹¹⁹. The presence of those genes might trigger bacteria into thinking that they have been infected with phages, leading to the activation of the SOS response and destruction of cells. I also observed that Bromden gp37, Tyrosine kinase, is showing signs of moderate toxicity (Figure 8 and Supplemental 1). This gene is implied in the site-specific integration of the phage genome within the host genome and is thus active in the establishment of the lysogenic cycle¹²⁰. Thus, this result is unexpected and shows that Bromden gp37 also possesses an action during the lytic cycle.

On the other hand, I observed little to no cytotoxicity effect coming from the HNH endonucleases present in Bromden (Figure 8 and Supplemental 1). This result is quite different from the result obtained previously, both by Heller and Amaya, as one of the HNH present in those phage genomes always exhibits high level of toxicity towards the host^{51,84}.

Finally, mCherry was observed for the majority of the non-toxic assays, providing a visual confirmation that our gene of interest was transcribed by the induction with aTc. However, despite this colour change, it can't be ruled out that these genes might be toxic when being expressed with others, in a synergetic manner during a normal phage infection⁵⁰. This would require adding more than one gene per plasmid, increasing our number of assays exponentially. Furthermore, Heller already hypothesized that some non-toxic genes might be insoluble in the cells and/or accumulate within them, making them inefficient against *M. smeg* cells⁵¹. Finally, the mCherry level was sometimes highly variable between 2 assays, which could indicate higher transcription of the gene. A reason for those changes could be linked to the freshness of our aTc plates. I believe that this may be the case because while usually being prepared 3 days before my experiment, some aTc plates were prepared up to one day before the cytotoxicity assay, which could represent higher levels of aTc, and thus higher induction. In addition, knowing that aTc is

UV-sensitive¹²¹, various levels of light, due to the presence of large windows in our lab and the use of fluorescent tube lights, may have impacted the efficiency of our aTc stock and/or plates. According to Baumschlager, aTc inactivation can happen within 3min of exposure to UVA light or hours under a blue light¹²¹. This result is most likely mitigated in real life, as rays are not directly aligned with aTc and windows/lights have some UV treatment, preventing most of those UV rays. However, the impact of the natural light over my plates has not been tested and could be a factor explaining this disproportionate activation.

Altogether, the results from this assay could be translated to about 3,500 phages, previously sequenced present on the phagesDB platform. This represents around 72% of the sequenced phages present on this database⁴².

Final repetition of Cytotoxicity assays

To ensure reproducibility of the results obtained and quality of my observations, most of the genes present in this thesis have been repeated at least two times. However, 31 genes need to be repeated to ensure proper confirmation of their phenotype. I'm on track to finish these assays by the end of the summer of 2024.

Bromden gp75: Nicotinate Ribosyltransferase

Being intrigued by the high level of cytotoxicity resulting from expressing Bromden 75 in *Mycobacterium smegmatis*, I decided to take a deeper look at Bromden gp75. Using HHPred^{122,123}, I found that its gene function is nicotinate ribosyltransferase, an enzyme studied in bacteria and yeast, but never within phages¹²⁴⁻¹²⁶. To uncover clues to understand the mechanisms of Bromden gp75 toxicity, I used AlphaFold2¹¹⁰, a protein folding software that

allowed us to obtain a predicted 3D structure of the protein. This predicted structure of gp75 matched a human protein, Nicotinamide Phosphoribosyltransferase (NAMPT), on the Dali server¹¹¹, matching previous HHPred data^{122,123}.

NAMPT is used to elevate cellular level of Nicotinamide adenine dinucleotide (NAD⁺)¹²⁷, one of the main energy currencies in cells. Mechanistically, NAMPT is converting nicotinamide (NAM) to nicotinamide mononucleotide (NMN), an NAD⁺ precursor using α -D-5-phosphoribosyl-1-pyrophosphate (PRPP) and Adenosine triphosphate (ATP). This conversion is the limiting factor of the biosynthesis of NAD⁺ in mammals. Decreases in NAD⁺ levels have been linked to aging and metabolic disorders.

Looking at the broader literature, this enzyme was found to enhance the human gut system against bacterial infection^{128,129}, which could explain why our host dies when increased level of NAD⁺.

Superimposing the predicted and solved structures reveals a large number of similarities between the two proteins, with good conservation within the active site and the four residues in the human NAMPT, that are required for NAM conversion: Asp219, Ser241, His247, Ser275. Residues Asp219, Ser241 and Ser275 are needed for the hydrogen bonding to NAM within the active site, while residue His247 catalyzes the transfer of a phosphoribosyl group from the PRPP to NAM. Based on this observation, I substituted alanine at the conserved residues Asp221, His247 and Ser276 in the plasmid expressing Bromden gp75 to test if gp75 activity is required for gp75 toxicity.

The three mutant plasmids were transformed into *M. smeg* and I observed that D221N and H247Y did not produce transformants, similar to what I observe with wild-type gp75 and

suggesting that either activity isn't required for toxicity, or that these individual mutants still retain sufficient activity to be lethal when expressed. Thus, I assume that those modifications do not modify the activity of our protein as they still lead to the complete death of the host. On the other hand, the S276A mutant did produce transformants supporting a model in which the activity of gp75 is needed for its toxicity (Figure 16).

This recovery indicates that the function of this residue might be conserved between species. If this is the case, this residue is in charge of the position of NAM, and changing it results in lower conversion of NAM to NMN, and thus, less NAD^+ that could have a toxic effect on *M. smeg*.

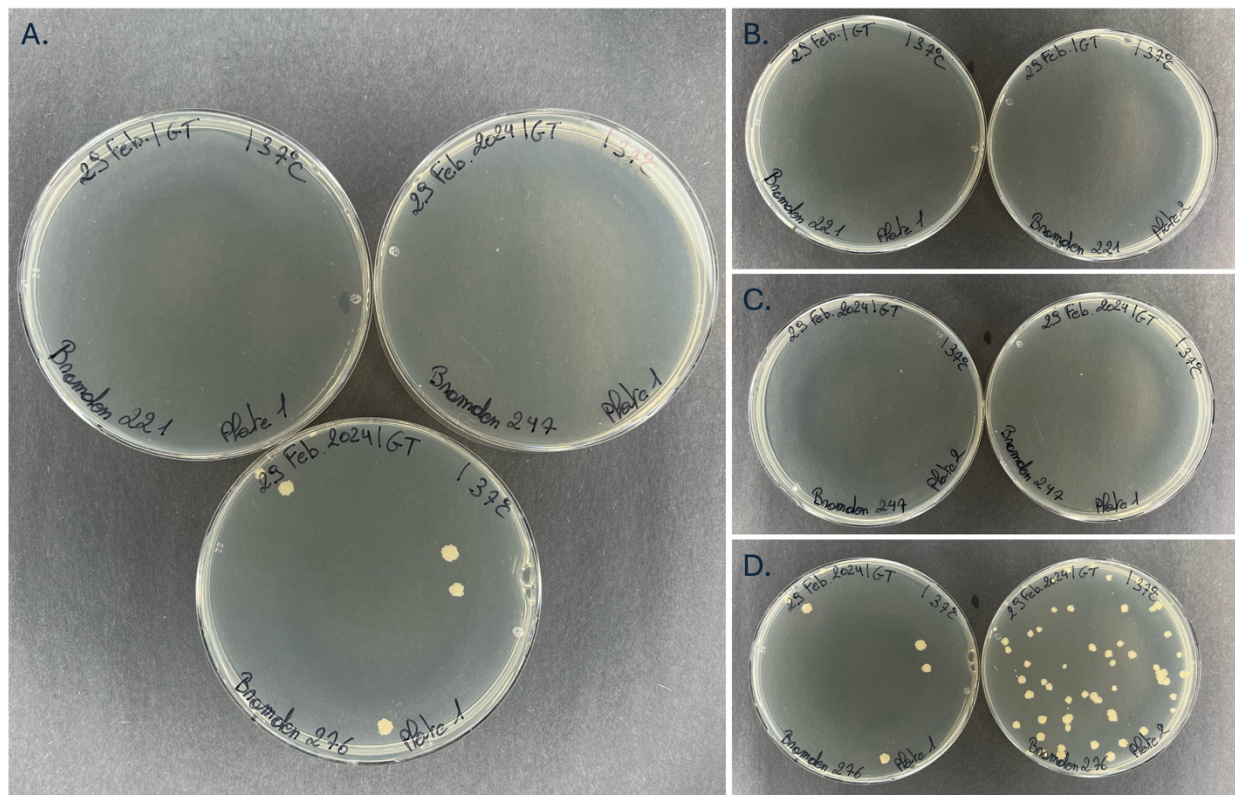


Figure 16. gp75 mutant S276A allows for partial *M. smeg* recovery. Panel A. is a combination of the electroporated mutants, while panel B presents mutation D221N, panel C. H247Y and panel D. S276A.

Bromden gp127: NKF

When transforming the plasmid that induced gp127, an orpham in Bromden, I noted this gene impacts the transformation in *M. smeg* by greatly reducing the size of the colonies formed prior to his induction (Figure 10).

While continuing with the cytotoxicity assay and inducing the plasmid, I obtained some conflicting data about the toxicity of gp127. My first assay showed that gp127 was mildly cytotoxic while the second showed no cytotoxicity at all (Supplemental 1). As this gene seems to impact bacterial growth without the presence of inducer, I relayed back to the possible leakiness of the promoter⁵¹. This evasion might not be complete, resulting in the variable phenotype observed. Since the gene function of gp127 is NKF, I can't gain any clues about the cytotoxicity by its gene function. Furthermore, this gene is unique to Bromden (ORPHAM protein). and thus, hasn't been characterized in any other phage.

Using the same techniques as gp75, I found no notable match for gp127 using AlphaFold2 (Figure 17) and Dali. I also produced a version of the plasmid, including a STOP codon, allowing us to ensure that the promotor is not leaky. This plasmid still has to be transformed in *M. smeg* to test my hypothesis.

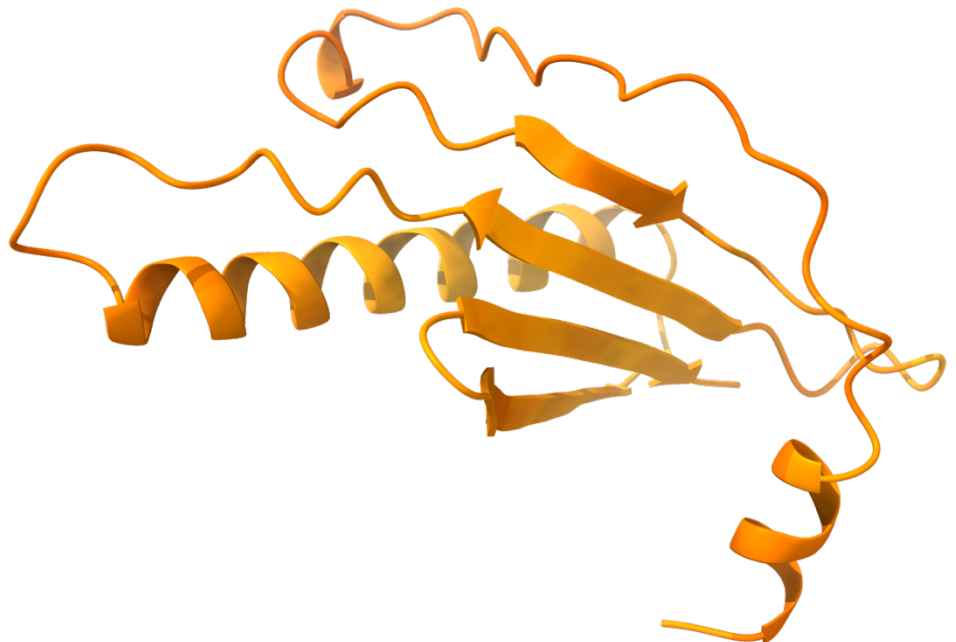


Figure 17. Protein folding of Bromden's gp127. Obtained using ChimeraX and its AlphaFold capabilities. The sequence of the protein was obtained throughout Bromden's NCBI entry (gene ID: 63209938).

Gain more knowledge about gene function.

This screening is only a first step in our quest to learn more about the gene function present in the Mycobacteriophage Bromden. 18/28 genes that showed moderate to high levels of toxicity against the host, such as gp53; gp73 or gp127, have not been characterized yet. Thus, more work is required to uncover mechanisms of toxicity, leading to the toxic phenotype that was observed. By finding protein interactors within the host and testing if the phage gene is essential for phage infection, I would obtain precious clues to help us define the role and importance of those genes.

Goal I: identify protein network linked to a gene of interest.

Phage proteins have a specific role. From being part of the structural integrity of the phage, to hijack the host machinery, numerous interactions within the host occur, which may lead to some toxicity. As I uncovered 18 out of 28 toxic genes with no known function, finding their target within the host is essential to understanding the role they play within the phage life cycle. Using techniques such as a 2-hybrid assay or Immunoprecipitation, I could uncover some of the key characteristics such as phage-host protein binding, which would give us clues about the function that said gene possesses.

Immunoprecipitation uses the presence of an affinity tag to allow for the pull-down of the tagged gene and associated proteins in the host. While this technique may be interesting, highly toxic genes, such as Bromden_75 won't be able to follow this workflow, as they lyse completely cells during the recovery process.

An alternative, such as the 2-hybrid assay uses interaction between the host protein, coming from a library of the host proteome, and the gene product of interest, in another host. The binding

of both proteins starts the transcription of antibiotic-resistant genes and the breakdown of X-gal, creating blues colonies. This work is currently ongoing in our lab, with the hope of understanding gp75's toxicity secret.

Goal II: modify Bromden's genome and observe phenotypic changes

As phage genomes range from 8kb to over 400kb, some of their genes have essential functions for the infection of the host and the survival of the virus. As we don't know the possible impact of NKF genes, a way to gain clues about their importance during the phage infection is to remove them from the phage genome and observe the phenotype changes that would occur.

CRISPY-BRED or CRIPSR-based Bacteriophage Recombineering of Electroporated DNA is a genetic technique allowing for the genetic manipulation of phage DNA^{130–132}. This tool will allow us to edit Bromden's genome, by mutating or removing genes and test the resulting infection. The phenotype that could be observed following a gene deletion/mutation in Bromden could be a) no changes in infection, meaning that the gene is not mandatory for phage life, but could lead to immunity interaction with bacteria to prevent further phage infection, b) partial reduction of the infection, showing that the gene would be important for phage life but not unreplaceable by another pathway, with functions ranging from phage replication, DNA binding, assembly of phage particles, c) total reduction of phage infection, indicating that this gene is essential for the phage, and that the resulting product is not viable, d) increase in the infection (more virulent/lytic phage) which could happen if you delete an important lysogeny gene.

I would like to use this tool to mutate Bromden 75 and observe if the resulting phage infection is impacted, as Nicotinate Ribosyltransferase showed an extreme level of toxicity against the host. I'm expecting that the mutant S276A, previously shown to allow for partial recovery of the host, will lead to a decrease in the infection of Bromden. Furthermore, testing the gene deletion of highly toxic genes would allow us to understand if those are key players in the phage infection.

2. Immunity

What is immunity?

Immunity genes are key players in phage infection and superinfection. When a phage infects its host and enters the lysogenic cycle, the expression of specific genes can provide prophage-mediated immunity which protects the lysogen from infection by other phages and increases the survival of the host. Some immunity genes, like the immunity repressor (Bromden 39) are well-defined and are required for maintenance of lysogeny^{29,120}. Other immunity mechanisms are poorly defined, so exploring and finding immunity genes is important to further our understanding of both phage life cycles.

Bromden infection at 37°C is unreliable

With poor Bromden infection on the uninduced plate at 37°C, and non-existent one when induced, it could be thought that Bromden shows signs of immunity against the tested gene present in Figure 13. However, the plasmid used, pExTra01 doesn't contain any gene, and thus should show no immunity against phage infection.

I discovered that the ideal infection temperature for Bromden is 32°C⁸⁷. I confirmed this by measuring the titer of our lysate, using a spot test and calculating the E.O.P. Bromden's infection is up to 100 more efficient at lower temperature, while also creating clearer plaques, indicating that the phage is acting in a less temperate manner.

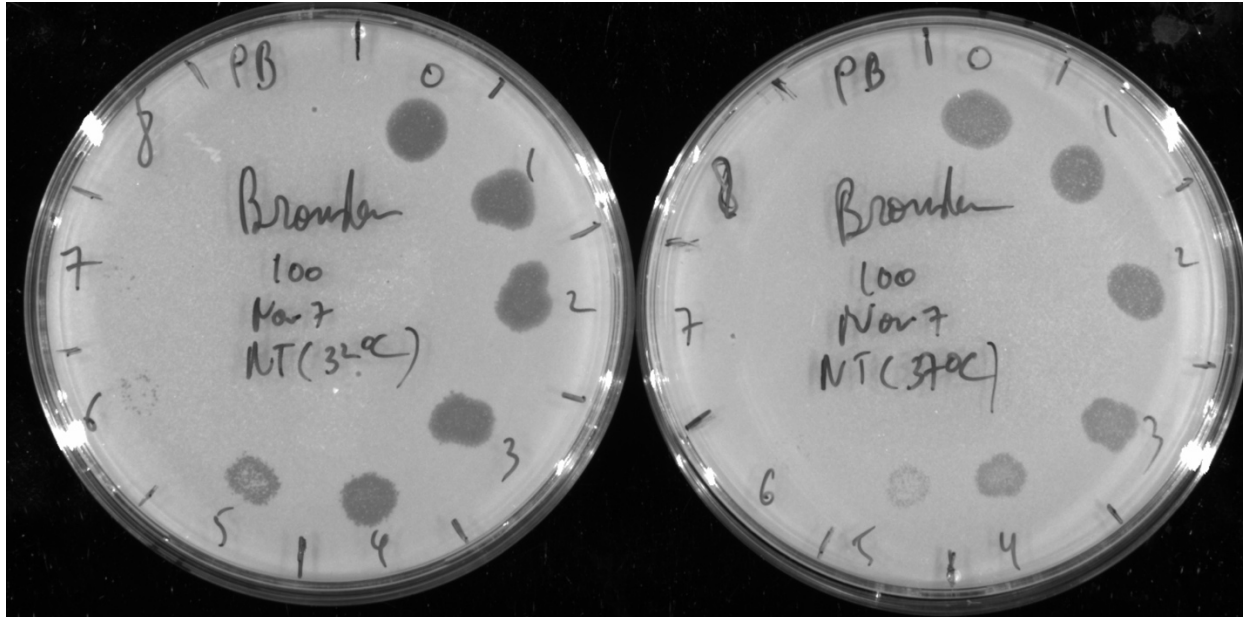


Figure 18. Spot test of Bromden's lysate at 32°C and 37°C on *M. smegmatis*. Both plates were created in parallel, using the same phage dilutions and incubated for 36h. The 32°C plate (on the left) shows clearer plaques and better infection (up to 10^{-7}), while the plate at 37°C (on the right) shows more turbid plaques, with infection up to 10^{-5} .

However, after reducing the temperature for my assay to 35°C, I observed a severe growth defect in *M. smeg* containing a plasmid at this temperature, which impacted both the reproducibility of the experiment and the calculation of the E.O.P. for all phages. I hypothesize that this growth defect is either caused by the requirement of the cells to maintain an episomal plasmid or due to the presence of kanamycin as the antibiotic selection. As a result, immunity results have been stopped to troubleshoot this assay.

Troubleshooting

I explored the possibility of an issue with the Kan resistance cassette present on the plasmid. As the temperature decreases, cells may get more sensitive to Kanamycin, which leads to an uneven bacterial lawn. This was quickly dismissed after comparing the pExTra plasmid with others present in the Jim Sun lab (pMS2¹³³; pMS2kan¹³³; pMN437¹³⁴ and pMV261^{105,106}), as all plasmids share the same Kan cassette (Figure 19), origin of replication and were commonly left to incubate at room temperature, still allowing for even bacterial growth.

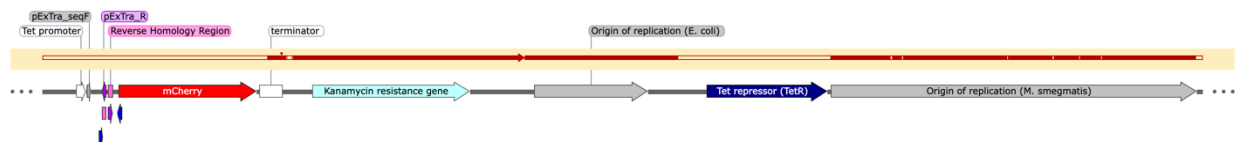


Figure 19. Alignment of pExTra with pMV261. Obtained using SnapGene¹¹². The top strand contains pMV261 sequence while pExTra is present at the bottom. With this alignment, we can observe that the Kanamycin Resistance gene is identical between both plasmids. Other attributes, such as the origin of replication in *E. coli* and *M. smeg* are also fairly similar, with some modifications present on the latest.

While creating more lysate for Bromden, I noticed a low frequency of clear plaques, which I suspected might be virulent mutants of Bromden (Figure 20). Anas Khoja, an honours student in the lab, has purified several independent clear plaques and confirmed they are genetic mutants. These mutants are not yet sequenced, but they offer a new hope to produce the immunity assays for Bromden's genes.

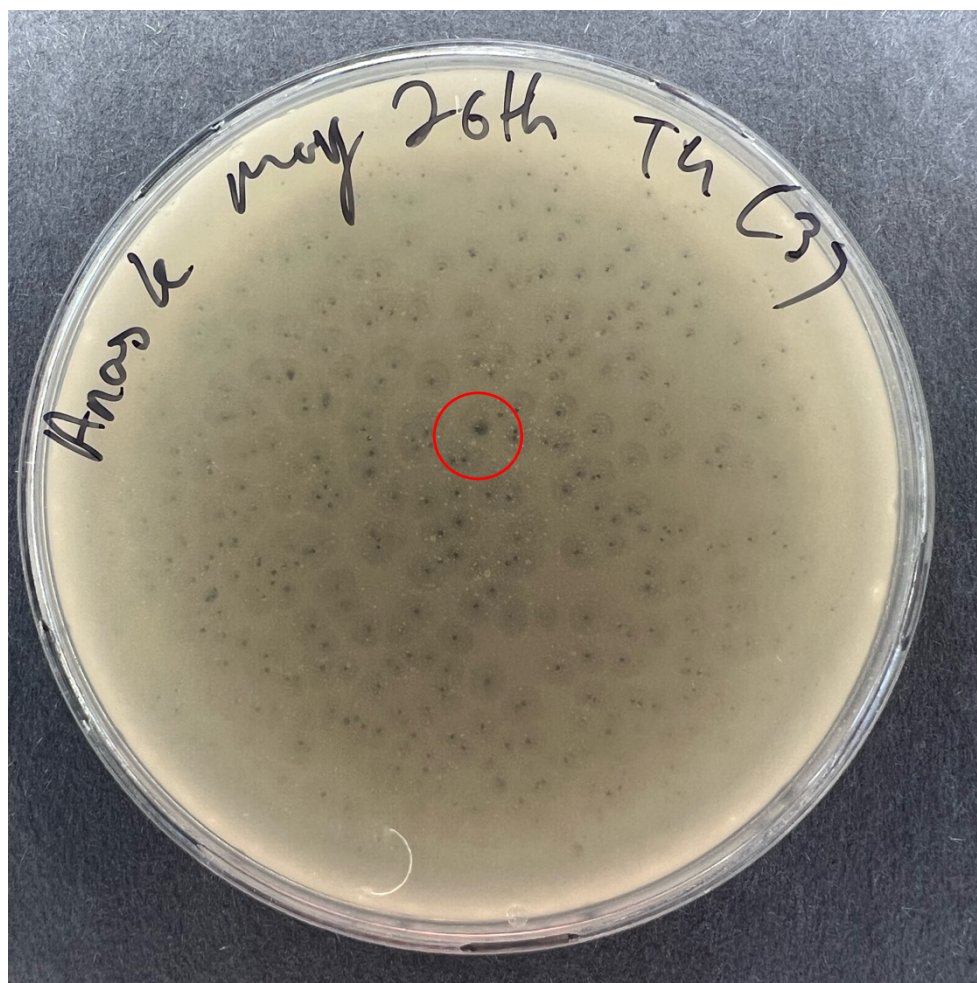


Figure 20. Bromden infection leads to the creation of clear plaque mutants. Image from Anas Khoja. While making a webbed plate to obtain more lysate, you can observe that some of Bromden's plaques are bigger and clearer than others (highlighted in red is one of the clear plaque mutants). This plaque was picked and went through our phage isolation protocol to obtain a pure lysate of those clear plaque mutants.

3. *Host range in Mycobacterium tuberculosis*

Phages showing potential against *M. tb* will be subject to further study, with an initial focus on sequencing these phages to determine if they are related to known therapeutic phages (ZoeJ and Muddy^{28,30}). One of the key analyses will be to find common characteristics between these phages and others previously used to treat *M. tb* infection. These phages could be great candidates for phage therapies and screening for mutants with an expanded host range on pathogenic isolates.

We observe that Bromden (#26 and #27 in Table 2) does not infect *M. tb*. However, one of the clear plaque mutants that was isolated while troubleshooting our immunity assays was able to infect *M. tb* lux, albeit with an EOP of 0.00001. I am currently testing whether this low level of infection reflects a low frequency of mutants with an expanded host range, or if the infection is simply caused by “killing from without”, a poorly understood phenomenon in which infection at high MOI causes host lethality, without a productive infection^{135,136}.

Confirm results in M. tb + test phages isolated between June 2023 and today

In addition to re-testing the previously isolated phages, an additional 30 Mycobacteriophages have been added to the uOttawa database since my assay in 2023. These phages have not been tested on *M. tb*, making their host range unknown. Furthermore, it would be interesting to expand the host range by testing other Mycobacterium (*M. abscessus*, *M. leprea*) which are known to cause diseases in humans, against which Mycobacteriophages have proven effective.

Conclusion

With this project, I was able to create a library of plasmid integrating the genes present in the bacteriophage Bromden, and I successfully screened that library against its host bacteria. Out of this screening, I found 14 genes moderately impacting bacterial growth, and 14 genes severely impacting the bacteria. Moreover, 2 genes were found to exhibit extreme cytotoxicity, impacting their host before their induction. These genes have been subjected to further studies, both by investigating the promoter and using Alphafold + Dali to uncover their protein folding^{110,111}.

I tried to complete the immunity assay for Bromden's genes but faced issues with Bromden infection. This issue is being addressed by obtaining a clear plaque mutant of this phage that could be used to test the phenotype.

With the help of Zach M., I successfully tested uOttawa phage library towards *M. tuberculosis* and found 7 phages able to infect *M. tb*, which could lead to future therapies. I found out that Bromden is not a suitable candidate for standalone phage therapy. However, a few candidates from our collection showed great potential to become possible treatment options.

Altogether, there is still a lot of work to be done before we can fully understand how Bromden works. However, the data in this thesis may help us understand about 3700 phages which contain genes homologous to those in Bromden.

Tables

Table 3. Bromden gene function list. Compiled from the data available on Bromden's NCBI GeneBank record (accession [MH576973](#)). tRNAs have been included in this table, but highlighted in grey as they were not part of this study.

Gene number	Function	Gene number	Function	Gene number	Function	Gene number	Function	Gene number	Function
1	Terminase, small subunit	20	Minor tail protein	41	Excise	78	RusA-like resolvase (endonuclease)	116	tRNA-Tyr
4	Terminase, large subunit	21	Minor tail protein	44	Membrane protein	79	WhiB transcriptional factor	118	tRNA-Leu
5	Portal protein	25	Hydrolase	47	Metallophosphoesterase	80	WhiB transcriptional factor	119	tRNA-His
6	Capsid maturation protease	27	Lysin A	50	Band-7-like membrane protein	82	DNA binding protein	120	tRNA-Ile
7	Scaffolding Protein	28	Lysin B	54	RNA-binding protein	84	SsDNA binding protein	121	tRNA-Gln
8	Major capsid protein	29	Holin	56	Membrane protein	88	RNA ligase	122	tRNA-Gly
9	Head to tail adaptor	30	Holin	60	Exonuclease	89	HNH endonuclease	123	tRNA-Lys
10	Head to tail stopper	31	Membrane protein	61	Endonuclease VII	90	ClpP-like protease	124	tRNA-Ala
12	Tail Terminator Protein	32	NrdH glutaredoxin	63	ERF family ssDNA binding protein	96	HNH endonuclease	125	Nuclease
13	HNH endonuclease	33	Membrane protein	64	Erf-like ssDNA annealing protein	99	DnaQ-like DNA polymerase III subunit	129	tRNA-Asn
14	Major tail protein	34	Nucleotidyl transferase	67	HNH endonuclease	103	HNH endonuclease		
15	Tail assembly chaperone	35	Lsr2-like DNA bridging protein	70	DnaB-like replicative helicase	104	endonuclease VII		
16	Tail assembly chaperone	37	Tyrosine integrase	71	Transcriptional regulator WhiB-like	110	tRNA-Leu		
17	Tape measure protein	38	Membrane protein	74	Phosphoribosyl transferase	111	tRNA-Thr		
18	Minor tail protein	39	Immunity repressor	75	Nicotinate ribosyl-transferase	114	tRNA-Pro		
19	Minor tail protein	40	Cro	76	Polynucleotide kinase	115	tRNA-Trp		

Table 4. Primer information for the amplification, clone verification and sequencing purposes of pExTra. Information provided by the SEA-GENES consortium.

Primer Name	Primer Sequence (5' to 3')	Use in Research
pExtra UniF	ATGAGCACGATCCGCATGCG	pExTra clone verification (PCR)
pExTra_UniR	CCCTTCGAGACCATAGATCTGTTCC	pExTra clone verification (PCR and sequencing)
pExTra SeqF	GTACCCGTGTGTACGACCAGC	pExTra clone verification (sequencing)
pExTra F	GTCGACACTCGAGTCGGATCCTG	Amplification of pExTra backbone
pExTra R	ATGGAAGTGATTCTCCGCATGC	Amplification of pExTra backbone

Table 5. List of primers used for the PCR amplification of Bromden's genes. This primer list was prepared by HHMI and sent to the Rudner lab as part of the Bromden package. Those primers were diluted to 10 μ M using mqH₂O and pre-mixed for their lab use.

Name	Forward primer sequence (5' to 3')	Name	Reverse primer sequence (5' to 3')
oBromden 1F	atgcggaggaaatcaactccatATGCCGCCACGGAAGAG	oBromden 1R	tgcaggatccgactcgaggtgcacTCAGCCCGCCTTCTCG
oBromden 2F	atgcggaggaaatcaactccatATGGGCGCGGTGATC	oBromden 2R	tgcaggatccgactcgaggtgcacTCATTCCGGGCAATCAATGC
oBromden 3F	atgcggaggaaatcaactccatATGCCCGAATGATGCAG	oBromden 3R	tgcaggatccgactcgaggtgcacTCAAACATCACCGAAGTCTCTC
oBromden 4F	atgcggaggaaatcaactccatATGGCAATGACCGCCGTC	oBromden 4R	tgcaggatccgactcgaggtgcacTCAAACCATCTGTACCGCTTCTTC
oBromden 5F	atgcggaggaaatcaactccatATGATCGAACTTCCCGACG	oBromden 5R	tgcaggatccgactcgaggtgcacTCATGCCCGCCTACTC
oBromden 6F	atgcggaggaaatcaactccatATGGCTGAGGACGAC	oBromden 6R	tgcaggatccgactcgaggtgcacTCAAGCGATCTTAGTTTCC
oBromden 7F	atgcggaggaaatcaactccatATGTCTGATCTAGATACTGATAACG	oBromden 7R	tgcaggatccgactcgaggtgcacTCACTGAGAGTCGCC
oBromden 8F	atgcggaggaaatcaactccatATGGCGCACATTTTGTGAAGC	oBromden 8R	tgcaggatccgactcgaggtgcacTCACGGAACGGTGACC
oBromden 9F	atgcggaggaaatcaactccatATGGCGGTCTAGCG	oBromden 9R	tgcaggatccgactcgaggtgcacTCACCAAGGGACCAC
oBromden 10F	atgcggaggaaatcaactccatATGAACGACGAGACTTTGACCGTC	oBromden 10R	tgcaggatccgactcgaggtgcacTCAGGCGTTACCCGCC
oBromden 11F	atgcggaggaaatcaactccatATGCCCGCTCGCGGTC	oBromden 11R	tgcaggatccgactcgaggtgcacTCAATTGAACAGCTCGCCATTACG
oBromden 12F	atgcggaggaaatcaactccatATGACGGTCTGTTTCCCGATTG	oBromden 12R	tgcaggatccgactcgaggtgcacTCATGAACCGCGCAAAGAC
oBromden 13F	atgcggaggaaatcaactccatATGGGGTGTGTTGGAATGC	oBromden 13R	tgcaggatccgactcgaggtgcacTCAATCGTGTAATAATCCGTCATC
oBromden 14F	atgcggaggaaatcaactccatATGACGGATTTTACACGATTAAGG	oBromden 14R	tgcaggatccgactcgaggtgcacTCAAGCGGTGACAGTG
oBromden 16F	atgcggaggaaatcaactccatATGGGTGAAGCTGAACCTTAGCGAG	oBromden 16R	tgcaggatccgactcgaggtgcacTCATAGGTAATCCACTCCGCAGC
oBromden 15F	atgcggaggaaatcaactccatATGGGTGAAGCTGAACCTAGC	oBromden 15R	tgcaggatccgactcgaggtgcacTCACTTCCGAAAAAAGTGCTCG
oBromden 17F	atgcggaggaaatcaactccatATGGCTGAATTTGTGCGGCC	oBromden 17R	tgcaggatccgactcgaggtgcacTCAAGCTTCGCGCAGG
oBromden 18F	atgcggaggaaatcaactccatATGACCGATTTTCTCAAGATCG	oBromden 18R	tgcaggatccgactcgaggtgcacTCAGCGCTGACGACGC
oBromden 19F	atgcggaggaaatcaactccatATGGCTAAACAATGGACCGACATTC	oBromden 19R	tgcaggatccgactcgaggtgcacTCAACTCTGAACAATGTGGACACCC
oBromden 20F	atgcggaggaaatcaactccatATGGCTAAAACGCAAGCAGAGTCC	oBromden 20R	tgcaggatccgactcgaggtgcacTCAGCCCTTACCCCGCTC
oBromden 21F	atgcggaggaaatcaactccatATGACGATGCCGAATGGC	oBromden 21R	tgcaggatccgactcgaggtgcacTCAAACACCGTCTGCCCCG
oBromden 22F	atgcggaggaaatcaactccatATGCTGAGTTTACGCAAAACCGTC	oBromden 22R	tgcaggatccgactcgaggtgcacTCACGCCGCCCGGTAC
oBromden 23F	atgcggaggaaatcaactccatATGAGCTGGGCGGTGAC	oBromden 23R	tgcaggatccgactcgaggtgcacTCATTGATAAGCCCGATACCAAGCC
oBromden 24F	atgcggaggaaatcaactccatATGGCTTTTACTACAGGCGCTAAG	oBromden 24R	tgcaggatccgactcgaggtgcacTCACGCCGCCCGCTAAG
oBromden 25F	atgcggaggaaatcaactccatATGCTGAGCGTCGGACCAATTATG	oBromden 25R	tgcaggatccgactcgaggtgcacTCAAACACCGTCTTAGCTTCCC
oBromden 26F	atgcggaggaaatcaactccatATGGGATTCATTGAGTCTCTAATTG	oBromden 26R	tgcaggatccgactcgaggtgcacTCACTTAAAAAAGCCGCCG
oBromden 27F	atgcggaggaaatcaactccatATGGCTCTATTGGGACAACCTGACG	oBromden 27R	tgcaggatccgactcgaggtgcacTCACGAGCGGCTTGTGCTG
oBromden 28F	atgcggaggaaatcaactccatATGGCTGCCGGTCCG	oBromden 28R	tgcaggatccgactcgaggtgcacTCAGAACTCTTAGATAATC
oBromden 29F	atgcggaggaaatcaactccatATGGTATATGACGAGCAGTTGCC	oBromden 29R	tgcaggatccgactcgaggtgcacTCACTACCTTCTTCTAGCTTCG
oBromden 30F	atgcggaggaaatcaactccatATGAAGATTTTCGGGCAACAAGCC	oBromden 30R	tgcaggatccgactcgaggtgcacTCACGCTCTTAGATAGAAGGCCG
oBromden 31F	atgcggaggaaatcaactccatATGAAGCGGAGATTGTTAGG	oBromden 31R	tgcaggatccgactcgaggtgcacTCACTTCAAATGTCCAATTCC
oBromden 32F	atgcggaggaaatcaactccatATGAAGTAACGGTTTACTCACCC	oBromden 32R	tgcaggatccgactcgaggtgcacTCAAGCCGCAAGTGGC
oBromden 33F	atgcggaggaaatcaactccatATGAATCCAGATGTTTCGTC	oBromden 33R	tgcaggatccgactcgaggtgcacTCACGCAAGACACCGG
oBromden 34F	atgcggaggaaatcaactccatATGAGATTAATCCAGTGATCGTGG	oBromden 34R	tgcaggatccgactcgaggtgcacTCATGCGGCGCATCC
oBromden 35F	atgcggaggaaatcaactccatATGGGTAAAGAAAGTACCG	oBromden 35R	tgcaggatccgactcgaggtgcacTCAAGACTGACCGGC
oBromden 36F	atgcggaggaaatcaactccatATGACCTTCTACGTGACATG	oBromden 36R	tgcaggatccgactcgaggtgcacTCAGTTGGTGTGCTCAACTGCTG
oBromden 37F	atgcggaggaaatcaactccatATGGCCGAAAGCGAAGC	oBromden 37R	tgcaggatccgactcgaggtgcacTCAAACGTTCCGGCAGCAG
oBromden 38F	atgcggaggaaatcaactccatATGATCGCTCAACTCAGCCACG	oBromden 38R	tgcaggatccgactcgaggtgcacTCAGACCGCGCCGTC
oBromden 39F	atgcggaggaaatcaactccatATGTGCCAGTCCGGAAAAACC	oBromden 39R	tgcaggatccgactcgaggtgcacTCACAGAGGGGCTATCTGTG
oBromden 40F	atgcggaggaaatcaactccatATGTGTATGTTTCTCTCGTGCCG	oBromden 40R	tgcaggatccgactcgaggtgcacTCACAGGATTTCGGGACAC
oBromden 41F	atgcggaggaaatcaactccatATGACTGCATCCACAGCGG	oBromden 41R	tgcaggatccgactcgaggtgcacTCACTGGTCTACCTCCAGG
oBromden 42F	atgcggaggaaatcaactccatATGACCACGAAAGTTGACGGCG	oBromden 42R	tgcaggatccgactcgaggtgcacTCATTCTGCTGCTCTCGTGTAC
oBromden 43F	atgcggaggaaatcaactccatATGATGTCCAGCCTTACCAGAAC	oBromden 43R	tgcaggatccgactcgaggtgcacTCAAGCACTTGATCGGACGTGAAC
oBromden 44F	atgcggaggaaatcaactccatATGCTTAGAGACAACGTTGTCTG	oBromden 44R	tgcaggatccgactcgaggtgcacTCACCATCCCGCGAAC
oBromden 45F	atgcggaggaaatcaactccatATGACCTTGAATACACGCTATGTAC	oBromden 45R	tgcaggatccgactcgaggtgcacTCAGATCAGGATTTGCGTC
oBromden 46F	atgcggaggaaatcaactccatATGACGGTTATTTACCTGG	oBromden 46R	tgcaggatccgactcgaggtgcacTCATAGGTAGTCTGTCCC
oBromden 47F	atgcggaggaaatcaactccatATGGAACCGGAAGT	oBromden 47R	tgcaggatccgactcgaggtgcacTCATTCTGCTGCTCAAC
oBromden 48F	atgcggaggaaatcaactccatATGGTGGAGGTCGCGG	oBromden 48R	tgcaggatccgactcgaggtgcacTCAGACAGGATTTGCTG
oBromden 49F	atgcggaggaaatcaactccatATGCAAGCGCGGTATCGG	oBromden 49R	tgcaggatccgactcgaggtgcacTCAACGGGTGAGCATGTGCG
oBromden 50F	atgcggaggaaatcaactccatATGGAAGGAAACCTTTGAACCG	oBromden 50R	tgcaggatccgactcgaggtgcacTCAGCGAGCCGGAGC
oBromden 51F	atgcggaggaaatcaactccatATGTCGAAAAAGGCC	oBromden 51R	tgcaggatccgactcgaggtgcacTCACAGCAAGCAAGAG
oBromden 52F	atgcggaggaaatcaactccatATGACTCTAAGACTTAAAGTCTTCG	oBromden 52R	tgcaggatccgactcgaggtgcacTCATGCGTTTCACTCTTAAC
oBromden 53F	atgcggaggaaatcaactccatATGAGCGTTAAGAAGCGCACACC	oBromden 53R	tgcaggatccgactcgaggtgcacTCACTTAGTGGCTTCGACGTGG
oBromden 54F	atgcggaggaaatcaactccatATGACTACCTTTTTCAG	oBromden 54R	tgcaggatccgactcgaggtgcacTCACGCAAGCATATTTGC
oBromden 55F	atgcggaggaaatcaactccatATGCCAGGACCAATGTCCG	oBromden 55R	tgcaggatccgactcgaggtgcacTCATTCTTGTGTCCTTTCCGCTC
oBromden 56F	atgcggaggaaatcaactccatATGAAGGCTCTCGTTATCGTGG	oBromden 56R	tgcaggatccgactcgaggtgcacTCAGCCATTGATCTTTAACTTC
oBromden 57F	atgcggaggaaatcaactccatATGGCTAGCAAGCTGGTTGTAGAG	oBromden 57R	tgcaggatccgactcgaggtgcacTCAGCCGCCCGCCTG
oBromden 58F	atgcggaggaaatcaactccatATGACGTACCGCGTTG	oBromden 58R	tgcaggatccgactcgaggtgcacTCAGCCATCTTTGTTTCTCTAAC

oBromden 59F	atgctggaggaatcaacttccatATGGCTAAGCCTGTTGGCAC	oBromden 59R	tcaggatccgactcgaggtgctgacTCAGTCTCGCTAACCTTCGAG
oBromden 60F	atgctggaggaatcaacttccatATGGACGCTCCGCG	oBromden 60R	tcaggatccgactcgaggtgctgacTCACCACTGGTAGTGATCTAGGTTTC
oBromden 61F	atgctggaggaatcaacttccatATGACCGCTGCCCGC	oBromden 61R	tcaggatccgactcgaggtgctgacTCATATAGACCCCTCTAATTCAACG
oBromden 62F	atgctggaggaatcaacttccatATGACCACTGATACGAGTTTGGC	oBromden 62R	tcaggatccgactcgaggtgctgacTCAGGCCCCGCTCTTTTG
oBromden 63F	atgctggaggaatcaacttccatATGACGGCACTAAGGCTGATC	oBromden 63R	tcaggatccgactcgaggtgctgacTCAGACGGTAACCGCCC
oBromden 64F	atgctggaggaatcaacttccatATGTTCAACAAACACAATTGCGGG	oBromden 64R	tcaggatccgactcgaggtgctgacTCACAGAGCCTGCCCTCG
oBromden 65F	atgctggaggaatcaacttccatATGACTGATGTTCTACCCG	oBromden 65R	tcaggatccgactcgaggtgctgacTCACCTTGACGAACCTCCAC
oBromden 66F	atgctggaggaatcaacttccatATGAGTGAATACAATCCGGTCTCCG	oBromden 66R	tcaggatccgactcgaggtgctgacTCACCACTCGCCACGG
oBromden 67F	atgctggaggaatcaacttccatATGGCGAGTGGTGAG	oBromden 67R	tcaggatccgactcgaggtgctgacTCAACCCCGTTCTCC
oBromden 68F	atgctggaggaatcaacttccatATGTCCAAAAGCCTCAGGGC	oBromden 68R	tcaggatccgactcgaggtgctgacTCATCCACGCGGAGAC
oBromden 69F	atgctggaggaatcaacttccatATGAGCGACTTCGGGAAAATC	oBromden 69R	tcaggatccgactcgaggtgctgacTCAAGCAGCAACTCTCCC
oBromden 70F	atgctggaggaatcaacttccatATGACCGAGCCTGACCGAG	oBromden 70R	tcaggatccgactcgaggtgctgacTCATGTGATGCGCTGATAGTGG
oBromden 71F	atgctggaggaatcaacttccatATGAGCGCTGAGATTAAGG	oBromden 71R	tcaggatccgactcgaggtgctgacTCAATCCGATTACGTAACGC
oBromden 72F	atgctggaggaatcaacttccatATGGACGCGTTACTG	oBromden 72R	tcaggatccgactcgaggtgctgacTCAATCACGTGGGTAG
oBromden 73F	atgctggaggaatcaacttccatATGGCTTACGCTTACAGCAATGC	oBromden 73R	tcaggatccgactcgaggtgctgacTCAGGCAACGCTCTAGTGG
oBromden 74F	atgctggaggaatcaacttccatATGGCCGTTCATCTAAGGGC	oBromden 74R	tcaggatccgactcgaggtgctgacTCAGTCATCACTAAGCCCTTAACC
oBromden 75F	atgctggaggaatcaacttccatATGACTAAGTTTGCGCCGTTG	oBromden 75R	tcaggatccgactcgaggtgctgacTCATCCCTGCTGAGCTAAAGTGC
oBromden 76F	atgctggaggaatcaacttccatATGACCTTGACTCTTACAGCAATGC	oBromden 76R	tcaggatccgactcgaggtgctgacTCAAAAATCTCCGGGCTGCAC
oBromden 77F	atgctggaggaatcaacttccatATGCGTTGGACGAACCTGGTC	oBromden 77R	tcaggatccgactcgaggtgctgacTCAGTCCCGACCTTGG
oBromden 78F	atgctggaggaatcaacttccatATGGAGCACCTGTCTAAC	oBromden 78R	tcaggatccgactcgaggtgctgacTCACGCCAATTCCGATC
oBromden 79F	atgctggaggaatcaacttccatATGACCGATCCGAATTGGC	oBromden 79R	tcaggatccgactcgaggtgctgacTCAGGCAATACCGCAATC
oBromden 80F	atgctggaggaatcaacttccatATGAGCAAGACACCGGGC	oBromden 80R	tcaggatccgactcgaggtgctgacTCAAGCAGCATTTTCCCCC
oBromden 81F	atgctggaggaatcaacttccatATGCTGCTTAGCACCACGATAAAGC	oBromden 81R	tcaggatccgactcgaggtgctgacTCACGCATCGCGCGG
oBromden 82F	atgctggaggaatcaacttccatATGGGATTAGAGCCGCGTTG	oBromden 82R	tcaggatccgactcgaggtgctgacTCAGGTGAACCGCCACG
oBromden 83F	atgctggaggaatcaacttccatATGCACAAACGCGGGG	oBromden 83R	tcaggatccgactcgaggtgctgacTCAGGCACTAATCTTCTCTTATC
oBromden 84F	atgctggaggaatcaacttccatATGCCTAACGCTGAAATTAC	oBromden 84R	tcaggatccgactcgaggtgctgacTCAGAACGCGGAGGAGC
oBromden 85F	atgctggaggaatcaacttccatATGTCTCACGAAAAAATTCCGTG	oBromden 85R	tcaggatccgactcgaggtgctgacTCAATCCCGCCACGAG
oBromden 86F	atgctggaggaatcaacttccatATGAGAGAACGTTGGGGC	oBromden 86R	tcaggatccgactcgaggtgctgacTCAAAGCAGCCTCAACC
oBromden 87F	atgctggaggaatcaacttccatATGACAACTAGGCGAATCTTAG	oBromden 87R	tcaggatccgactcgaggtgctgacTCATTCGCTCTCACATTTG
oBromden 88F	atgctggaggaatcaacttccatATGAGCTTAGACGCGCCAG	oBromden 88R	tcaggatccgactcgaggtgctgacTCAGGCGGCTGACGTAAC
oBromden 89F	atgctggaggaatcaacttccatATGAATAAGCCCTTCAAGCGGATAG	oBromden 89R	tcaggatccgactcgaggtgctgacTCACGAGTCATCTTCTAAATCC
oBromden 90F	atgctggaggaatcaacttccatATGACGGTCGATATTAAGGACGC	oBromden 90R	tcaggatccgactcgaggtgctgacTCAGCTAGTCCCTCAATCC
oBromden 91F	atgctggaggaatcaacttccatATGGCATATGACGATTACTTTGG	oBromden 91R	tcaggatccgactcgaggtgctgacTCAGGCAATAGCCGC
oBromden 92F	atgctggaggaatcaacttccatATGGCTGATCGGTTTGCATGG	oBromden 92R	tcaggatccgactcgaggtgctgacTCAATTCGTCCTCCACATCG
oBromden 93F	atgctggaggaatcaacttccatATGAGGTACACGCTTCTAGCTGG	oBromden 93R	tcaggatccgactcgaggtgctgacTCAGGCGGCTCTCGGCC
oBromden 94F	atgctggaggaatcaacttccatATGTCGCTAGGTGG	oBromden 94R	tcaggatccgactcgaggtgctgacTCAGCCATTACTCCCC
oBromden 95F	atgctggaggaatcaacttccatATGGCTAGCTTGGAACTTAAGCAC	oBromden 95R	tcaggatccgactcgaggtgctgacTCAGAGAGGCTTCTCAAGCATCAC
oBromden 96F	atgctggaggaatcaacttccatATGTGGATTGATAGCCCG	oBromden 96R	tcaggatccgactcgaggtgctgacTCAAATGCTTCTGGAAGCTG
oBromden 97F	atgctggaggaatcaacttccatATGAAGCCGTCAGACTTTGC	oBromden 97R	tcaggatccgactcgaggtgctgacTCAATTTCTTCCCCAAGCG
oBromden 98F	atgctggaggaatcaacttccatATGACCACTAAGGACGCTATTAAAC	oBromden 98R	tcaggatccgactcgaggtgctgacTCACGCGAACGCCCCG
oBromden 99F	atgctggaggaatcaacttccatATGAGCGCCCGCTC	oBromden 99R	tcaggatccgactcgaggtgctgacTCAGATGGCTCAATTCGGTAG
oBromden 100F	atgctggaggaatcaacttccatATGAAGCCGTTGCGC	oBromden 100R	tcaggatccgactcgaggtgctgacTCACTTGATCCAGCAGGAG
oBromden 101F	atgctggaggaatcaacttccatATGTCCGGCCCGAAGTTTG	oBromden 101R	tcaggatccgactcgaggtgctgacTCAACGAGCAGGCTTAAC
oBromden 102F	atgctggaggaatcaacttccatATGAGTAAGAGGCGAG	oBromden 102R	tcaggatccgactcgaggtgctgacTCACGCTTGTCTTCT
oBromden 103F	atgctggaggaatcaacttccatATGCCAGGGAATCTCCGC	oBromden 103R	tcaggatccgactcgaggtgctgacTCACGCAAAACAGTCCCG
oBromden 104F	atgctggaggaatcaacttccatATGAGGCAACCCACGTC	oBromden 104R	tcaggatccgactcgaggtgctgacTCAAGCGTCCCGTTCTTCT
oBromden 105F	atgctggaggaatcaacttccatATGTCTGACGAATACGATCC	oBromden 105R	tcaggatccgactcgaggtgctgacTCACTCATCAGCGAATC
oBromden 106F	atgctggaggaatcaacttccatATGAGTGAGGTTTACAAGTC	oBromden 106R	tcaggatccgactcgaggtgctgacTCAAGTGCATTTCGGC
oBromden 107F	atgctggaggaatcaacttccatATGCCGAATGCACTTAACCTTTGGG	oBromden 107R	tcaggatccgactcgaggtgctgacTCACTCCCTCCGACG
oBromden 108F	atgctggaggaatcaacttccatATGGCGTTGATTTCTGATCCGC	oBromden 108R	tcaggatccgactcgaggtgctgacTCAGTGTCTTAACGTGGTTCAGCG
oBromden 109F	atgctggaggaatcaacttccatATGTGTGGTGAGCCTGAGG	oBromden 109R	tcaggatccgactcgaggtgctgacTCACCGCTTCTCTCGCG
oBromden 112F	atgctggaggaatcaacttccatATGAGTATTCAGAAGCCCG	oBromden 112R	tcaggatccgactcgaggtgctgacTCATACACGCGGAGG
oBromden 113F	atgctggaggaatcaacttccatATGGGTTTGATTTCTGATCCCG	oBromden 113R	tcaggatccgactcgaggtgctgacTCAGTTGCGGACCTTCG
oBromden 117F	atgctggaggaatcaacttccatATGTCTACGTTACAATTACCGCG	oBromden 117R	tcaggatccgactcgaggtgctgacTCACGTACACGCGCTG
oBromden 125F	atgctggaggaatcaacttccatATGTCTGAGGTTAAGAGCCTTACG	oBromden 125R	tcaggatccgactcgaggtgctgacTCAAGCTCAACCGCC
oBromden 126F	atgctggaggaatcaacttccatATGGGCGGTCGCGGTG	oBromden 126R	tcaggatccgactcgaggtgctgacTCAATTTTCTTCTTACCGCGC
oBromden 127F	atgctggaggaatcaacttccatATGGGAGCGTTTCGAGAAC	oBromden 127R	tcaggatccgactcgaggtgctgacTCACAAAACACAAACAGTTGCGTAC
oBromden 128F	atgctggaggaatcaacttccatATGTGGGTTGTTGGATGAAGACG	oBromden 128R	tcaggatccgactcgaggtgctgacTCAATTTCTCTCCATCCACAGC
oBromden 130F	atgctggaggaatcaacttccatATGACCGCACACCCCTTAC	oBromden 130R	tcaggatccgactcgaggtgctgacTCAAAAGTCTCTTATCCACTTGT
oBromden 131F	atgctggaggaatcaacttccatATGGCATGGCGTAGCGC	oBromden 131R	tcaggatccgactcgaggtgctgacTCAATCCACACAGCCCAAC

Table 6. List of primers designed to mutate Bromden's genes. Primers for gp75 and gp127 stop mutants are included in this table (STOP site is present on red). Those primers were diluted to 10 μ M using mqH₂O for downstream applications.

Name	Forward primer sequence (5' to 3')	Name	Reverse primer sequence (5' to 3')
gp75STOP_F	atgctggaggaatcaacttccatATG TAG AAGTTTGCGCCGCTTGCGG	oBromden 75R	tcaggatccgactcgaggtgctgacTCATCCCTGCTGAGCTAAAGTGC
gp75 D221N_F	CACAGGGACGAATTCGCTGGTGG	gp75 D221N_R	CCACCAGCGAATTCGCTCCCTGTG
gp75 S241A_F	GAGTACGTAGCCGGGCGCCGTCGCCGCTACCG	gp75 S241A_R	CGGTAGCGGGCACGGCGCCGCTACGTACTC
gp75 H247Y_F	CGCTACCGAGTACTCGGTGATGT	gp75 H247Y_R	ACATACCGGAGTACTCGGTAGCG
gp75 S276A_F	CGTCCGGAATCGTCGCGGTGGTTAGCGACACG	gp75 S276A_R	CGTGTGCTAACCACGGCGACGATTCCGGACG
gp127STOP_F	atgctggaggaatcaacttccatATG TAG GCGTTTCGAGAACGCCCTTCT	oBromden_127R	tcaggatccgactcgaggtgctgacTCACAAAACACAAACAGTTGCGTAC

Table 7. Primers used for internal sequencing. Includes only Bromden's gene inserts superior to 1500 base pairs. Primers constructed with the help of Primer3¹⁰⁰⁻¹⁰² and NCBI GenBank⁹⁵ Bromden record (Accession MH576973.1).

Primer Name	Primer sequence (5' to 3')
gp4 F sequencing	CCGAGGATTCCGAGATGCTG
gp5 F sequencing	GGTCGAGTATCTGGTGCCTG
gp17 F sequencing 1	TGAAGATTTGAACGCCAGC
gp17 F sequencing 2	CGGTTTCGACGGGTTTCATTGA
gp17 F sequencing 3	CTTAAGGGCGAGATGGACGG
gp17 F sequencing 4	GGACACGACGCTACAGAACT
gp17 F sequencing 5	TTAGTCCTGGGGTTGCTTCG
gp17 F sequencing 6	GGGTTACAACGATCCGCAGT
gp19 F sequencing	ATTGGTCGGATTGGCAGTGT
gp21 F sequencing 1	CGGACGGCACAATTAAGGAC
gp21 F sequencing 2	TTTCCTCGACCTGCTTTGGG

Table 8. PCR reactions guidelines for reactions. Following the adjustments made to the NEB and SEA-GENES protocols available for those enzymes. These reactions were used for the construction of the plasmid library of Bromden.

	Q5 DNA Polymerase		Taq DNA Polymerase	
Use	PCR amplification of the inserts		PCR confirmation of inserts post-assembly	
PCR Step	Temperature	Duration	Temperature	Duration
a. Initial Denaturation	95 °C	1 min	94 °C	5 min
b. Denaturation	95 °C	10 sec	94 °C	15 sec
c. Annealing	60 °C	10 sec	60 °C	15 sec
d. Extension	72 °C	30 sec/kb	68 °C	1 min/kb
Repeat PCR Steps b -d for 31 cycles				
e. Final Extension	72 °C	5 min	68 °C	5 min
f. Hold	4-12 °C	infinite	4-12 °C	infinite

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Contributions of Collaborators

This thesis represents my work, with the addition of various collaborators over the past few years.

First, I would like to thank Danielle Heller and the HHMI team for their previous work, guidance and support along this project. Their various protocols were the blueprint of this work and helped streamline many of our experiments. Furthermore, she provided essential component, such as the pExTra plasmids, controls and consumables (enzymes, *E. coli* cells) as well as her time in the form of a short bootcamp to get us up to speed with the SEA-GENES project.

Some of the controls such as the pExTra01; 02 and 03 + the backbone were prepared by Elizabeth Williams, our TMM lab tech. She also helped me manage the plates required for the various experiments, helping me focus on other tasks such as creating more plasmids, creating datacards or taking a deeper look at the literature.

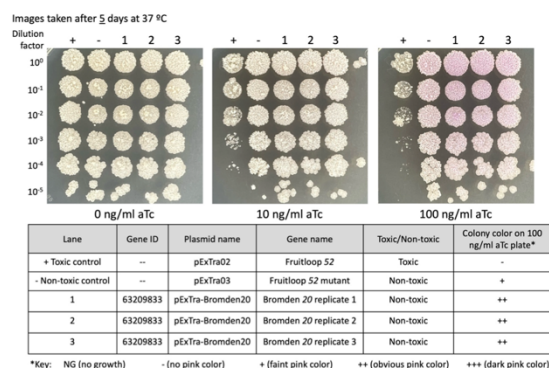
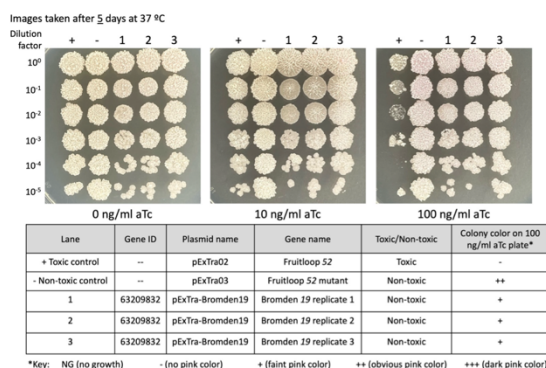
I would also like to give credit to Erika Z. and the SEA-GENES rotation students 2023 in the Rudner lab for their help in the construction of plasmid for Bromden's library. The production of their plasmid, and data cards for the PCR cloning and *E. coli* transformation of inserts offered me the ability to complete my work within a 2-year time frame.

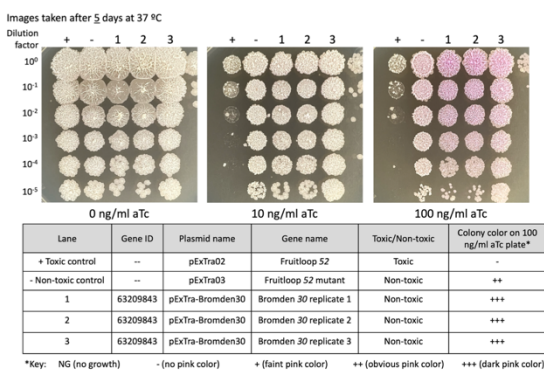
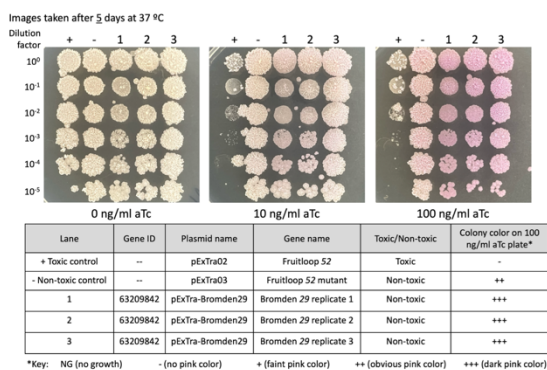
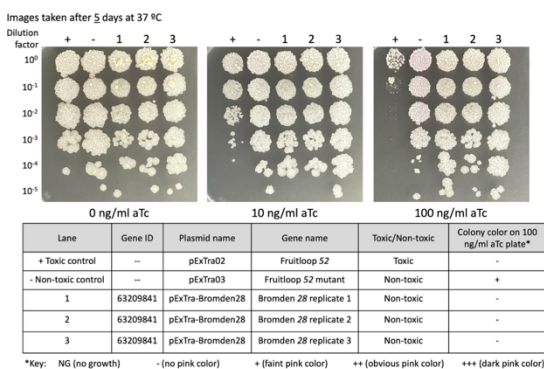
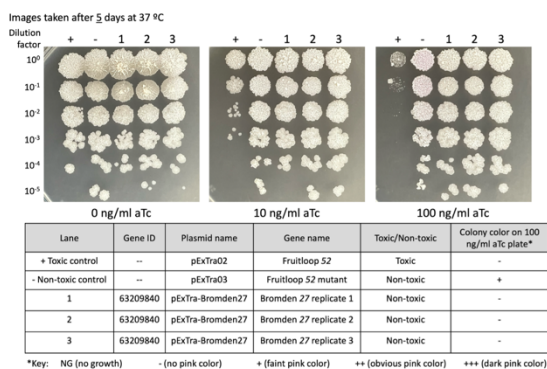
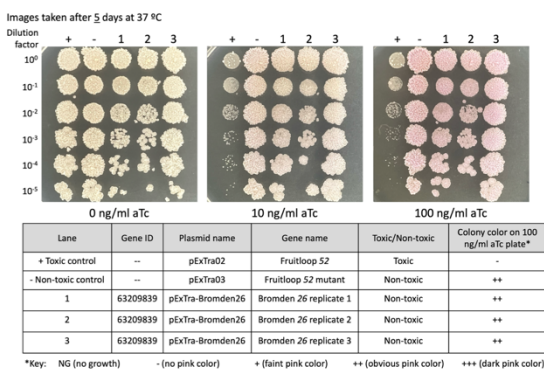
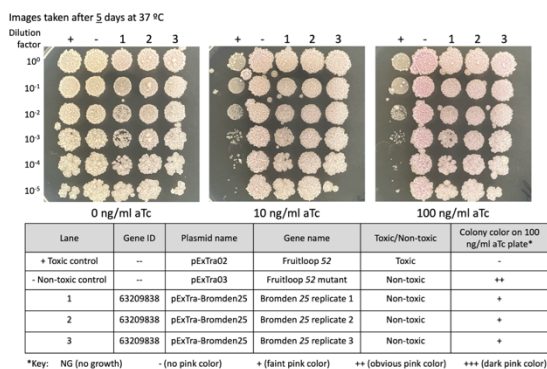
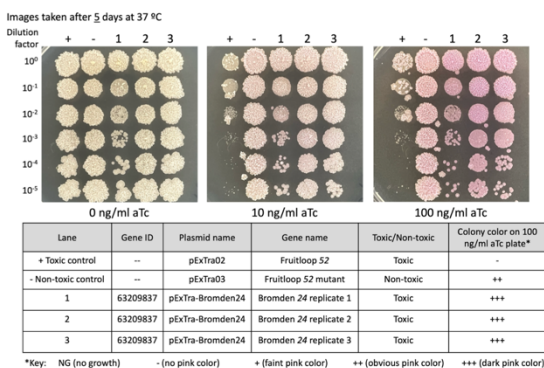
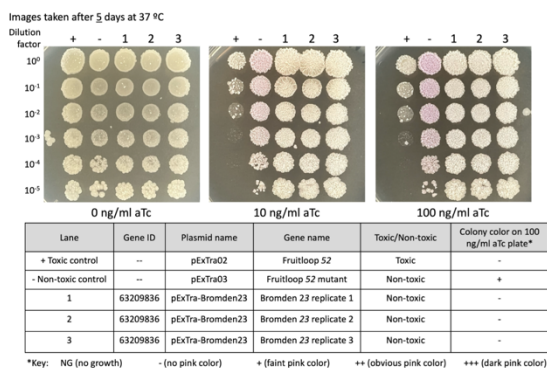
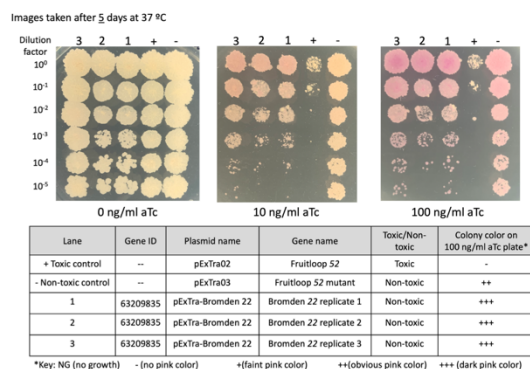
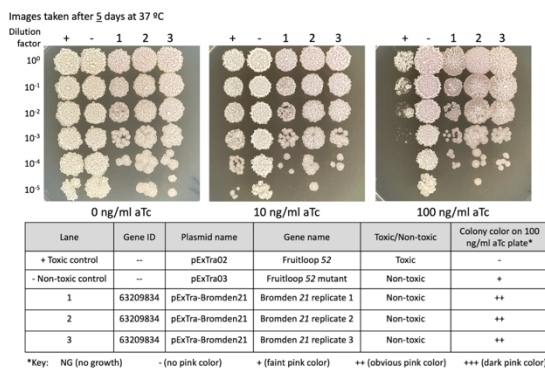
Continuing on collaborative work, I'm deeply recognisant to Jim Sun and his lab for allowing us to test our phage repertory in *M. tuberculosis*. This work would not have been possible without their logistics and expertise using this pathogenic host. I also have to thank Zach M. for all the time he spent in the biosafety cabinet to plate our phages and keeping a look on our pathogenic cultures.

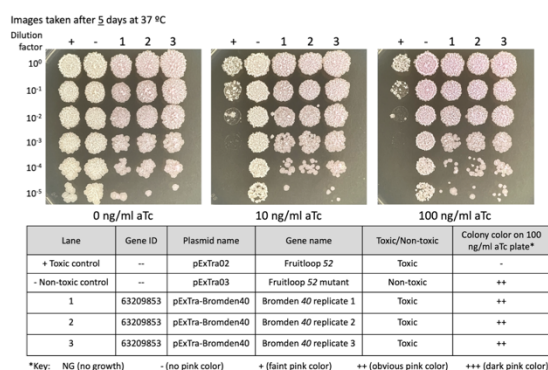
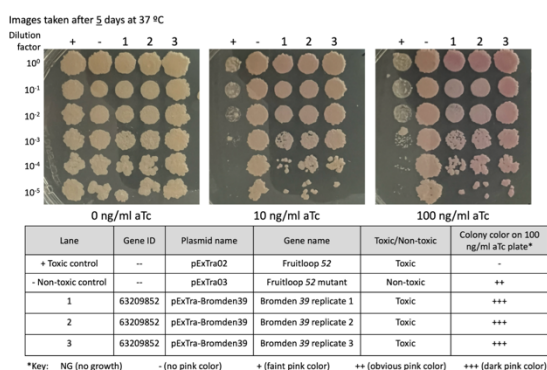
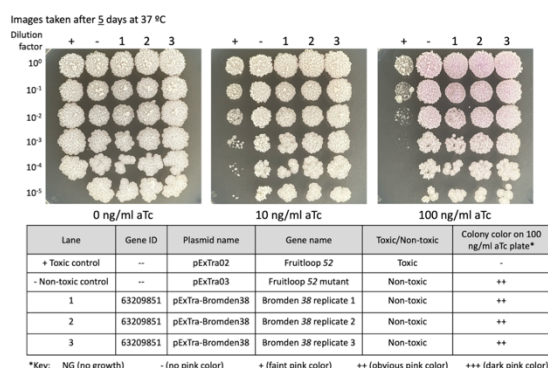
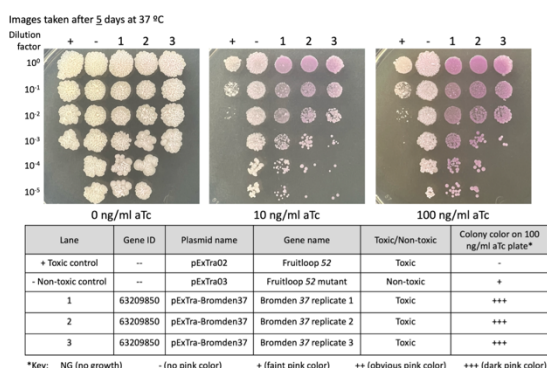
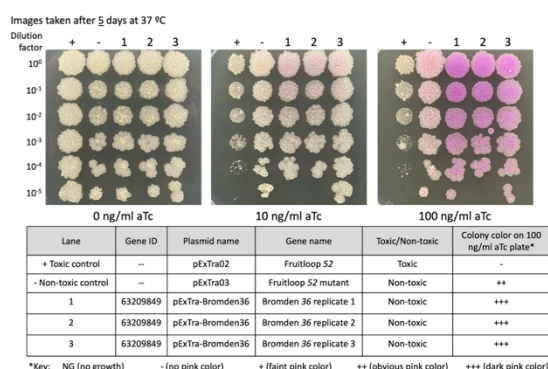
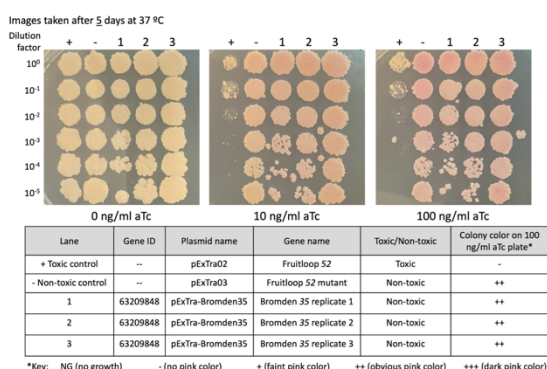
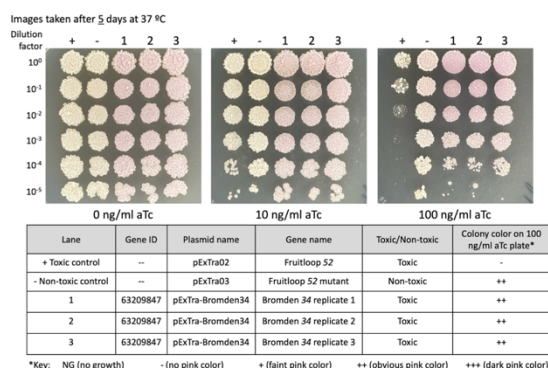
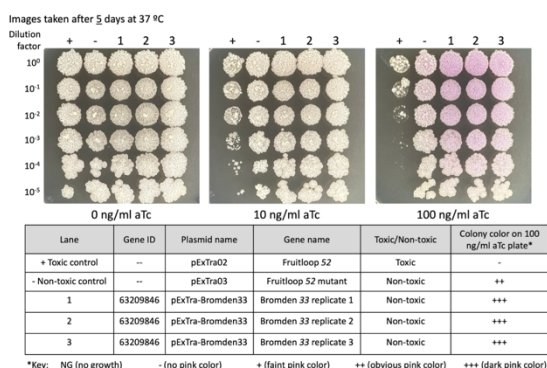
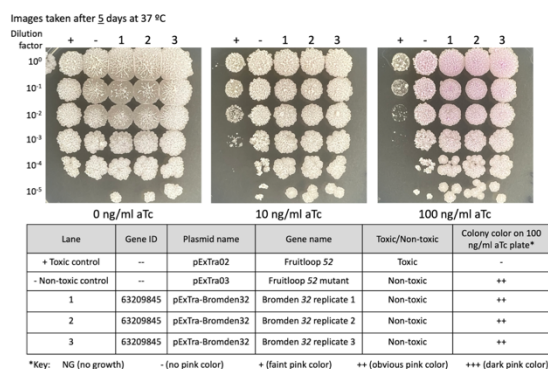
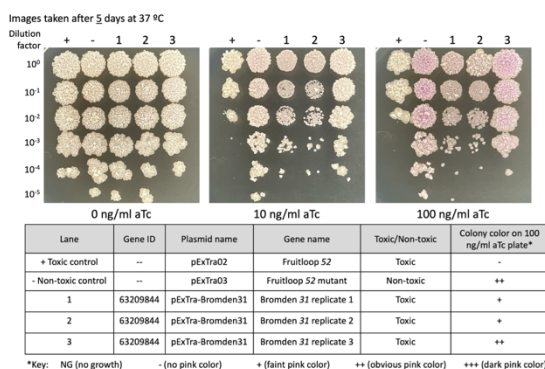
Sequencing of our plasmids was done by the researchers at Azenta and Plasmidsaurus, while I manually confirmed our clones using SnapGene and the data present on Bromden's NCBI entry.

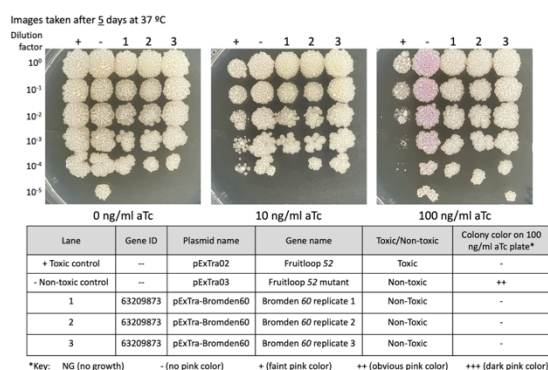
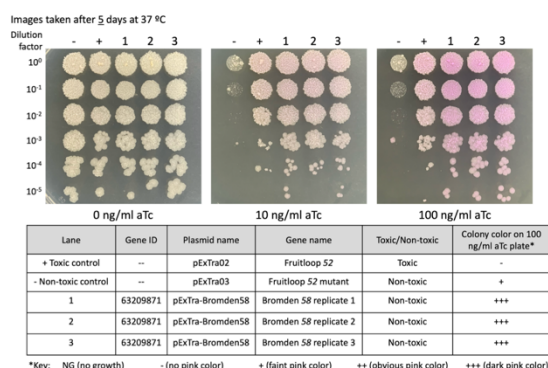
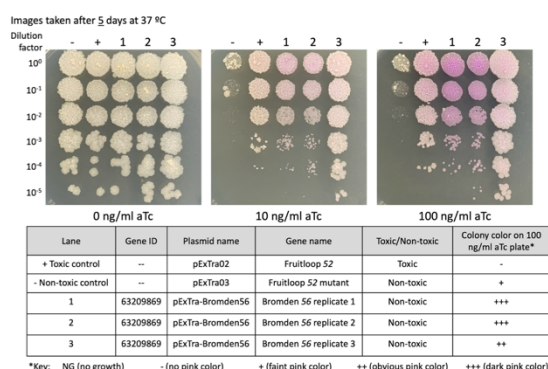
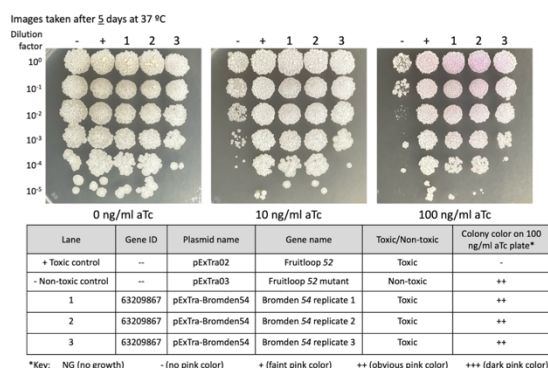
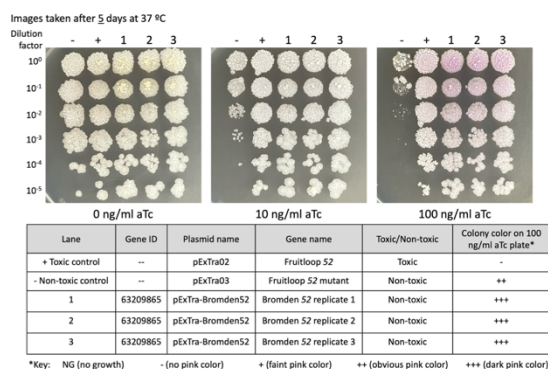
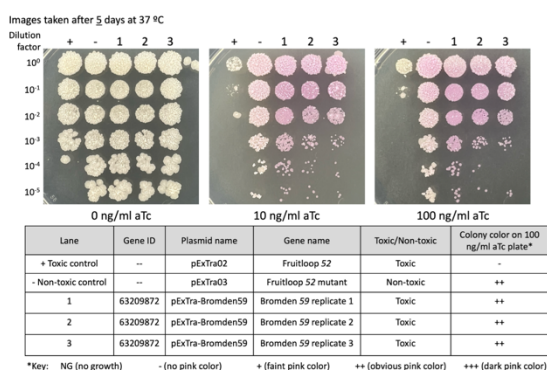
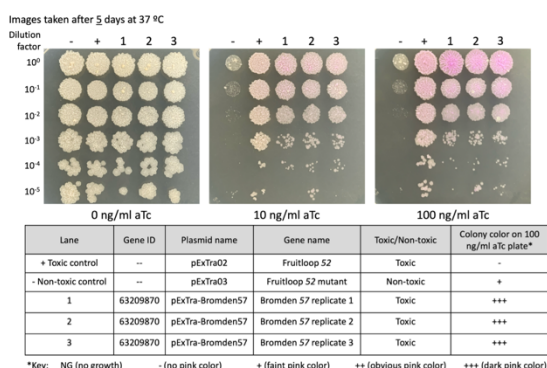
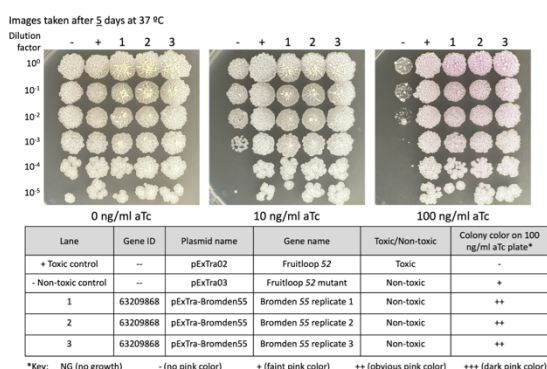
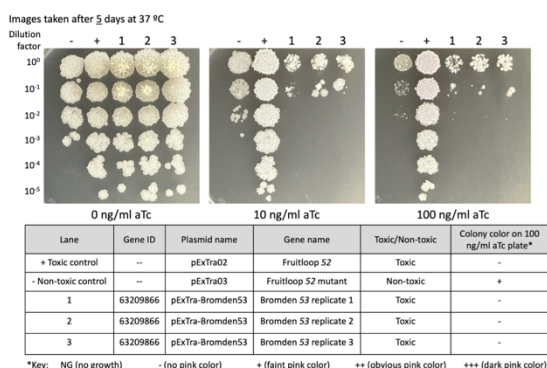
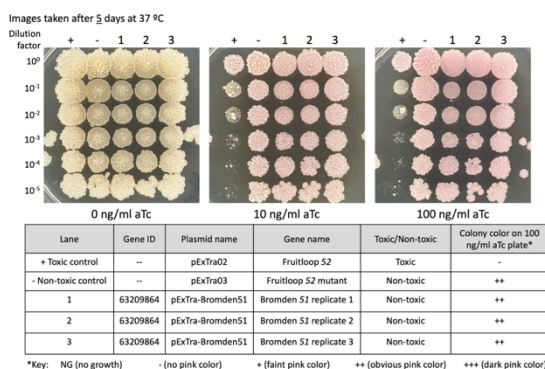
Supplemental

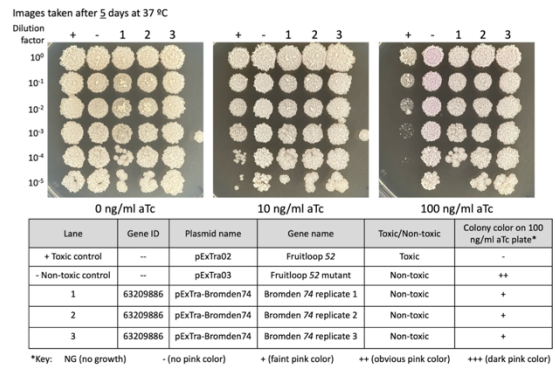
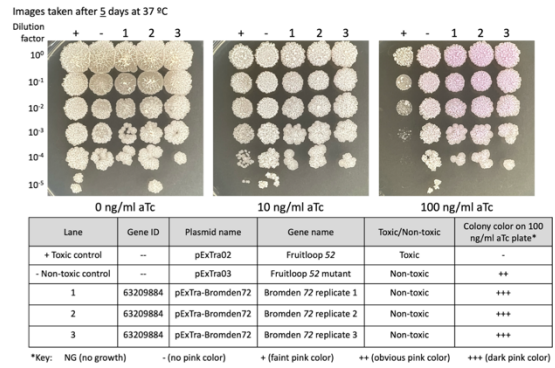
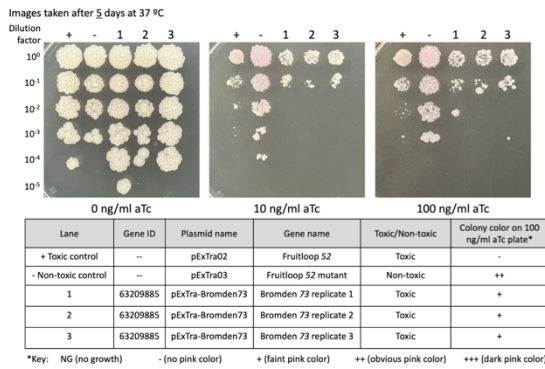
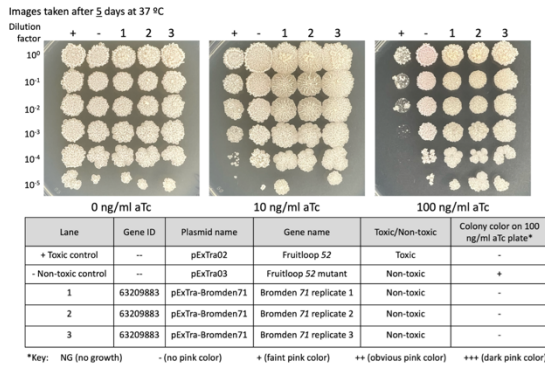
Supplemental 1. Cytotoxicity datacards collected of Bromden. All the data cards have been constructed in Microsoft PowerPoint. Plates were photographed directly after the assay and the level of mCherry induction were consigned in the lab notebook before being transferred to the table. Each cytotoxic gene has been repeated and a copy of the second data card is available upon request.



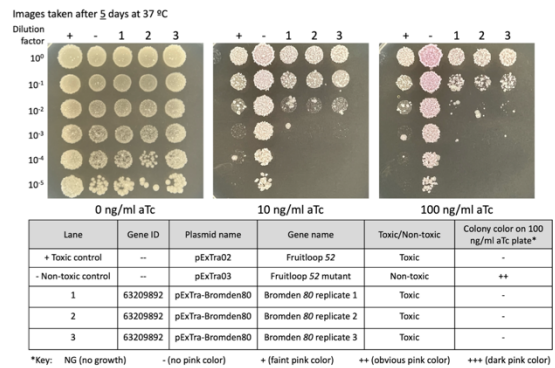
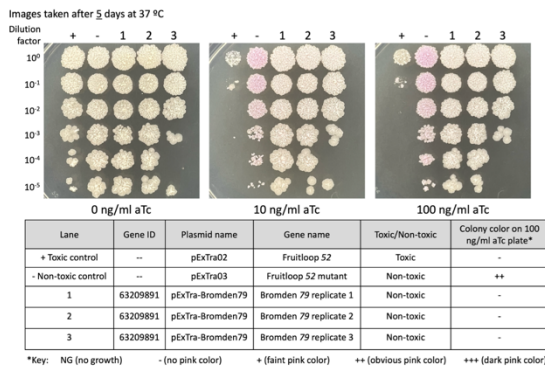
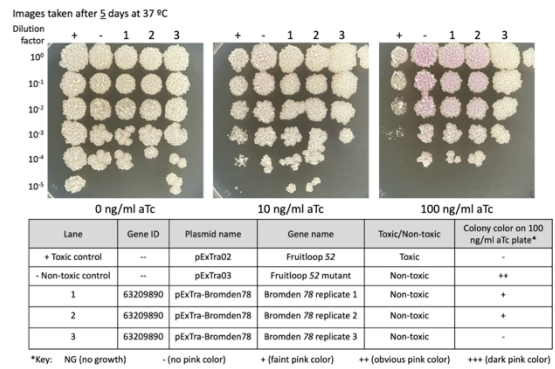
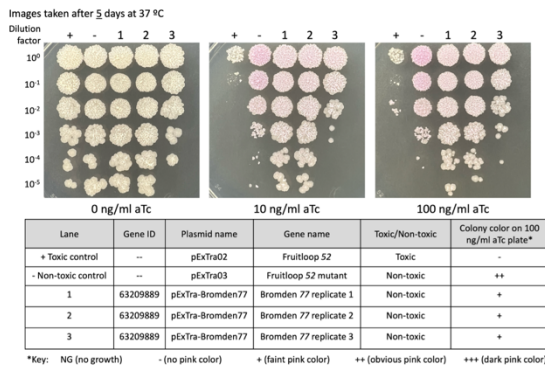
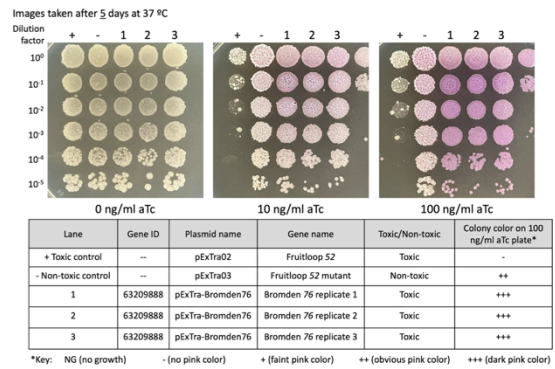








Gp75
N/A



0 ng/ml a/c		10 ng/ml a/c		100 ng/ml a/c	
Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic	Colony color on 100 ng/ml a/c plate*
+ Toxic control	–	pExTra2	Fruitleep 52	Toxic	–
- Non-toxic control	–	pExTra3	Fruitleep 52 mutant	Non-toxic	++
1	63209903	pExTra-Bromden91	Bromden 91 replicate 1	Toxic	++
2	63209903	pExTra-Bromden91	Bromden 91 replicate 2	Toxic	++
3	63209903	pExTra-Bromden91	Bromden 91 replicate 3	Toxic	++

0 ng/ml a/c		10 ng/ml a/c		100 ng/ml a/c	
Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic	Colony colour on 100 ng/ml a/c plate*
+ Toxic control	--	pExTra02	Fruitleop 52	Toxic	--
- Non-toxic control	--	pExTra03	Fruitleop S2 mutant	Non-toxic	++
1	63209905	pExTra-Bromden93	Bromden 93 replicate 1	Toxic	++
2	63209905	pExTra-Bromden93	Bromden 93 replicate 2	Toxic	++
3	63209905	pExTra-Bromden93	Bromden 93 replicate 3	Toxic	++

0 ng/ml a/c		10 ng/ml a/c		100 ng/ml a/c	
Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic	Colony color on 100 ng/ml a/c plate*
+ Toxic control	--	pExTra2	Fruitleep 52	Toxic	
- Non-toxic control	--	pExTra3	Fruitleep 52 mutant	Non-toxic	**
1	63209907	pExTra-Bromden95	Bromden 95 replicate 1	Toxic	+++
2	63209907	pExTra-Bromden95	Bromden 95 replicate 2	Toxic	+++
3	63209907	pExTra-Bromden95	Bromden 95 replicate 3	Toxic	+++

0 ng/ml a/c		10 ng/ml a/c		100 ng/ml a/c	
Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic	Colony color on 100 ng/ml a/c plate*
+ Toxic control	–	pExTra2	Fruitleep 52	Toxic	
- Non-toxic control	–	pExTra3	Fruitleep 52 mutant	Non-toxic	++
1	63209909	pExTra-Bromden97	Bromden 97 replicate 1	Non-toxic	++
2	63209909	pExTra-Bromden97	Bromden 97 replicate 2	Non-toxic	++
3	63209909	pExTra-Bromden97	Bromden 97 replicate 3	Non-toxic	++

0 ng/ml a.i.		10 ng/ml a.i.		100 ng/ml a.i.	
Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic	Colony color on 100 ng/ml a.i. plate*
+ Toxic control	–	pExTra2	Fruitleep 52	Toxic	–
- Non-toxic control	–	pExTra3	Fruitleep 52 mutant	Non-toxic	+
1	63209911	pExTra-Bromden99	Bromden 99 replicate 1	Toxic	–
2	63209911	pExTra-Bromden99	Bromden 99 replicate 2	Toxic	–
3	63209911	pExTra-Bromden99	Bromden 99 replicate 3	Toxic	–

	0 ng/ml a/c	10 ng/ml a/c	100 ng/ml a/c	
Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic
+ Toxic control	--	pTxTra2	Fruitleep52	Toxic
- Non-toxic control	--	pTxTra3	Fruitleep52 mutant	Non-toxic
1	63209904	pTxTra-Bromden92	Bromden92 replicate 1	Non-toxic
2	63209904	pTxTra-Bromden92	Bromden92 replicate 2	Non-toxic
3	63209904	pTxTra-Bromden92	Bromden92 replicate 3	Non-toxic

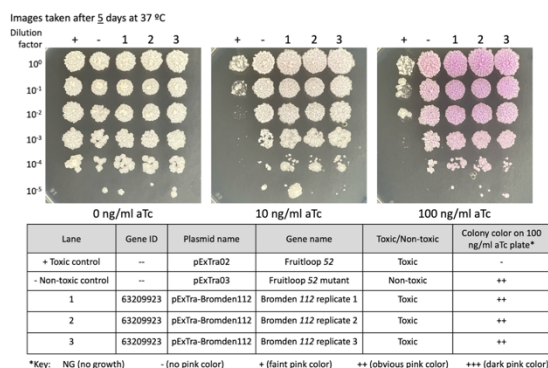
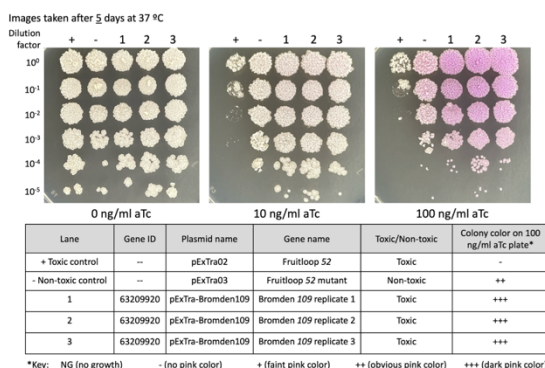
0 ng/ml a/c		10 ng/ml a/c		100 ng/ml a/c	
Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic	Colony color on 100 ng/ml a/c plate*
+ Toxic control	--	pXtra02	Fruitleop52	Toxic	-
- Non-toxic control	--	pXtra03	Fruitleop52 mutant	Non-toxic	++
1	63209906	pLxtra-Bromden94	Bromden94 replicate 1	Toxic	-
2	63209906	pLxtra-Bromden94	Bromden94 replicate 2	Toxic	-
3	63209906	pLxtra-Bromden94	Bromden94 replicate 3	Toxic	-

0 ng/ml a/c		10 ng/ml a/c		100 ng/ml a/c	
Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic	Colony color on 100 ng/ml a/c plate*
+ Toxic control	--	pTxTra2	Fruitleep52	Toxic	+
- Non-toxic control	--	pTxTra3	Fruitleep52 mutant	Non-toxic	+
1	63209908	p6xTra-Bromden96	Bromden 96 replicate 1	Non-toxic	+
2	63209908	p6xTra-Bromden96	Bromden 96 replicate 2	Non-toxic	+
3	63209908	p6xTra-Bromden96	Bromden 96 replicate 3	Non-toxic	+

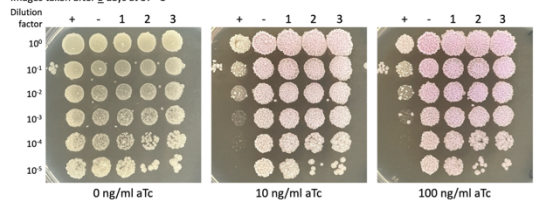
0 ng/ml a/c		10 ng/ml a/c		100 ng/ml a/c	
Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic	Colony color on 100 ng/ml a/c plate*
+ Toxic control	--	pExTra02	Fruitleep52	Toxic	-
- Non-toxic control	--	pExTra03	Fruitleep52 mutant	Non-toxic	+
1	63209910	pExTra-Bromden98	Bromden 98 replicate 1	Non-toxic	-
2	63209910	pExTra-Bromden98	Bromden 98 replicate 2	Non-toxic	-
3	63209910	pExTra-Bromden98	Bromden 98 replicate 3	Non-toxic	-

0 ng/ml a/c		10 ng/ml a/c		100 ng/ml a/c	
Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic	Colony color on 100 ng/ml a/c plate*
+ Toxic control	--	pExTra2	Fruitlet52	Toxic	-
- Non-toxic control	--	pExTra3	Fruitlet52 mutant	Non-toxic	++
1	63209912	pExTra-Bromden100	Bromden 100 replicate 1	Non-toxic	+++
2	63209912	pExTra-Bromden100	Bromden 100 replicate 2	Non-toxic	+++
3	63209912	pExTra-Bromden100	Bromden 100 replicate 3	Non-toxic	+++

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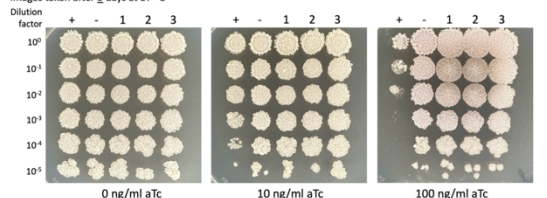
Images taken after 5 days at 37 °C



Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic	Colony color on 100 ng/ml aTc plate*
+ Toxic control	--	pExTra02	Fruitloop 52	Toxic	-
- Non-toxic control	--	pExTra03	Fruitloop 52 mutant	Non-toxic	++
1	63209924	pExTra-Bromden113	Bromden 113 replicate 1	Non-toxic	++
2	63209924	pExTra-Bromden113	Bromden 113 replicate 2	Non-toxic	++
3	63209924	pExTra-Bromden113	Bromden 113 replicate 3	Non-toxic	++

*Key: NG (no growth) - (no pink color) + (faint pink color) ++ (obvious pink color) +++ (dark pink color)

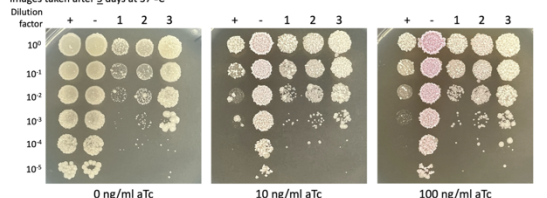
Images taken after 5 days at 37 °C



Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic	Colony color on 100 ng/ml aTc plate*
+ Toxic control	--	pExTra02	Fruitloop 52	Toxic	-
- Non-toxic control	--	pExTra03	Fruitloop 52 mutant	Non-toxic	+
1	63209936	pExTra-Bromden125	Bromden 125 replicate 1	Non-toxic	+
2	63209936	pExTra-Bromden125	Bromden 125 replicate 2	Non-toxic	+
3	63209936	pExTra-Bromden125	Bromden 125 replicate 3	Non-toxic	+

*Key: NG (no growth) - (no pink color) + (faint pink color) ++ (obvious pink color) +++ (dark pink color)

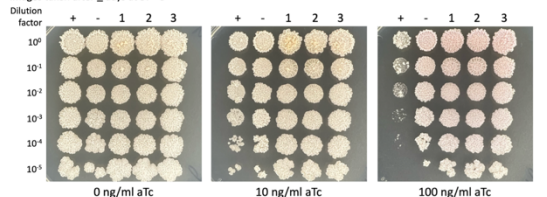
Images taken after 5 days at 37 °C



Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic	Colony color on 100 ng/ml aTc plate*
+ Toxic control	--	pExTra02	Fruitloop 52	Toxic	-
- Non-toxic control	--	pExTra03	Fruitloop 52 mutant	Non-toxic	++
1	63209938	pExTra-Bromden127	Bromden 127 replicate 1	Non-toxic	-
2	63209938	pExTra-Bromden127	Bromden 127 replicate 2	Non-toxic	-
3	63209938	pExTra-Bromden127	Bromden 127 replicate 3	Non-toxic	-

*Key: NG (no growth) - (no pink color) + (faint pink color) ++ (obvious pink color) +++ (dark pink color)

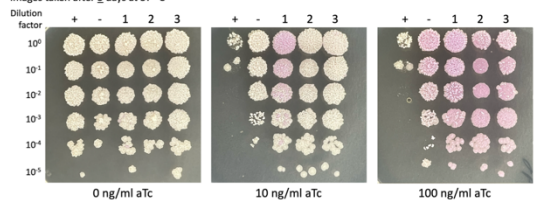
Images taken after 5 days at 37 °C



Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic	Colony color on 100 ng/ml aTc plate*
+ Toxic control	--	pExTra02	Fruitloop 52	Toxic	-
- Non-toxic control	--	pExTra03	Fruitloop 52 mutant	Non-toxic	++
1	63209941	pExTra-Bromden130	Bromden 130 replicate 1	Non-toxic	++
2	63209941	pExTra-Bromden130	Bromden 130 replicate 2	Non-toxic	++
3	63209941	pExTra-Bromden130	Bromden 130 replicate 3	Non-toxic	++

*Key: NG (no growth) - (no pink color) + (faint pink color) ++ (obvious pink color) +++ (dark pink color)

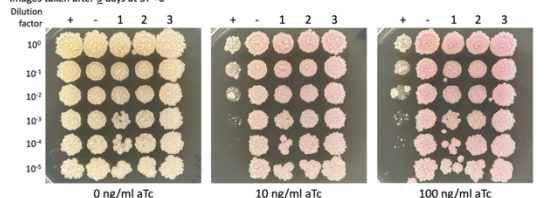
Images taken after 5 days at 37 °C



Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic	Colony color on 100 ng/ml aTc plate*
+ Toxic control	--	pExTra02	Fruitloop 52	Toxic	-
- Non-toxic control	--	pExTra03	Fruitloop 52 mutant	Non-toxic	++
1	63209928	pExTra-Bromden117	Bromden 117 replicate 1	Non-toxic	++
2	63209928	pExTra-Bromden117	Bromden 117 replicate 2	Non-toxic	++
3	63209928	pExTra-Bromden117	Bromden 117 replicate 3	Non-toxic	++

*Key: NG (no growth) - (no pink color) + (faint pink color) ++ (obvious pink color) +++ (dark pink color)

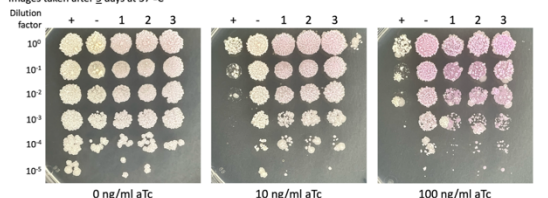
Images taken after 5 days at 37 °C



Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic	Colony color on 100 ng/ml aTc plate*
+ Toxic control	--	pExTra02	Fruitloop 52	Toxic	-
- Non-toxic control	--	pExTra03	Fruitloop 52 mutant	Non-toxic	++
1	63209937	pExTra-Bromden126	Bromden 126 replicate 1	Non-toxic	++
2	63209937	pExTra-Bromden126	Bromden 126 replicate 2	Non-toxic	++
3	63209937	pExTra-Bromden126	Bromden 126 replicate 3	Non-toxic	++

*Key: NG (no growth) - (no pink color) + (faint pink color) ++ (obvious pink color) +++ (dark pink color)

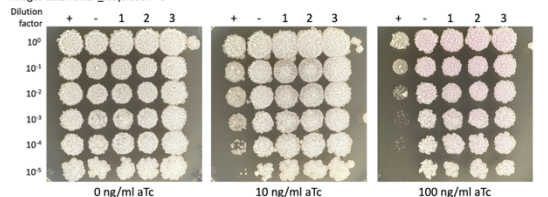
Images taken after 5 days at 37 °C



Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic	Colony color on 100 ng/ml aTc plate*
+ Toxic control	--	pExTra02	Fruitloop 52	Toxic	-
- Non-toxic control	--	pExTra03	Fruitloop 52 mutant	Non-toxic	++
1	63209939	pExTra-Bromden128	Bromden 128 replicate 1	Toxic	++
2	63209939	pExTra-Bromden128	Bromden 128 replicate 2	Toxic	++
3	63209939	pExTra-Bromden128	Bromden 128 replicate 3	Toxic	++

*Key: NG (no growth) - (no pink color) + (faint pink color) ++ (obvious pink color) +++ (dark pink color)

Images taken after 5 days at 37 °C



Lane	Gene ID	Plasmid name	Gene name	Toxic/Non-toxic	Colony color on 100 ng/ml aTc plate*
+ Toxic control	--	pExTra02	Fruitloop 52	Toxic	-
- Non-toxic control	--	pExTra03	Fruitloop 52 mutant	Non-toxic	++
1	63209942	pExTra-Bromden131	Bromden 131 replicate 1	Non-toxic	++
2	63209942	pExTra-Bromden131	Bromden 131 replicate 2	Non-toxic	++
3	63209942	pExTra-Bromden131	Bromden 131 replicate 3	Non-toxic	++

*Key: NG (no growth) - (no pink color) + (faint pink color) ++ (obvious pink color) +++ (dark pink color)

Supplemental 2. Bromden Immunity datacards collected. All the data cards have been constructed in Microsoft PowerPoint. Plates were photographed directly after the assay and EOP were calculated.

Images taken after 2 days at 37 °C

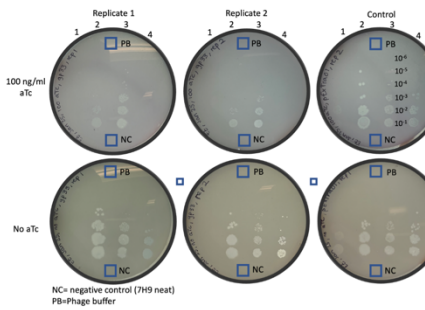


Table 1: M. smegmatis lawns			
Replicates	Plasmid name	Gene name	Gene ID
100 ng/ml aTc	pEXtra-63209846	Bromden_33	63209846
No aTc	pEXtra-63209846	mCherry only	---

Table 2: Phage lysates			
Phage name	Bromden	Archie	Finny
Titer on control plate*	~5x10 ⁸ pfu/ml	~5x10 ⁸ pfu/ml	~1.5x10 ⁸ pfu/ml
E.O.P. (replicate #1)	0	~0.02	~2
E.O.P. (replicate #2)	0	~0.1	~2

* The 100 ng/ml aTc plate results were used to calculate E.O.P.
E.O.P.= replicate plate titer/ control plate titer

Images taken after 2 days at 37 °C

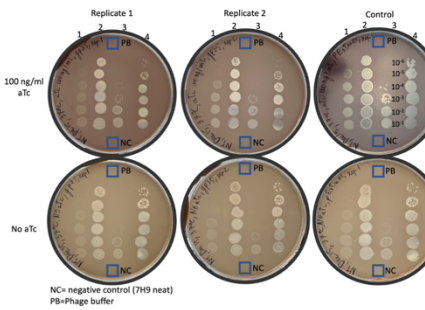


Table 1: M. smegmatis lawns			
Replicates	Plasmid name	Gene name	Gene ID
100 ng/ml aTc	pEXtra-63209848	Bromden_35	63209848
No aTc	pEXtra-63209848	mCherry only	---

Table 2: Phage lysates			
Phage name	Bromden	Archie	Finny
Titer on control plate*	~3x10 ⁸ pfu/ml	~10 ⁸ pfu/ml	~1.3x10 ⁸ pfu/ml
E.O.P. (replicate #1)	~2.5	N/A	~3.5
E.O.P. (replicate #2)	~1	N/A	~3

* The 100 ng/ml aTc plate results were used to calculate E.O.P.
E.O.P.= replicate plate titer/ control plate titer

Images taken after 2 days at 37 °C

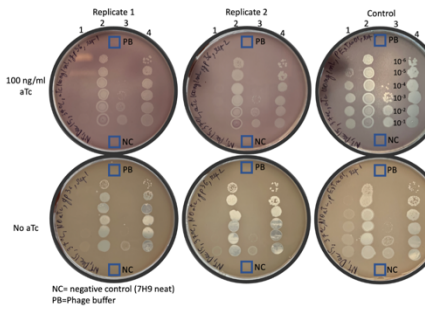


Table 1: M. smegmatis lawns			
Replicates	Plasmid name	Gene name	Gene ID
100 ng/ml aTc	pEXtra-63209849	Bromden_36	63209849
No aTc	pEXtra-63209849	mCherry only	---

Table 2: Phage lysates			
Phage name	Bromden	Archie	Finny
Titer on control plate*	~3x10 ⁸ pfu/ml	~10 ⁸ pfu/ml	~1.3x10 ⁸ pfu/ml
E.O.P. (replicate #1)	~1/3	N/A	~1
E.O.P. (replicate #2)	~0.5	N/A	~1

* The 100 ng/ml aTc plate results were used to calculate E.O.P.
E.O.P.= replicate plate titer/ control plate titer

Images taken after 2 days at 37 °C

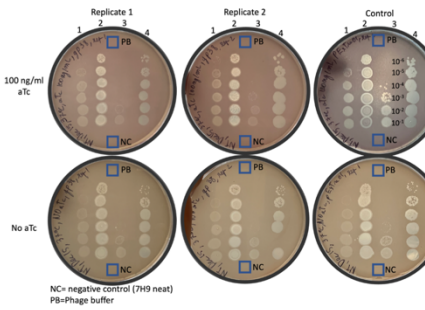


Table 1: M. smegmatis lawns			
Replicates	Plasmid name	Gene name	Gene ID
100 ng/ml aTc	pEXtra-63209851	Bromden_38	63209851
No aTc	pEXtra-63209851	mCherry only	---

Table 2: Phage lysates			
Phage name	Bromden	Archie	Finny
Titer on control plate*	~3x10 ⁸ pfu/ml	~10 ⁸ pfu/ml	~1.3x10 ⁸ pfu/ml
E.O.P. (replicate #1)	~3	N/A	~1
E.O.P. (replicate #2)	~3.5	N/A	~1/2

* The 100 ng/ml aTc plate results were used to calculate E.O.P.
E.O.P.= replicate plate titer/ control plate titer

Images taken after 2 days at 37 °C

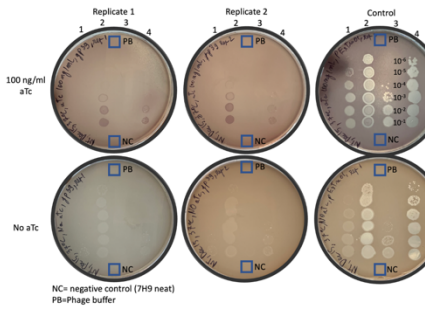


Table 1: M. smegmatis lawns			
Replicates	Plasmid name	Gene name	Gene ID
100 ng/ml aTc	pEXtra-63209852	Bromden_39	63209852
No aTc	pEXtra-63209852	mCherry only	---

Table 2: Phage lysates			
Phage name	Bromden	Archie	Finny
Titer on control plate*	~3x10 ⁸ pfu/ml	~10 ⁸ pfu/ml	~1.3x10 ⁸ pfu/ml
E.O.P. (replicate #1)	~7x10 ³	0	~7x10 ³
E.O.P. (replicate #2)	~4x10 ³	0	~1.4x10 ⁴

* The 100 ng/ml aTc plate results were used to calculate E.O.P.
E.O.P.= replicate plate titer/ control plate titer

Images taken after 2 days at 37 °C

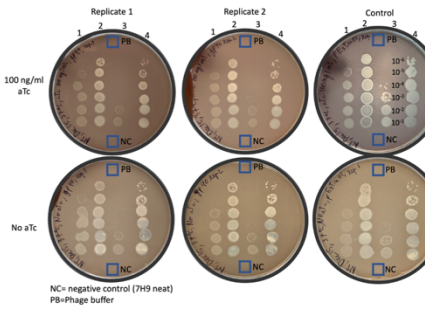


Table 1: M. smegmatis lawns			
Replicates	Plasmid name	Gene name	Gene ID
100 ng/ml aTc	pEXtra-63209853	Bromden_40	63209853
No aTc	pEXtra-63209853	mCherry only	---

Table 2: Phage lysates			
Phage name	Bromden	Archie	Finny
Titer on control plate*	~3x10 ⁸ pfu/ml	~10 ⁸ pfu/ml	~1.3x10 ⁸ pfu/ml
E.O.P. (replicate #1)	~3	N/A	~1
E.O.P. (replicate #2)	~4	N/A	~1

* The 100 ng/ml aTc plate results were used to calculate E.O.P.
E.O.P.= replicate plate titer/ control plate titer

Images taken after 2 days at 37 °C

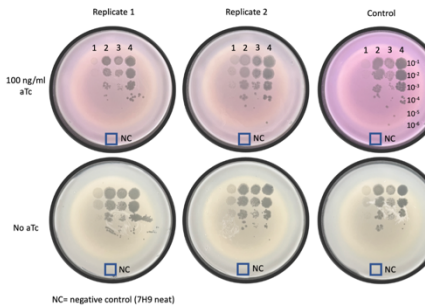


Table 1: M. smegmatis lawns			
Replicates	Plasmid name	Gene name	Gene ID
100 ng/ml aTc	pEXtra-63209863	Bromden_50	63209863
No aTc	pEXtra-63209863	mCherry only	---

Table 2: Phage lysates			
Phage name	Bromden	Archie	Finny
Titer on control plate*	~1.2x10 ⁸ pfu/ml	~1.6x10 ⁸ pfu/ml	~1.8x10 ⁸ pfu/ml
E.O.P. (replicate #1)	~10	~0.5	~5
E.O.P. (replicate #2)	~10	~0.67	~5

* The 100 ng/ml aTc plate results were used to calculate E.O.P.
E.O.P.= replicate plate titer/ control plate titer

Images taken after 2 days at 37 °C

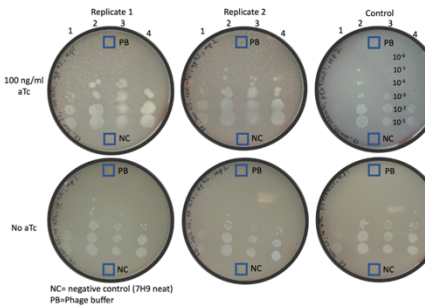


Table 1: M. smegmatis lawns			
Replicates	Plasmid name	Gene name	Gene ID
100 ng/ml aTc	pEXtra-63209855	Bromden_42	63209855
No aTc	pEXtra-63209855	mCherry only	---

Table 2: Phage lysates			
Phage name	Bromden	Archie	Finny
Titer on control plate*	~3x10 ⁸ pfu/ml	~10 ⁸ pfu/ml	~1.3x10 ⁸ pfu/ml
E.O.P. (replicate #1)	~1	~0.3	~0.5
E.O.P. (replicate #2)	~2	~1	~0.4

* The 100 ng/ml aTc plate results were used to calculate E.O.P.
E.O.P.= replicate plate titer/ control plate titer

Images taken after 2 days at 37 °C

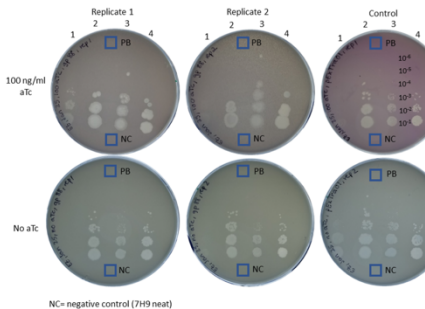


Table 1: M. smegmatis lawns			
Replicates	Plasmid name	Gene name	Gene ID
100 ng/ml aTc	pEXtra-63209900	Bromden_48	63209900
No aTc	pEXtra-63209900	mCherry only	---

Table 2: Phage lysates			
Phage name	Bromden	Archie	Finny
Titer on control plate*	4x10 ⁸ pfu/ml	8x10 ⁸ pfu/ml	1.2x10 ⁹ pfu/ml
E.O.P. (replicate #1)	0.9	0.1	3.3
E.O.P. (replicate #2)	0.5	0.035	33.3

* The 100 ng/ml aTc plate results were used to calculate E.O.P.
E.O.P.= replicate plate titer/ control plate titer

Images taken after 2 days at 37 °C

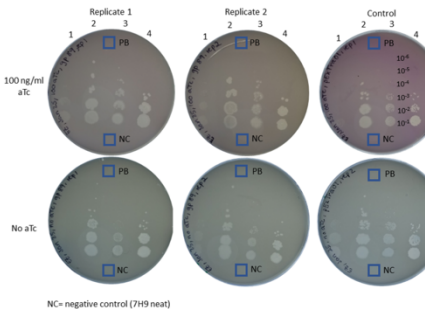


Table 1: M. smegmatis lawns			
Replicates	Plasmid name	Gene name	Gene ID
100 ng/ml aTc	pEXtra-63209901	Bromden_49	63209901
No aTc	pEXtra-63209901	mCherry only	---

Table 2: Phage lysates			
Phage name	Bromden	Archie	Finny
Titer on control plate*	4x10 ⁸ pfu/ml	8x10 ⁸ pfu/ml	1.2x10 ⁹ pfu/ml
E.O.P. (replicate #1)	3	5	3.3
E.O.P. (replicate #2)	5	1.5	2

* The 100 ng/ml aTc plate results were used to calculate E.O.P.
E.O.P.= replicate plate titer/ control plate titer

Images taken after 2 days at 37 °C

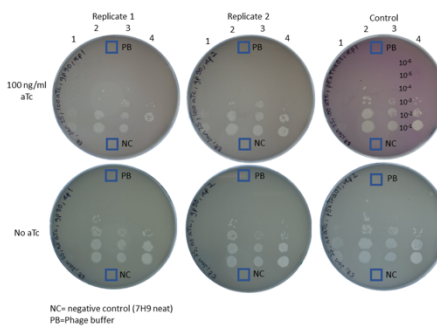


Table 1: M. smegmatis lawns			
	Plasmid name	Gene name	Gene ID
Replicates	pLxTra-63209902	Bromden_30	63209902
Control	pLxTra01	mCherry only	--

Table 2: Phage lysates				
Phage name	1	2	3	4
Bromden	4x10 ⁶ pfu/mL	8x10 ⁵ pfu/mL	1.2x10 ⁵ pfu/mL	3.2x10 ⁴ pfu/mL
Archie				
Finney				
LeBron				
Titer on control plate*	2	0.5	2.7	0.5
E.O.P. (replicat e 1)				
E.O.P. (replicat e 2)	2	0.1	1	0.25

* The 100 ng/ml aTc plate results were used to calculate E.O.P.
E.O.P.=replicate plate titer/ control plate titer

Images taken after 2 days at 37 °C

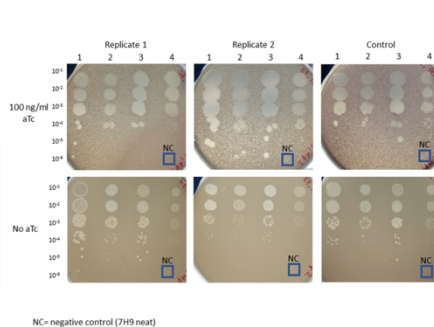


Table 1: M. smegmatis lawns			
	Plasmid name	Gene name	Gene ID
Replicates	pLxTra-63209905	Bromden_31	63209905
Control	pLxTra01	mCherry only	--

Table 2: Phage lysates				
Phage name	1	2	3	4
LeBron				
Finney				
Archie				
Bromden				
Titer on control plate*	4x10 ⁷ pfu/mL	1.2x10 ⁷ pfu/mL	4x10 ⁷ pfu/mL	8x10 ⁷ pfu/mL
E.O.P. (replicat e 1)	10	10	0.3	1.5
E.O.P. (replicat e 2)	2	66.7	5	1.5

* The 100 ng/ml aTc plate results were used to calculate E.O.P.

Images taken after 2 days at 37 °C

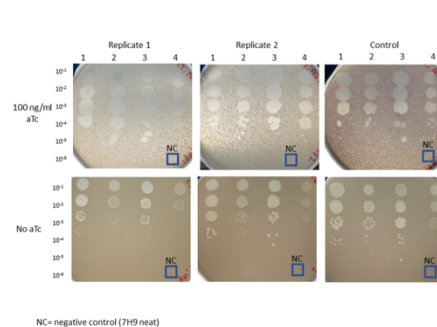


Table 1: M. smegmatis lawns			
	Plasmid name	Gene name	Gene ID
Replicates	pLxTra-63209906	Bromden_34	63209906
Control	pLxTra01	mCherry only	--

Table 2: Phage lysates				
Phage name	1	2	3	4
LeBron				
Finney				
Archie				
Bromden				
Titer on control plate*	4x10 ⁷ pfu/mL	1.2x10 ⁷ pfu/mL	4x10 ⁷ pfu/mL	8x10 ⁷ pfu/mL
E.O.P. (replicat e 1)	0.4	6.7	5	0.7
E.O.P. (replicat e 2)	0.4	6.7	0.4	1

* The 100 ng/ml aTc plate results were used to calculate E.O.P.

Images taken after 2 days at 37 °C

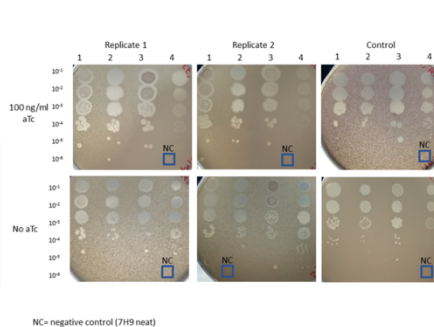


Table 1: M. smegmatis lawns			
	Plasmid name	Gene name	Gene ID
Replicates	pLxTra-63209908	Bromden_36	63209908
Control	pLxTra01	mCherry only	--

Table 2: Phage lysates				
Phage name	1	2	3	4
LeBron				
Finney				
Archie				
Bromden				
Titer on control plate*	4x10 ⁷ pfu/mL	1.2x10 ⁷ pfu/mL	4x10 ⁷ pfu/mL	8x10 ⁷ pfu/mL
E.O.P. (replicat e 1)	1	33.3	2	5
E.O.P. (replicat e 2)	10	3.3	1	5

* The 100 ng/ml aTc plate results were used to calculate E.O.P.