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# BACTERIOPHAGE HOST-RANGE EXPANSION TO INCLUDE TWO STRAINS OF CLOSTRIDIUM SPOROGENES

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**by**

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THESIS

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## ABSTRACT

Bacteriophages are viruses that infect bacteria, which have been used to treat bacterial infections in people for decades. They are also now being used as FDA approved preservatives as a means to prevent the growth of spoiling bacteria in many ready-to-eat foods. Bacteriophages tend to infect a very narrow range of bacterial hosts, some only a single sub-strain. When used for therapeutics or food treatment however, it is desirable to use phages that are promiscuous in their host range. This has led to attempts to broaden the host range of the most useful phages. By passaging phages in co-cultures of their natural host with increasing ratios of a target-host, we were able to expand the host range of two phages specific to *Clostridium sporogenes.* This adaptation was performed in both the presence and absence of a mutagen. Of twenty-eight total mutants, six were sufficiently stable to persist in target-host culture alone. Of these six, four were chosen for further analysis of their infective activity in their natural and target hosts. Most phage populations that resulted from each passage of each co-culture infection lost infective activity against their initial host and also failed to show infecting activity in the target-host. On the other hand, there were several phage populations that demonstrated a gain of activity against one or

both hosts. In some cases the gained infectivity exceeded the infective activity that was measured for the starting host at the beginning of the project. Though mutations causing host-expansion events in phages seem to occur randomly, there was a notable increase in the number of successful mutations in the presence of a mutagen as opposed to without.

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## NOMENCLATURE

| | |
|---|---|
| ATCC® | American Type Culture Collection: Established in 1925, the ATCC is a private, nonprofit biological resource center and research organization, which acquires, authenticates, produces, preserves, develops and distributes standard reference microorganisms, cell lines and other materials for research in the life sciences. |
| ATCC® numbers | ATCC designations for viruses or organism strains arranged in groupings based on how they were acquired by the ATCC |
| ATCC® 17886 | Gram positive *Clostridium sporogenes* (Metchnikoff) Bergey et al; established host for bacteriophages ATCC® 17886-B1 and ATCC® 7955-B3<br>Biosafety level 1 |
| ATCC® 17886-B1 | Bacteriophage deposited by McClung, L.S. isolated from Chicken feces<br>Biosafety level 1 |
| ATCC® 17886-B3 | Bacteriophage deposited by McClung, L.S. isolated from Chicken feces<br>Biosafety level 1 |
| ATCC® 7955 | Gram positive *Clostridium sporogenes* (Metchnikoff) Bergey et al; not known to host bacteriophages ATCC® 17886-B1 or ATCC® 7955-B3 but is considered the "Target-Host" in this project used in the host-expansions experiments described herein.<br>Biosafety level 1 |
| BD | Becton, Dickinson and Company©: Medical technology company from which culturing reagents and anaerobic chamber systems were acquired. |
| Fixed Mutation | A mutation in a bacteriophage genome which allows the phage to infect the target-host organism and allow the phage to continue to propagate in culture containing 100% target-host. |
| PES | polyethersulfone; hydrophilic filter material |
| PFU | Plaque Forming Unit |
| RCB | Reinforced Clostridial Broth medium |
| RT | Room Temperature (21-25°C) |

| | |
|---|---|
| SPM | Single Productive Mutation |

# Chapter 1

## Introduction

Bacteriophage, viruses that infect prokaryotes, comprise the largest virus group currently known (Ackerman, H. W., 2001 & 2011; Keary et. al 2013; Seed et. al 2013). With over 100 billion viral particles per liter of sea water, they outnumber bacteria10:1 both in number and diversity within their shared environments. It is therefore not surprising that at least 100 novel bacteriophage genera are described each year (Ackerman, H. W., 2001 & 2011; Keary et. al 2013; Sorek, et. al 2008). Currently, known phages span one order and ten families but as they are an area of active study, more phages are becoming known, causing phylogenetic trees for them change often. Phage genomes can consist of DNA or RNA and are either single stranded or double stranded. Of the 13 phage families, which are known to infect 140+ bacterial genera, most (96%) use double strand DNA (Ackerman, H. W., 2001).

Having been engaged in a veritable arms race with their bacterial hosts for the past 3.4-3.6 billion years, since just after bacteria appeared, bacteriophage have become ubiquitous and serve as a key component of the biosphere (Ackerman, H. W., 2001 & 2011; Keary et. al 2013). With an estimated $(10)^{25}$ new viral infections initiated every second, phages tend to take on genetic mutations at a faster rate than anything on Earth and consequently, serve as a formidable competitor with their respective bacterial hosts (Ackerman, H. W., 2001 & 2011).

Bacteriophages were discovered independently by Frederick Twort in 1915 and Felix d'Herelle in 1917 (Ackerman, H. W., 2001 & 2011). Felix d'Herelle is noted for naming these viruses "Bacteriophage", meaning "bacteria eater" in Greek (Keary et. al 2013; Maura & Debarbieux 2012). Shortly after his discovery, d'Herelle pursued using bacteriophage as therapy for bacterial infections, first working with *Salmonella* infected chickens (Keary et. al

2013). In his experiment, 100% of phage-treated chickens survived whereas 75% of untreated birds died (Keary et. al 2013). D'Herelle and others were soon providing phage treatments for dysentery, plague, cholera, and gas gangrene in soldiers and civilians in the Soviet Union during WWII. There was moderate to good success (Keary et. al 2013). Host specificity for phage was not well understood at the time however, and the limitations of phage therapy in clearing all kinds of infections indiscriminately made phage therapy seem fickle when compared to the use of antibiotics that were being developed in the 1940's (Keary et. al 2013). Because of this, phage therapy research and treatment languished in the West and was replaced with antibiotics as the premiere antimicrobial treatment (Keary et. al 2013). Though antibiotics at this time were seen as a better answer to bacterial infection than phage therapy, as it turned out, the broad use of antibiotics came with a cost (Thiel 2004; Keary et. al 2013). After applying a myriad of different antibiotics to countless applications such as treating chronic bacterial infections in human and animals, as prophylactic treatments for bacterial infection, and for surface decontamination, many drug resistant strains of bacteria have emerged and become a difficult problem to combat with the continued use of antibiotics (Thiel 2004; Keary et. al 2013; Sebaihia, M., et. al 2006).

The use of phages alone or in conjunction with current antibiotics presents a large range of application possibilities (Keary et. al 2013). They can be used to combat drug resistant bacterial infections and have been shown to be useful in bacterial genome manipulation in the development of vaccines. Phages can provide antibiotic-free bacterial infection treatments when antibiotics may not be a viable option for the patient. They can also fill a niche in bacterial surface decontamination of hospital spaces (Taylor & Oppenheim 1998). Building and testing cocktails of phage variants for human or veterinary therapy can allow

for direct targeting of harmful bacteria, leaving helpful enteric bacteria and eukaryotic cells unscathed (Thiel 2004; Keary et. al 2013). Antibiotic treatments, in contrast, tend to be indiscriminate and can present adverse side-effects to the patient (Keary et. al 2013, Thiel 2004).

Phage cocktails have also been shown to be easier and faster to develop than antibiotics and do not tend to cause the side-effects brought on by antibiotics, such as allergic reactions or problems in the gastrointestinal systems of patients (Keary et. al 2013). Further, where antibiotics tend to dilute out as they travel through the patient's body and arrive at a site of infection, phages will proliferate and increase at the site of infection until the target bacteria are eliminated, at which point the remaining phages are eliminated by the body through normal clearing processes (Keary et. al 2013). Endotoxins released by the resulting multitude of lysed bacterial cells could pose a potential problem for the patient but no more than with the use of antibiotics (Keary et. al 2013).

The advantages of phage therapy have not been lost on industry. The George Eliava Institute of Bacteriophage, Microbiology and Virology, founded in 1923 and located in Tbilisi, Georgia, for example, continues to research phage therapy and treat patients using a large library of phages that they have discovered and collected over the years (Eliava 2014; Keary et. al 2013). Another example is the product, AgriPhage™. This product is a phage cocktail developed by the U.S. based company OmniLytics™ to combat *Xanthomonas campestris* and *Pseudomonas syringae,* both bacterial threats to tomato and chili crops (AgriPhage 2014). AgriPhage™ was approved by the U.S. Environmental Protection Agency (EPA) in 2005 to be used on U.S. crops (AgriPhage 2014). Moreover, crops grown devoid of chemical pesticides but treated with bacteriophage cocktails fall within the

guidelines of "organically grown food" and may be sold as such (AgriPhage 2014). Since the approval of AgriPhage™, more phage cocktail products have become available. In 2006, the U.S. Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA) approved the use of LISTEX™, a phage cocktail made by Micreos that targets *Listeria monocytogenes,* to be used as a phage surface-treatment on many ready-to-eat foods and also as a preservative in foods such as yogurt and cheese (Ingram 2009; Micreos 2014). This approval was followed by the 2011 FDA approval of the EcoShield™, a phage cocktail manufactured by Intralytix, Inc. to fight *E. coli* O157:H7, for use in processed foods, the 2013 FDA approval of SalmoFresh™ to prevent *Salmonella enterica* growth in foods, and then the 2014 FDA approvals to use ListShield™ by Intralytix, Inc. targeting *Listeria monocytogenes* and SALMONELEX™ by Micreos targeting *Salmonella enterica*, in ready-to-eat foods as well (Intralytix 2014; FDA 2014). Many of these cocktails have also been approved for use as surface decontaminants during routine cleaning and the decontamination performed in food processing plants (Intralytix 2014). In addition, phage cocktails produced by Intralytix, Inc., PLSV-1™ and INT-401™, targeting *Salmonella* and *Clostridium perfingens,* respectively, have been approved to apply directly to livestock in order to prevent the spread of infection through animal groups such as chicken flocks (Intralytix 2014).

Bacteriophage host specificity can target and prevent the infection of a specific bacterial species or even serotype (Keary et. al 2013). However, the extreme host specificity can also limit their current and potential usefulness (Keary et. al 2013). Given the narrow host specificity of phage, and the broad range of bacterial serotype diversity present in any given infection, many different serotypes of phages must be used in order to fully eliminate all the harmful bacteria (Keary et. al 2013). In order to achieve complete bacterial elimination, the

phages that are used must overwhelm the bacteria that comprise the infection; if not, the phages that have been introduced will simply engage in a typical arms race with the bacteria, reminiscent of a long standing co-evolution between the two, where they both mutate and counter-mutate in order to maintain their survival (Villion & Moineau 2013; Bondy-Denomy et. al 2013; Garneau et. al 2010; Maura & Debarbieux 2012; Seed, et. al 2013; Bondy-Denomy, et. al 2013; Sorek, et. al 2008). Phages can overwhelm a culture or infection in one of two ways; 1) outnumbering the bacteria causing the infection, or 2) through more efficient infection of the bacteria. Phage cocktails are an assembly of different variants of phages, each of which is specific to different bacterial strains or serotypes (Keary et. al 2013). By including many phage types, each with a specific bacterial host strain or serotype, the hope is to deliver enough of the specific phage variant to a given system to overwhelm the bacteria and resolve the infection (Keary et. al 2013).

Given the host specificity limitations of phage, the primary aim of this project will be to induce and/or, "capture" genomic mutations within phages that have caused a host-expansion event where the phage of interest continues to infect their original host and are also able to infect another, pre-determined bacterial host. To date, the phage cocktails that are commercially available target only a few of the bacterial pathogens that cause common problems in human health and industry (Keary et. al 2013; Taylor & Oppenheim 1998). By expanding their host range or increasing their current infecting efficiency, phage cocktails could be produced that are more simplistic, easier to derive, and faster and cheaper to develop. Additionally, through the host expansion techniques described here, existing phages may be viewed as more versatile for use and phage cocktails could then be made to eliminate richly diverse bacterial contaminations and infections (Thiel 2004).

## Chapter 2

## Materials & Methods

**Biomaterials Used:** Two commercially available bacteriophages, Cs-17886-B1 and Cs-17886-B3, were obtained from the American Type Culture Collection (ATCC). Both are known to infect a specific strain of the Gram positive anaerobe *Clostridium sporogenes* (ATCC 17886) with high efficacy. The goal of these experiments was to generate and retain mutant in phage that could productively infect a previously un-infectable "target host" strain of *C. sporogenes* (ATCC 7955). Both *C. sporogenes* strains were also obtained from the ATCC.

**Culture Medium:** Both Cs-17886 and Cs-7955 were propagated in Becton, Dickinson (BD) Reinforced Clostridial Medium (BD 215192) and assayed via plaque assay to quantify the existing infectivity and titer against the pre-established phage-host (*C. sporogenes* ATCC 17886) as well as the infectivity and titer of potentially mutated bacteriophage against the target host *C. sporogenes* ATCC 7955.

**Anaerobic chambers:** Liquid cultures and plaque assays were statically incubated overnight at 37°C in BD GasPak™ 100/150 anaerobic systems manufactured by Becton, Dickinson and Company (Franklin Lakes, New Jersey).

**Producing host and target-host working stocks:** 10ml aliquots of BD Reinforced Clostridial broth in 15 ml glass tubes were capped with aluminum foil, steam sterilized at 121°C for 15 min., and inoculated with 100 µl of frozen stock of either *C. sporogenes* ATCC 17886 or ATCC 7955. Frozen bacterial stocks were kept at -80°C in 50% sterile glycerol. Inoculated cultures were statically incubated under anaerobic conditions at 37°C for 12-15

hours. Cultures kept for more than two days at 4°C after initial incubation were not used for assay or phage propagation purposes.

**Plaque assay:** Using a method modified from Betz and Anderson (1963), Reinforced Clostridial Medium containing 1.2% agar was poured into 4" petri dishes and allowed to cool and solidify to create a base agar layer. These plates were conditioned in an anaerobic environment at room temperature (RT) for no less than 15 hours before use. 100 µl of bacteriophage sample was then mixed with 300 µl of *C. sporogenes* culture in a 14ml plastic test tube (Fisher 14-959-11B) using a pipette and incubated at RT for 10-20min. After incubation the infected culture was mixed with 4ml of BD Reinforced Clostridial Medium with 0.7% agar held at 52°C in a thermoblock, and then poured over the solid base-agar layer in a petri dish. After allowing the top agar containing infected bacteria to cool and solidify, assay plates were then statically incubated at 37°C under anaerobic conditions for 12-15hrs. A Reichert Darkfield Quebec™ Colony Counter was used to count plaques.

**Titer Assay:** Figure 1 gives an example of titer assays which include serially diluting phage samples in SM Buffer and performing a plaque assay on each of the diluted samples. The resulting plaques in each assay are then counted and the phage concentration of the initial sample may be calculated in Plaque Forming Units (PFU) per ml.

**Phage genomic DNA isolation and quantification:** Phage genome isolation was performed using the ZR Viral DNA/RNA™ kit (cat# D7020) by Zymo Research. DNA quantification was performed using the Invitrogen Qubit® 2.0 Fluorometer and genome size was estimated using the 0.8% agarose E-Gel® EX electrophoresis Kit by Invitrogen.

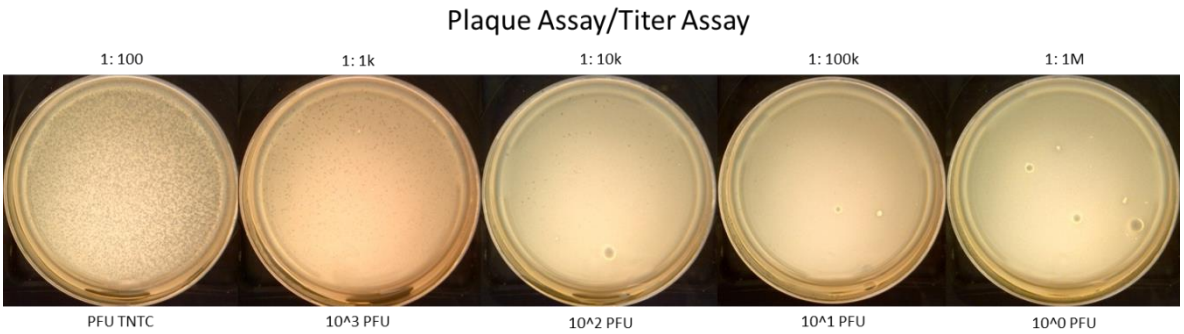



Fig. 1 Plaque assays of null-mutagen B3 mutant bacteriophage that have been serially diluted (by log ) and assayed against target host *C. sporogenes* 7955. The number of Plaque Forming Units (PFUs) drop by order of magnitude as they have been diluted by order of magnitude. Plaque assays of serially diluted phage isolated from co-culture infections, are used to determine the presence and titer of mutant phages active against the target host.

**Experiment:** Upon receipt of *C. sporogenes* ATCC 17886 and *C. sporogenes* 7955, bacterial strains master and working stocks of each were propagated in BD Reinforced Clostridial Broth Medium (RCB), mixed with glycerol to 50%, flash-frozen in LN2, and stored at -80°C. Bacteriophages *C. sporogenes* 17886-B1 and *C. sporogenes* 17886-B3 were propagated separately in *C. sporogenes* ATCC 17886 cultures and the phage activity and concentrations of each was determined via plaque assay technique against the host *C. sporogenes* 17886 and target-host *C. sporogenes* 7955 (Fig. 1). This ensured that each of the initial phage stocks was highly active against their shared host, *C. sporogenes* 17886, but not active against the target-host, *C. sporogenes* 7955.

To isolate host expansion mutant phage by co-culture with host bacteria, each bacterial strain was first cultured separately and then subsequently mixed to create ratios of Cs-17886:Cs-7955 as 1:0, 9:1, 1:1, 1:9, and 0:1 on 10 ml cultures. Two ml of each initial B1 and B3 bacteriophage stock was independently added to each co-culture and allowed to incubate under static, anaerobic conditions at 37°C for 12-15 hours. The resulting progeny phage from each co-culture were purified by centrifugation (Eppendorf 5810) at 3220 x g for 20 min. and the supernatant filtered through a 0.22 µm polyethersulfone (PES) filter

(Millipore Steriflip™ ). Fresh sets of co-cultures containing bacteria naïve to the phage, were then infected with the newly purified bacteriophage progeny. Bacteriophage cultivated in each co-culture ratio was passaged in this manner six times under the same conditions. The PFU that infect the host and target-host, respectively, in the phage population derived from co-culture infections was determined by titer assay. Titer assays were used to determine the resulting infectivity against the target-host as well as the original host (Table 1).

Mutant phages infecting the target-host, *C. sporogenes* ATCC 7955 were isolated from agar plaques by diffusion in 100 µl/PFU of sterile SM buffer. These samples were then centrifuged 3220 x g for 10-20min. to separate out the agar. The resulting phage-rich supernatant was then used in another plaque assay containing only target host for the purpose of increasing the phage population from the plaque(s). By growing the mutant phage population the mutation is then "fixed" in that an entire population now carries the desired mutation instead of only a small number of viral particles. Though many plaque isolates failed to propagate, the mutations which were successfully fixed were further propagated using the same method against only the target-host until enough active phages existed that the genomic DNA could be extracted, purified and sequenced.

To increase the frequency of mutations, Mitomycin C was also added to cultures in some experiments. Mitomycin C causes pyrimidine dimers in DNA and is often used to mimic the genomic mutations associated with exposure to UV-C radiation (Levine 1960). Mitomycin C was added to each co-culture upon phage passaging to a final concentration of 1µg/ml (Levine 1960; Kiritani, et. al 1973).

# Chapter 3

## Results

Stocks of Cs-17886-B1 and Cs-17886-B3 phages were each propagated using *C. sporogenes* ATCC 17886 as host in liquid cultures. The resulting phage stocks were purified and used in phage genomic DNA isolations and in host-expansion experiments. Initial B1 and B3 phage stocks were verified to be infectious against *C. sporogenes* ATCC 17886 but not to *C. sporogenes* ATCC 7955 using plaque assays. The concentrations of the initial B1 and B3 phages were determined to be 3.4x10^7pfu/ml and 1.9x10^8pfu/ml, respectively by titer assay.

Each phage stock was individually incubated in *C. sporogenes* co-cultures as previously described, harvested, and used to infect naïve, bacterial co-cultures for a total of six passages. After each passage, the phage products were passed through a 0.22 µm filter and stored at 4°C. Titer assays on each phage product using the target-host only were performed to screen for phage mutants (Table 1).

Plaques that appeared when only the target-host strain was used revealed mutant phages that had expanded their host range but the reproducibility in some of the mutants that were harvested was limited. A slight bias in all mutants toward co-cultures containing higher concentrations of natural-host was observed however no bias was seen in the number of passages required to gain or lose mutant phage (Table 1). This random mutation pattern lends itself to a low predictability of the appearance of Single Productive Mutations (SPMs). A more pronounced bias of B3 SPM's than that of B1 toward co-culture infections with higher concentrations of natural-host was seen (Table 1 & 2).

**Table 1**
**-MC Co-culture Infections Against Cs-7955**

| | **100% 17886** *(0% 7955)* | **90% 17886** *(10% 7955)* | **50% 17886** *(50% 7955)* | **10% 17886** *(90% 7955)* | **0% 17886** *(100% 7955)* |
|---|---|---|---|---|---|
| B1 x 1 | **12** | 0 | 0 | 0 | 0 |
| B1 x 2 | 0 | 0 | 0 | 0 | 0 |
| B1 x 3 | **1** | 0 | 0 | **2** | 0 |
| B1 x 4 | 0 | 0 | 0 | 0 | 0 |
| B1 x 5 | 0 | 0 | 0 | 0 | 0 |
| B1 x 6 | 0 | 0 | 0 | 0 | 0 |
| | | | | | |
| B3 x 1 | 0 | 0 | 0 | 0 | 0 |
| B3 x 2 | **23** | **21** | 0 | 0 | 0 |
| B3 x 3 | 0 | 0 | 0 | **42** | **17** |
| B3 x 4 | **TNTC** | **TNTC** | 0 | 0 | 0 |
| B3 x 5 | 0 | 0 | No Lawn | 3 | 0 |
| B3 x 6 | 0 | 0 | No Lawn | 0 | 0 |

Table 1: Results of plaque assays using 100% target-host to screen for mutants contained in a sample taken from the product of each phage passage into cultures containing no Mitomycin C. Screen assays containing plaques, and thus mutant phage, are highlighted in yellow. Results from each co-culture infection using B (passages 1-6) are listed in the top six rows and that of B3 are listed in the bottom six rows. *Too Numerous To Count (TNTC)

Adding Mitomycin C (MC) to a final concentration of 1µg/ml along with the phages being passaged caused SPM's of B1 and B3 to occur more frequently and to follow a pronounced bias toward co-cultures with higher concentrations of host strain (Table 2). Additionally, more total plaques were seen in each co-culture infection screening. This suggests a correlation between the presence of Mitomycin C and the frequency of phage mutation producing SPMs (Table 2).

As host-expanded mutant phages were discovered, they were separated from the agar-based plaque and allowed to propagate further in 100% target-host culture. Plaques were removed by forcing the tip of a sterile glass Pasteur pipette through the top agar layer and

expelling the resulting plug into a small conical vial containing SM buffer. Phages were then allowed time to escape the agar matrix and diffuse into the buffer. After incubating at room

**Table 2**
**+MC Co-culture Infections Against Cs-7955**

| | **100% 17886** *(0% 7955)* | **90% 17886** *(10% 7955)* | **50% 17886** *(50% 7955)* | **10% 17886** *(90% 7955)* | **0% 17886** *(100% 7955)* |
|---|---|---|---|---|---|
| B1 x 1 | 0 | 0 | 1 | 1 | 0 |
| B1 x 2 | 0 | 0 | 0 | 0 | 0 |
| B1 x 3 | 0 | 0 | 1 | 0 | 0 |
| B1 x 4 | 0 | 0 | 0 | 0 | 0 |
| B1 x 5 | 0 | 0 | 0 | 0 | 0 |
| B1 x 6 | TNTC | 0 | 3 | 0 | 0 |
| | | | | | |
| B3 x 1 | 10 | 26 | 1 | 0 | 0 |
| B3 x 2 | 0 | 5 | 0 | 0 | 0 |
| B3 x 3 | 2 | 6 | 0 | 0 | 0 |
| B3 x 4 | 4 | 16 | 0 | 0 | 0 |
| B3 x 5 | 1 | 27 | 0 | 1 | 0 |
| B3 x 6 | TNTC | TNTC | 0 | 0 | 0 |

Table 2: Results of plaque assays using 100% target-host to screen for mutants contained in a sample taken from the product of each phage passage into cultures containing 1µg/ml Mitomycin C. Screen assays containing plaques, and thus mutant phage, are highlighted in yellow. Results from each co-culture infection using B (passages 1-6) are listed in the top six rows and that of B3 are listed in the bottom six rows. *Too Numerous To Count (TNTC)

temperature for 2-4 hours, the buffer was removed and used in another plaque assay so to propagate the mutant phage population further. This was repeated until the mutant phage population had grown large enough to perform a successful liquid infection in a pure target-host liquid culture. These liquid infections were then centrifuged, the supernatant passed through a 0.22 µm filter, and assayed to determine the mutant phage concentration. These routine titer assays not only confirmed the continued phage activity against the target-host but also provided a means to determine the mutant phage concentration within each mutant phage expansion.

A total of 28 mutant phage variants were detected, six of which were successfully "fixed" as mutants, able to propagate in 100% target-host culture (Table 3). Of the six fixed mutants, four were chosen for further analysis. The four phage mutants that were chosen represent both B1 and B3 phage mutants as well as each of the co-culture environments where MC was added and where it was not (Tables 3 &4).

**Table 3**
**Co-culture Infections Resulting in Mutant Plaque Forming Units**

| -MC | | +MC | | |
|---|---|---|---|---|
| B1 | B3 | B1 | B3 | |
| B1x1 100% | B3x2 100% | B1x1 50% | B3x1 100% | B3x4 90% |
| B1x3 100% | B3x2 90% | B1x1 10% | B3x1 90% | B3x5 100% |
| B1x3 10% | B3x3 10% | B1x3 50% | B3x1 50% | B3x5 90% |
| | B3x3 0% | B1x6 100% | B3x2 90% | B3x5 10% |
| | B3x4 100% | B1x6 50% | B3x3 100% | B3x6 100% |
| | B3x4 90% | | B3x3 90% | B3x6 90% |
| | B3x5 10% | | B3x4 100% | |

Table 3: Phage mutants discovered from plaque assays (100% target-host) performed on the filtered product from each co-culture passage. Left: mutants discovered from co-cultures containing no MC. Right: mutant phage discovered from co-cultures containing MC. B1-MC yielded 3 mutant phage where B1+MC yielded 5. B3-MC yielded 7 mutant phage where B3+MC yielded 13.

The two selected mutants that were derived from -MC conditions include B1 from the third passage of 100% host culture and B3 from the fourth passage of the 90%host: 10% target-host co-culture infection. The two selected mutants acquired from +MC conditions include B1 from the sixth passage of 50% host: 50% target host co-culture, and B3 from the fifth passage of 90% host: 10% target-host co-culture.

Concentrations of phage populations from each passage was determined through titer assays of 100% host-culture infections. As a result, growth fluctuations with similar trends

were seen across phage populations and were used to determine phage infectivity gain or loss to the original-host (Fig. 2-6). These titer assays, using 100% host, were performed using the product of each –MC and +MC passage for B1 and B3 (Fig. 2). This growth cycle was used

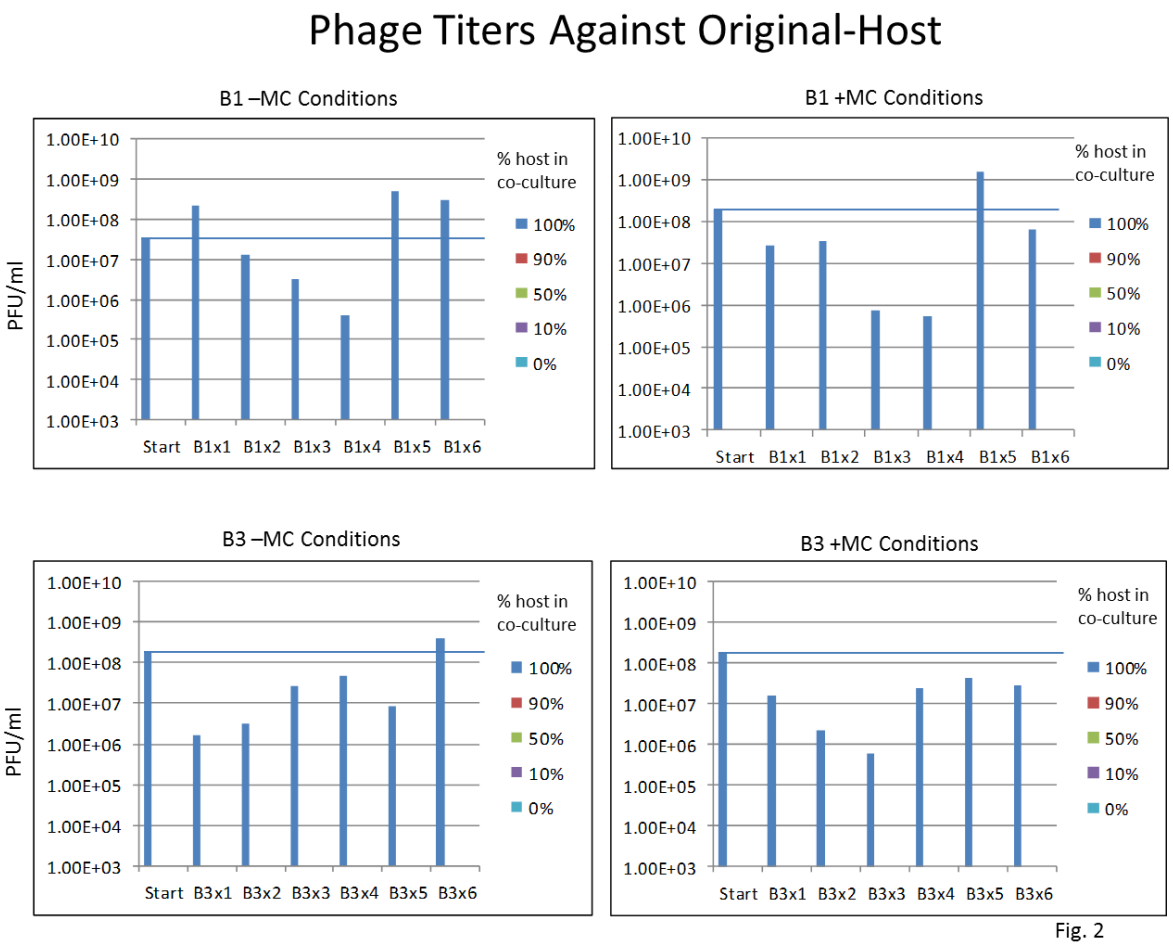



Fig. 2

(B1ϕ starting titer 3.4E+7pfu/ml) **Left:** B1 (top), B3 (bottom) Phage propagated under no artificial mutagenic stress. **Right:** B1 (top), B3 (bottom) Phage propagated in the presence of 1µg/ml Mitomycin C. All show the natural infectivity cycle of each phage when passaged into 100% natural-host

as a benchmark to compare the growth cycles observed in the other phage populations. It should be noted that where the –MC and +MC B1 growth cycles are similar, those of B3 are nearly completely out-of-phase.

As seen in Figures 3-5, the trends followed by phage populations passaged in 100% original-host culture are similar to the corresponding phage populations passaged in each co-

culture. Since naïve bacterial cells were used in each passaging event, this leaves the phage populations themselves as the only component to have participated in each passage. More, phage populations from each chain of co-culture passages were separate from one another yet they still followed similar growth cycles as their cohort populations.

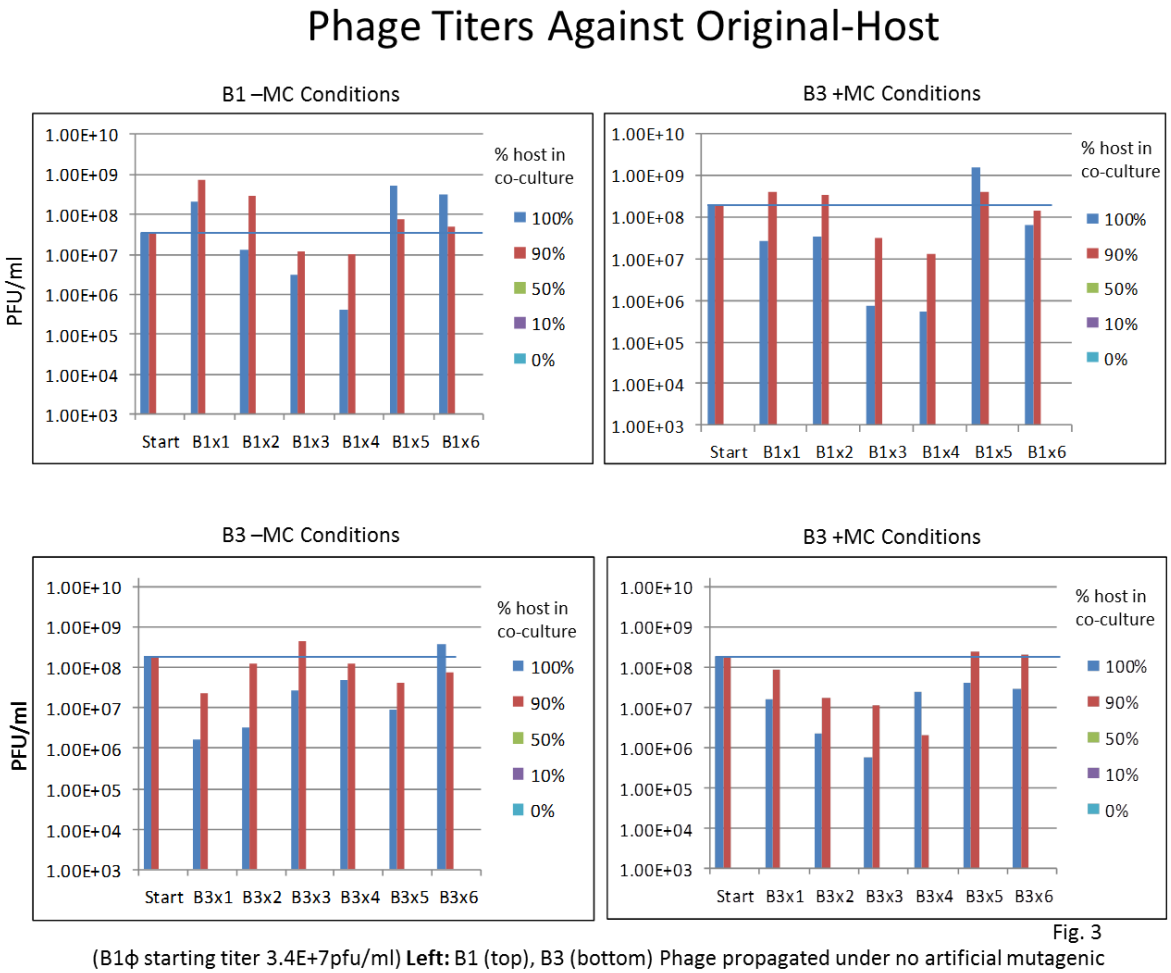



Fig. 3

(B1ϕ starting titer 3.4E+7pfu/ml) **Left:** B1 (top), B3 (bottom) Phage propagated under no artificial mutagenic stress. **Right:** B1 (top), B3 (bottom) Phage propagated in the presence of 1µg/ml Mitomycin C. All 90% host with 10% target-host co-cultures follow the natural infectivity cycle of their perspective phage.

Both B1 and B3 phage concentrations increased dramatically in co-cultures containing 50% or greater concentration of the host organism (Fig. 2&3). They also gained infectivity to the target host either comparable or greater than that to the host (Fig. 2&3). In In contrast, decreases in host-infecting phage concentrations was shown in co-cultures

containing 50% or less host in the co-cultures. Phage propagation in 100% host cultures sharply increased after the first round of infection, declined to near zero through passages 2-4, increased to over 15 times the starting phage titer, and then dropped after the sixth passage. In the presence of MC however, B1 concentrations sharply increased to 22- times from where it started and then dropped to a concentration comparable to the starting

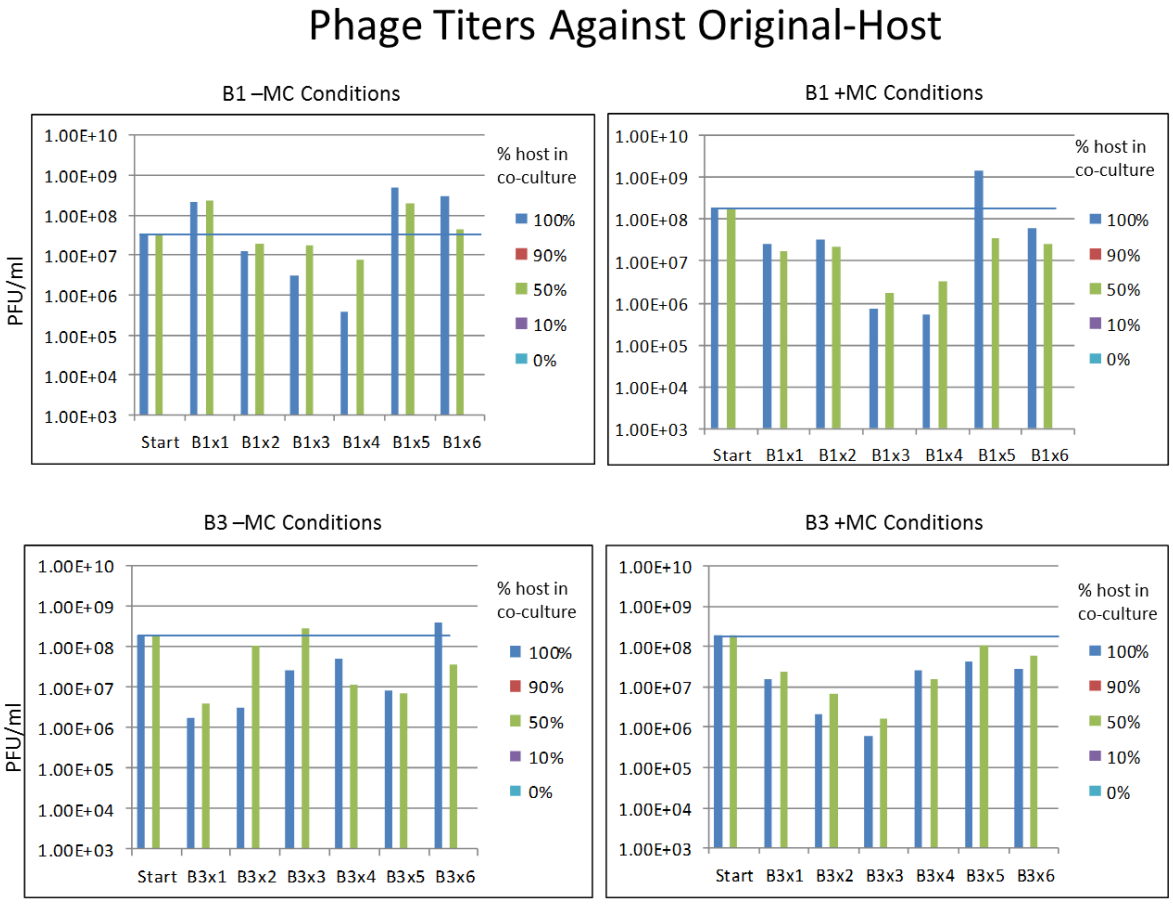



Fig. 4

(B1ϕ starting titer 3.4E+7pfu/ml) **Left:** B1 (top), B3 (bottom) Phage propagated under no artificial mutagenic stress. **Right:** B1 (top), B3 (bottom) Phage propagated in the presence of 1µg/ml Mitomycin C. All 50% host with 50% target-host co-cultures follow the natural infectivity cycle of their perspective phage.

concentrations (Fig. 3). Cultures devoid of MC, that included 90% host, increased in phage concentration after the first round of infection, waned after the second passage, fell below the starting concentration over two passages, and rebounded to concentrations above the starting

titer through the last two passages. With MC present, B1 phage titers followed the same trend but had markedly higher concentrations in later passages (Fig. 4). The large spike seen in the fifth passage of B1 +MC could be due to a population rebound caused by a bacterial resistance to MC in that specific culture. Bacterial resistance could cause more bacterial growth, causing more phage propagation, resulting in the larger phage population seen in Fig. 4. Since, all of the potentially resistant bacteria from that round were removed and

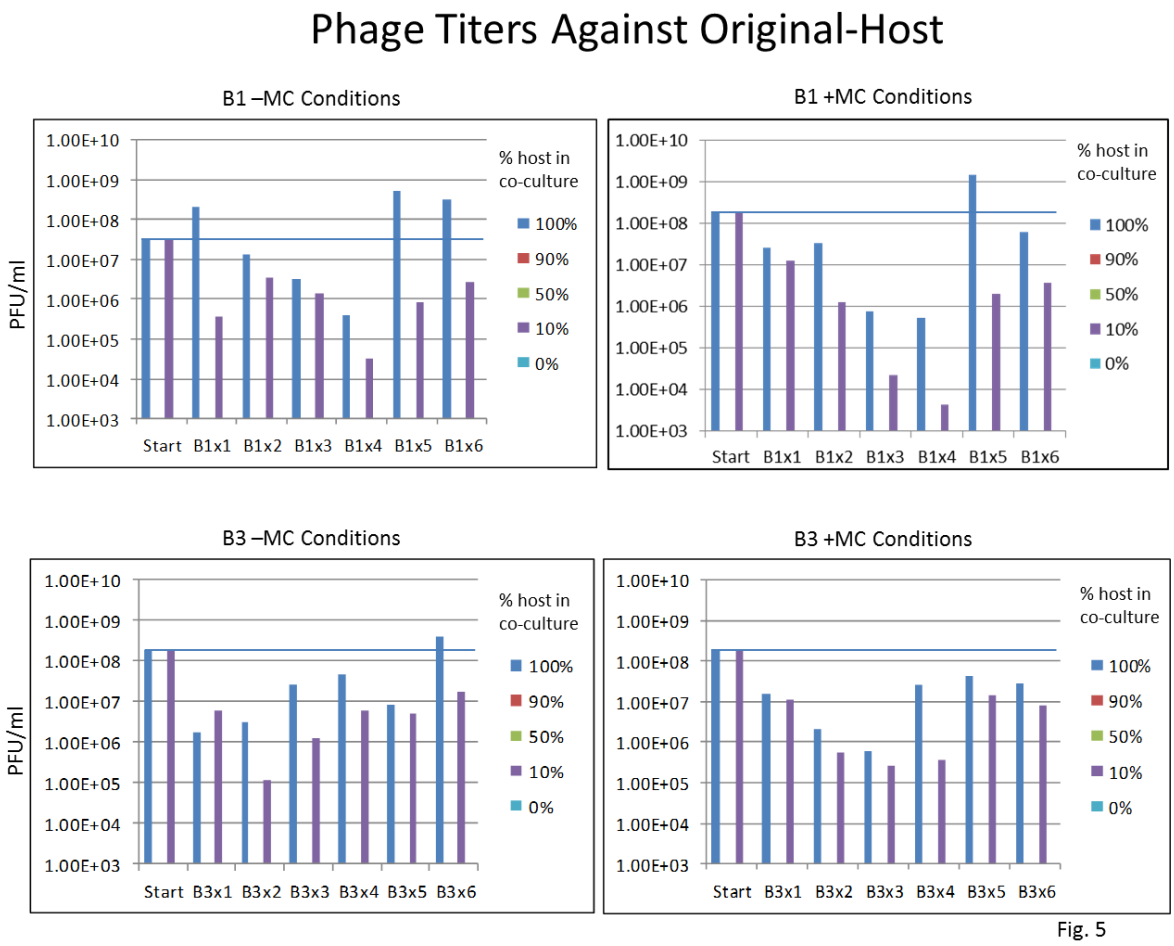



Fig. 5

(B1ϕ starting titer 3.4E+7pfu/ml) **Left:** B1 (top), B3 (bottom) Phage propagated under no artificial mutagenic stress. **Right:** B1 (top), B3 (bottom) Phage propagated in the presence of 1µg/ml Mitomycin C. All 10% host with 90% target-host co-cultures follow the natural infectivity cycle of their perspective phage.

replaced by new bacteria, naïve to the phage being used, the resulting bacterial culture would decline, as was seen in the following passage (Fig. 5). B3 did not show increased concentrations with the host organism as large as B1 (Fig. 3). Though starting with a concentration one order of magnitude higher than that of B1, B3 populations fell below the starting titers in almost every instance. B3 grew to concentrations well above their starting point in -MC infections containing 50% or more host. B3 populations from +MC conditions only grew titers past their starting point in cultures containing 90% host and only after several iterations of co-culture infections.

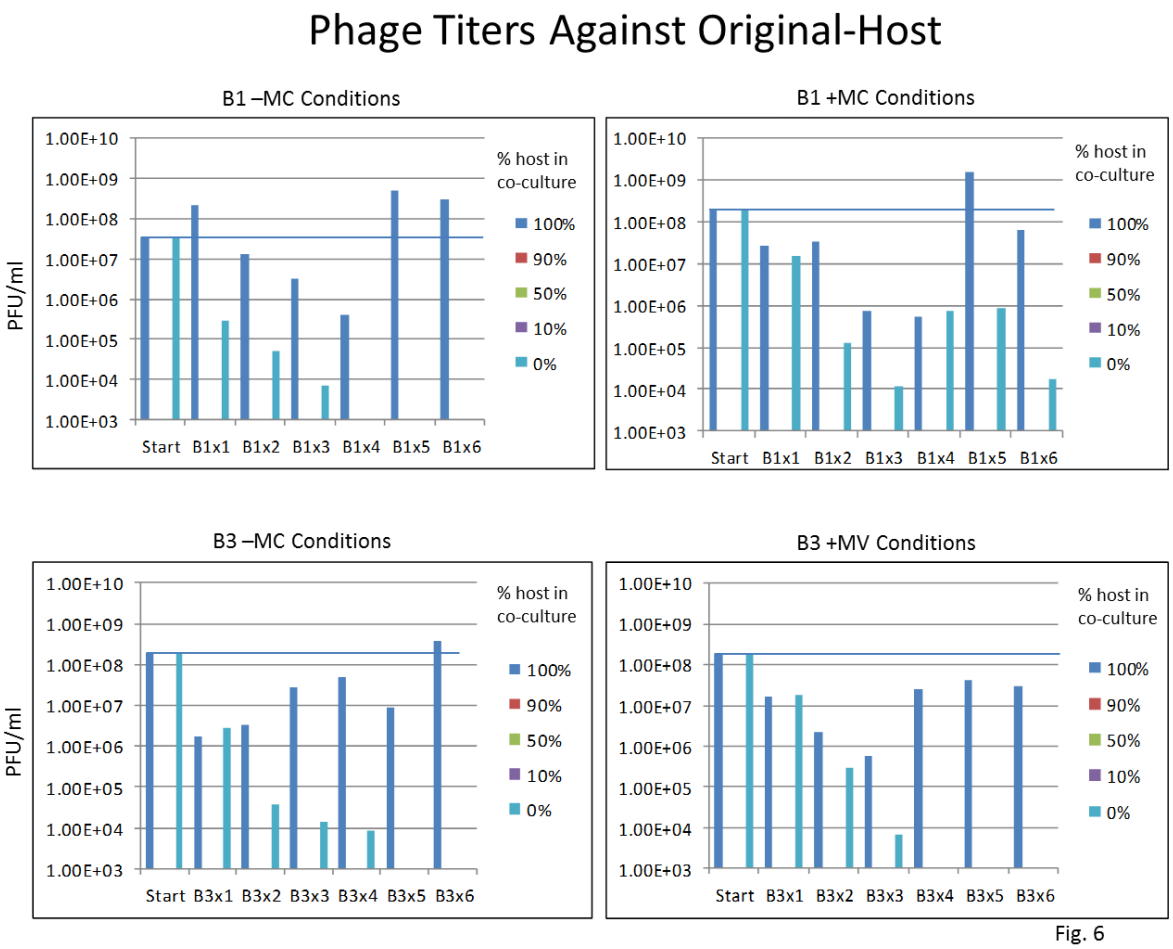



Fig. 6

(B1ϕ starting titer 3.4E+7pfu/ml) **Left:** B1 (top), B3 (bottom) Phage propagated under no artificial mutagenic stress. **Right:** B1 (top), B3 (bottom) Phage propagated in the presence of 1µg/ml Mitomycin C. Phage populations show simple dilution between passages except for the Cs-B1 phage under Mutagen-Added conditions where a population rebound is shown.

Comparing the infectivity of each of the four selected mutant phage populations against the host and target host, B3 from +MC is noted for having lost 93% activity against the host than before the first passage and gaining 0.1% against the target-host (Fig. 7). This notwithstanding, the mutant B3 was still able to propagate in 100% target-host culture. Under +MC conditions however, B3 was found to have increased host infection activity 110% to that of its initial host activity and became roughly 250-times that, more infective to the target-host (Fig. 7). B1 mutants however, appear to have taken a different path. The infectivity of B1 from –MC fell by 86%, and also fell by 24% under +MC conditions (Fig. 7). B1 infectivity to the target-host under -MC conditions was only 4% that of its initial activity against the host but increased to 165% that of target-host activity (Fig. 7).

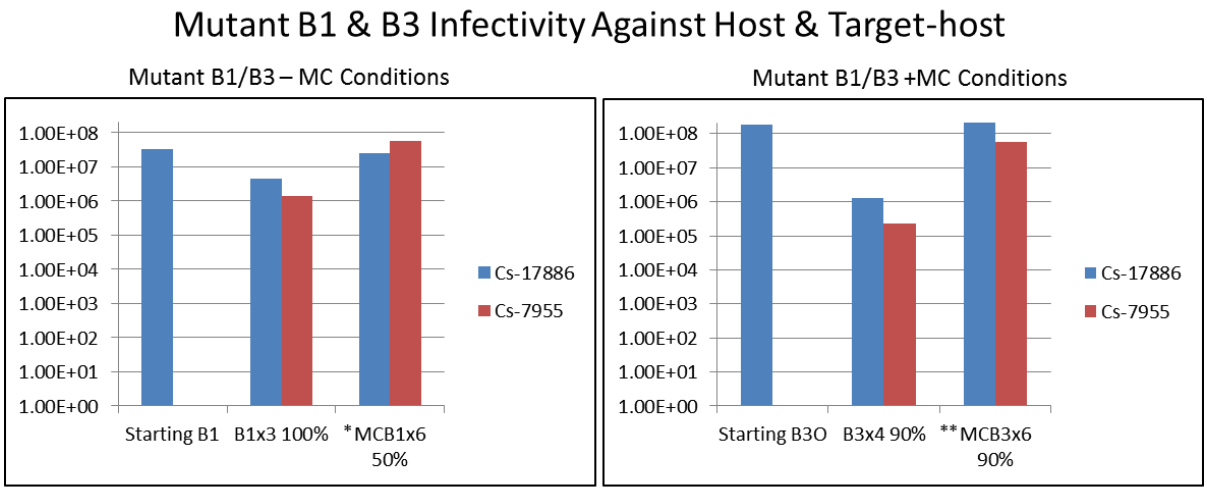



Fig. 7

(B1ϕ starting titer 3.4E+7pfu/ml - B3ϕ starting titer 1.9E+8pfu/ml) Mutant B1, propagated in 100% target host without the presence of Mitomycin C, lost 86% infecting activity against the host where B3 lost 93%, but each fixed mutant is active against the target host by 4% and 0.001% of their original activity against the host. In the presence of mutagen, B1 lost 24% infective activity against the host where B3 increased by 10%. B1 gained 65% more infecting activity against the target host compared to its starting activity against the host organism and B3 gained 30% activity against the target host.
*MCB1x6 50% is the B1 mutant phage derived from the sixth passage in the 50% host: 50% target-host co-culture ration in +MC conditions.
**MCB3x6 90% is the B3 mutant phage derived from the sixth passage in 90% host: 10% target-host co-culture ratio in +MC conditions

To better understand the specific mutations that allowed the mutant phages to infect the target host, we sought to sequence their genomes. Viruses are known to carry double stranded or single stranded genomes of RNA or DNA (Ackerman, H. W., 2001 & 2011).

First, we eliminated the possibility of either genome being RNA. Aliquots of the original phage samples were first analyzed for dsDNA, ssDNA, and RNA using the Invitrogen Qubit® 2.0 Fluorimeter, with its associated DNA and RNA dye-assay kits, to measure the amount of contaminating DNA and RNA was present in the purified phage samples (Table 6). Aliquots of B1 and B3 were then treated with RNAse in order to eliminate all possible RNA not associated with either of the phages and reanalyzed (Table 6). The genomes of each were isolated and purified using a ZR Viral DNA/RNA™ kit (cat# D7020) by Zymo Research and analyzed again (Table 6). RNA was present before phage lysis but absent directly after the RNAse treatment indicating that all of the RNA contaminant was successfully removed. RNA was also absent after the phage genomes were isolated and purified demonstrating that neither the B1 nor B3 genome is RNA-based (Table 6).

**Table 4**
**B1/B3 Phage Genome Determination/Isolation**

| **Total RNA Present** | B1 | B3 |
|---|---|---|
| Before RNAse Treatment | 28.6ng/µL | 18.1 ng/µL |
| After RNAse Treatment | <20ng/mL | <20ng/mL |
| After Genome Isolation | <20ng/mL | <20ng/mL |
| **Total dsDNA Present** | | |
| Before DNAse Treatment | 3.69 ng/µL | 5.16 ng/µL |
| After DNAse Treatment | 1.06 ng/µL | 3.52 ng/µL |
| After Genome Isolation | 0.186 ng/µL | 0.171 ng/µL |
| Miniprep from crude sample (no other treatment) | 4.36 ng/µL | 6.28 ng/µL |
| **Total ssDNA Present** | | |
| Before DNAse Treatment | 10.3 ng/µL | 6.64 ng/µL |
| After DNAse Treatment | 10.4 ng/µL | 3.78 ng/mL |
| After Genome Isolation | 0.572 ng/µL | 0.564 ng/µL |
| Miniprep from crude sample (no other treatment) | 16.1 ng/µL | 32.4 ng/µL |

Table 4: Shows the amount of total RNA, dsDNA, and ssDNA present after treating purified phage samples with various endonucleases and exonucleases.

Since the vast majority of known phage genomes are dsDNA, sequencing under this assumption of both phages was attempted (Ackerman 2011). After obtaining sequence data from B1 but not B3, the B3 genome was suspected to be ssDNA. To compare the genomes of B1 and B3, aliquots of each were treated with DNAse I (New England Biolabs) and analyzed for both dsDNA and ssDNA prior to phage lysis (Table 7). Contaminating ssDNA and dsDNA were present in both phage samples before treating with DNAse but largely absent after. This suggests the contaminating DNA was successfully eliminated from the purified phage samples (Table 6). After the phage genomes of the DNAse-treated phage samples were isolated, the concentrations of dsDNA and ssDNA extracted from B1 comparable. With the B1 genome shown to be dsDNA, these results provide a point of comparison for the B3 genome. From the DNA shearing that occurs during the genome isolation process, the presence of ssDNA in the B1 sample should be expected. Three DNA isolation kits were used to isolate DNAse pretreated B3 genome and all agreed that the B3 genome contains >200% ssDNA content than that of dsDNA (Fig. 5). Since ssDNA is known to loop around and anneal to itself over semi-complementary regions of the strand forming regions of dsDNA, seeing dsDNA would be expected for a ssDNA phage genome.

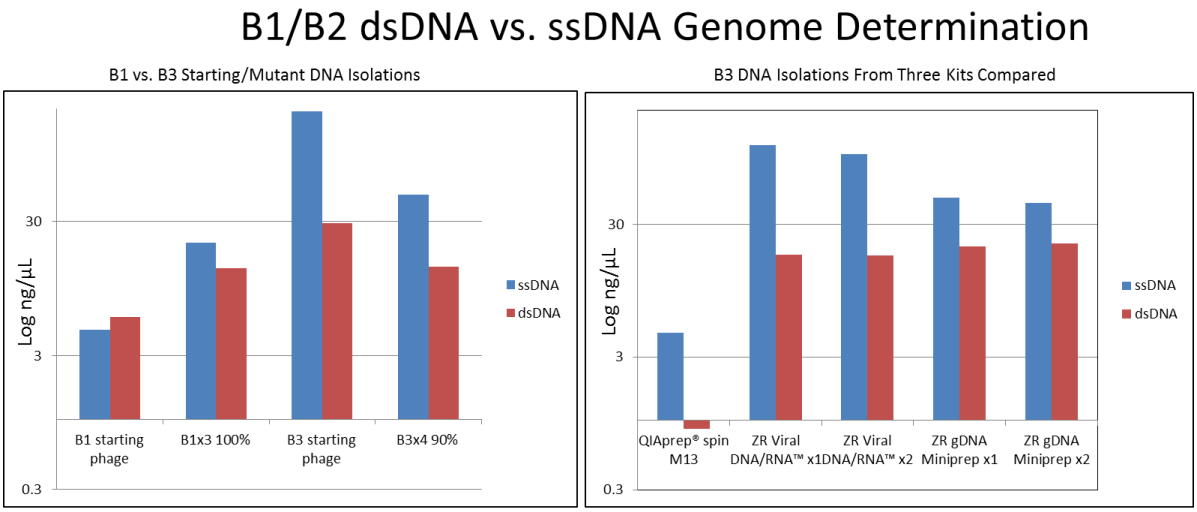



Fig. 8

**Left:** Both B1 gDNA isolations resulted in very little difference between ssDNA and dsDNA concentrations where in both isolations from B3 reflect the presence of much more ssDNA than dsDNA. **Right:** DNA isolations from three different kits all show many times more ssDNA present than dsDNA with a significantly larger yield from the ZR Viral DNA/RNA™ kit from both reactions that were run.

To provide further evidence that the B3 genome is ssDNA, B3 genome isolates were independently digested by endonuclease or exonuclease enzymes to eliminate either dsDNA or ssDNA and then analyzed for the presence of both ssDNA and dsDNA (Table 7). Purified B3 samples were digested with DNase I to remove DNA not associated with the phage in the purified sample. The presence of both was found in the purified B3 phage before and after the DNase I treatment. B3 isolated genomes were split into two aliquots where one was treated with T7 Endonuclease I, to eliminate dsDNA, and the other treated with Exonuclease T, to eliminate ssDNA. Each sample was then analyzed for ssDNA and dsDNA concentrations (Table 7).

**Table 5**
**B3 Phage Genome Determination**
**(averaged standardized values)**

| **Total dsDNA Present** | **ng/µl** |
|---|---|
| Before DNAse Treatment | 3.32 |
| After DNAse Treatment | 1.44 |

| | |
|---|---|
| After Genome Isolation | 1.49 |
| After T7 Endo I Digest (digests dsDNA) | 0.85 |
| After Exo T Digest (digests ssDNA) | 1.54 |

| **Total ssDNA Present ng/µl** | ng/µl |
|---|---|
| Before DNAse Treatment | 28.35 |
| After DNAse Treatment | 4.27 |
| After Genome Isolation | 9.77 |
| After T7 Endo I Digest (digests dsDNA) | 10.03 |
| After Exo T Digest (digests ssDNA) | 5.5 |

Table 5: Shows dsDNA and ssDNA concentrations present in B3 genome isolates before and after enzyme digestions made to eliminate either form of DNA. B3 is likely to have a ssDNA genome.

The purified phage sample contained 8.5 times more ssDNA than dsDNA and dropped to only 3 times more after being treated with DNase I where it remained after the genome isolation (Table 8). 11.8 times more ssDNA than dsDNA was found in the sample where dsDNA was eliminated and 3.6 times more ssDNA than dsDNA was found in the sample where ssDNA was eliminated (Table 8). Assuming that many phages within a population make new genetic material but fail to assemble properly and that many phages may simply fall apart and release their genomes for one reason or another, a large amount of phage genome is expected to be present within the purified culture.

Phage genome size was also estimated prior to our attempts to sequence them through gel electrophoresis. The B3 genome isolates from the three DNA isolation kits were compared using the Invitrogen Qubit® 2.0 Fluorometer and an 0.8% agarose E-Gel® EX electrophoresis Kit (Fig 9.). In addition to estimating genome sizes this also helped to

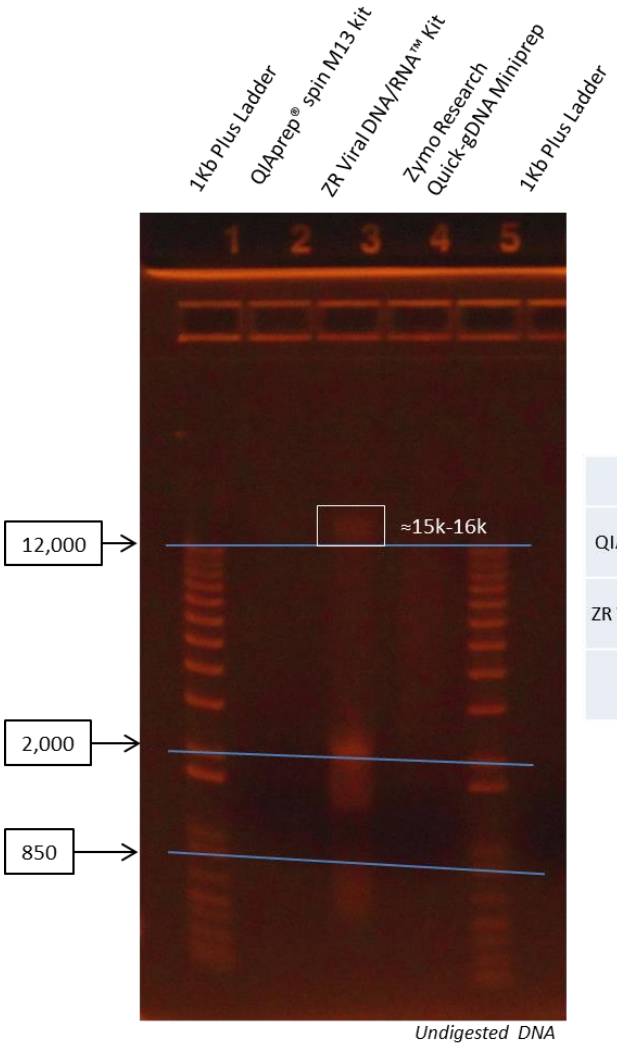
**Table 6**
**B3 Phage Isolation**
**ssDNA:dsDNA**

| | |
|---|---|
| Untreated sample | 8.5 |
| dsDNA and ssDNA digest | 3 |
| Genome Isolation | 6.6 |

| | |
|---|---|
| dsDNA digest | 11.8 |
| ssDNA digest | 3.6 |

Table 6: shows data from Table 7 in a ratio format between amount of ssDNA present in B3 genome isolates to dsDNA.

determine the from the ZR Viral DNA/RNA™ Kit and gel electrophoresis shows a sharp band at roughly 14-15kb along with small fragments of DNA or RNA (Fig. 9). It is yet to be determined if the genomes of either phage is circular or linear and since no attempt was made to enzymatically linearize either, the 14-15kb genome length seen in Fig. 9 and 10 should only be viewed as an estimated genome size. Since DNA also exists in a supercoiled state, these genomes could be circular and supercoiled and so these data only show that each genome is larger than 14kb. After receiving the sequence data from the B1 genome and learning that the Cs-17886-B1 phage is the same phage as the previously sequenced Cs-8074-B1 phage, the genome size of the B1 here is 47,595-bp long (Mayer et. al 2012). Considering the known B1 genome size and the similarity of the electrophoresis data between B3 and B1, the B3 genome could also be between 14k and 47k-b long as well.




*Undigested DNA*

## DNA Isolation Kit Efficacy Comparison Electrophoresis

| | ssDNA | dsDNA |
|---|---|---|
| QIAprep® spin M13 kit (cat#27704) | 4.6ng/ul | 0.864ng/ul |
| | 0.314ng/ul | 0.0524ng/ul |
| ZR Viral DNA/RNA™ Kit (cat#D7020) | 120ng/ul | 17.7ng/ul |
| | 102ng/ul | 17.5ng/ul |
| Zymo Research Quick-gDNA Miniprep (cat#D3025) | 48.0ng/ul | 20.4ng/ul |
| | 44.0ng/ul | 21.6ng/ul |

Undigested B3 genome isolates from 3 different isolation kits run on a 0.8% agarose Invitrogen E-Gel® EX with a 1Kb Plus ladder.

Fig. 9

# Chapter 4

## Discussion

To investigate the nature of the specific mutations that occurred in each of the two phages we thought it necessary to examine genomes of the B1 and B3 phages. Since viruses in general are known to carry either single strand or double strand, RNA or DNA, it was necessary to determine the genome type of each phage first. After eliminating the possibility of an RNA genome for each, it was assumed that they would both carry a dsDNA genome as does the vast majority of all known phages. Sequencing was attempted based on this assumption. After receiving large areas of the B1 genome sequence they were searched in BLAST and determined to be the same sequence as the *C. sporogenes* ATCC 8074-B1 phage which is commercially offered as a phage separate from ATCC 17886-B1.

Since the B3 genome isolation was treated as though it was dsDNA and no sequence data was generated, further investigation into its genome type was initiated. With this outcome along with having already eliminated RNA as a genome possibility, we suspected the B3 genome to be ssDNA. Upon comparing data from the known B1 dsDNA genome and the suspected ssDNA B3 genome we found large differences in the quantity of ssDNA vs. dsDNA between their genome isolates (Fig. 7). Comparable amounts of ssDNA and dsDNA was found in B1 genome isolations but markedly more ssDNA than dsDNA was found in that of B3. This not only supports the idea that the B3 genome is ssDNA but also could help explain why B3 produced more total mutants and why more B3 mutants were able to be fixed. This difference in genome makeup could also help explain why B3 was able to gain infectivity in the host where B1 did not and why B3 was able to become more active in the target host, by orders of magnitude, than it started against its original host.

Aside from the differences found between the B1 and B3 genomes, this study demonstrated that the passage of phages in co-cultures of host strains generated mutants that had achieved host range expansion into a target host (Table 1). Some but not all of these mutant phage populations were able to be propagated on the target host in a plaque assay. The inability of many of the mutants to productively infect the target host, speaks to the variance in their stability. Since many mutants were not able to persist in a culture containing 100% target host and others were able to thrive, perhaps many different mutations routinely occur, some causing efficient host expansion and others not. Additionally, in the -MC co-culture infections, host expanding mutations did not correlate to the number of passages they underwent but did show a minor correlation to the presence of small concentrations of target host within the co-cultures. Mutations leading to host expansion appeared to have occurred largely at random. To support this, host expanding mutations were found to occur in culture passages that contained no target host at all. With no impetus to drive such a mutation, clearly these mutations occurred randomly but were simply discovered by screening against the desired target host. This suggests that many different types of mutations occur at random but the desired mutations seem to occur with higher frequency in co-cultures containing at least 10% target host. With a wealth of natural host available, more phage propagation is able to occur providing more opportunities for mutation. With the addition of the target host, it then would be readily available within the co-culture to propagate those phages which happened to mutate in such a way as to infect the target host. By adding MC to the co-culture a higher number of initially productive mutations occur in co-cultures containing a small amount of target host (Table 2). The +MC experiments also had more overall mutations when compared to -MC experiments. The

likelihood that the genome of B3 is ssDNA a plausible explanation for why we see more mutations for B3 than for B1since ssDNA is less stable than dsDNA and more prone to mutation. Many productive mutations were routinely seen through passaging but more were seen in infections with an added mutagen and a small amount of the target host. The mutagen causes a higher frequency of overall mutation and the presence of the target host helps propagate the mutant phage that happen to be able to infect.

Harvesting plaques harboring the desired host expansion mutations presents a problem. Each individual plaque forming unit could conceivably represent a unique mutation event within the phage genome, indeed many genetically unique phage variants could exist within a single plaque. By treating the plaques in each assay as a Single Productive Mutation however, and placing them in an environment that contains 100% target-host, each phage variant must compete for survival and to dominate the phage population. Out of this competition, comes a consensus population of the mutant phages that carry the mutations which caused them to out-compete the other mutant phages within the same environment.

Of the 28 phage mutants discovered only six were able to propagate in 100% target host culture. Four of the six fixed mutants were selected to represent both phage-types under both +MC and -MC conditions. It is worth noting that almost twice the mutations for each phage type (B1 and B3) were seen under +MC conditions than in the absence of a mutagen. This further indicates that the addition of MC speeds the host-expansion process for phages. Further, in both environments, the B3 phage represented over 130-260% more fleeting mutants than the B1 phage, illustrating a dramatic mutation rate difference between the two phage types. In other words, B1 mutates less often than the B3 phage, probably due to its

more stable dsDNA genome. This may indicate that phage-types with ssDNA genomes may be more prone to directed host expansion than others with dsDNA, making them better candidates for industrial use. On the other hand, these data do not tell us how stable those mutations are, or if they are also more prone to lose their lytic ability toward a desired bacterial host. More work needs to be done in this area to answer these questions.

The phage product from every co-culture infection was assayed to determine the concentration of phage present that still infects the original host. The populations of phage naïve to the target host and only ever passaged in cultures containing 100% original host, increased and decreased in a cyclic pattern (Fig. 2). This fluctuation was used as a standard of growth and attrition to compare the growth changes in the other co-culture infections against. All of the phage populations being passaged between the co-culture environments followed the same cyclic trend, with the exception of the culture containing 100% target host (Figs. 3-5). Since phage populations were passaged in only one culture ratio and never intermingled with the phage populations passaged in the other co-culture ratios, the growth fluctuations seen in each population is truly separate from the others. Still, the growth cycles seen in each phage population follow the same growth cycle trend as the phage propagated in 100% original host culture. Since the growth cycles for each phage population is independent of one another, the fact that they are all similar to one another is a reproducible phenomenon and can be used as a tool for comparing the overall infectivity across mutant phage populations.

In comparing the growth patterns for both phage populations, it is clear that passaging phage in cultures containing a small percentage of target host causes the overall phage population to remain higher than without target host. As the target host percentage increases

however, the overall phage population declines until we see nothing more than a simple dilution curve in those passages from and into 100% target host. This sort of population growth is expected from a selective pressure that is not great enough to overwhelm the phage population. With no host available to the non-mutated phage, replication ceases eliminating the possibility of mutation events to occur and so the dilution curves seen in Fig. 6. It should be noted that all of the growth fluctuation patterns seen in the co-culture infections of B3 -MC are different than those of B1 and B3 in the +MC experiments and B1 in the –MC experiments (Fig. 2-5). Where passages 3 and 4 in the B3 -MC experiments show an increasing population trend, all of the others show a decreasing population trend. If the addition of the mutagen caused this growth cycle phase-shift in B3, it clearly did not cause the same result in the B1 phage populations. In contrast, B3 propagated in 100% target host, phage populations under both conditions where simply diluted out by passaging (Fig. 6). The same trend was shown for B1 under -MC conditions but no dilution curve is seen for B1 populations in the +MC conditions. Though this trend is indicative of a rebounding population of phages that could have undergone host expansion, they went undetected in the initial screening where only 1% of each phage product volume was screened against the target host and therefore went unrecorded in this study. Still, if the addition of the mutagen caused this phage population rebound, it failed to do the same for both phage types. It should also be noted that that the sinusoidal B3 population cycles from each co-culture ratio under the -MC condition appears to be completely out of phase relative to those of the B3 from +MC for all of the co-culture ratios except one. The exception is in the B3 growth cycle form co-culture infections containing 90% target host which appears to be more similar to the growth cycles found in the B3 +MC experiments (Fig. 5). Again, more work needs to be

done to understand the growth cycles themselves but the fact that this difference seems to be correlated to the addition of MC is interesting and noteworthy.

## Chapter 5

## Conclusions

This study describes a novel method of bacteriophage host-range expansion which can be used as a tool to produce new phage and phage cocktails, as well as to improve existing phage cocktails used in human health and industry. This study demonstrates that bacteriophage genomes can incur many random mutations during replication and that it is possible to detect and capture desired mutations. Further, we have demonstrated that by adding a small concentration of a mutagen at the onset of culture infection mutations we can induce more increasing the overall yield of the desired stable mutations that can be stably propagated into large populations. These techniques may be useful in creating efficacious novel phages for use in commercial industry and in human health. We have also shown that phages created under these mutagenic conditions have greater lytic efficacy toward both their natural and target hosts. Using this method, the lytic activity of known phage may be improved on their natural host and expanded into new hosts.

More work needs to be done to fully understand and refine this process. An investigation of the genetic mutations that occur and how they map to phenotypic expression would allow us to determine the parts of the phage anatomy most important to host range expansion. Little research has been done to unlock the full potential of host range expansion in bacteriophage. Questions regarding how many different hosts can a phage have and how taxonomically diverse can those hosts be must be answered. Several studies have demonstrated that phage may infect bacteria of different genera. Crill, et. al (2000) demonstrated that bacteriophage ϕX174 could shift between *Escherichia coli* C and

*Salmonella enterica.* In addition, serotypes of the T4 *e. coli* phage were found to not only infect *Escherichia coli* and closely related *Shigella* species, but also infect the evolutionally distant bacterium *Yersinia pseudotuberculosis* I (Tétart, et. al 1996 & 1998). Adding to the potential of host range expansion, Tétart, et. al (1996) suggests that through recombination within a given phage genome, even the exchange of tail fiber sequences between phages, could allow for host range expansion without any loss of previous host specificity. One example: genes encoding host recognition proteins located on the tips of each tail fiber are flanked by G-rich DNA motifs exchanges involving these sequences can change the host specificity of the mutated phage, allowing it to cross species boundaries and infect taxonomically distant hosts (Tétart, et. al 1996).

In this study, we demonstrated that the two phage types responded differently to the addition of Mitomycin C. Other phage might not be affected at all, or may be rendered completely non-functional under these conditions. Additional mutagens need to be investigated in order to allow this process to be generalized to the wide array of known and yet undiscovered phage.

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