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LDRD PROJECT NUMBER: 178849

LDRD PROJECT TITLE: A Novel Application of Synthetic Biology and Directed Evolution to Engineer Phage-based Antibiotics

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ABSTRACT:

The emergence of multiple drug resistant bacteria poses threats to human health, agriculture and food safety. Annually over 100,000 deaths and up to \$20 billion loss to the U.S. economy are attributed to multiple drug resistant bacteria. With only four new chemical antibiotics in the drug development pipeline, we are in dire need of new solutions to address the emerging threat of multiple drug resistance. We propose a paradigm-changing approach to address the multi-drug resistant bacteria problem by utilizing Synthetic Biology (SynBio) methodologies to create and evolve “designer” bacteriophages or phages – viruses that specifically infect bacteria – to infect and kill newly emerging pathogenic bacterial strains WITHOUT the need for chemical antibiotics. A major advantage of using phage to combat pathogenic bacteria is that phages can co-evolve with their bacterial host, and Sandia can be the first in the world to establish an industrial scale Synthetic Biology pipeline for phage directed evolution for safe, targeted, customizable solution to bacterial drug resistance. Since there is no existing phage directed evolution effort within or outside of Sandia, this proposal is suitable as a high-risk LDRD effort to create the first pipeline for such an endeavor. The high potential reward nature of this proposal will be the immediate impact in decontamination and restoration of surfaces and infrastructure, with longer term impact in human or animal therapeutics. The synthetic biology and screening approaches will lead to fundamental knowledge of phage/bacteria co-evolution, making Sandia a world leader in directed evolution of bacteriophages.

INTRODUCTION:

Synthetic Biology is an emerging engineering discipline at the intersection of Biology, Chemical Engineering, Chemistry, Electrical engineering, or Computer Science¹. In layman’s terms, SynBio is “genetic engineering on steroids” - where traditional genetic engineering examines one mutant at a time, SynBio screens billions of

mutants in an iterative fashion to select for desired traits. SynBio is made possible by recent advancements in ultra-high throughput technologies, some of which are developed and used at Sandia, including enzyme directed evolution and ultra-high throughput enzyme screening platform²⁻⁴. Sandia’s existing SynBio efforts have been focused on enzyme engineering and metabolic engineering for bioenergy applications. Using Sandia’s existing expertise in SynBio as a launching pad, this LDRD project will be the first in the SynBio field to establishing a phage directed evolution pipeline (figure 1).

Phage therapy date back to the 1900's, but early attempts were hampered by the lack of basic understanding of phage biology, and was replaced by chemical antibiotics⁵. Fast forward 100 years - bacteria have evolved resistance to chemical antibiotics, and continue to evolve faster than new chemical antibiotics can be developed. Now, phages are enjoying renewed interest in their uses as detection⁶, decontamination⁷ and delivery⁸, adjuvant⁹, and antibiotic agents¹⁰. Our differentiating approach is to use the phage itself as the antibiotic, and leverage Sandia's exiting engineering and Synthetic Biology expertise to make "evolvable" antibiotics that can be tailored to newly emerging drug resistant bacteria strains.

Most importantly, since phages co-evolve with their bacterial hosts, they present an opportunity for human intervention to direct phage evolution at a

pace faster than bacteria can evolve resistance. The directed evolution of phages is only possible if the proper SynBio pipeline is developed, and we will attempt to develop the blueprint for such a pipeline using M13 filamentous phage and its natural host *E. coli* as a model system.

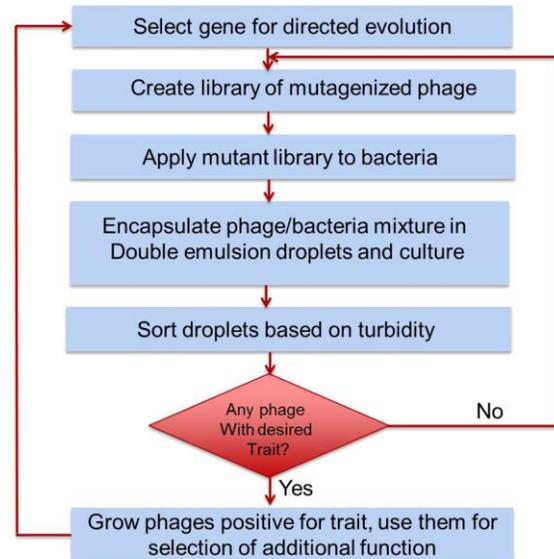


Figure 1. Proposed workflow for SynBio Phage Directed Evolution pipeline.

DETAILED DESCRIPTION OF EXPERIMENT/METHOD:

First, to prove the principle that phages can be evolved *in vitro* to expand host range and increase lethality, we will use M13 filamentous phage and its natural host *E. coli* as a model system. M13 is a non-lytic bacteriophage with a single stranded DNA genome of ~6400 bases that encodes 11 proteins, naturally infecting F-factor carrying *E. coli* strains. M13 infects *E. coli* with an F pilus (F⁺) by binding its p3 minor coat protein to the tip of the F pilus to gain entrance into the bacteria (figure 2, left). To expand M13 host range, we will mutagenize the p3 minor coat protein sequence in hopes of creating a mutant M13 phage that infect bacteria without F-pilus (figure 2, right). The mutant p3 M13 library will be created using error-prone PCR, and screened for any replication in F⁻ *E. coli*. The read-outs of the screen will be OD₆₀₀ measurements, Colony Forming Unit determination, and Sanger sequencing of any positive phage plaques. Any enriched infectious mutant M13 sequence will be compared to a standard curve, and the top "hits" will be phages that can infect an unnatural host, and by virtue of its infectivity to all *E. coli* regardless of F factor, have increased lethality. The goal of the first set of experiments is to establish rules-of-thumb for designing mutant phage libraries as antibiotics, and lay the groundwork for developing sophisticated microfluidic ultra-high throughput iterative screening platform in with possible follow-on funding.

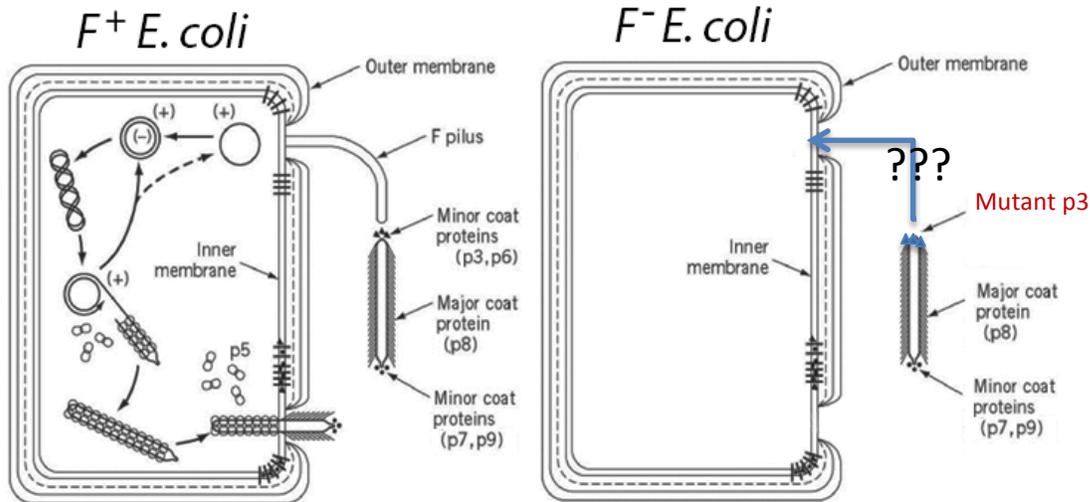
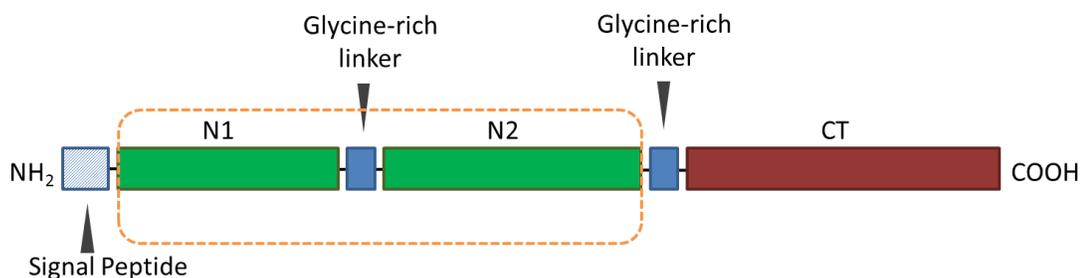


Figure 2. M13 phage uses its minor coat protein p3 to bind the tip of the F pilus and initiate the infection process (left). Mutant M13 libraries with mutated p3 proteins will be added to *F⁻ E. coli* to screen for propagation of any infectious mutant M13 phages.

Mutant M13 Phage library generation

To perform random mutagenesis using error-prone PCR of p3 protein, detailed analysis of p3 structure/function was performed to select the optimal regions for mutagenesis (figure 3). Figure 4 shows the primer sequences and their location on the p3 gene. The resultant mutant fragment comprises of both N1 and N2 domains with the glycine rich linker in between, and a total of 678 base pairs in size. The N terminal leader sequence and the C terminus region were left untouched.

M13 PIII structure and function



- Signal Peptide (1-18 a.a.) – for molecule processing
- N1 (19-85 a.a.) – Forms complex with toIA
- N2 (105-235 a.a.) – Interaction with F-pilus
- CT (275-424 a.a.) – required for release of viral particles from host and proper insertion of PIII and PVI into mature virion.
- region of random mutagenesis by error-prone PCR

Figure 3. M13 P3 structure and function. P3 is a modular protein, with domains N1 and N2 involved in host recognition and infection, and the CT domain involved in viral particle release and proper P3 and P6 insertion into mature virion. Primers flanking N1 and N2 domains (orange dash) were designed and optimized for the purpose of random mutagenesis by error-prone PCR.

p3 sequence and mutagenesis location

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> 0 M13KE_g3p
GTGAAAAAATTATTATTCGCAATTCCTTTAGTGGTACCTTTCTATTCTCACTCGGCCGAAACTGTTGAAA
GTTGTTTAGCAAATCCCATACAGAAAATTCATTTACTAACGTCTGGAAAGACGACAAAACCTTTAGATCG
TIACGCTAACTATGAGGGCTGTCTGTGGAAATGCTACAGGCGTTGTAGTTTGTACTGGTGACGAAACTCAG
TGTACGGTACATGGGTTCTATTGGGCTTGCTATCCCTGAAAATGAGGGTGGTGGCTCTGAGGGTGGCG
GTTCTGAGGGTGGCGTTCTGAGGGTGGCGGTACTAAACCTCTGAGTACGGTGATACACCTATTCCGGG
CTATACTTATCAACCCCTCTCGACGGCACTTATCCGCCTGGTACTGAGCAAACCCCGCTAATCCTAAT
CCTTCTCTGAGGAGTCTCAGCCTCTAATACTTTTCATGTTTCAGAATAATAGGTTCCGAAATAGGCAGG
GGGCATTAAGTGTATACGGGCACTGTTACTCAAGGCACTGACCCGTTAAAACCTATTACCAGTACAC
TCCTGTATCATCAAAGCCATGTATGACGCTTACTGGAACGGTAAATTCAGAGACTGCGCTTCCATTCT
GGCTTAAATGAGGATTAATTTGTTTGTGAATATCAAGGCCAATCGTCTGACCTGCCTCACCTCCTGTCA
ATGCTGGCGGCGGCTCTGGTGGTGGTTCTGGTGGCGGCTCTGAGGGTGGTGGCTCTGAGGGTGGCGGTTCT
TGAGGGTGGCGGCTCTGAGGGAGGCGGTTCCGGTGGTGGCTCTGGTTCCGGTGATTTTGATTATGAAAAG
ATGGCAAACGCTAATAAGGGGGCTATGACCGAAAATGCCGATGAAAACGCGCTACAGTCTGACGCTAAAG
GCAAACCTGATTCTGCTGCTACTGATTACCGTGCTGCTATCGATGGTTTCATTGGTGACGTTCCCGGCT
TGCTAATGGTAATGGTCTACTGGTATTTTGTGGCTCTAATCCCAAATGGCTCAAGTCCGGTGACGGT
GATAATTCACCTTAAATGAATAATTTCCGCTCAATATTTACCTTCCCTCCCTCAATCGGTTGAATGTCGCC
CTTTTGCTTTGGCGCTGGTAAACCATGAATTTCTATTGATTGTGACAAAATAAACTTATCCGTGG
TGCTTTGCGTTTCTTTTATATGTTGCCACCTTTATGTATGATTTTCTACGTTTGCTAACATACTGCGT
ATAAAGGAGTCTTAA
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Red – mutagenesis primers	Bold – region for random mutagenesis,
Green – g3p-C terminus region	contains N1 and N2 regions, plus glycine
Mutagenized fragment = 678 bp	Rich linker in between.

Figure 4. Mutagenesis Primer sequence and location.

Mutant M13KE phagemid library generation

For the purpose of convenience, M13KE phagemid was purchased from New England Biolabs for the initial mutagenesis studies. M13KE contains Lac Z promoter and sequence, and can be used for blue/white phage plaque assays for screening. The random mutagenesis of P3 was performed using error-prone polymerase supplied in the GeneMorph Domain Mutagenesis kit (Agilent, Santa Clara, CA), and Diversify Random Mutagenesis kit (Clontech). Three mutagenesis frequencies were selected for library generation using the GeneMorph kit: 9 mutations/kb (medium), 16 mutations/kb (high), and highest, >20 mutations/kb. Each mutation frequency was achieved through adjustment of input M13KE DNA, and number of PCR cycles. After the error-prone PCR, mutant P3 fragments were gel purified and quantified, subsequently used in EZ clone reaction to generate Replicative Form (RF) M13KE mutant phagemids. Figure 5 demonstrates the EZ clone reaction, and subsequent initial screens of mutant libraries.

Genemorph EZ clone reaction

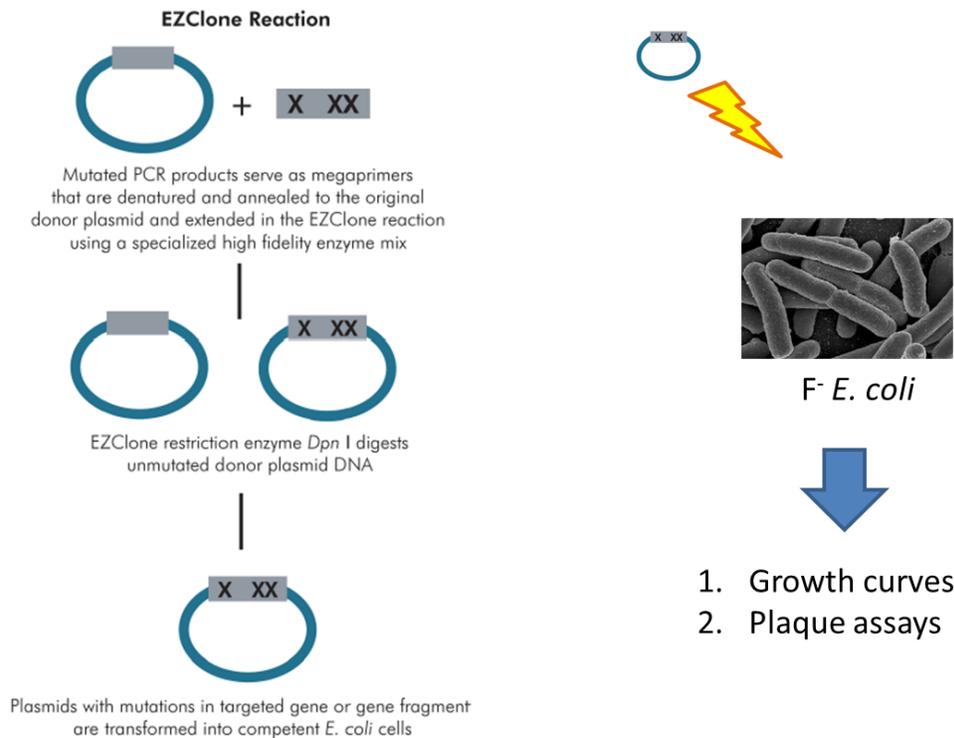


Figure 5. The EZ clone reaction is illustrated on the left (Adapted from Agilent product insert). The mutant PCR products generated by error-prone PCR is used as a megaprimer and annealed to donor M13KE phagemid, and extended in the EZClone reaction. Following amplification, *Dpn I*, a restriction enzyme that digests methylated DNA is added to destroy the non-mutated donor phagemid, leaving only synthetic double stranded M13KE phagemids containing the mutated P3 sequences. The mutant libraries were then electroporated into *F⁻ E. coli* cells and their growth is monitored by OD₆₀₀ readings and blue/white plaque assays.

Electrocompetent *F⁻* bacteria generation

Make electrocompetent *E. coli* TB-1 cells. These are *F⁻*, and have Lac Z promoter. Will be used for screening of mutant libraries.

Procedure:

1. Inoculate 2 ml of a fresh overnight *E. coli* culture into 4x 200 ml of LB in 500 mL flask.
2. Cool rotor, centrifuge bottles, water, and 10% glycerol to 4°C or place on ice.

3. Grow the cells at 37°C shaking at 250 rpm to an OD600 of approximately 0.5–0.7. The best results are obtained with cells that are harvested at early- to mid-log phase; the appropriate cell density depends on the strain and growth conditions but should be about $4\text{--}5 \times 10^7$ cells/ml.
4. Chill the cells on ice for ~15 min. For all subsequent steps, keep the cells as close to 0°C as possible (in an ice/water bath) and chill all containers in ice before adding cells. Transfer the cells to a sterile, cold 250 ml centrifuge bottle and centrifuge at 4000 x g for 15 minutes at 4°C.
5. Carefully pour off and discard the supernatant. It is better to sacrifice yield by pouring off a few cells than to leave any supernatant behind.
6. Gently resuspend the pellet in 200 ml of ice-cold water. Centrifuge at 4000 x g for 15 minutes at 4°C; carefully pour off and discard the supernatant. Repeat once.
7. **Resuspend each pellet in ~5-10 ml of ice-cold 10% glycerol** (used 50 mL TV to resuspend by mistake). Combine into 2x 50 mL falcon tubes. Centrifuge at 4000 x g for 20 minutes at 4°C.
8. Had some trouble pouring off supernatant without losing more cells, especially with second tube. Left or added ~4 mL 20% glycerol to each, and spun again for 20 min.
9. Resuspend each cell pellet in a final volume of 1 ml of ice-cold 10% glycerol (because lost some cells, decreased volume slightly). Total final volume of cells was about 3 mL. The cell concentration should be about $1\text{--}3 \times 10^{10}$ cells/ml.
10. Freeze resuspended cells in 100 uL aliquots for transformation. Keep tubes on ice, then freeze on dry ice before storing at -80°C. Also made 2x 20 uL aliquots for test transformations.

Phage plaque Assay

1. Re-streak *E.coli* on no antibiotic plate. Grow at RT over the weekend.
2. Pick one colony and inoculate 5 ml of LB.
3. Melt top agar in microwave, make 5x 3mL aliquots for plaque assays. Keep at about 50C until ready to use.
4. Prepare dilutions of phage in LB, as shown in table 1 below. Samples in bold will be used for phage infections.

Table 1. Dilutions of phage for plaque assay.

Sample	Cells	Dilution	Serial Dilution	LB (uL)	Phage (uL)	Total Volume (uL)
Stock	-	0	0	-	-	-
A	ER2537 (F ⁻)	1x10 ²	100	49.5	0.5	50
B	ER2537 (F ⁻)	1x10 ⁴	100	198	2	200
C	ER2537 (F ⁻)	1x10 ⁶	100	198	2	200
D	ER2537 (F⁻)	1x10⁸	100	198	2	200
E	ER2537 (F⁻)	1x10⁹	10	90	10	100
F	ER2537 (F⁻)	5x10¹⁰	2	50	50	100
Negative (no phage)	ER2537 (F⁻)	0	0	0	0	50

5. When the culture in Step 1 reaches mid-log phase, dispense 200 µl into microfuge tubes, one for each phage dilution.
6. To carry out infection, add 10 µl of each phage dilution to each tube, mix briefly, and incubate at room temperature for 5 minutes.
7. Transfer the infected cells one infection at a time to culture tubes containing warm Top Agar. Vortex briefly and IMMEDIATELY pour culture onto a LB/IPTG/X-gal agar plate. Gently tilt and rotate plate to spread top agar evenly.
8. Allow the plates to cool for 30 minutes, invert, and incubate overnight at 37°C.
9. Count plaques. Calculate titer.

RESULTS:

Mutagenesis Primer Optimization

5 sets of mutagenesis primers (table 2) were designed and tested to find the optimal sequences and annealing temperatures for error-prone PCR. The primers were purchased from Integrated DNA Technologies (Coralville, IA).

Table 2. M13 P3 mutagenesis primer sets.

g3p_Nterm_1F	TCGCAATTCCTTTAGTGGTACCTT
g3p_Nterm_1R	CAAATCACC GGAACCAGAGC
g3p_Nterm_2F	GCAATTCCTTTAGTGGTACCTTTCT
g3p_Nterm_2R	AATCACC GGAACCAGAGCCA
g3p_Nterm_3F	TTCGCAATTCCTTTAGTGGTACCTT
g3p_Nterm_3R	AAAATCACC GGAACCAGAGC
g3p_Nterm_4F	GCAATTCCTTTAGTGGTACCTTTC
g3p_Nterm_4R	AAAATCACC GGAACCAGAGCC
g3p_Nterm_5F	GTGGTACCTTTCTATTCTCACTCG

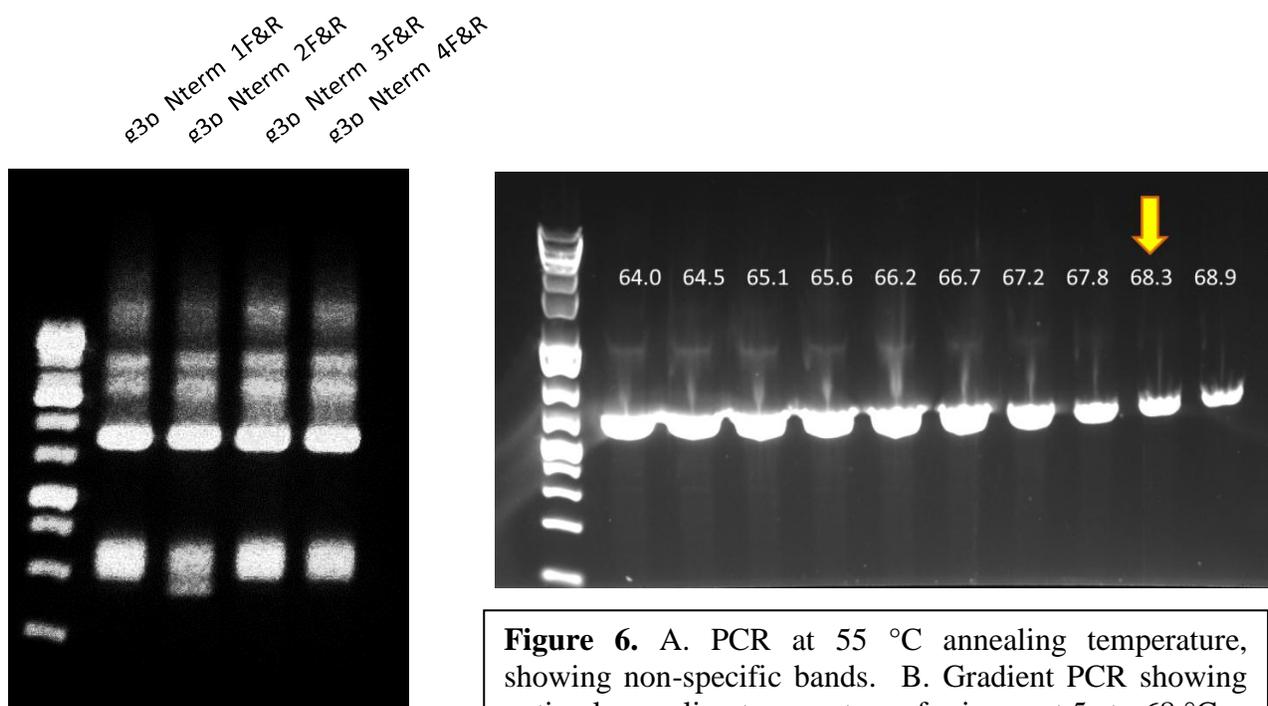
g3p_Nterm_5R

GCCAGCATTGACAGGAGGTT

At 55°C annealing temperature (figure 6A), all 4 sets of primers generated non-specific PCR bands, so gradient PCR was used to test annealing temperatures to find the optimal annealing temperature at ~ 68°C to generate the desired P3 fragment for error-prone PCR mutagenesis. Mutagenesis (figure 6B).

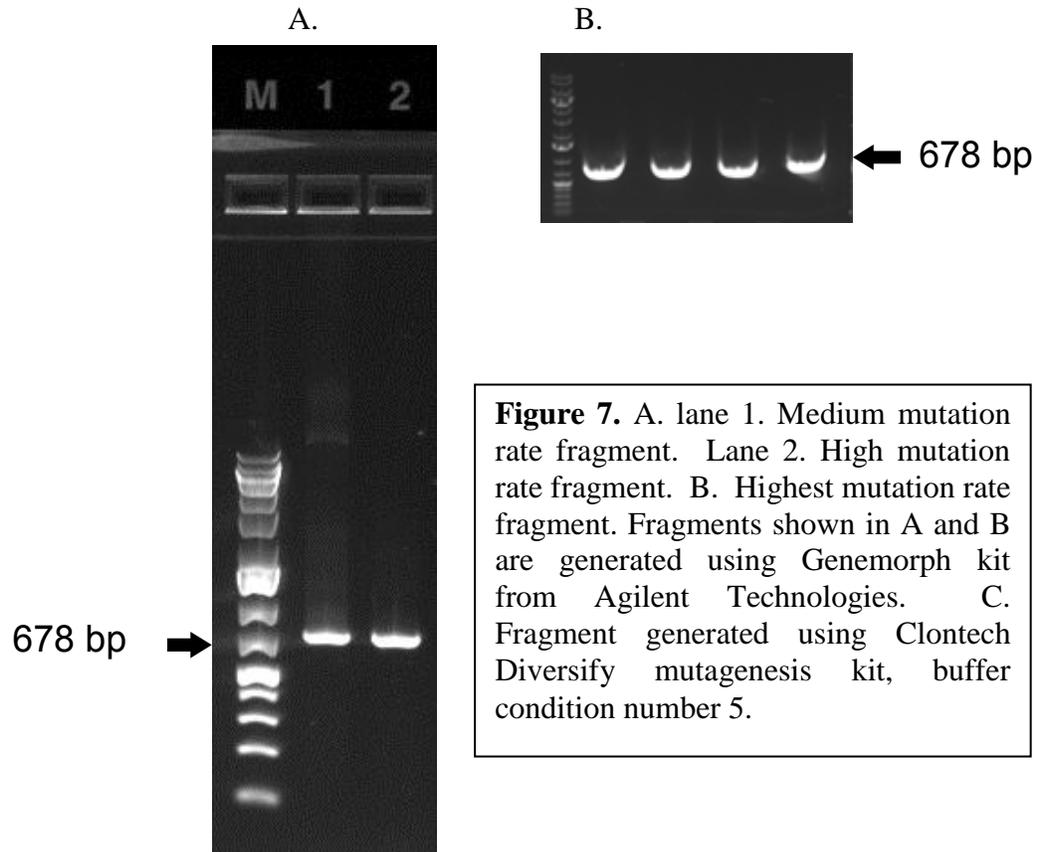
Library generation and purification

Since we do not know which mutational frequency will produce infectious mutant M13 phages, we selected three mutation rates according to manufacturer's suggestions. For the Genemorph Domain Mutagenesis kit, we chose 9 mutations/kb (medium), 16 mutations/kb (high), and >20 mutations/kb(highest) by varying input DNA amounts and cycle numbers. Medium: 100ng input, 30 cycles = 900ng M13KE; High: 10ng input, 30 cycles = 90ng M13KE; Highest: 10ng input, 30 cycles, 2nd round of PCR with 10ng input at 30 cycles, do second PCR using 10ng of High mutation PCR as input. For the Clontech Random Mutagenesis kit, Buffer condition number 5 was selected as the optimal condition for mutagenesis PCR. One band at 678bp was produced as expected.



Annealing Temp = 55°C

Figure 6. A. PCR at 55 °C annealing temperature, showing non-specific bands. B. Gradient PCR showing optimal annealing temperature of primer set 5 at ~68 °C.



EZ Clone Phagemid generation

EZ Clone reaction from the Genemorph kit was used to generate double stranded replicative form (RF) of M13KE phagemid for all libraries. The megaprimer reaction was digested with Dpn1 to destroy all methylated double stranded M13KE phagemid, leaving only synthetic RF M13KE with mutant megaprimer incorporated. Figure 8 below shows all 4 mutant libraries before and after digestion with Dpn1 restriction enzyme.

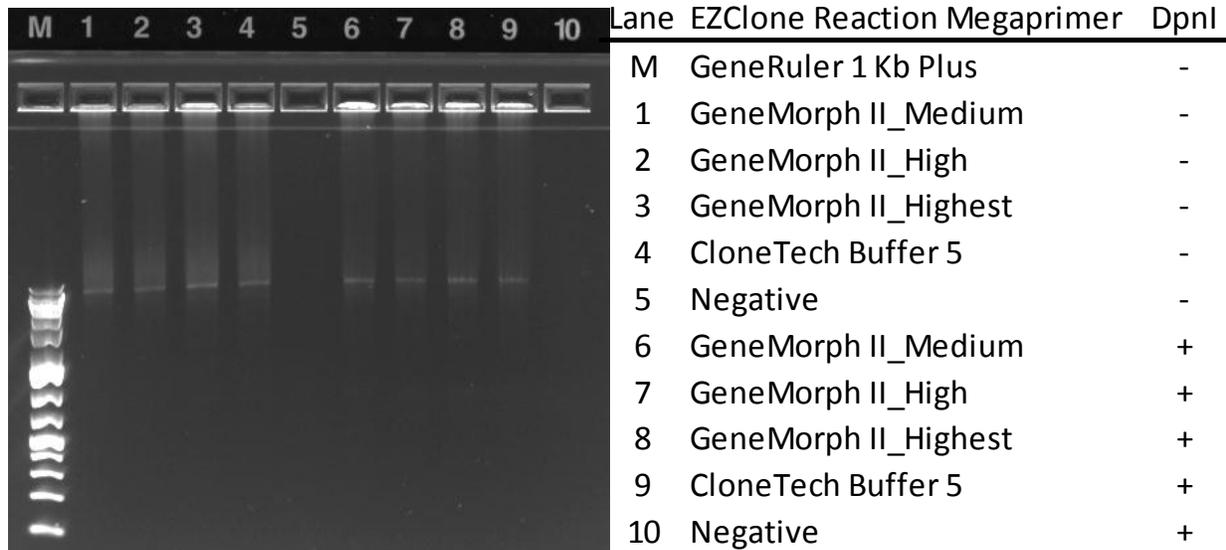


Figure 8. lanes 1-4 EZClone megaprimer reaction with M13KE phagemid used as template DNA. Lanes 6-9, the same EZClone megaprimer reaction after digestion with DpnI.

Testing baseline infectivity on F⁻ *E. coli* strains

The M13KE phage particles were tested in plaque assays against ER2738 and ER2537 *E. coli* strains. The ER2738 strain is NEB's recommended host strain for phage display, and it bears the F pilus. The ER2537 strain was obtained by special request from NEB and presumed to be F⁻, and otherwise identical to ER2738. The plaque assay protocol detailed in the previous section as used for both strains, and the results are shown as below in figure 9. Both ER2738 and ER2537 showed identical calculated Pfu values, suggesting that either ER2537 is not F⁻, or that it is contaminated with F⁺ bacteria. As a result of this finding, an additional three strains of known F⁻ *E. coli* were tested for their susceptibility to M13KE infection.

Plaque assays testing other F⁻ *E. coli* strains

E. coli strains TB1, CAG597, and BL-21 were tested using plaque assays with M13KE phage particles, and compare to ER2738. TB1 and CAG597 are both K12 derivatives, and BL-21 is a B strain derivative. The results of the plaque assays are shown in figure 10. All three strains showed no susceptibility against M13KE, and TB1 was selected as the host strain of choice due to its compatibility with blue-white screening.

Sample	Cells	Phage Dilution	Plaques	Titer (PFU/mL)	Average	Notes
E	K12 ER2738 (F ⁺)	10 ⁹	328	2.81 x 10 ¹²	3.2 x 10 ¹²	Chunky top agar
F	K12 ER2738 (F ⁺)	10 ¹⁰	36	3.6 x 10 ¹²		Chunky top agar
E	ER2537 (F ⁻)	10 ⁹	289	2.89 x 10 ¹²	3.2 x 10 ¹²	
F	ER2537 (F ⁻)	10 ¹⁰	36	3.6 x 10 ¹²		Chunky top agar

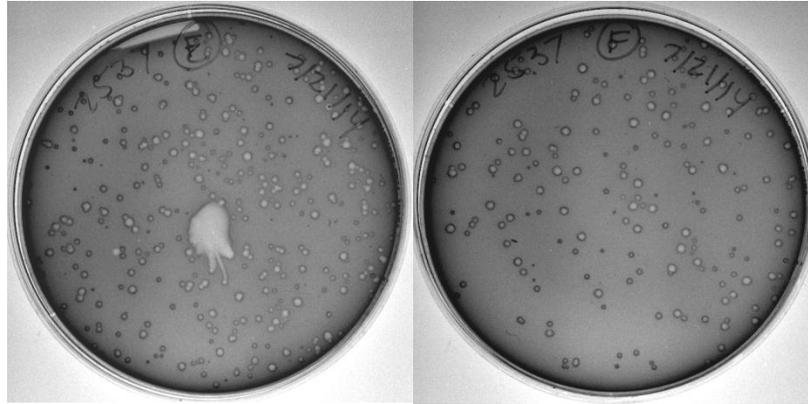
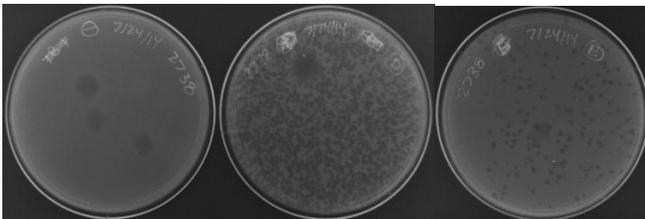


Figure 9. Plaque assay indicating ER2537 is not F⁻. The calculated Pfu for ER2738 (F⁺) is equivalent to Pfu for ER2537.

ER2738 on LB plates:



TB1 on x-gal plates:



BL21 on LB plates:



CAG597 on LB plates:



Figure 10. Plaque assays showing TB1, BL21, and CAG591 are all unsusceptible to M13KE infection. ER2738 serves as positive control for M13KE infection.

K12 derivative TB-1 as F⁻ strain host of choice for screening. Electrocompetent TB-1 cells were generated and tested for transformation efficiency (TE).

TE = (# colonies) / (ug DNA) / (fraction of total recovery vol plated = colony forming units/ ug DNA

$$\begin{aligned}
 &= 78/0.0005 \text{ ug DNA}/ (1/1020) = 1.6 \times 10^9 \text{ cfu/ug} \\
 &= 133/0.0005 \text{ ug DNA}/ (2/1020) = 1.36 \times 10^9 \text{ cfu/ug} \\
 &= 311/0.0005 \text{ ug DNA}/ (5/1020) = 1.27 \times 10^9 \text{ cfu/ug}
 \end{aligned}$$

average TE $\sim 1.41 \times 10^9$ cfu/ug

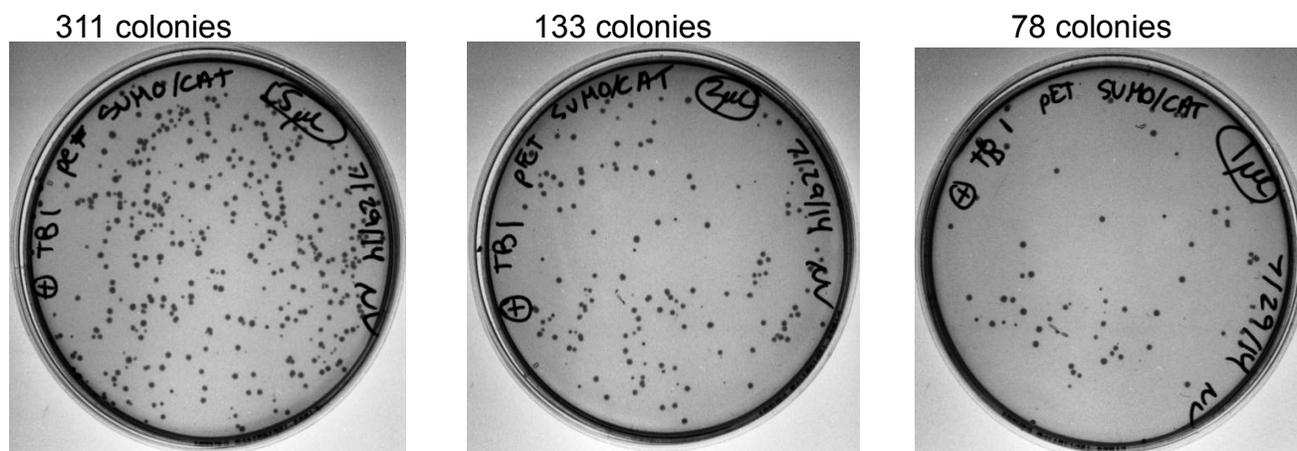
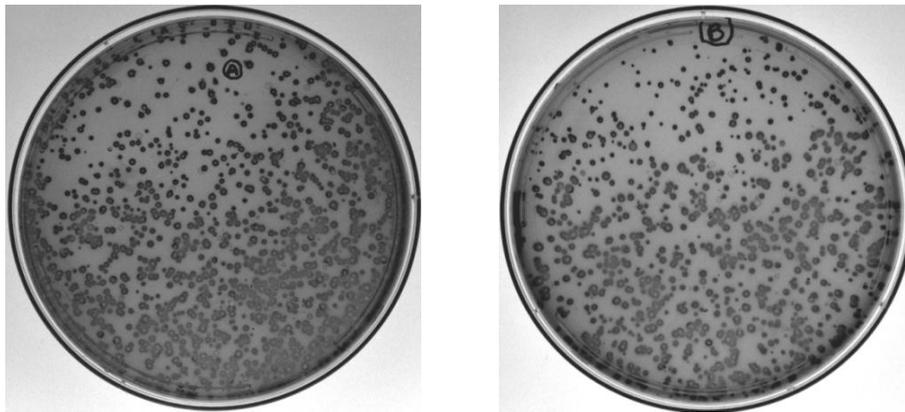


Figure 11. Electrocompetent TB-1 cells were tested for their transformation efficiency by electroporation with pET SUMO/CAT on Kanamycin resistant plates. The average TE of the TB-1 electrocompetent cells was calculated to be $\sim 1.41 \times 10^9$ cfu/ug.

M13KE mutant library screening

A novel method was developed for screening of mutant phages. The mutant phagemid library was transformed by electroporation into host TB1 cells and one round of phage particle production was expected. The supernatant from the TB1 cells was recovered and tested using Plaque assay against ER2738 bacteria to check for presence of phage particles. As indicated in row A of figure 12, there was live, infectious phages present in the supernatant. For screening the entire phage particle library, the supernatant was concentrated using Amicon columns and applied to top agar containing TB1 cells to screen for presence of phage plaques formed by mutant phage that can infect F⁻ TB1 E. coli. So far, no positive mutant has been found .

Sample	Volume Added (uL)	Cell Type	Notes
A Sup	0.5	ER 2738	Control, show at least 1 round of phage is produced from transformation. 
B conc (1:105 dil)	0.5	ER 2738	Control, show sample is not lost during concentration. Diluted to match concentration of A. 
C conc	100	TB1	screening for TB1 infection.
D conc	100	TB1	screening for TB1 infection.
E conc	100	TB1	screening for TB1 infection.
F conc	80	TB1	screening for TB1 infection.



C-F did not show any infection.

Figure 12. “High” mutation frequency library screen. Electroporation of mutant phagemid library resulted in one round of phage particle production. The library contained infectious particles that can infect F⁺ ER2738 E. coli (A, B), but no plaques were found against TB1 (C-F).

DISCUSSION:

Mutagenesis of M13KE P3 protein and significance of mutational frequency

The purpose of this small 25K LDRD project is perform proof-of-principle studies using SynBio and directed evolution to evolve bacteriophages to expand host range and increase lethality in order to create a new class of evolvable antibiotics to combat the emergence of multiple drug resistant bacteria. A theoretical pipeline for generating synthetic phages was proposed in figure 1, and the work performed was to test and improve upon this theoretical pipeline and establish rules-of-thumb in the directed evolution of bacteriophages. The model organisms used in this

LDRD effort was M13KE, a modified version of M13 coliphage that naturally infects F⁺ *E. coli* bacteria. M13 naturally infects host by binding its P3 coat protein to the conjugative or F pilus, and as the pilus retracts into the cell body, the M13 is brought along with it and domains of the P3 protein makes contact with TolA protein in the bacterial membrane and initiates the translocation process by which the M13 phage enters the host and begins its replication process. In the beginning of the project, existing structure/function information regarding the P3 protein was used to select for the most relevant region in the P3 for randomized mutagenesis to produce a mutant phage that can bind and infect a host without the presence of the F pilus (Figure 2). The P3 protein itself is a modular protein as indicated in figure 3, with a leader sequence that is required for expression, 2 N-terminal domains and a C terminal domain, all separated by glycine-rich linkers. The two N-terminal domains are known to be involved in host recognition and infections, whereas the C-terminal domain is known to be essential for proper mature P3 protein insertion into progeny phages at the end of the phage reproductive cycle. Since no previous attempts at random mutagenesis and directed evolution of M13 phages have been attempted, the logical starting point for random mutagenesis was the two N-terminal domains. Mutagenesis primers were designed to flank the two N-terminal domains and the glycine-rich linker in between, with total length of 678 bp (figure 4), and mutant M13KE phagemid libraries containing mutated P3 gene was successfully done using the EZClone reaction supplied by the GeneMorph kit (Figure 5). The mutagenesis primers were optimized to produce one visible product band (figure 6), and error-prone PCR reactions were used to generate randomly mutated libraries of P3 mutant N-terminal fragments (figure 7). What was not obvious at the beginning of the project was that multiple mutational frequencies would need to be made in order to screen for the desired mutant. According to Agilent, selection of the appropriate mutation frequency is very important to the success of a particular application. For analyzing protein structure-function relationships, the desired mutation frequency is very low, at 1-2 nucleotides/gene. In directed evolution studies, 2-7 nucleotide/gene is commonly used, and sometimes, highly mutagenized libraries with >20 mutations/gene have yielded improved protein function. Hence, for P3 directed evolution studies in this effort, mutational frequencies of 9/kb (medium), 16/kb(high), and >20/kb (highest) were chosen to optimize the chance of creating the desired mutant infectious M13KE phage. Clontech's suggested mutational frequency for directed evolution echoed that of Agilent. Clontech mutagenesis rate for directed evolution was suggested to be 2-6 mutations/gene, and a mutant library with 4.6 mutations/kb was generated with the Clontech diversify random mutagenesis kit. All four mutant phagemid libraries were incorporated into M13KE using the EZClone reaction, and digested using DpnI to eliminate any WT M13KE in the library (figure 8) prior to electroporation into *E. coli*. Since four libraries needs to be screened to ascertain the optimal mutagenesis frequency for M13 directed evolution for future mutagenesis efforts, the screening process became much more labor-intensive.

Choice of F⁻ *E. coli* host

The choice of an F⁻ host *E. coli* strain that is not naturally infected by M13 was made with consultation with New England Biolabs (NEB). The strain ER2537 is reportedly a parental F⁻ strain to the F⁺ ER2738 which NEB recommends as the host strain of choice for M13KE phage display studies. However, upon initial experiments using phage plaque assays to establish baseline infectivity using both ER2537 and ER2738 (Table 1, figure 9). Since ER2738 and ER2537 showed the same Pfu values, the only conclusion is that either ER2537 is F⁺, or it is contaminated with F⁺ bacteria, rendering it useless in this study. A significant effort was put

forth to test other F⁻ *E. coli* strains to find a suitable host strain that is not naturally infected by M13, and use it as target host for directed evolution of mutant M13KE. This unexpected interlude cost the project valuable time and resource, and the lesson here is not to rely on commercial vendors to provide reliable reagents when no contract or payment has been put in place to ensure accountability on the part of the vendor. Three additional F⁻ strains: TB-1, BL-21, and CAT597 were tested and compared to ER2738 for their susceptibility to M13KE infection using plaque assays (figure 10). All three strains tested showed no plaques even at the highest phage concentration, and the K12 derivative TB-1 was selected as F⁻ strain of choice due to its suitability for blue/white screening. TB-1 cells were grown overnight, and prepared as electrocompetent stocks, and their transformation efficiency was tested by electroporation of pET SUMO/CAT (Invitrogen) plasmid bearing Kanamycin resistance (Figure 11). The average TE of plasmid was calculated to be $\sim 1.41 \times 10^9$ cfu/ μ g.

Screening of M13KE mutant library

When the phagemid library was electroporated into TB-1 *E. coli* host, the RF mutant phagemids produced one cycle of phages to yield phage particles that can be measured using phage plaque assay. The resultant phage particles were concentrated using Amicon spin column concentrators, and applied to bacterial lawns for plaque assays. The mutant library contained infectious phage plaques when applied to the F⁺ ER2738 strain (A, B figure 12), but no infectivity was found against the TB-1 strain (C-F Figure 12). Since the LDRD project budget was only a modest 25K, we only had 10 weeks to perform all the work described in this report. We did not have time to complete screening for all four libraries, and the diversity of each mutant library far exceeds the volume of transformation we were able to manually perform. In addition, for the sake of convenience, we chose to use M13KE phages for the initial pilot experiments, and since M13KE is a modified phage used for phage display and sequencing applications, it is not a wildtype M13 coliphage, and therefore has lower infectivity than the natural M13 bacteriophage. In the future, only natural phages should be used for mutagenesis and directed evolution to ensure that the resultant mutants have the highest chance of acquiring desired traits.

ANTICIPATED IMPACT:

Scientific lessons learned

Mutagenesis and mutant library preparation is very easily accomplished using the newest Directed Evolution reagents.

The anticipated impact of this 25K LDRD includes technical lessons learned regarding the suitability of bacteriophages as subjects of directed evolution. At least with the case of the DNA bacteriophage M13, bacteriophages can be seamlessly incorporated into the newest SynBio methodologies available from commercial sources. The existing reagents and methodologies employed to enhance enzyme catalytic activity can be directly used, without modification, to randomly mutagenize any selected region of phage genome without the need to isolate and purify phage genes and proteins. This alone is a significant finding that opens the door to large scale, industrial level SynBio efforts for phage directed evolution. Hands-on manipulation time for the mutagenesis and library preparation can be accomplished easily within 1-2 weeks, and existing

mutazymes and cloning kits easily produces replicative phage genomes that can be translated into functional phage particles within a day.

Ultra high-throughput technology for mutant phage library screening

Another significant technical finding is that the sheer volume of phage screening required for even a small evolutionary change in phage infection phenotype is overwhelming when conducted by traditional plaque assay methods and manual manipulations. An ultra high-throughput technology for the library screens will be absolutely necessary. A candidate technology that was proposed for a larger LDRD budget, but could not be accommodated by the smaller approved budget of 25K was the use of microfluidic double emulsion droplets. The key technological advance will be the development and implementation of ultra-high throughput double-emulsion microfluidic droplet platform, which will generate water-in-oil-in-water (W/O/W) droplets that can encapsulate phage and

bacteria for culture and automated screening and sorting in a commercial flow cytometer (figure 13). The use of double emulsion droplets as microreactors of cell studies have been reported in the literature^{11,12}, but none have been reported for use in phage directed evolution. The double emulsion generator will be fabricated using fused silica, and the droplet generator chip will be compatible with Sandia's MICA platform^{13,14}, capitalizing on the precise fluidic controls and automated valves attributed to MICA. The droplet-based microfluidic platform will allow us to rapidly screen billions of candidate phages and isolate lytic phage, bypassing laborious low-throughput manual plaque assays. After several rounds of automated mutagenesis/culture/ screening, candidate phages will be sequenced, and BLASTed to identify reoccurring genetic motifs and uncover patterns of co-evolution that will provide insights for rationally designing phages that will specifically target a range of pathogenic bacteria strains without causing harm to the beneficial microbiome. In addition, incremental directed evolution of phages that can survive exposure to various body fluids can also be performed using the microfluidic droplet platform to select for designer phages that can be safely and effectively delivered to multiple organs.

After development of the double emulsion droplet screening platform, directed evolution of the “designer” phages enabled by microfluidic droplet platform can be used to test “designer” phage’s ability to infect and lyse clinical isolates of pathogenic *E. coli* from UC Davis’s infectious disease clinic. Ultimately, our goal is to expand designer phage development for combating different species of multiple drug resistant select agent bacteria.

Potential External Sponsors

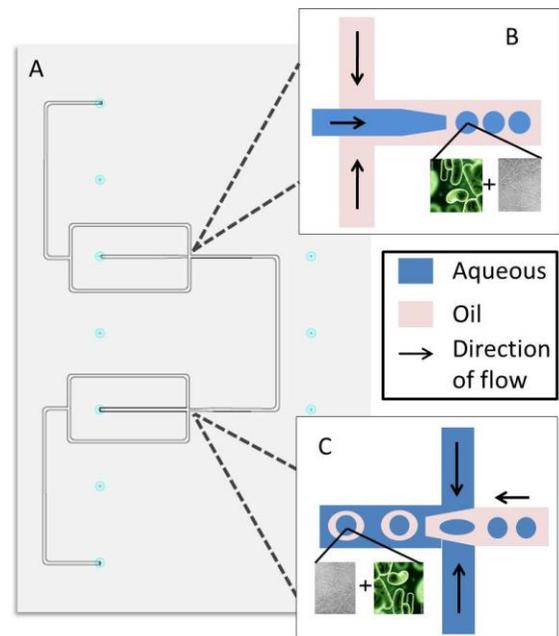


Figure 13. Double emulsion droplet generator chip. A. A glass chip with 2 T-junctions for forming double emulsions. B. First T-junction to form water-in-oil droplets containing bacteria/phage mixture. C. Second junction to form W/O/W droplets.

As it stands, Defense Threat Reduction Agency (DTRA) is the most likely external sponsor to be interested in funding the development of synthetic phages for biodefense applications. The preliminary data generated with the 25K project will be combined with the design of the double-emulsion droplet microfluidic platform to generate quad chart and white paper to be used for solicitation of sponsorship from DTRA. I will be working closely with IAT lead Blake Simmons and Senior Scientist Paula Imbro to craft strategies for finding external sponsors at DTRA.

CONCLUSION:

In conclusion, the key accomplishments of this LDRD are as follows:

1. The establishment of the SynBio pipeline for the directed evolution of bacteriophages with increased host range and infectivity for applications as novel evolvable antibiotics
2. The establishment of rules-of-thumb when designing phage mutagenesis experiments for directed evolution.

At the beginning of the project, a theoretical pipeline was proposed (figure 14, left) as the methodology for systematic design and screening of mutant M13 phages to infect unnatural host. After the initial studies conducted in during the course of this LDRD project, the original flowchart was modified to include the need for multiple mutational frequencies when generating libraries, the substitution of Sanger sequencing for Next-generation sequencing as the most appropriate method of sequence analysis for long (>600bp) mutagenized phage gene fragments, and the need for ultra-high throughput screening technology to accommodate the size of mutant libraries required for directed evolution. The modified flowchart for Synthetic Phage directed evolution is shown in figure 14, right.

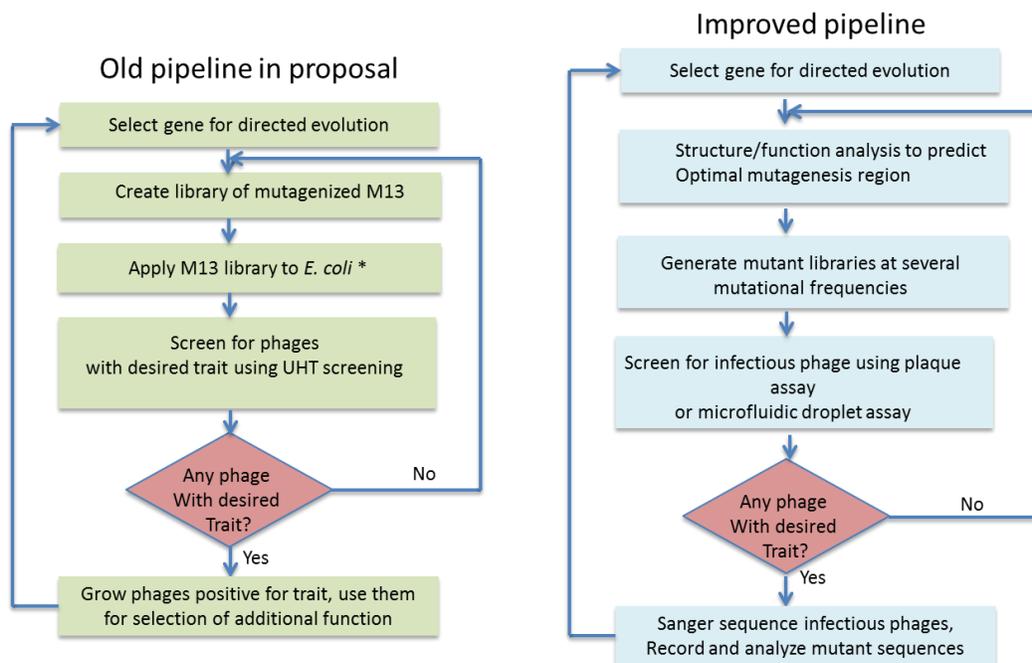


Figure 14. Left, the theoretical pipeline in the LDRD proposal. Right, the improved pipeline after the LDRD experiments were performed.

The rules-of-thumb for designing synthetic phage directed evolution studies learned from these initial pilot experiments include:

- 1). Use natural phage backbone instead of modified phagemids in order to preserve maximal infectivity of phages.
- 2). The selection of mutagenesis target in the phage genome requires substantial knowledge of the structure/function of that segment.
- 3). Multiple mutational frequencies need to be used in order to maximize the probability of finding the mutant with desirable characteristics.
- 4). The screening efforts to provide adequate coverage of the mutation library diversity is beyond that of traditional manual plaque assay. Ultra high-throughput methodologies **MUST** be developed and employed. A continuous mutagenesis and screening platform that minimizes manual manipulations will be key to establishing a successful implementation of Synthetic Phage genesis.

The initial feasibility studies into Synthetic Phage directed evolution produces some valuable results that promises a true solution to the emerging threat of bacterial drug resistance. The fact that phages genes can be easily mutated, inserted, and translated into infectious phage particles using common commercial mutagenesis reagents is very promising in terms of establishing a new area in SynBio to include whole organism directed evolution in a potentially rapid and automatable fashion. The emergence of multiple drug resistance in bacteria due to the overuse and misuse of chemical antibiotics is exacerbated by the lateral gene transfer mechanisms that confer new antibiotic resistance across multiple species of pathogenic bacteria, and what was once opportunistic infections in hospitals are now becoming community acquired infections in healthy people. The underlying obstacle in developing new effective countermeasure against bacterial pathogens is the bacteria's ability to evolve and respond to evolutionary pressure exerted by the chemical antibiotics we have used heavily for the past 8 decades. The speed at which bacteria can evolve resistance far exceeds our ability to decipher the genetic and biochemical mechanisms behind their resistance and generate new chemical antibiotics as alternative treatments. Since evolution and the ability to adapt is key to the bacteria's survival, an obvious conclusion is that in order to win the war of evolution, we must employ an arsenal capable of can co-evolve and out-evolve the bacterial enemy. Bacteriophages naturally infect and either kill or retard bacteria growth, and specifically only infect bacteria, they make the perfect candidates for human intervention and usage as the evolvable antibiotics we will need to permanently address the pathogenic bacteria problem. The results from this LDRD indicate that bacteriophages can be easily manipulated and subjected to SynBio efforts and be subject of automated directed evolution process to become the custom tailored "live" antibiotics that are safe the human and animal patients.

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